# B Cell Help Provided by Human γδ T Cells

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# Dedications

This thesis is dedicated to my family, especially to my dear mother, brothers and sisters who believed in me and supported me wholeheartedly throughout all my studies.

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# Summary

Vγ9Vδ2 T cells are a minor subset of T cells in human blood that differ from all other lymphocytes by their specific responsiveness to (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), a metabolite produced by a large range of microbial pathogens. Vγ9Vδ2 T cells can be skewed towards distinct effector functions, in analogy to, and beyond, the emerging plasticity of $CD4^+$ T cells. Depending on the microenvironment, Vγ9Vδ2 T cells can assume features reminiscent of Th1, Th2, Th17 and Treg cells as well as professional antigen presenting cells (APCs). The main focus of this PhD was to investigate the role of the follicular B helper T (Tfh) cell derived cytokine IL-21 in enhancing the ability of human Vγ9Vδ2 T cells in providing B cell help.

In order to try to mimic the physiological conditions in the GC, an *in vitro* system of autologous Vγ9Vδ2 T cells and B cells from tonsils or blood, the microbial metabolite HMB-PP and IL-21 was used. HMB-PP induced up-regulation of IL-21 receptor on Vγ9Vδ2 T cells. In return, IL-21 played a co-stimulatory role in the expression of the B cell-attracting chemokine CXCL13, the CXCL13 receptor CXCR5, the co-stimulatory molecules inducible co-stimulator (ICOS), OX40 and CD70 by activated Vγ9Vδ2 T cells. IL-21 also enhanced the ability of activated Vγ9Vδ2 T cells to support antibody production by B cells. Furthermore, Vγ9Vδ2 T cells not only themselves became highly activated APC marker expressing cells but also modified activation and APC marker expression on B cells.

Findings presented in this thesis provide evidence that IL-21 contributes to the acquisition of B cell helper functions by human Vγ9Vδ2 T cells. In secondary lymphoid tissues, the interaction between HMB-PP-responsive Vγ9Vδ2 T cells, IL-21-producing Tfh cells and B cells is likely to impact on the generation of high affinity, class-switched antibodies in microbial infections.

# Publications and Presentations

## Publication

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Bansal RR, Meuter S, Moser B, Eberl M. (2008). **IL-21 induces the B cell attracting chemokine CXCL13 in human γδ T cells** (Poster presentation)**.** *British Society of Immunology congress 2008.* Scottish Exhibition and Conference Centre, Glasgow, UK.

# Contents List

**Declaration** ..... **2**
**Dedications** ..... **3**
**Acknowledgements** ..... **4**
**Summary** ..... **5**
**Publications and Presentations** ..... **6**
**Contents List** ..... **8**
**List of Figures** ..... **13**
**List of Abbreviations** ..... **17**

**Chapter 1: Introduction** ..... **20**
*1.1 General features of innate and adaptive immunity* ..... *21*
1.1.1 Features of innate immunity ..... 21
1.1.2 Features of adaptive immunity ..... 24
*1.2 T cells and cellular immunity* ..... *28*
1.2.1 Major T cell subsets ..... 28
1.2.2 MHC restriction and antigen processing ..... 29
1.2.2.1 MHC class I pathway ..... 29
1.2.2.2 MHC class II pathway ..... 30
1.2.2.3 Cross-presentation ..... 30
1.2.2.4 CD1 antigen presenting pathway ..... 31
1.2.3 T cell co-stimulation and activation ..... 31
1.2.3.1 Co-stimulation via CD28 ..... 32
1.2.3.2 Delivery of co-inhibitory signals via CTLA-4 ..... 33
1.2.4 T cell receptor signalling and activation ..... 33
1.2.5 $CD4^+$ T helper subsets ..... 35
1.2.6 T cell memory subsets ..... 38
*1.3 B cells and humoral immunity* ..... *39*

1.3.1 Structure and function of antibodies ..... 39
1.3.1.1 IgM ..... 40
1.3.1.2 IgG ..... 42
1.3.1.3 IgA ..... 42
1.3.1.4 IgE ..... 43
1.3.1.5 IgD ..... 43
1.3.2 Generation of B cells and B cell receptor ..... 43
1.3.3 B cell antigens ..... 45
1.3.4 Mature B cell populations ..... 46
1.3.4.1 Marginal zone B cells ..... 46
1.3.4.2 B1 B cells ..... 47
1.3.4.3 Follicular B cells ..... 47
1.3.5 The germinal centre response ..... 49
1.3.6 Memory B cells ..... 51
1.3.7 Plasma cells ..... 52
*1.4 Tfh cells and molecules involved in Tfh and B cell interactions ..... 53*
1.4.1 Tfh cell differentiation ..... 55
1.4.2 Bcl6 ..... 55
1.4.3 The chemokine receptor CXCR5 and ligand CXCL13 ..... 56
1.4.4 Co-stimulatory molecules ..... 58
1.4.4.1 CD40L ..... 59
1.4.4.2 OX40 ..... 60
1.4.4.3 ICOS ..... 61
1.4.4.4 CD70 ..... 63
1.4.4.5 PD-1 ..... 63
1.4.5 IL-21 ..... 64
1.4.6 IL-21 receptor ..... 65
*1.5 Vγ9Vδ2 T cells and role in immune responses ..... 68*
1.5.1 γδ T cells in humans ..... 68
1.5.2 Activators of Vγ9Vδ2 γδ T cells ..... 69
1.5.2.1 (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate ..... 69

1.5.2.2 Isopentenyl pyrophosphate ..... 72
1.5.2.3 Alkylamines ..... 72
1.5.2.4 Aminobisphosphonates ..... 72
1.5.3 Recognition and pathogen release of HMB-PP ..... 73
1.5.4 Vγ9Vδ2 T cell migratory profile ..... 74
1.5.5 Vγ9Vδ2 T cell memory subsets ..... 75
1.5.6 Vγ9Vδ2 T cell effector functions ..... 76
1.5.7 APC function of Vγ9Vδ2 T cells ..... 76
1.5.8 Vγ9Vδ2 T cell involvement in B cell responses ..... 77
*1.6 Hypothesis and aims* ..... *80*
**Chapter 2: Materials and Methods ..... 81**
*2.1 Cell isolation* ..... *82*
2.1.1 Isolation of peripheral blood mononuclear cells ..... 82
2.1.2 Isolation of mononuclear cells from lymphoid tissue ..... 83
2.1.3 Isolation of mononuclear cells from peritoneal dialysis fluid ..... 83
*2.2 Cell sorting* ..... *84*
2.2.1 Cell sorting for co-culture experiments ..... 84
2.2.2 Cell sorting for functional assays ..... 87
*2.3 Cell counting* ..... *91*
*2.4 Cell culture* ..... *93*
2.4.1 Phenotypic assays ..... 93
2.4.2 CXCL13 and cytokine production assays ..... 94
2.4.3 Functional assays ..... 94
*2.5 Flow cytometry* ..... *97*
2.5.1 Antibodies and cell staining ..... 97
2.5.2 Flow cytometry analysis ..... 101
2.5.3 Gating strategy ..... 101
*2.6 Immunofluorescence microscopy* ..... *103*
*2.7 ELISA* ..... *103*
*2.8 Health and safety* ..... *104*
*2.9 Statistical analysis* ..... *104*

**Chapter 3: IL-21 co-stimulates expression of key molecules involved in B cell help on peripheral HMB-PP activated Vγ9Vδ2 T cells ..... 105**

*3.1 Chapter specific hypothesis and aims ..... 106*

*3.2 Results ..... 107*

3.2.1. Freshly isolated peripheral Vγ9Vδ2 T cells express the receptor for IL-21 ..... 107

3.2.2. IL-21R is significantly up-regulated on HMB-PP activated peripheral Vγ9Vδ2 T cells ..... 109

3.2.3. IL-21 enhances expression of CD25 and CD69 activation markers on HMB-PP stimulated peripheral Vγ9Vδ2 T cells ..... 115

3.2.4. IL-21 in the presence of HMB-PP induces expression of the B cell-attracting chemokine CXCL13 by peripheral Vγ9Vδ2 T cells ..... 120

3.2.5. IL-21 in the presence of HMB-PP stimulation does not induce expression of CXCR5 on peripheral Vγ9Vδ2 T cells. ..... 129

3.2.6. IL-21 and HMB-PP stimulation induces expression of ICOS, OX40 and CD70 co-stimulatory molecules on peripheral Vγ9Vδ2 T cells ..... 133

3.2.7. HMB-PP stimulation induces expression of CCR7, CD40, CD86 and HLA-DR on peripheral Vγ9Vδ2 T cells ..... 145

3.2.8. Proliferation and differentiation of Vγ9Vδ2 T cell subsets in response to IL-21 and HMB-PP stimulation ..... 149

**Chapter 4: Up-regulation of key molecules involved in B cell help on tonsillar Vγ9Vδ2 T cells ..... 156**

*4.1 Chapter specific hypothesis and aims ..... 157*

*4.2 Results ..... 158*

4.2.1 Presence of activated Vγ9Vδ2 T cells in lymphoid tissue ..... 158

4.2.2 Freshly isolated tonsillar Vγ9Vδ2 T cells do not express CXCL13 but express IL-4, TNF-α and IFN-γ ..... 166

4.2.3 Freshly isolated tonsillar Vγ9Vδ2 T cells express CXCR5, ICOS, OX40 and CD40L ..... 170

4.2.4. Freshly isolated tonsillar Vγ9Vδ2 T cells express CCR7, CD40 and HLA-DR ..... 173

4.2.5 Tonsillar Vγ9Vδ2 T cell memory subsets differ from peripheral blood ..... 175

4.2.6 Freshly isolated tonsillar Vγ9Vδ2 T cells express the receptor for IL-21 .....177
4.2.7. HMB-PP stimulation enhances expression of CD25 and CD69 activation markers on tonsillar Vγ9Vδ2 T cells.....179
4.2.8. IL-21 co-stimulation enhances expression of CXCR5 on HMB-PP stimulated tonsillar Vγ9Vδ2 T cells.....182
4.2.9. HMB-PP stimulation enhances expression of ICOS, OX40, CD70 and CD28 co-stimulatory molecules on tonsillar Vγ9Vδ2 T cells .....184
4.2.10. IL-21 and HMB-PP stimulation enhances expression of CCR7, CD40, CD86, and HLA-DR APC markers on tonsillar Vγ9Vδ2 T cells .....189
4.2.11. Selective proliferation of tonsillar Vγ9Vδ2 T cells in response to HMB-PP stimulation .....194
**Chapter 5: IL-21 enhances the ability of HMB-PP activated Vγ9Vδ2 T cells to provide B cell help.....197**
*5.1 Chapter specific hypothesis and aims.....198*
*5.2. Results.....200*
5.2.1. Phenotypic analysis of freshly isolated tonsillar and peripheral blood B cells.200
5.2.2. Tfh cells and IL-21 induce B cell IgM, IgG and IgA antibody production .....205
5.2.3. Peripheral Vγ9Vδ2 T cells provide help for tonsillar B cell antibody production .....209
5.2.4. IL-21 dependent tonsillar Vγ9Vδ2 T cells provide help for tonsillar B cell IgM antibody production.....213
5.2.5. IL-21 and HMB-PP stimulated peripheral Vγ9Vδ2 T cells induce peripheral B cell activation, APC marker expression and antibody production .....223
**Chapter 6: Discussion.....233**
*6.1 Future directions.....245*
**References.....248**
**Appendix: Peer reviewed publication.....268**

# List of Figures

Figure 1.1. Primary and secondary lymphoid organs. ..... 26
Figure 1.2. $CD4^+$ T helper subsets. ..... 36
Figure 1.3. Schematic structure of a monomeric antibody molecule. ..... 41
Figure 1.4. Schematic structure of a lymph node. ..... 48
Figure 1.5. The germinal centre reaction. ..... 50
Figure 1.6. Important molecules for Tfh cell function. ..... 54
Figure 1.7. IL-21R signalling pathways. ..... 67
Figure 1.8. MEP and mevalonate pathways for isoprenoid biosynthesis. ..... 71
Figure 1.9. Murine γδ T cells support the formation of germinal centres in severe combined immunodeficiency (SCID) mice. ..... 78
Figure 1.10. Clusters of human γδ T cells found within a reactive germinal centre. ..... 78
Figure 2.1. Schematic diagram showing gridlines on a haemocytometer grid. ..... 92
Figure 2.2. Example of gating strategy for identifying CD25 and OX40 positive Vγ9Vδ2 T cell populations. ..... 102
Figure 3.1. Freshly isolated peripheral blood Vγ9Vδ2 T cells express the receptor for IL-21. ..... 108
Figure 3.2. HMB-PP induced up-regulation of IL-21R expression on peripheral blood Vγ9Vδ2 T cells. ..... 111
Figure 3.3. Sustained CD25 expression on HMB-PP activated peripheral blood Vγ9Vδ2 T cells. ..... 113
Figure 3.4. IL-21R expression is up-regulated on HMB-PP activated $CD25^+$ Vγ9Vδ2 T cells. ..... 114
Figure 3.5. Flow cytometric purity analysis of freshly isolated Vγ9Vδ2 T cells, B cells and monocytes. ..... 116
Figure 3.6. HMB-PP activated Vγ9Vδ2 T cells in both PBMCs and in co-cultures co-express CD25 and CD69. ..... 118
Figure 3.7. CD25 and CD69 expression on Vγ9Vδ2 T cells is dependent on the concentration of HMB-PP. ..... 119

Figure 3.8. IL-21 induces secretion of CXCL13 by HMB-PP activated Vγ9Vδ2 T cells...123
Figure 3.9. Detection of intracellular CXCL13 in Vγ9Vδ2 T cells by flow cytometry. .....126
Figure 3.10. Detection of intracellular CXCL13 in Vγ9Vδ2 T cells by immunofluorescence microscopy.....127
Figure 3.11. CXCL13 positive Vγ9Vδ2 T cells express IL-4 and IFN-γ. .....128
Figure 3.12. Freshly isolated peripheral Vγ9Vδ2 T cells do not express CXCR5. .....131
Figure 3.13. IL-21, HMB-PP or PHA stimulation does not induces CXCR5 expression in peripheral Vγ9Vδ2 T cells. .....132
Figure 3.14. Freshly isolated peripheral Vγ9Vδ2 T cells do not express CD40L. .....135
Figure 3.15. Freshly isolated peripheral Vγ9Vδ2 T cells do not express ICOS or OX40...136
Figure 3.16. Freshly isolated peripheral Vγ9Vδ2 T cells express CD28 and CD27. .....137
Figure 3.17. IL-21 does not induce CD40L expression by HMB-PP activated peripheral Vγ9Vδ2 T cells. .....139
Figure 3.18. IL-21 maintains higher expression of OX40 and ICOS on HMB-PP activated peripheral Vγ9Vδ2 T cells. .....141
Figure 3.19. IL-21 induces co-expression of ICOS and OX40 on HMB-PP activated peripheral Vγ9Vδ2 T cells. .....142
Figure 3.20. CD70, CD28 and CD27 co-stimulatory molecule expression on non-activated and HMB-PP activated peripheral Vγ9Vδ2 T cells. .....144
Figure 3.21. HMB-PP stimulation up-regulates CCR7, CD40, CD86 and HLA-DR expression on peripheral Vγ9Vδ2 T cells. .....147
Figure 3.22. HMB-PP stimulation induces co-expression of CCR7, CD40 and HLA-DR on peripheral Vγ9Vδ2 T cells. .....148
Figure 3.23. The effects of IL-21 and HMB-PP stimulation on Vγ9Vδ2 T cell memory subsets, based on CCR7 expression. .....151
Figure 3.24. The effects of IL-21 and HMB-PP stimulation on Vγ9Vδ2 T cell memory subsets, based on CD27 expression. .....153
Figure 3.25. Peripheral blood Vγ9Vδ2 T cells proliferate in response to IL-21+HMB-PP stimulation. .....155
Figure 4.1. Vγ9Vδ2 T cells are detectable in a variety of human lymphoid tissue. .....161

Figure 4.2. Vγ9Vδ2 T cells from colon biopsies, appendix and tonsils express surface CD69. ..... 164
Figure 4.3. Comparison of CD69 and CD25 expression on peripheral blood and tonsillar Vγ9Vδ2 T cells. ..... 165
Figure 4.4. CD25 expression on tonsillar Vγ9Vδ2 T cells is up-regulated upon *in vitro* PMA and ionomycin stimulation. ..... 165
Figure 4.5. Tonsillar $CD4^+$ T cells express intracellular CXCL13. ..... 168
Figure 4.6. Tonsillar γδ and $CD4^+$ T cells express IFN-γ, TNF-α and IL-4. ..... 169
Figure 4.7. CD40L expression on tonsillar Vγ9Vδ2 and $CD4^+$ T cells is up-regulated upon *in vitro* PMA and ionomycin stimulation. ..... 172
Figure 4.8. Tonsillar Vγ9Vδ2 and $CD4^+$ T cells express CCR7, CD40 and HLA-DR. ..... 174
Figure 4.9. Higher percentage of Vγ9Vδ2 T cells express CCR7, CD40 and HLA-DR in tonsils in comparison to peripheral blood. ..... 174
Figure 4.10. Vγ9Vδ2 and $CD4^+$ T cell memory subsets in tonsils. ..... 176
Figure 4.11. Tonsillar memory Vγ9Vδ2 T cell populations differ from peripheral blood. . 176
Figure 4.12. Freshly isolated tonsillar Vγ9Vδ2 T cells express the receptor for IL-21. ..... 178
Figure 4.13. HMB-PP induces up-regulation of CD25 and CD69 activation markers on tonsillar Vγ9Vδ2 T cells. ..... 181
Figure 4.14. IL-21 enhances expression of CXCR5 on HMB-PP stimulated tonsillar Vγ9Vδ2 T cells. ..... 183
Figure 4.15. HMB-PP induces up-regulation of ICOS, OX40, CD70 and CD28 co-stimulatory molecules on tonsillar Vγ9Vδ2 T cells. ..... 186
Figure 4.16. HMB-PP or IL21 does not induce up-regulation of ICOS, OX40, CD70 or CD28 co-stimulatory molecules on tonsillar $CD4^+$ T cells. ..... 188
Figure 4.17. HMB-PP induces up-regulation of CCR7, CD40, CD86 and HLA-DR on tonsillar Vγ9Vδ2 T cells. ..... 191
Figure 4.18. HMB-PP and IL-21 does not induce up-regulation of CCR7, CD40, CD86 or HLA-DR on tonsillar $CD4^+$ T cells. ..... 193
Figure 4.19. Tonsillar Vγ9Vδ2 T cells proliferate in response to HMB-PP stimulation. ..... 196
Figure 5.1. Comparison of B cell subsets in tonsils and peripheral blood. ..... 202

Figure 5.2. Cell surface phenotype of freshly isolated B cells from peripheral blood and tonsils. ..... 204
Figure 5.3. Flow cytometric purity analysis of isolated tonsillar Tfh and $CXCR5^+$ B cells. ..... 207
Figure 5.4. IL-21 induces a dose-dependent effect on IgM, IgG and IgA antibody production. ..... 208
Figure 5.5. Peripheral Vγ9Vδ2 T cells induce antibody production during co-culture with tonsillar B cells. ..... 212
Figure 5.6. Flow cytometric purity analysis of isolated tonsillar Vγ9Vδ2 positive T cells. 214
Figure 5.7. HMB-PP and IL-21 dependent tonsillar Vγ9Vδ2 T cells induce potent IgM antibody production during co-culture with tonsillar B cells. ..... 217
Figure 5.8. Comparison of IL-21 and IL-2 stimulation on tonsillar Vγ9Vδ2 T cells help in antibody production. ..... 219
Figure 5.9. Tonsillar Vγ9Vδ2 T cells do not further enhance antibody production induced by Tfh cells. ..... 221
Figure 5.10. Titration of tonsillar Vγ9Vδ2 T cells does not enhance antibody production induced by Tfh cells. ..... 222
Figure 5.11. HMB-PP activated peripheral γδ T cells in co-cultures up-regulate activation and APC marker expression on B cells. ..... 225
Figure 5.12. HMB-PP activated peripheral γδ T cells in co-cultures up-regulate activation and APC marker expression. ..... 226
Figure 5.13. HMB-PP and IL-21 stimulated Vγ9Vδ2 T cells do not induce B cell differentiation. ..... 228
Figure 5.14. HMB-PP and IL-21 stimulated peripheral Vγ9Vδ2 T cells induce potent antibody production during co-culture with peripheral B cells. ..... 231
Figure 5.15. HMB-PP and IL-21 stimulated peripheral Vγ9Vδ2 T cells provide dose dependent help on antibody production. ..... 232
Figure 6.1. Proposed interaction between γδ T cells, B cells and Tfh cells in secondary lymphoid tissue. ..... 244

# List of Abbreviations

| | |
|---|---|
| Ab | Antibody |
| AF488 | Alexa fluor488 |
| AID | Activation induced cytidine deaminase |
| APC | Allophycocyanin |
| APC | Antigen presenting cells |
| APC-H7 | Allophycocyanin-Hi10lite®7-BD |
| BALT | Bronchus associated lymphoid tissue |
| BCR | B cell receptor |
| BFA | Brefeldin A |
| Blimp1 | B lymphocyte induced maturation protein 1 |
| BrHPP | Bromohydrin pyrophosphate |
| CAPD | Continuous ambulatory peritoneal dialysis |
| CCR7 | CC-chemokine receptor 7 |
| CD | Cluster of differentiation |
| CD40L | CD40 ligand |
| CDR | Complementarity determining region |
| CLP | Common lymphocyte progenitors |
| CLRs | C-type lectin receptors |
| COSHH | Control of substances hazardous to health |
| CSR | Class-switch recombination |
| CTLA-4 | Cytotoxic T lymphocyte antigen-4 |
| CTLs | Cytotoxic T lymphocytes |
| CVID | Common variable immunodeficiency |
| CXCL13 | CXC-chemokine ligand 13 |
| CXCR5 | CXC-chemokine receptor 5 |
| D | Diversity |
| DAPI | 4'-6-Diamidino-2-phenylindole |
| DCs | Dendritic cells |
| ECD | Also known as PE-Texas Red |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| FACS | Fluorescence activated cell sorting |
| FcR | Fc receptors |
| FCS | Foetal calf serum |
| FDCs | Follicular dendritic cells |
| FITC | Fluorescein isothiocyanate |
| FOXP3 | Forkhead box protein P3 |
| FPP | Farnesyl pyrophosphate |
| FSC | Forward scatter |
| GALT | Gut-associated lymphoid tissue |

| | |
|---|---|
| GATA-3 | GATA binding protein-3 |
| GC | Germinal centre |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |
| GMFI | Geometric mean fluorescence intensity |
| Gy | Gray |
| HEVs | High endothelial venules |
| HLA | Human leukocyte antigen |
| HMB-PP | (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate |
| ICAM-1 | Intercellular adhesion molecule-1 |
| ICOS | Inducible co-stimulator |
| ICOSL | inducible co-stimulator ligand |
| IFNs | Interferons |
| IFN-γ | Interferon-gamma |
| Igs | Immunoglobulins |
| IL-10 | Interleukin-10 |
| IL-2 | Interleukin-2 |
| IL-21 | Interleukin-21 |
| IL-21R | Interleukin-21 receptor |
| IL-2R | Interleukin-2 receptor |
| IL-4 | Interleukin-4 |
| ILs | Interleukins |
| IPP | Isopentenyl pyrophosphate |
| ITAMs | Immunoreceptor tyrosine-based activation motifs |
| J | Joining |
| Jak | Janus activated kinase |
| LAT | Linker of activated T cells |
| LFA-1 | Lymphocyte function associated antigen-1 |
| LPS | Lipopolysaccharide |
| mAb | Monoclonal antibody |
| MACS | magnetic activated cell sorting |
| MALT | Mucosa-associated lymphoid tissue |
| MAPK | Mitogen-activated protein kinase |
| MCP-1 | Macrophage chemoattractant protein-1 |
| MEP | 2-C-methyl-D-erythritol 4-phosphate |
| MFI | Mean fluorescence intensity |
| MHC | Major histcompatibility complex |
| MZ | Marginal zone B cells |
| NK | Natural killer cells |
| NKT | Natural killer T cells |
| NLRs | NOD-like receptors |
| OX40L | OX40 ligand |
| PAMPs | Pathogen-associated molecular patterns |
| PB | Pacific Blue |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PD1 | Programmed death 1 |
| PD-L1 | Programmed death 1 Ligand |

| | |
|---|---|
| Pe | Phycoerythrin |
| PeCy5 | Phycoerythrin-Cy5 |
| PeCy7 | Phycoerythrin-Cy7 |
| PerCp-Cy5.5 | Peridinin Chlorophyll Protein Complex-Cy5.5 |
| PHA | Phytohaemagglutinin |
| PI3K | Phosphatidylinositol 3-kinase |
| PKC | Protein kinase C |
| PMA | Phorbol myristate acetate |
| PNA | Peanut agglutinin |
| PRRs | Pattern recognition receptors |
| Rad | Radiation absorbed dose |
| RLRs | RIG-I like receptors |
| RORγt | Retinoid-related orphan receptor γt |
| RPMI | RPMI-1640 media |
| RT | Room temperature |
| SAC | Staphylococcus aureus Cowan strain 1 |
| SAP | Signalling lymphocytic activation molecule (SLAM)-associated protein |
| SCID | Severe combined immunodeficiency |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SHM | Somatic hypermutation |
| sIgA | Secretary IgA |
| SLAM | Signalling lymphocytic activation molecule |
| SLP-76 | SH2-containing leukocyte protein 76 kd |
| SSC | Side scatter |
| STAT | Signal transducer and activator of transcription |
| TAP | Transporter associated protein |
| TCM | T central memory |
| TCR | T cell receptor |
| TD | T cell dependent |
| TEM | T effector memory |
| TEMRA | Terminally differentiated effector |
| Tfh | Follicular B helper T cell |
| TGF-β | Transforming growth factor-beta |
| Th | T helper |
| TI | T cell independent |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| TNFR | Tumour necrosis factor receptor |
| TNF-α | Tumour necrosis factor-alpha |
| Treg | T regulatory |
| ULBPs | UL16-binding proteins |
| V | Variable |
| ZAP-70 | Zeta-associated protein 70 kd |
| α-GalCer | α-galactosylceramide |
| αβ | Alphabeta |
| γδ | Gammadelta |

# Chapter 1:

## Introduction

## 1.1 General features of innate and adaptive immunity

The body's immune system has evolved to protect the host from a large array of infectious pathogens. The ability to recognise, respond and eliminate many non-self (foreign) substances while remaining tolerant to the body's own self-molecules is important. Failure in maintaining self-tolerance can manifest as autoimmunity (Abbas et al. 2007; Manicassamy and Pulendran 2011). The immune response relies on the complex interactions between organs, tissues, cells and molecules (Abbas et al. 2007; Chaplin 2010; Moser and Leo 2010). In vertebrates, the immune response is divided into two major branches called innate (or natural) and adaptive (or acquired) immunity. These divisions are not mutually exclusive, as an effective immune response requires the coordinated action of both systems (Abbas et al. 2007; Luster 2002; Turvey and Broide 2010).

### 1.1.1 Features of innate immunity

The innate immune system uses non-specific defence mechanisms that pre-exist in all individuals to protect the host from infection or disease. These innate defence mechanisms act within minutes of infection and provide the initial response of rapid sensing and elimination of pathogens. Innate immunity can be seen to comprise four types of defensive barriers: anatomical, physiological, phagocytic and inflammatory (Abbas et al. 2007; Bonilla and Oettgen 2010; Janeway et al. 2001).

Anatomical barriers include intact skin and mucous membranes. Physiological barriers include temperature, pH and soluble factors (lysozyme, interferons, complement proteins, collectins) (Abbas et al. 2007; Murphy et al. 2008; Turvey and Broide 2010). Phagocytic cells such as neutrophils, monocytes, macrophages and dendritic cells (DCs) rapidly accumulate at sites of infection and tissue injury and function to identify, ingest and destroy

pathogenic microbes by the process of phagocytosis (Abbas et al. 2007; Chaplin 2010). Other cellular components of innate immunity include natural killer (NK) cells, eosinophils, basophils, mast cells, natural killer T (NKT) cells, γδ T cells, CD8αα T cells, B1 B cells and marginal zone (MZ) B cells (Abbas et al. 2007; Borghesi and Milcarek 2007; Mogensen 2009; Turvey and Broide 2010). Many of these cell populations are also involved in adaptive immunity, thus link innate and adaptive immune responses (Abbas et al. 2007; Borghesi and Milcarek 2007).

Components of innate immunity recognise pathogens using germ line-encoded pattern recognition receptors (PRRs). PRRs are expressed on the surface or inside various cell types and are also present as circulating soluble proteins (Abbas et al. 2007). Examples of PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I like receptors (RLRs), and NOD-like receptors (NLRs) (Janeway and Medzhitov 2002; Manicassamy and Pulendran 2011). PRRs recognise and bind to a limited range of common pathogenic structures termed pathogen-associated molecular patterns (PAMPs), which are shared by a broad range of microbes (but not present on mammalian cells). PAMPs are unique structures essentially required for the survival of microbial organisms. Some examples of PAMPs include lipopolysaccharide (LPS) from Gram-negative cell wall of bacteria, lipotechoic acids from the Gram-positive cell wall of bacteria, bacterial and viral DNA, and viral RNA (Abbas et al. 2007; Bonilla and Oettgen 2010; Janeway 1989; Janeway and Medzhitov 2002; Mogensen 2009).

Within minutes of infection or tissue damage a local response called inflammation is induced. The inflammatory response attempts to rapidly eliminate toxic agents and repair injury, thus preventing spread of infection to other organs. Inflammation results in leakage of vascular fluid containing serum proteins with antibacterial activity, influx of phagocytic cells, and release of small signalling proteins called cytokines and chemokines, as well as other inflammatory mediators into the tissue site of infection (Abbas et al. 2007; Janeway et al. 2001; Murphy et al. 2008; Turvey and Broide 2010).

Cytokines are a large group of low molecular weight signalling proteins produced by a wide variety of immune and non-immune cells. Most cytokines act on the cell type that produced the cytokine (autocrine) or on nearby cells (paracrine). Some cytokines can exert activities at sites distant from their synthesis, thus act in an endocrine manner (Janeway et al. 2001). Cytokines evoke a series of biological activities after binding to specific cytokine receptors on responsive target cells. Cytokines such as interleukins (ILs) and interferons (IFNs) act primarily as chemical messengers that influence the development of a cellular and humoral immune inflammatory response, as well as survival, proliferation, differentiation and homeostasis of lymphoid cells (Habib et al. 2003; Monteleone et al. 2009; Søndergaard and Skak 2009).

Tissue resident macrophages, which have matured from blood monocytes migrating into tissue, are the first to encounter pathogens. Binding of pathogen by PRRs on macrophages induces secretion of a range of cytokines that mediate the inflammatory response. Among the cytokines released include a family of chemokines, these target all types of leukocytes and are produced by a wide variety of cell types (Janeway et al. 2001; Moser 2003). Chemokines play important roles in leukocyte traffic control in both inflammatory and homeostatic conditions (Moser et al. 2004). The major function of chemokines is to act as a chemoattractant for guiding leukocyte migration in and out of specific organs/tissue sites (Moser 2003). Leukocytes expressing the appropriate chemokine receptors are attracted towards the source of the chemokine and follow a gradient of increasing chemokine concentration. For example, during the inflammatory response macrophages secrete the inflammatory chemokine IL-8 (also known as CXCL8), this attracts neutrophils (expressing the receptors for IL-8) towards the source of IL-8, and as a result neutrophils are recruited from the blood to the tissue site of infection. Similarly, secretion of the chemokine macrophage chemoattractant protein-1 (MCP-1; CCL2) recruits more monocytes, which differentiate into tissue macrophages (Janeway et al. 2001; Moser 2003; Moser and Willimann 2004). In contrast, homeostatic chemokines regulate leukocyte migration during hematopoiesis (in the bone marrow and thymus) and during initiation of adaptive immune responses (in secondary lymphoid tissues) (Moser 2003; Moser et al. 2004).

Recruitment of leukocytes from the blood vessels to tissue (termed extravasation) not only depends on the recognition of a chemoattractant gradient but also on their adhesive interaction with the extracellular matrix (Moser 2003; Moser and Willimann 2004). During extravasation, circulating leukocytes adhere reversibly to the vessel wall; this allows them to roll along the endothelium. Chemokine receptor triggering arrests rolling and allows leukocytes to be firmly attached to vessel walls through the actions of integrins. This enables leukocytes to transmigrate through the endothelial barrier and enter tissue sites (Bromley et al. 2008; Förster et al. 2008; Moser and Willimann 2004). During the inflammatory response this allows circulating phagocytes to encounter an infecting microorganism (Janeway et al. 2001).

When a pathogen resists, escapes or overwhelms the defence mechanisms of the innate immune system the more powerful adaptive immune system is triggered. The type of adaptive immune response triggered is influenced by the innate response and depends on the type of microbe (i.e. bacteria or virus) involved (Abbas et al. 2007; Luster 2002). Several days are required for an adaptive immune response to develop, during this time the innate response functions to contain the infection (Janeway et al. 2001).

### 1.1.2 Features of adaptive immunity

The adaptive immune system is unique to jawed vertebrates and provides the second line of defence, which can take several days to develop and mount. The initial-primary response is slow but improves (more rapid and powerful) upon repeated exposures to the same pathogen. Two major hall-marks of adaptive immunity are the ability to mount antigen specific responses and the formation of immunological memory during a primary response (Abbas et al. 2007; Murphy et al. 2008; van Zelm et al. 2007).

Generation of an effective adaptive immune response relies on antigen presenting cells (APCs), lymphocytes, effector molecules (cytokines and chemokines) and the anatomic organisation of cells, lymphoid tissues and organs (Abbas et al. 2007; Bonilla and Oettgen 2010; Moser and Leo 2010). The main mediators of the adaptive immune response are T lymphocytes (T cells) and B lymphocytes (B cells). Both cell types arise from common lymphoid progenitor cells within the bone marrow. Precursor T cells migrate to and mature in the thymus whilst precursor B cells continue to mature in the bone marrow (Abbas et al. 2007; Chaplin 2010) (**Figure 1.1**).

T and B cell maturation involves generation of highly diverse T cell receptors (TCRs) and B cell receptors (BCRs), that are capable of recognising an infinite number of microbial and non-microbial foreign substances called antigens. During generation of antigen receptors lymphocytes undergo a process of positive and negative selection, ensuring that only self tolerant T cells and B cells emerge. The vast majority of lymphocytes that recognise self antigens is eliminated by a process called clonal deletion (Abbas et al. 2007; Manicassamy and Pulendran 2011; Murphy et al. 2008).

Diversity of T and B cell receptors is generated by random genetic rearrangements and mutation of a germline set of sequences that encode variable (V), diversity (D) and joining (J) regions. As a consequence each lymphocyte expresses a unique antigen receptor. This process known as V(D)J recombination or gene rearrangement creates a diverse repertoire (in the order of $>10^{14}$) of antigen specific receptors. Newly generated T and B lymphocytes expressing antigen specific receptors exit the primary lymphoid organs and enter the periphery as mature but antigen-naive cells (Abbas et al. 2007; Alam and Gorska 2003; Bonilla and Oettgen 2010; Moser and Leo 2010).

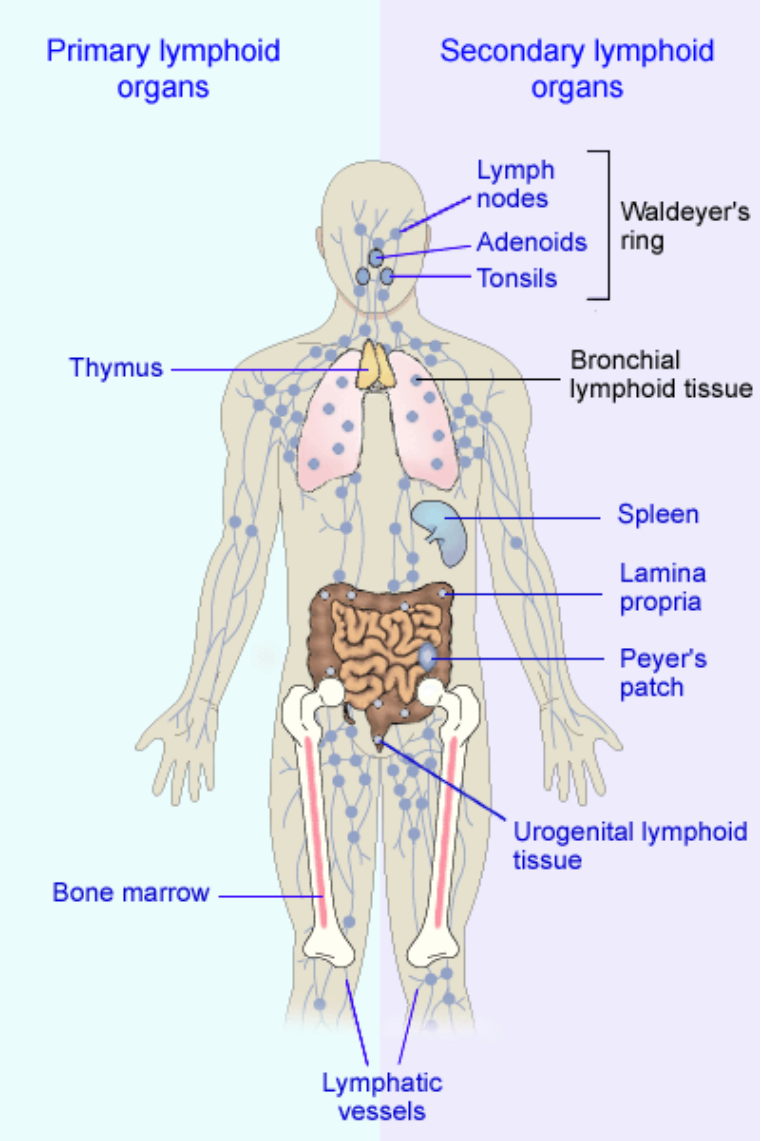


**Figure 1.1. Primary and secondary lymphoid organs.**
The primary lymphoid organs (thymus and the bone marrow) are sites where lymphocytes are generated and differentiate into mature naïve lymphocytes. Secondary lymphoid organs are the tissues in which immune responses are initiated, i.e., tonsils, adenoids and lymph nodes scattered throughout the body; bronchial area (lungs), spleen, gastrointestinal area (gut) urogenital tract. Taken from (St Georges University of London).

The percentage of antigen-specific lymphocytes in peripheral blood is very low (<0.001%) (Alam and Gorska 2003). To increase the chances of encountering specific antigen, resting naïve T and B lymphocytes continually recirculate between blood and secondary lymphoid organs (**Figure 1.1**). Whilst trafficking through the lymph nodes and spleen, lymphocytes get exposed to antigens collected by the lymph or blood, respectively (Abbas et al. 2007; Bonilla and Oettgen 2010). Recognition of antigen by a naïve lymphocyte specific for the antigen leads to cell activation and proliferation resulting in clonal expansion of lymphocytes expressing receptors of the same specificity, in a process called clonal selection. This process allows many clones of lymphocytes with different antigen specificities to be generated (Abbas et al. 2007; Sadofsky 2001).

Stimulation by specific antigen induces naïve lymphocytes to differentiate into effector cells, followed by the generation of a pool of memory cells. Antigen specific effector T cells undergo clonal expansion to eliminate pathogen. After clearance of infection, the majority of effector cells are removed by apoptosis restoring the immune system to its resting basal state. However, a small proportion of effector cells are retained as long-lived memory cells, which remain present for many years in a functionally quiescent state in the absence of antigen. In contrast to naïve cells, memory cells develop a higher affinity for antigen and require a lower threshold for activation. Thus, upon re-encounter with the same antigen during secondary immune responses, memory cells are able to respond more rapidly and effectively in eliminating the pathogen (Abbas et al. 2007; Appay et al. 2008; Boesteanu and Katsikis 2009; Moser and Leo 2010; Sallusto and Lanzavecchia 2009; Sallusto et al. 1999). T and B cell antigen receptors recognise antigen in very different ways and each cell type plays unique roles in adaptive immunity. T lymphocytes mediate the cellular response whilst B lymphocytes mediate the humoral response of adaptive immunity (Alam and Gorska 2003; Bonilla and Oettgen 2010).

## 1.2 T cells and cellular immunity

### 1.2.1 Major T cell subsets

Distinct populations of leukocyte subsets can be identified based on surface expression of membrane proteins. Cell membrane proteins (also referred to as markers, molecules or antigens) are detected by their binding of specific monoclonal antibodies and are assigned cluster of differentiation (CD) numbers (Abbas et al. 2007; Chaplin 2010). CD3 is a surface molecule non-covalently linked to the T cell receptor (TCR) and is commonly used to identify T cells (Bonilla and Oettgen 2010; Wang et al. 2009; Yang et al. 2005).

Two types of functionally distinct T lymphocytes exist based on the type of TCR expressed. T cells either bear heterodimeric, transmembrane glycoprotein TCR comprised of disulfide-bonded α and β polypeptide chains (αβ T cells) or of γ and δ polypeptide chains (γδ T cells) (Ciofani and Zúñiga-Pflücker 2010; O'Brien et al. 2007; Yang et al. 2005). In human adults the peripheral T lymphocyte population is composed of approximately 95% of αβ T cells and 0.5 to 5% of γδ T cells (Chaplin 2010; Hayday 2000). During maturation in the thymus αβ T cells differentiate into two major subpopulations based on selective surface expression of CD4 or CD8 membrane proteins, known as $CD4^+$ T cells and $CD8^+$ T cells (Chaplin 2010).

Encounter with specific antigen stimulates naïve $CD4^+$ and $CD8^+$ αβ T cells to clonally expand and differentiate into cells with effector functions. Effector $CD4^+$ T cells are known as T helper (Th) lymphocytes and effector $CD8^+$ T cells as cytotoxic T lymphocytes (CTLs). Each subset plays distinct functional roles in host defence (Chaplin 2010; Rock and Shen 2005). $CD8^+$ CTLs primarily kill tumour/transformed or pathogen infected cells by inducing cell apoptosis through secretion of effector proteins such as perforin, granzymes and granulysin (Chaplin 2010; Harty et al. 2000). Conversely, $CD4^+$ Th cells

orchestrate different arms of the immune response, including helping B cells make antibody as well as recruiting a large array of cell types to sites of infection (Bedoui and Gebhardt 2011; Chaplin 2010; Davis et al. 2007; Zhu and Paul 2008).

## 1.2.2 MHC restriction and antigen processing

$CD4^+$ and $CD8^+$ αβ T cells recognise foreign antigen via their TCR in the form of peptide fragments only if presented in association with specialised molecules called major histocompatibility complex (MHC) (Chaplin 2010). MHC molecules also known as human leukocyte antigens (HLA) are composed of an extracellular peptide binding groove attached to immunoglobulin like domains and transmembrane and cytoplasmic domains (Abbas et al. 2007). There are 2 major types of MHC molecules which differ in structure, expression, and function. MHC class I molecules (HLA-A, HLA-B, and HLA-C) expressed by all nucleated cells are restricted to presenting peptides only to $CD8^+$ T cells. MHC class II molecules (HLA-DR, HLA-DQ, and HLA-DP) expressed predominantly by APCs (DCs, macrophages and B cells) can only present peptides to $CD4^+$ T cells (Alam and Gorska 2003; Bonilla and Oettgen 2010; Chaplin 2010). CD4 and CD8 co-receptors on T cells interact with non-polymorphic regions of class II and class I MHC molecules, respectively and are essential for recognition of the MHC antigen complex (Abbas et al. 2007; Medema and Borst 1999). The MHC groove can only bind short linear peptides, thus antigen must be processed before it can be bound to MHC molecules (Alam and Gorska 2003; Chang et al. 2006; Moser and Leo 2010). Each class of MHC samples different pools of antigen, which are processed and presented using distinct pathways.

### 1.2.2.1 MHC class I pathway

MHC class I molecules bind and present endogenously synthesised viral and mutated proteins as well as normal intracellular proteins. CTLs are activated in response to viral and mutated proteins derived from the cytosol of virus infected and tumour cells, respectively,

but not in response to normal cellular proteins (Abbas et al. 2007; Alam and Gorska 2003). MHC class I associated peptides are produced by the proteolytic degradation of cytosolic proteins by an enzyme complex found in the cytoplasm called the proteasome. The transporter associated protein (TAP) transports peptides from the cytosol to the endoplasmic reticulum (ER), inside the ER peptides are bound to newly synthesised class I molecules. The peptide-MHC class I complex is transported to the cell surface for presentation to cytotoxic $CD8^+$ T cells (Abbas et al. 2007; Rock and Shen 2005).

#### 1.2.2.2 MHC class II pathway

In contrast, MHC class II bind and present antigens from the extracellular environment which have been internalised by APCs using phagocytosis/and or endocytosis. APCs found in tissues throughout the body actively sample and internalise extracellular components such as microbial pathogens, dead or dying cells and immune complexes (Rossi and Young 2005). Internalised protein antigens get localised inside intracellular membrane bound vesicles called endosomes and lysosomes, which contain enzymes that degrade proteins into small peptides. MHC class II molecules associated with a protein called the invariant chain are transported out from the ER in exocytic vesicles. Endosomes containing processed peptides fuse with vesicles transporting MHC class II molecules. The invariant chain occupying the MHC peptide-binding groove is exchanged for antigenic peptides (Abbas et al. 2007). The peptide-MHC class II complex is transported to the cell surface for presentation to helper $CD4^+$ T cells. Mature DCs express high levels of MHC and co-stimulatory molecules thus are the most potent APCs for stimulation of naïve T cells. Macrophages and B cells present antigen to differentiated effector and helper T cells (Abbas et al. 2007; Bedoui and Gebhardt 2011; Lewinsohn et al. 2011).

#### 1.2.2.3 Cross-presentation

The MHC class I and II antigen-processing and presenting pathways are not entirely exclusive. Studies have shown ingested extracellular proteins to escape from endosomal compartments into the cystosol and enter the class I pathway of antigen presentation. This

feature enables APCs to present extracellularly obtained microbial proteins or tumour antigens to CTLs and elicit a response towards infected tissue and tumour cells, respectively. This phenomenon is known as 'cross-presentation' and is generally restricted to specialised APCs such as DCs (Segura et al. 2009), and more recently has been demonstrated in γδ T cells (Brandes et al. 2009; Brandes et al. 2005; Himoudi et al. 2012; Landmeier et al. 2009; Meuter et al. 2010; Moser and Eberl 2011; Wu et al. 2009). Equally, endogenous proteins from the cytosol can be presented in association with MHC class II proteins, however, such processes are yet ill-defined (Chaplin 2010; Moser and Leo 2010).

#### 1.2.2.4 CD1 antigen presenting pathway

A third type of antigen presenting pathway involves the presentation of both foreign and self-derived lipid antigens by a MHC-like molecule called cluster of differentiation 1 (CD1). CD1 glycoproteins are a family of antigen presenting molecules which are expressed on the surface of a variety of APCs. Similar to extracellular proteins, lipids are processed in endosomes and lysosomal compartments and presented to the TCR of CD1-restricted T cells (Alam and Gorska 2003; Brigl and Brenner 2004; Gumperz 2006; Lawton and Kronenberg 2004; Sugita et al. 2004). For example, NKT cells recognise CD1d-presented natural self or microbial glycolipids as well as a range of synthetic glycosylceramides. The synthetic antigen α-galactosylceramide (α-GalCer) is known to selectively activate NKT cells in the context of CD1d molecules (Borg et al. 2007; Cohen et al. 2009; Godfrey and Rossjohn 2011).

### 1.2.3 T cell co-stimulation and activation

The activation of lymphocytes is a carefully controlled event, which allows inactive cells to progress from $G_0$ into $G_1$ phase of the cell cycle. T cell activation and survival requires sustained signalling from both the antigen specific TCR and from co-stimulatory molecules (Daoussis et al. 2004; Shi et al. 2009).

The αβ chains of the TCR are non-covalently linked with transmembrane CD3 signalling chains consisting of γ, δ, ε and a ζ-ζ homodimer. Interaction of TCR/CD3 complex and CD4/CD8 molecules with MHC-bound antigenic peptide provides only a partial signal for T cell activation. Complete and productive activation requires a second signal, which is referred to as co-stimulation and is provided by trans-membrane protein molecules called co-stimulators (Bonilla and Oettgen 2010; Chaplin 2010). Protein expression of surface co-stimulatory molecules is tightly regulated by negative (co-inhibitory) signals to avoid unnecessary lymphocyte activation and autoimmunity (Daoussis et al. 2004; Fife and Pauken 2011).

A growing list of cell-associated receptor/ligand pair co-stimulatory molecules exist, which provide positive co-stimulatory signals (Leitner et al. 2010). The majority of co-stimulatory molecules belong to one of four superfamilies based on their structural characteristics. These include the B7 and tumour necrosis factor (TNF) superfamily members, which interact with the CD28 and TNF receptor (TNFR) superfamily members, respectively (Frauwirth and Thompson 2002; Leitner et al. 2010; Ribot et al. 2011; Sharpe 2009).

### 1.2.3.1 Co-stimulation via CD28

Full activation of naive T cells is dependent on co-stimulation provided via the T cell surface receptor CD28, which is constitutively expressed on resting naïve and newly activated αβ T cells (Boesteanu and Katsikis 2009; Chaplin 2010; Rudd et al. 2009; Sansom et al. 2003). B7.1 (CD80) and B7.2 (CD86) expressed on the surface of APCs are the ligands for CD28; both are up-regulated following APC activation (Abbas et al. 2007; Bhatia et al. 2006; Moser and Leo 2010; Sansom et al. 2003). During initial antigen priming of naive T cells, engagement of CD28 receptor on T cells by CD80 or CD86 on DCs delivers signals necessary for optimal T cell activation (Bhatia et al. 2006; Sansom et al. 2003). Upon activation, signals delivered by the TCR and co-stimulatory molecules stimulate the secretion of the T cell growth factor IL-2 and up-regulate surface expression of

the IL-2 receptor (IL-2R). Binding of IL-2 to its receptor drives the T cell to enter the cell cycle inducing cell survival and proliferation (Létourneau et al. 2009; Murphy et al. 2008). Conversely, interaction of peptide-MHC with the TCR in the absence of co-stimulation induces a state of T cell non-responsiveness called anergy or cell death. However, memory and effector T cells are less dependent on CD28:CD80/86 co-stimulation (Bjørgo and Taskén 2010; Chaplin 2010; Frauwirth and Thompson 2002; Schwartz 2003).

#### 1.2.3.2 Delivery of co-inhibitory signals via CTLA-4

The activating effects of CD28 are counterbalanced by co-inhibitory signals delivered by structurally similar molecule called cytotoxic T lymphocyte antigen-4 (CTLA-4 or CD152). Surface expression of CTLA-4 is only induced on T cells in response to the co-stimulatory signals delivered by CD28 (Bhatia et al. 2006; Leitner et al. 2010). CTLA-4 expressed on activated T cells binds to CD80/86 molecules with much higher affinity in comparison to CD28 (Sansom et al. 2003). Engagement of CTLA-4 with CD80/86 inhibits T cell activation, cell cycle progression and IL-2 production, thus counteracting the effects of CD28 co-stimulation (Frauwirth and Thompson 2002; Leitner et al. 2010; Sharpe 2009).

### 1.2.4 T cell receptor signalling and activation

T cell activation requires sustained TCR signalling which can only be achieved through hours of stable contact between the APC and T cell (Dustin et al. 2010). Antigen recognition by the TCR supported by co-stimulatory molecules, leads to rapid clustering of TCR and associated molecules (i.e. CD3 chains, CD4 or CD8 and CD28) at the contact zone between the T cell and APC (Bonilla and Oettgen 2010). This results in the formation of a junction between the two cells called the immunologic synapse, which is stabilised by adhesion molecules such as lymphocyte function associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) (Krummel and Cahalan 2010). During the formation of the synapse the TCR and co-stimulatory molecules bind with their counter

ligands on the APC, this brings the cytosolic domains of these molecules into proximity (Alam and Gorska 2003; Bonilla and Oettgen 2010).

TCR signalling is initiated by enzymes known as tyrosine kinases (i.e. Lck, Fyn and ZAP-70), which attach phosphate groups to certain tyrosine residues on proteins converting them into an active state. The αβ chains of the TCR are devoid of enzymatic activity. Signals from the TCR are transmitted via associated CD3 (γ, δ, ε) and ζ signalling chains which contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains (Abbas et al. 2007; Chaplin 2010; Rodríguez-Fernández et al. 2010). Upon clustering of TCR and co-receptors, Lck (or Fyn) associated with the cytoplasmic domain of CD4 or CD8 molecule becomes activated and phosphorylates the tyrosines in the ITAMs of the CD3 and ζ chains. This recruits ζ-associated protein 70 kd (ZAP-70), which docks on to the phosphorylated ITAM and becomes activated upon Lck-mediated phosphorylation (Abbas et al. 2007; Chaplin 2010). Activated ZAP-70 in turn phosphorylates adapter proteins such as linker of activated T cells (LAT) and SH2-containing leukocyte protein 76 kd (SLP-76) (Abbas et al. 2007; Bonilla and Oettgen 2010; Chaplin 2010). As a result distinct downstream signalling pathways are initiated, such as: Ras-MAP (mitogen associated protein), protein kinase C (PKC) and Calcium-Calcineurin pathways (Abbas et al. 2007). Activation of these pathways induces activation of NFκB, NFAT and AP-1 transcription factors, which control the expression of genes that encode IL-2, cyclins and Bcl-2 family members (Abbas et al. 2007; Ribot et al. 2011).

Simultaneous to TCR signalling, engagement of CD28 by CD80 or CD86 molecules triggers a cascade of signalling events downstream of CD28. These signalling events enhance TCR signalling leading to optimal activation of transcription factors, allowing complete T cell activation to proceed (Bjørgo and Taskén 2010; Boomer and Green 2010). Synergistic signalling via TCR and CD28 promotes T cell survival (by inducing expression of anti-apoptotic proteins), production of growth factors/interleukins (most significantly IL-2), cell-cycle progression, proliferation and differentiation (Bonilla and Oettgen 2010; Boomer and Green 2010; Chaplin 2010; Crispín and Tsokos 2009).

## 1.2.5 $CD4^+$ T helper subsets

$CD4^+$ T cells exert most of their effector functions through secreted cytokines. Naïve $CD4^+$ T cells abundantly produce the cytokine IL-2 and upon encounter with antigen have the capacity of developing into one of many functionally distinct effector subsets. Some of the major types of $CD4^+$ T cell subsets include: Th1, Th2, Th17, T regulatory (Treg), follicular B helper T (Tfh) cells and the more recently discovered, Th9 and Th22. $CD4^+$ T cell subsets are categorised based on the types of cytokines produced, transcription factors and cell surface molecules expressed. Differentiation into a particular subset is determined by the pattern of cytokines present in the microenvironment at the time of T cell activation (Chaplin 2010; Deenick et al. 2011; Li et al. 2011; Sallusto and Lanzavecchia 2009; Zhu et al. 2010) (**Figure 1.2**).

IL-12 and interferon-γ (IFN-γ) up-regulate expression of the intracellular molecules signal transducer and activator of transcription (STAT) 1 and STAT4, which in turn stimulate expression of the transcription factor T-bet (also denoted as T-box 21; TBX21), this selectively polarises cells toward the Th1 cell differentiation program. Th1 cells produce the inflammatory cytokines IFN-γ and TNF-α, as well as lymphotoxin and IL-2 (Chaplin 2010; Li et al. 2011; Sallusto and Lanzavecchia 2009; Zhu et al. 2010).

IL-4 up-regulates expression of the transcription factors STAT 6 which in turn stimulates expression of the transcription factor GATA binding protein 3 (GATA-3), this promotes Th2 cell differentiation. Th2 cells produce a wide range of cytokines including IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and granulocyte macrophage-colony stimulating factor (GM-CSF). Th1 secreted cytokines inhibit Th2 mediated reactions and Th2 secreted cytokines inhibit Th1 mediated reactions. IL-2 and IL-4 act as autocrine growth factors on Th1 and Th2 cells, respectively (Abbas et al. 2007; Bonilla and Oettgen 2010; Chaplin 2010; Sallusto et al. 2004; Sallusto and Lanzavecchia 2009; Zhu and Paul 2008; Zhu et al. 2010).

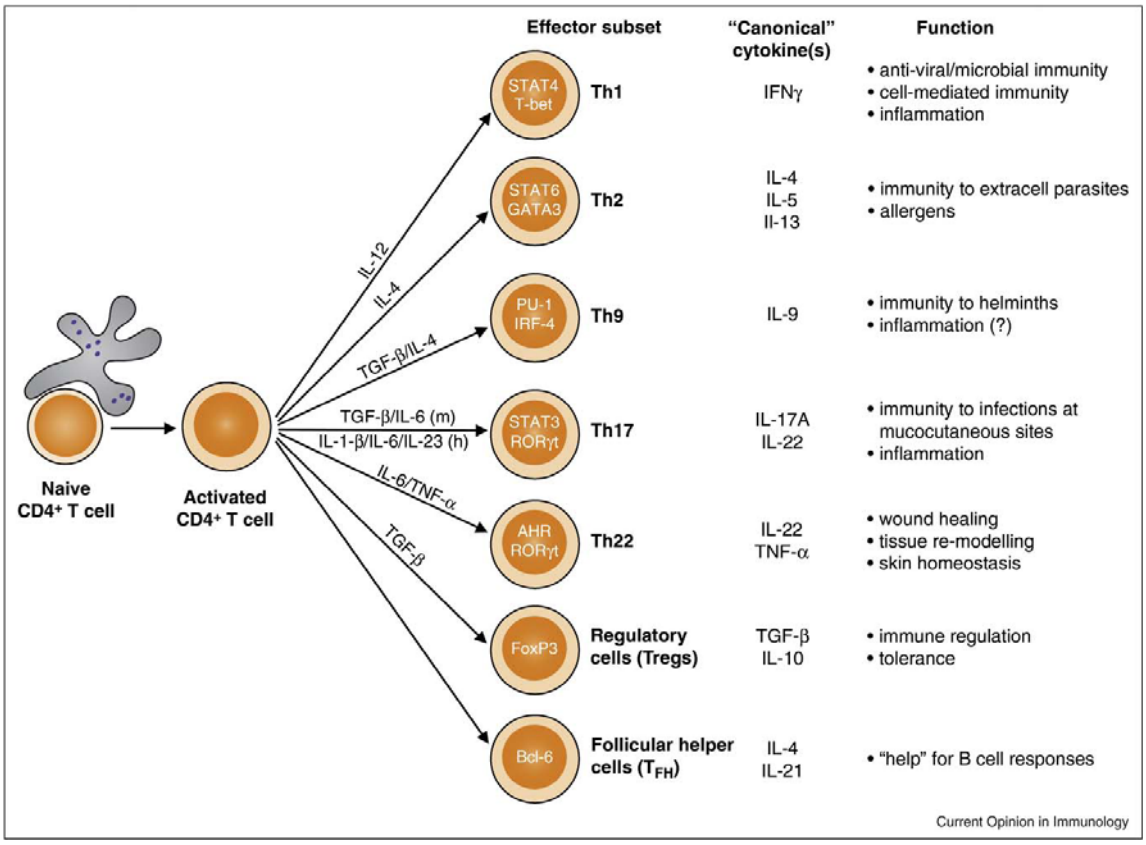


**Figure 1.2. CD4$^+$ T helper subsets.**
Differentiation of naïve CD4$^+$ T cells into effector subsets is regulated by cytokines and transcription factors. Each subset can be uniquely identified based on the set of 'canonical' cytokines produced, which allows cells to perform specific functions; (h): human; (m): mouse. Taken from (Deenick et al. 2011).

The different cytokines secreted reflect the different biological functions of a subset. The main function of Th1 cells is to enhance phagocyte-mediated defence against intracellular microbes. Th1 secreted cytokines act on macrophages, neutrophils, B cells and NK cells. The main function of Th2 cells is to promote IgE and eosinophil/mast cell mediated immune reactions in response to extracellular parasites and allergens. IL-4 secreted by Th2 cells can act on B cells to stimulate production of IgG and IgE antibodies (Abbas et al. 2007; King et al. 2008; Sallusto and Lanzavecchia 2009). IL-4 producing Th2 cells have long been viewed as the main subset that supports B cell antibody production. Recently, a bona fide subset of $CD4^+$ T cells expressing the CXC-chemokine receptor 5 (CXCR5) termed Follicular B helper T (Tfh) cells have been established as a subset distinct from Th2 or Th1 cells (Moser et al. 2002; Schaerli et al. 2001; Schaerli et al. 2000). Tfh cells are now known as true helper cells for B cells (Crotty 2011; Haynes 2008; King 2009; King et al. 2008). Tfh cells are discussed in further detail later in this chapter.

A third effector T helper cell subset called Th17 cells, are characterised by the production of IL-17A, IL-17F and IL-22. Th17 cells also secrete IL-21 which feeds back on developing Th17 cells. These cells do not express GATA3 or T-bet but express high levels of the transcription factors retinoid-related orphan receptor γt (RORγt), which is induced by transforming growth factor-β (TGF-β) together with IL-6, IL-1, or other pro-inflammatory cytokines. Th17 cells play an important role in the clearance of pathogens such as extracellular bacteria and fungi and induce tissue inflammation in various autoimmune diseases (Annunziato and Romagnani 2009; Bonilla and Oettgen 2010; Chaplin 2010; Korn et al. 2009; Sallusto et al. 2004; Sallusto and Lanzavecchia 2009; Zhu et al. 2010).

More recently identified effector T helper cell subsets include Th9 and Th22 cells. Th9 cells selectively secrete high amounts of IL-9 and express the transcription factor PU.1 which is required for Th9 cell development. Differentiation of activated $CD4^+$ T cells into Th9 cells requires balanced signals from both TGF-β and IL-4. The precise pathophysiological role of Th9 cells remains unclear, evidence suggests Th9 cells to contribute to inflammation in several autoimmune diseases (Annunziato and Romagnani 2009; Chang et al. 2010; Deenick et al. 2011; Jabeen and Kaplan 2012; Tan and Gery

2012). Th22 cells express high levels of aryl hydrocarbon receptor (AHR) and IL-22. Differentiation of Th22 cells is dependent upon the presence of both IL-6 and TNF-α. Th22 cells are thought to be involved in wound healing, tissue re-modelling and skin homeostasis (Annunziato and Romagnani 2009; Deenick et al. 2011; Witte et al. 2010).

Briefly, Tregs, defined as $CD4^+$ T cells that constitutively express IL-2Rα (also known as CD25) and the transcription factor forkhead box protein P3 (FOXP3), play an indispensable role in mediating and maintaining immune tolerance (Corthay 2009; Vignali et al. 2008).

### 1.2.6 T cell memory subsets

Following clearance of infection the majority of effector T cells are removed by apoptosis, while a small fraction is retained as antigen primed circulating memory cells. Memory T cells are further divided into central memory ($T_{CM}$) and effector memory ($T_{EM}$) subsets. This division is defined based on the expression of homing receptors and effector functions. Similar to naïve T cells, $T_{CM}$ cells co-express lymph node homing receptor CC-chemokine receptor 7 (CCR7) and adhesion receptor L-selectin (CD62L), but lack immediate effector functions (Moser and Loetscher 2001; Rosen 2004; Sallusto et al. 1999). In contrast, $T_{EM}$ cells do not express lymph node homing receptors but express other homing receptors which allow migration to peripheral tissues (i.e. skin and the gut). $T_{EM}$ cells can rapidly display effector functions such as cytokine secretion and cytotoxicity (Boyman et al. 2009; Moser and Loetscher 2001; Sallusto et al. 2004; Sallusto and Lanzavecchia 2009).

Naive, $T_{CM}$, $T_{EM}$ and terminally-differentiated effector T cells ($T_{EMRA}$) subsets are commonly distinguished based on the differential expression of cell surface molecules CD45RA (or CD45RO), CCR7, CD27, CD62L and CD28 (Appay et al. 2008; Moser and Loetscher 2001; Sallusto et al. 2004; Sallusto and Lanzavecchia 2009). However, phenotypic characteristics of subsets can change upon cell activation. For example, upon

re-stimulation, differentiating $T_{CM}$ cells lose CCR7, while $T_{EM}$ transiently up-regulate CCR7 (Sallusto et al. 2004).

## 1.3 B cells and humoral immunity

### 1.3.1 Structure and function of antibodies

Immunoglobulins (Igs) are a primary product of B lymphocytes and plasma cells (Bonilla and Oettgen 2010; Vale and Schroeder 2010). Immunoglobulins exist either as surface BCR anchored in the membrane of B cells, or as circulating effector molecules (antibodies) secreted by plasma cells within lymph nodes, spleen and bone marrow. Secreted antibodies circulate in blood, mucosal secretions and in the interstitial fluid of tissues (Abbas et al. 2007; Schroeder and Cavacini 2010).

The basic structure of an antibody molecule is composed of four polypeptide chains (two identical heavy chains and two identical smaller light chains) linked by disulfide bonds. Each of the four chains contains one $NH_2$-terminal variable and one or more COOH-terminal constant regions (domains). There are two types of light chain, lambda (λ) and kappa (κ); a B cell will produce antibodies either expressing Igλ or Igκ, but not both. The variable domains of both the heavy and light chains contain 3 hypervariable regions also known as complementarity determining regions (CDRs) that are brought together to form an antigen-binding site (containing 6 CDR regions), which contribute to the diversity of the antibody molecules. Thus, two identical antigen-binding sites are formed for each antibody molecule (Abbas et al. 2007; Chaplin 2010).

Elimination of bound antigen requires the interaction of antibody with effector cells and proteins. Two heavy chains containing two to three constant domains pair to form the Fc fragment (Mix et al. 2006). The Fc fragment binds to complement proteins and Fc receptors (FcR) on effector cells, such as mononuclear phagocytes, NK cells and mast cells (Abbas et al. 2007; Radaev and Sun 2002; Schroeder and Cavacini 2010). This triggers several effector mechanisms such as: neutralisation of microbes and toxins, opsonisation (coating) of pathogens, activation of complement proteins, antibody-dependent cell-mediated cytotoxicity and immediate hypersensitivity (Abbas et al. 2007; Mix et al. 2006; Schroeder and Cavacini 2010) (**Figure 1.3**).

Antibody molecules can be divided into distinct classes (or isotypes) and subclasses, based on the amino acid sequences in the constant region of the heavy chains. The five major classes are IgM, IgD, IgG, IgA, and IgE which are denoted by the corresponding lower-case Greek letter (μ, δ, γ, α, and ε, respectively). In humans IgG is further divided into four subclasses (IgG1, IgG2, IgG3 and IgG4) and IgA into two subclasses (IgA1 and IgA2). In mice the IgG subclasses are known as IgG1, IgG2a, IgG2b and IgG3. The different antibody isotypes and subtypes mediate different immune functions important for control of a diverse range of pathogens (Crotty 2011; Mix et al. 2006; Schroeder and Cavacini 2010).

#### 1.3.1.1 IgM

IgM comprises approximately 10% of total serum Ig. IgM is the first Ig to be expressed on the surface of developing B cells. Low affinity IgM (or natural) antibodies are produced at the early stage of an antibody response. Circulating molecules of IgM form pentamers (or hexamers) expressing many antigen-binding sites. This makes IgM highly efficient at opsonising multivalent antigens such as bacterial capsular polysaccharides for destruction and complement binding (Abbas et al. 2007; Schroeder and Cavacini 2010).

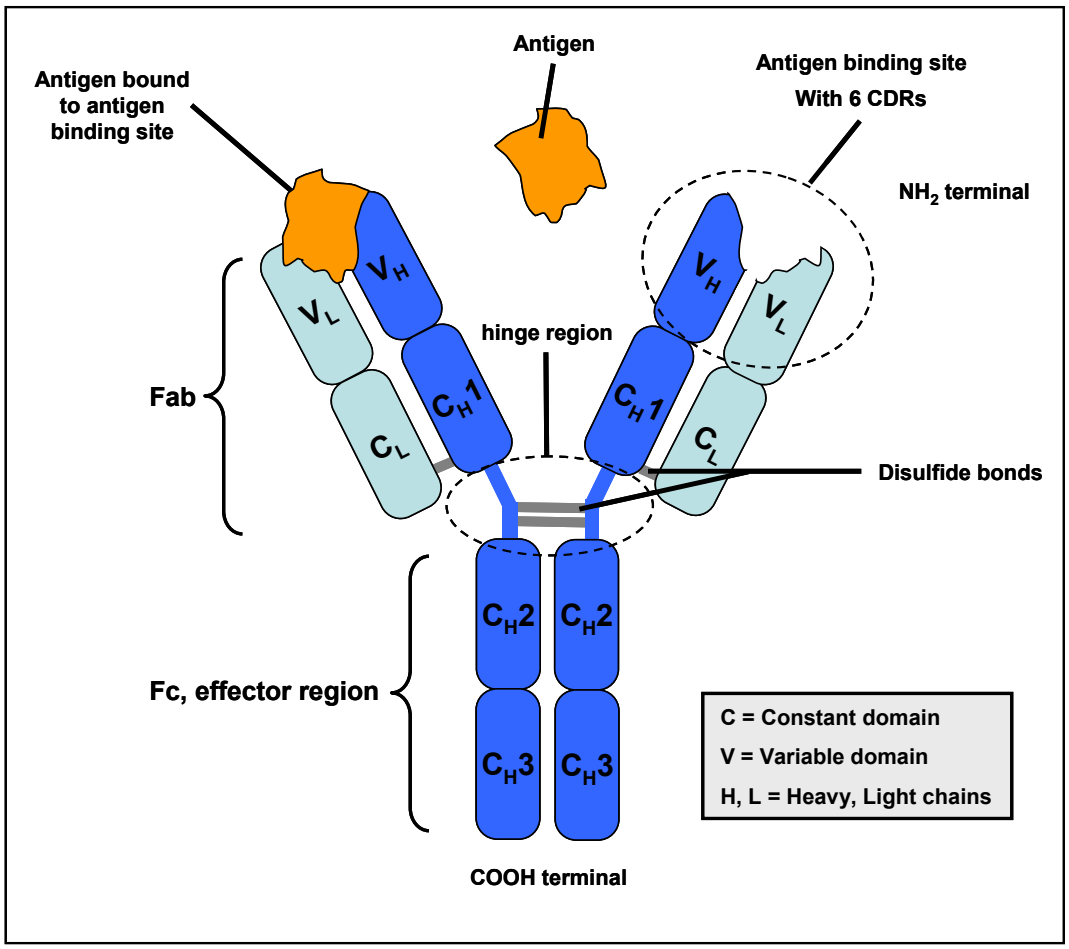


**Figure 1.3. Schematic structure of a monomeric antibody molecule.**

### 1.3.1.2 IgG

With the longest serum half-life out of the five Ig isotypes, IgG makes up approximately 75% of total serum Ig (Schroeder and Cavacini 2010). High-affinity IgG antibodies are abundantly produced during secondary immune responses to both bacterial and viral infections. IgG antibodies are predominantly found in the blood and extracellular fluid. IgG can diffuse into tissue, enter mucosal sites and be transported across the human placenta. Some of the major functions of IgG include blocking entry of microbes into host cells, opsonisation of pathogens for engulfment by phagocytes, activation of the complement system and release of granule enzymes from neutrophils that can directly neutralise viruses. IgG subclasses are numbered in order (IgG1 > IgG2 > IgG3 > IgG4) of their serum levels in healthy individuals and perform different biologic functions. IgG1 and IgG3 antibodies bind to protein antigens, whereas IgG2 and IgG4 antibodies bind to polysaccharide antigens (Abbas et al. 2007; Schroeder and Cavacini 2010; Spiegelberg 1989).

### 1.3.1.3 IgA

IgA antibodies are major players in mucosal immunity. In contrast to the low levels found in serum (15% of total Ig), IgA makes up two thirds of Igs found in extravascular secretions. As a monomer in serum, IgA is transported across the epithelium and released as a dimer (or trimer) at mucosal surfaces and in secretions (tears, saliva, breast milk, bronchial and digestive tract mucus). Secretary IgA (sIgA) protects mucosal surface from commensal bacteria, toxins, viruses and bacteria by direct neutralisation or by inhibiting adherence to epithelial surface (Brandtzaeg 2007; McGhee et al. 2007; Schroeder and Cavacini 2010). IgA is mainly produced by activated B cells in mucosa-associated lymphoid tissue (MALT) such as the gut-associated lymphoid tissue (GALT) (i.e. Peyer's patches, appendix and numerous isolated lymphoid follicles), lymphoepithelial structures such as palatine tonsils, adenoids and bronchus associated lymphoid tissue (BALT) (Brandtzaeg 2007).

#### 1.3.1.4 IgE

Despite its very low concentration in serum (0.01% of total) and a short half-life of approximately 2 days, monomeric IgE functions as a very potent antibody. IgE protects against parasitic infections and is involved in hypersensitivity and allergic reactions. Serum concentration of IgE is found at low concentrations because the majority become rapidly bound to cell surface Fc receptors. Antigen crosslinking of IgE bound to high affinity Fc receptors (FcεRI) on mast cells and basophils triggers the release of inflammatory mediators, which can expel infectious agents. IgE can also binds to low-affinity receptor (FceRII; CD23) expressed on B cells T cells, Langerhans cells, macrophages, monocytes, eosinophils, and platelets (Rindsjö and Scheynius 2010; Schroeder and Cavacini 2010; Stone et al. 2010).

#### 1.3.1.5 IgD

Membrane IgD is expressed on mature B lymphocytes and is thought to regulate B cell fate and early development. With a short half-life, circulatory monomeric IgD constitutes only 0.05% of total serum Ig, the function of which remains unknown (Schroeder and Cavacini 2010).

### 1.3.2 Generation of B cells and B cell receptor

Generation of B cells is a highly regulated process, which begins in the foetal liver before birth and proceeds throughout life in the bone marrow (Hardy and Hayakawa 2001). In these organs, B cell development progresses in a sequential antigen-independent manner (Moura et al. 2008). Hematopoietic stem cells differentiate into common lymphocyte progenitors (CLP), which differentiate further into pro-B cells, pre-B cells and then into immature B cells. In the bone marrow, generation of B cells is supported by stromal cells and cytokines such as IL-7 (Carter 2006; Ollila and Vihinen 2005; Zhang et al. 2004).

B cell surface bound antibodies are used as receptors for recognition of specific antigen, cell signalling and cell activation (Schroeder and Cavacini 2010). A functional surface Ig receptor is assembled during early B cell development, by random rearrangement of V(D)J gene segments encoding the Ig heavy and Ig light chains (Schroeder and Cavacini 2010; Vale and Schroeder 2010; van Zelm et al. 2007).

At the pro-pre-B cell stage a precursor BCR is formed after Ig heavy gene rearrangement resulting in the synthesis of Igμ heavy chains which associate with surrogate light chains. This precursor BCR is expressed on the cell surface of pre-B cells. Similar to the TCR, the very short cytoplasmic domain of the BCR can not transduce signals into the cell. Thus, the BCR is expressed in association with two transmembrane signalling proteins designated Igα (CD79a) and Igβ (CD79b), which have ITAM domains in their cytoplasmic regions (Benschop and Cambier 1999; Cambier et al. 2007; Chaplin 2010; Herzog et al. 2009). Pre-B cell receives signals via the BCR complex to undergo proliferation, during which IgL chain genes are rearranged and synthesised to form a complete IgM molecule. This drives Pre-B cells to differentiate into immature B cells expressing surface IgM as their antigenic receptor. Development proceeds through sequential molecular checkpoints that assure selection of only those B cells that do not react to self antigens. B cells which express self-reactive BCR enter apoptosis or undergo receptor editing (Ollila and Vihinen 2005; Vaughan et al. 2011; Zhang et al. 2004).

Negatively selected B cells exit the bone marrow and enter the blood as immature/transitional cells. These newly generated B cells home to the spleen, where development continues into IgM and IgD expressing mature (or naïve) B cells. Naïve B cells re-enter the circulation and home to lymph nodes, where development continues in an antigen-dependent manner (Bonilla and Oettgen 2010; Cariappa et al. 2007; Carter 2006).

### 1.3.3 B cell antigens

Newly formed mature antigen-naive B cells survive for only days or weeks unless stimulated with antigen. Unlike the TCR, the antigen specific BCR can directly bind to a wide range of small and large protein and non-protein antigens. Depending on the size and nature of the antigen B cell responses occur in a T cell dependent (TD) or T cell independent (TI) manner (Abbas et al. 2007; Vale and Schroeder 2010).

The B cell response to protein antigens such as viruses is dependent on cognate help provided by T cells. The BCR cannot bind to native proteins as a whole, but instead binds to specific surface segments called antigenic determinants or conformational epitopes (Abbas et al. 2007). Once bound, protein antigens are internalised into endosomal vesicles by receptor mediated endocytosis. The antigen is processed inside vesicles and presented on MHC class II or class I molecules to $CD4^+$ or $CD8^+$ T cells, respectively (Alam and Gorska 2003; Fairfax et al. 2008; Vale and Schroeder 2010).

Antigens with particular structural and/or compositional properties, i.e. non-protein antigens such as polysaccharides, lipids and nucleic acids are able to stimulate the differentiation of B cells into antibody-secreting cells in the absence of cognate T cell help. These antigens are known as TI antigens and are further divided into TI-1 and TI-2. T1-1 antigens are B cell mitogens capable of stimulating B cells independently of the BCR. An example of a TI-1 antigen is LPS, a major constituent of the cell wall of Gram-negative bacteria. B cells recognise such pathogen specific structures via expression of various TLRs, e.g. LPS is detected via TLR4 (Oracki et al. 2010). In contrast, TI-2 antigens deliver prolonged and persistent signalling through the BCR. TI-2 antigens include macromolecules such as polysaccharides and lipids, these are known as polyvalent antigens because they have repeating epitopes. Polyvalent antigens induce cross-linking and clustering of the BCR, this initiates intracellular signalling leading to B cell activation. TI antibody responses are

mainly initiated in the spleen, bone marrow, peritoneal cavity and at mucosal sites (Abbas et al. 2007; Mond et al. 1995; Oracki et al. 2010).

Furthermore, B cells recognise and produce antibodies against different epitopes on an antigen; this is known as a polyclonal response (Abbas et al. 2007). B cells can be stimulated polyclonally with superantigens, such as microbial or viral toxins. Superantigens interact with the BCR outside the antigen binding site and stimulate a larger number (approximately 500-fold increase) of lymphocytes in comparison to conventional peptide antigens (McGhee et al. 2007; Silverman 1998). A membrane protein produced by *Staphylococcus aureus* called protein A has the properties of a superantigen. Soluble protein A does not stimulate B cells alone but only when fixed on to bacteria. *Staphylococcus aureus* Cowan strain 1 (SAC) is a powerful surface Ig cross-linker commonly used for *in vitro* TI stimulation of human B cells (Goding 1978; Shantz 2000; Silverman 1998).

## 1.3.4 Mature B cell populations

In the spleen, immature B cells develop into one of three functionally and anatomically distinct population of mature B cells, which respond to preferentially different types of antigens (Abbas et al. 2007; Vale and Schroeder 2010).

### 1.3.4.1 Marginal zone B cells

A small population of mature B cells home to the marginal zone of the spleen and reside there as non-circulating Marginal zone (MZ) B cells (Spencer et al. 1998; Weill et al. 2009). MZ B cells initiate early TI and TD immune responses, thus are thought to play an important role in linking innate and adaptive immune responses. This distinct population of splenic B cells are specialised at mounting TI low-affinity antibody responses towards

blood-borne antigens, such as bacterial polysaccharides (Batista and Harwood 2009; Carter 2006; Pillai and Cariappa 2009; Vale and Schroeder 2010).

### 1.3.4.2 B1 B cells

B1 B cells represent a unique but minor population of B cells that are mainly found residing in mucosal tissue and the peritoneum of mice. B1 B cells produce non-specific natural IgM antibodies in response to non-specific stimuli such as TLR agonists. Studies carried out in mice support the existence of B1 B cell cells; whether B1 B cells exist in humans is still a matter of debate (Abbas et al. 2007; Batista and Harwood 2009; Hardy 2006; Vaughan et al. 2011).

### 1.3.4.3 Follicular B cells

The majority of mature B cells in the body exist as re-circulating or follicular B cells that continuously traffic from blood to follicles of secondary lymphoid organs such as lymph nodes, spleen, Peyer's patches and tonsils (Batista and Harwood 2009; Cyster 2010; Klein and Dalla-Favera 2008) (**Figure 1.1**). Follicular B cells critically rely on an intricate cellular collaboration with Tfh cells in generating a diverse repertoire of high-affinity class-switched antigen-specific antibodies, essential for the clearance of a plethora of different protein antigens. These tightly controlled processes take place within specialised structures called germinal centres (GCs), which are formed within the central region of primary B cell follicles in response to TD antibody responses (Allen et al. 2007; Batista and Harwood 2009; Crotty 2011; Cyster 2010; Gatto and Brink 2010; King et al. 2008; Vale and Schroeder 2010) (**Figure 1.4**).

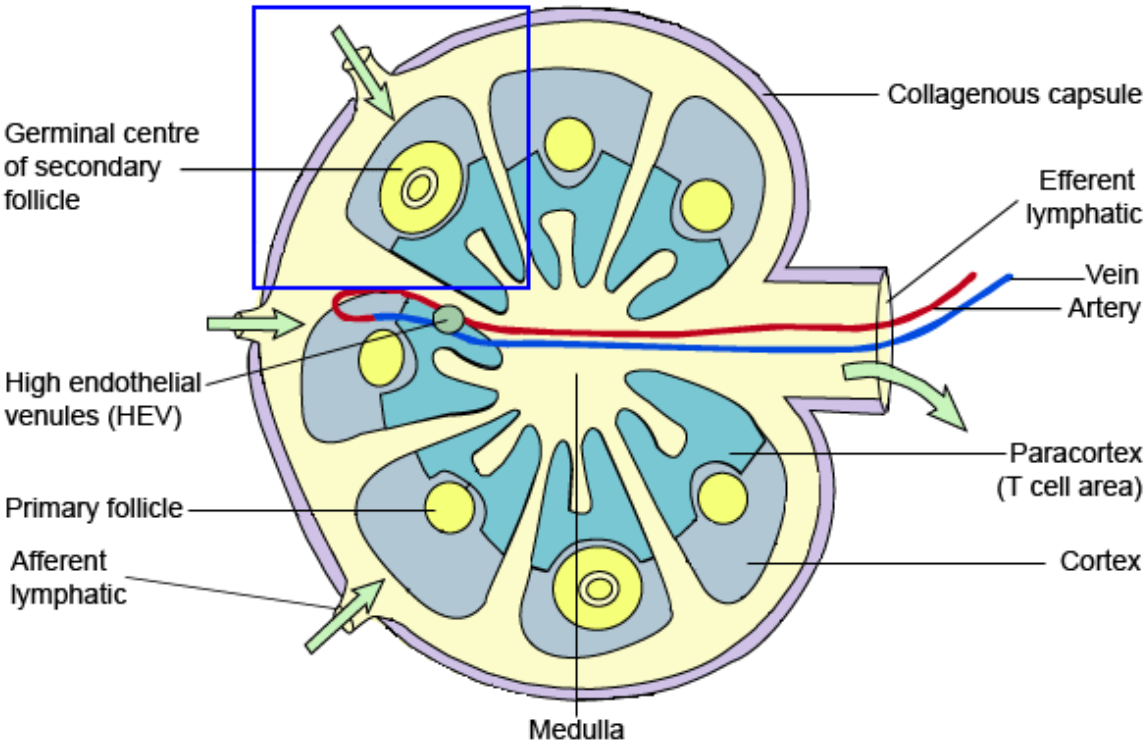


**Figure 1.4. Schematic structure of a lymph node.**
The lymph node structure is organised into defined B cell (follicles) and T cell areas (paracortex). Proliferating B cells are concentrated in germinal centres, which form within the central region of primary B cell follicles in response to T-dependent antibody responses. Taken from (St Georges University of London).

### 1.3.5 The germinal centre response

The GC is a dynamic microenvironment where antigen-activated B cells undergo a number of important modifications such as clonal expansion, somatic hypermutation (SHM), selection of high-affinity B cells and class-switch recombination (CSR). The GC is segmented into two major zones referred to as the dark zone and light zone. The dark zone is densely packed with rapidly proliferating large GC B cells known as centroblasts which migrate to the light zone and give rise to smaller non-proliferating centrocytes. In the light zone centrocytes are intermixed with tingible body macrophages, Tfh cells (known as GC Tfh cells) and follicular dendritic cells (FDCs) displaying intact antigen via immune complexes (Allen et al. 2007; Batista and Harwood 2009; Good-Jacobson and Shlomchik 2010; Pistoia and Cocco 2009; Vinuesa et al. 2010) (**Figure 1.5**).

To increase antibody diversity and affinity, expanding centroblasts undergo SHM, a step-wise process that permits Ig variable genes to undergo point mutations at a very high rate (hypermutation). In the light zone, only those centrocytes that bind to the original antigen on FDCs with the highest affinities are selected for survival and expansion. Important survival signals are also provided by GC Tfh cells. Selected high affinity antigen-reactive B cells may undergo CSR of constant region genes, a molecular mechanism which allows heavy chain isotype switching from IgM to IgG, IgA, or IgE whilst maintaining antigenic specificity (isotype-switching) (Allen et al. 2007; Good-Jacobson and Shlomchik 2010; Klein and Dalla-Favera 2008; Pistoia and Cocco 2009; Vinuesa et al. 2010). SHM and CSR require the presence of a key enzyme called activation-induced cytidine deaminase (AID) which initiates DNA strand-breaks in Ig variable regions and is expressed by proliferating centroblasts (Klein and Dalla-Favera 2008; Natkunam 2007; Vinuesa et al. 2009).

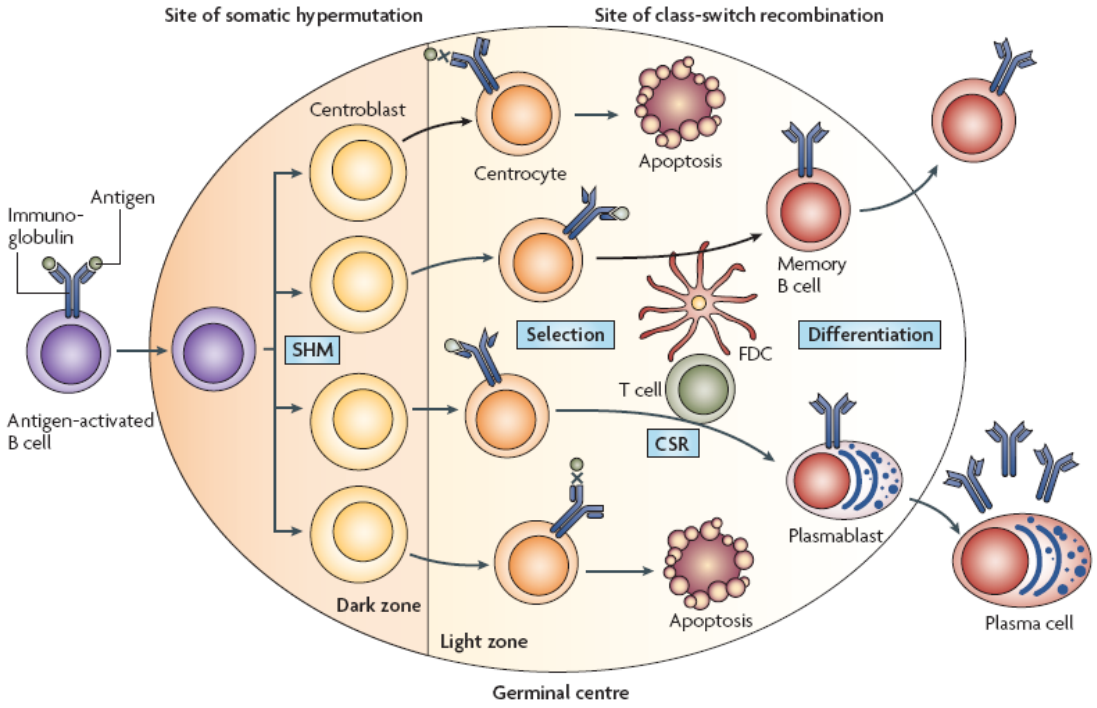


**Figure 1.5. The germinal centre reaction.**
SHM (somatic hypermutation); CSR (class-switch recombination); FDCs (follicular dendritic cells). Taken from (Klein and Dalla-Favera 2008).

## 1.3.6 Memory B cells

The purpose of the GC reaction is to enhance host protection for long periods of time by forming long-lived, high-affinity, class-switched, antigen-specific antibodies. Long-lived serological memory (antibodies that persists for decades) is formed when surviving antigen-reactive GC B cells differentiate into either memory B cells or long-lived plasma cells **(Figure 1.5)**. The factors that control which differentiation path a B cell takes or the quantity of memory and plasma cell populations generated are poorly understood. Both memory and plasma cells persist in the absence of sustained antigenic challenge and can directly or indirectly provide effector functions (Elgueta et al. 2010; Good-Jacobson and Shlomchik 2010; Tangye and Tarlinton 2009).

Memory B cells are antigen-experienced long-lived, quiescent B cells that express high-affinity surface antibodies. Compared to antigen-inexperienced naive B cells, memory B cells are capable of eliciting rapid and robust responses upon re-encounter with the pathogen. Memory B cells can rapidly proliferate and differentiate into short- and long-lived plasma cells, as well as perform other immediate effector and regulatory functions such as: antigen-presentation, T cell and DC regulation and cytokine and chemokine production (Good-Jacobson and Shlomchik 2010; Klein and Dalla-Favera 2008; Tangye and Tarlinton 2009).

Memory B cells in peripheral blood and secondary lymphoid organs exist as a phenotypically and functionally heterogeneous population (Good-Jacobson and Shlomchik 2010; Tangye and Tarlinton 2009). CD27, a TNFR family member, is as a transmembrane homodimer found expressed on all memory B cells, the majority of T cells, but not on naïve B cells (Agematsu et al. 2000). In humans, the CD27 positive memory B cell population can be subdivided into isotype-switched and non-switched (IgM positive) cells. Naive and memory B cells are commonly identified based on the differential expression of IgD and CD27 or IgD and CD38 surface markers (Good-Jacobson and Shlomchik 2010; Sanz et al.

2008; Tangye and Good 2007; Tangye and Tarlinton 2009). In comparison to CD27 negative B cells, CD27 positive B cells are more rapidly activated and produce higher levels of IgA, IgM, and IgG antibodies in response to *in vitro* stimulation with SAC plus IL-2, (Agematsu et al. 2000; Tangye and Good 2007). Furthermore, memory B cells are more sensitive to *in vitro* stimulation with the co-stimulatory molecule CD40 ligand (CD40L) plus IL-21 in producing IgG, IgA and IgM antibodies and in differentiating into Ig-secreting plasma cells (Konforte et al. 2009).

### 1.3.7 Plasma cells

Plasma cells are defined as terminally differentiated non-proliferating, antibody secreting cells. Plasma cells can either arise from extrafollicular regions or from GCs. Extrafollicular plasma cells are short-lived plasma cells that mount early response to antigen by secretion of low-affinity antibodies, predominantly IgM. In contrast, the GC response allows the generation of long-lived plasma cells, which secrete high-affinity antibodies (Elgueta et al. 2010; Oracki et al. 2010; Wols 2005). Down-regulation of the chemokine receptors CXCR5 and CCR7 guides plasma cells to exit GCs and secondary lymphoid organs, respectively. Depending on the expression of adhesion molecules, chemokines, and their receptors immature plasma cells home to various tissue sites in the body, such as the splenic red pulp, lymph node medulla, bone marrow, tonsils, gut and to sites of inflammation. However, the majority of plasma cells reside in bone marrow niches, which provide the contact (stromal cells) and growth factors (APRIL and IL-6) required for survival of long-lived plasma cells. Plasma cells are rarely found in the circulation, any cells found are likely to be precursors called plasmablasts, emigrating to their organ of choice (Fairfax et al. 2008; Ingold et al. 2005; Wols 2005).

B cell maturation to plasma cells is promoted by the induction of transcriptional repressor B lymphocyte induced maturation protein 1 (Blimp1). Plasma cells can be divided into immature and mature cells on the basis of Blimp1 expression levels, with higher levels

found in mature cells. Furthermore, Plasma cells can be distinguished from mature B cell subsets based on cell size, morphology and surface marker expression. On differentiation into plasma cells B cells down-regulate surface expression of CXCR5, CD19, B220, MHC class II, CD79, CD86, CD22, CD21, surface Ig in mouse and additionally CD20 in human. Surface markers which are up-regulated or remain expressed on human plasma cells include Syndecan-1 (Synd-1; CD138), CD38 and CD27 (Fairfax et al. 2008; Oracki et al. 2010; Wols 2005).

## 1.4 Tfh cells and molecules involved in Tfh and B cell interactions

Human Tfh cells are defined by the expression of the following characteristic markers: the transcriptional repressor B cell lymphoma 6 (Bcl6), CXCR5 (also known as Burkitt lymphoma receptor 1; BLR-1), the CXCR5 ligand CXC-chemokine ligand 13 (CXCL13; also known as B cell-attracting chemokine 1; BCA-1), the co-stimulatory molecules inducible co-stimulator (ICOS; CD278), CD40L (CD152), co-inhibitory molecule programmed death 1 (PD1; CD279), the signalling lymphocytic activation molecule (SLAM)-associated protein (SAP; also known as SH2D1a), cytokines IL-21, IL-4 and IL-10; IL-21R and IL-6R (Crotty 2011; King 2009; King et al. 2008; Ribot et al. 2011) (**Figure 1.6**). Murine Tfh cells express the same panel of markers with the exception of CXCL13 (Crotty 2011). Heightened expression of a combination of these molecules allows Tfh cells to be superior in facilitating B cells for antibody production (Yu et al. 2009). Some of the above Tfh cell-associated molecules are often used as markers to identify and distinguish Tfh cells from other Th subsets (Yu and Vinuesa 2010). The significance and function of the most key and best characterised Tfh cell-expressed molecules, in the context of B cell help are discussed in further detail below.

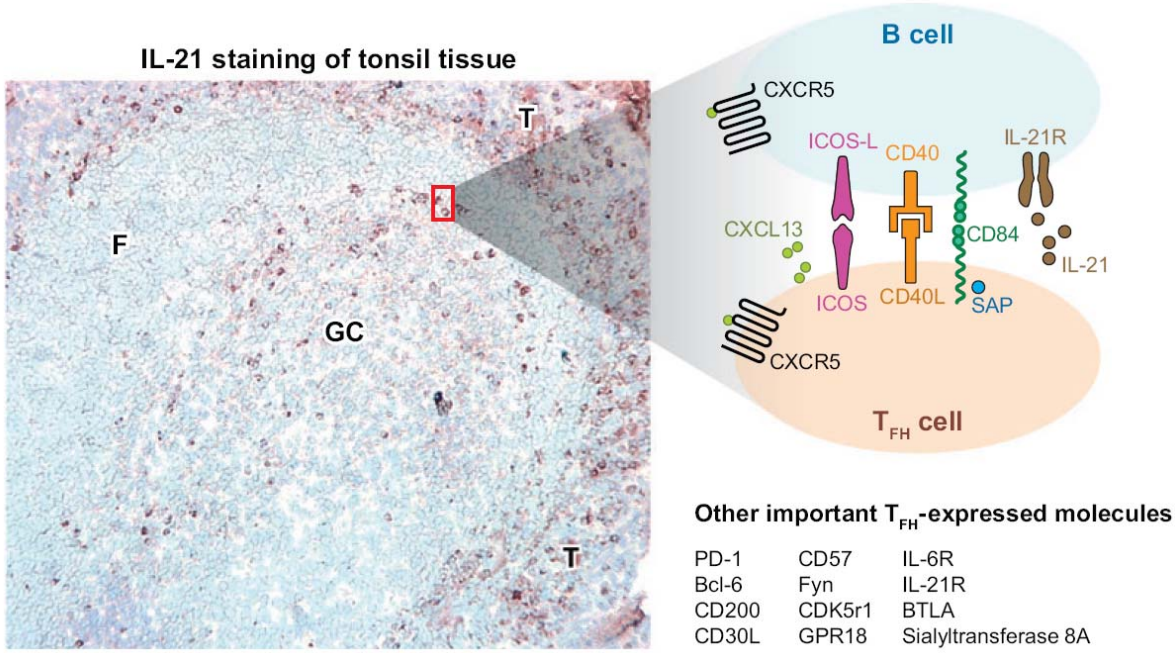


**Figure 1.6. Important molecules for Tfh cell function.**
(*Left*) Human tonsil stained for IL-21 expression, cells positive for IL-21 shown stained in dark purple. Regions of the tonsil indicated as T cell areas (T), the B cell follicle (F), and the germinal centre (GC). Note the positioning of IL-21-expressing cells at the border of the GC. (*Right*) Some of the best-characterised molecules involved in Tfh and B cell interactions. Listed below are numerous other molecules over-expressed in Tfh cells that most likely play an important role in their function. Taken from (King et al. 2008).

### 1.4.1 Tfh cell differentiation

Where and when a Th cell becomes a Tfh cell is controversial and remains to be defined. At present, four models have been proposed: Model 1) a distinct subset directly induced by cytokine (IL-6 or IL-21); Model 2) a distinct subset directly induced by B cells; Model 3) a non-distinct subset differentiated from Th1/Th2/Th17 cells; Model 4) Tfh differentiation is initially induced by antigen-presenting DCs and maintained by cognate interaction with B cells (Crotty 2011; Fazilleau et al. 2009; Yu and Vinuesa 2010). In support of the third model, studies have shown Tfh cells to develop from other Th subsets under certain conditions (Zaretsky et al. 2009). The fourth model of Tfh differentiation is based on more recent findings which suggest prolonged antigen presentation by DC to be critical in the generation of Tfh cells (Deenick et al. 2011). This new model considers Tfh cells as a non-restricted subset that can secrete moderate amounts of Th1, Th2, or Th17 cytokines, required for B cell antibody class switching (Crotty 2011).

### 1.4.2 Bcl6

Bcl6 has been identified as the transcription factor that specifically drives Tfh cell formation. Expression of Bcl6 is thought to be induced by the cytokines IL-21 and IL-6 through their ability to activate STAT3. Tfh cells selectively express high levels of Bcl6 whilst other Th subsets (Th1, Th2, or Th17 cells) express high levels of Blimp1. Bcl6 and Blimp1 act as transcriptional repressors; one inhibits the transcription of the other. Bcl6 controls Tfh differentiation by suppressing the activity of Blimp1 and other master transcription factors required for Th1, Th2 and Th17 generation (Crotty 2011; King 2009). Tfh cells can be distinctly characterised from other Th subsets by the high expression of both Bcl6 and CXCR5. In the absence of Bcl6 activated Th cells fail to express Tfh cell–associated molecules (Kassiotis and O'Garra 2009). Other transcription factors such as c-

Maf, Batf, and Bcl6-binding partners also play fundamental roles in the development and function of Tfh cells (Crotty 2011).

### 1.4.3 The chemokine receptor CXCR5 and ligand CXCL13

Tfh cells were first described in 2000 and 2001, as a subset of $CD4^+$ T cells found localised within GCs of human tonsils expressing high levels of CXCR5 (Breitfeld et al. 2000; Kim et al. 2001; Schaerli et al. 2001; Schaerli et al. 2000). Since then CXCR5 has been the canonical phenotypic marker for Tfh cells. CXCR5 is a G protein-coupled seven transmembrane chemokine receptor, which specifically allows migration of cells towards the follicular chemokine ligand CXCL13 (Cyster et al. 2000; Förster et al. 1994). In peripheral blood surface CXCR5 is uniformally expressed on all mature B cells and on a subpopulation of memory ($CD45RO^+$) T cells but not on naïve T ($CD45RA^+$) or $T_{EMRA}$ cells. The majority of blood $CXCR5^+$ T cells are $CD4^+$ (90%) and a minority $CD8^+$ (10%). In the $CD4^+$ $CD45RO^+$ memory population about 20% are found to express CXCR5, these cells are thought to be resting Tfh cells (Förster et al. 1994; Schaerli et al. 2001; Schaerli et al. 2000).

The CXCR5-CXCL13 axis plays a unique role in bringing together $CXCR5^+$ Tfh cells and B cells within secondary lymphoid organs, which is critical for T-B interactions (Allen and Cyster 2008; Crotty 2011; Ebert et al. 2005; Moser et al. 2002; Rasheed et al. 2006). In humans, CXCL13 is produced in abundance in the light zone of GCs by FDCs and Tfh cells (Allen and Cyster 2008; Crotty 2011).

Secondary lymphoid organs such as the lymph nodes are divided into two major regions called follicles enriched by B cells and the paracortex or T cell zone enriched predominantly by T cells (**Figure 1.4 and 1.6**). Follicles and T cell zone also contain FDCs and resident and migratory DCs, respectively (Oracki et al. 2010). Entry of circulating T and B cells into

lymph nodes is guided by adhesion receptor CD62L and the chemokine receptor CCR7. Expression of CCR7 on peripheral blood naïve T and B cells as well as TCM cells, permits entry into lymph nodes by passing through specialised vessels called high endothelial venules (HEVs) located in the paracortex (**Figure 1.4**). Lymphocytes bearing CCR7 are selectively attracted towards homeostatic chemokines ELC/CCL19 and SLC/CCL21, which are expressed on the luminal side of HEVs and are produced by stromal cells in the T cell zone (Batista and Harwood 2009; Bromley et al. 2008; Ebert et al. 2005; Moser and Loetscher 2001; Moser et al. 2002). CCR7 retains naïve $CD4^+$ T cells in the T cell zone, which is adjacent to B cell follicles. Within the T cell zone, T cells co-localise with antigen-loaded mature DCs. T cells specific for antigen become activated and receive signals from DCs to down-regulate CCR7 and up-regulate CXCR5. Expression of CXCR5 makes $CD4^+$ T cells (or pre-Tfh cells) responsive to CXCL13, this permits relocation from the T cell zone towards the B cell follicle where CXCL13 is produced at high concentration. In contrast, antigen-stimulated B cells up-regulate CCR7 and down-regulate CXCR5, this enables B cells to migrate from the follicle towards the T cell zone. Consequently, both cell types meet at the boundary between the B cell follicle and T cell zone (**Figure 1.6**). Here, activated $CXCR5^+$ $CD4^+$ T cells interact with B cells presenting processed antigen. Cognate recognition of antigen drives development of Tfh cells and permits delivery of T cell help to B cells. Recognition of the same antigen by both cell types is critical in TD antibody responses. Antigenic peptide recognised by the T cell must also be associated with the antigen recognised by the B cell. Upon receiving T cell help (i.e. via co-stimulatory molecules and cytokines), fully activated B cells can either migrate into extrafollicular areas and become short-lived plasma cells or colonise primary follicles to form GCs. At the same time, signals delivered via co-stimulatory molecules CD28, OX40, and ICOS on Tfh cells induces up-regulation of CXCR5 expression, allowing Tfh cells to enter into follicles. Some of these Tfh cells go on to support GC formation and the production of long-lived antibodies (Allen et al. 2007; Deenick et al. 2011; Ebert et al. 2005; King et al. 2008; Rasheed et al. 2006) (**Figure 1.5**).

The ontogeny of blood $CXCR5^+$ $CD4^+$ T cells and their relationship with tissue derived Tfh cells remains to be solved (Crotty 2011). In humans, the majority of studies on $CXCR5^+$

$CD4^+$ T cells have been carried out using peripheral blood and tonsillar derived cells (Ebert et al. 2004; Förster et al. 1994; Kim et al. 2001; Quigley et al. 2007; Rasheed et al. 2006; Schaerli et al. 2001; Schaerli et al. 2000). The T cell zone and B cell follicles of inflamed tonsils are found to be highly enriched with $CXCR5^+$ $CD4^+$ T cells. In comparison to the 20% of memory $CD4^+$ T cells found to express CXCR5 in blood, about 90% of memory $CD4^+$ T cells found in tonsils are $CXCR5^+$ (Ebert et al. 2004). Peripheral blood $CXCR5^+$ $CD4^+$ T cells lack the expression of activation markers (CD25, CD69 and HLA-DR) and co-stimulatory molecules (CD40L, OX40 and ICOS), thus are in resting state. In contrast tonsillar derived GC Tfh cells do express activation (CD69 and HLA-DR) and co-stimulatory molecules markers (ICOS) and are highly active at providing B cell help *in vitro* (Schaerli et al. 2000). Surface CXCR5 expression can be induced by *in vitro* stimulation on naïve T cells with superantigen-loaded DCs and on CXCR5-negative memory $CD4^+$ T cells with PHA or anti-CD3 (Schaerli et al. 2001). However CXCR5 expression on both tissue derived and peripheral $CD4^+$ T cells is rapidly lost *in vitro*. This transient expression of CXCR5 makes it a challenging marker for *in vitro* studies (Ebert et al. 2005; Schaerli et al. 2001; Schaerli et al. 2000). *In vivo* studies using IL-21-knock out mice have helped reveal the importance of IL-21 in promoting and maintaining CXCR5 surface expression on $CD4^+$ T cells (Lüthje et al. 2012; Silver and Hunter 2008; Spolski and Leonard 2010; Vogelzang et al. 2008).

### 1.4.4 Co-stimulatory molecules

The Tfh cell subset not only express appropriate cell surface molecules required for T cell priming by DCs (i.e. CD28:CD80/CD86) but also express specific molecules required for B cell help (Rolf et al. 2010). The specialised capacity of Tfh cells to provide B cell help requires co-localisation of both cell types and the delivery of signals to B cells via Tfh expressed co-stimulatory molecules and cytokines (Crotty 2011; King 2009; Spolski and Leonard 2010) (**Figure 1.6**). Tfh cells express high levels of transmembrane co-stimulatory molecules such as CD40L, ICOS, OX40 and CD70 that interact with counter receptors/ligands (CD40, ICOSL, OX40L and CD27, respectively) on DCs and B cells

(Crotty 2011). These and several other receptor/ligand pairs provide positive signals and allow extended cell-cell contacts to form, which is essential for bidirectional communication, mutual co-stimulation and activation of cells (Frauwirth and Thompson 2002). The co-stimulatory molecules CD40L, OX40, ICOS, CD70 and co-inhibitory molecule PD-1 in Tfh:B cell interactions, are discussed in more detail below.

#### 1.4.4.1 CD40L

CD40L (CD154), a TNF family member, is an integral membrane glycoprotein transiently expressed on the surface of recently activated $CD4^+$ T cells. CD40, the receptor for CD40L, is constitutively expressed mainly on the surface of APCs and is required for their activation (Daoussis et al. 2004). Sustained TCR ligation and CD28 co-stimulation provided by DCs during T cell priming induce up-regulation of CD40L on $CD4^+$ T cells. Subsequent binding of CD40 on DCs by CD40L on T cells results in DC activation, secretion of cytokines, and further up-regulation of MHC and co-stimulatory molecules. In turn, activated DCs provide specific signals (e.g. secretion of IL-12) which activate and stimulate naïve $CD4^+$ T cells to become pre-Tfh cells (Deenick et al. 2011; Leitner et al. 2010; Ma and Clark 2009).

At the edge of B cell follicles, pre-Tfh cells interact with B cells via CD40L:CD40 interactions, respectively. In B cells, CD40 signalling induces GC B cell activation, survival, proliferation and in the presence of specific cytokines isotype switching (Daoussis et al. 2004; King 2009; Yong et al. 2009). Stimulation provided by both CD40L and cytokines (IL-21 or IL-4) maintains B cell proliferation but inhibits plasma cell differentiation. However, reduction or elimination of CD40L signals with continued cytokine stimulation (most potently with IL-21) allows B cells to differentiate into IgM secreting plasma cells (Crotty 2011).

The regulation of CD40L expression both at the mRNA and protein level has been studied *in vitro*. CD40L can be induced upon PMA and ionomycin stimulation of peripheral blood human T cells. Peak levels of CD40L have been found following 4-6 hours of T cell

stimulation, which were rapidly lost and found undetectable by 16 hours, demonstrating a tight regulation of CD40L on T cells (Daoussis et al. 2004). Expression of other co-stimulatory molecules such as CD28 and ICOS are thought to assist CD40L expression. Furthermore, cytokines such as IL-2, IL-12, and IL-15 up-regulate CD40L expression, while IL-4 and IL-10 down-regulate CD40L (Daoussis et al. 2004; King 2009; Ma and Clark 2009; Yong et al. 2009).

It is important to note that both CD40L and CD40 can be reciprocally expressed. CD40L is also found on activated B cells and DCs and CD40 can be detected on activated αβ T cells (Ma and Clark 2009) and on Vγ9Vδ2 T cells (Brandes et al. 2005; Moser and Eberl 2011).

#### 1.4.4.2 OX40

OX40 (CD134), a TNFR family member, is expressed at high levels on activated Tfh cells including GC Tfh cells and under certain conditions on activated $CD8^+$ T cells and Tregs (Croft et al. 2009; Crotty 2011; King 2009). OX40 is not only required during DC:T and T:B cell-cell interactions, its expression is also vital for survival of activated T cells (Crotty 2011; Deenick et al. 2011). Unlike CD28, OX40 is not found on resting naïve or memory T cells, but is transiently induced within the first hours of T cell activation. Peak levels of OX40 can be detected both *in vitro* and *in vivo* between 1-5 days after initial stimulation. Although transient expression of OX40 can be induced with TCR and CD3 signals alone, sustained expression requires CD28:CD80/86 interactions, which can be further prolonged in the presence of specific cytokines (i.e. IL-1β, IL-2, and TNF) (Croft et al. 2009; Salek-Ardakani and Croft 2006).

Correspondingly, OX40 ligand (OX40L, CD252) is also induced within 24 hours of DC and B cell activation, this is secondary to CD40:CD40L interactions (Croft et al. 2009; Deenick et al. 2011; Salek-Ardakani and Croft 2006). The extent and duration of OX40L expression is also determined by CD28:CD80/86 interactions. A chronological sequential engagement of CD28 followed by OX40 is essential for T cell survival and proliferation. OX40

promotes T cell survival and division by inducing anti-apoptotic molecules (Croft et al. 2009; Crotty 2011; Salek-Ardakani and Croft 2006). Expression of OX40 and OX40L on T cells and APCs, respectively, regulates cytokine production and cytokine receptor signalling (Croft et al. 2009). Due to the limited number of studies, the exact role of OX40 in Tfh function remains unknown (King 2009). Initial studies suggested OX40 signalling to be important in Tfh cell formation and for the expression of CXCR5 on $CD4^+$ T cells (Deenick et al. 2011; King 2009; King et al. 2008). However, more recent *in vivo* murine studies showed that the absence of OX40 signalling had no effect on CXCR5 expression, Tfh differentiation, GC development and antibody titers (Crotty 2011). Overall, published literature suggests OX40 signals to play an important role in driving B cell differentiation toward antibody-secreting plasma cells and long-lived memory B cells (King 2009; King et al. 2008; Ma and Clark 2009).

#### 1.4.4.3 ICOS

ICOS (CD278) is a CD28-like T cell co-stimulatory molecule expressed at low levels on the surface of resting naïve T cells, which is rapidly up-regulated following TCR engagement and CD28 signalling. Highest expression of ICOS is found on Tfh cells, particularly on GC Tfh cells. However, ICOS is not restricted to Tfh cells, as it is also expressed on other helper subsets such as Th1, Th2, Th17 and Tregs (Hutloff et al. 1999; King et al. 2008; Simpson et al. 2010). ICOS binds to its specific ligand, ICOS-L (also known as CD275) expressed at low levels on APCs. ICOS-L is up-regulated following activation of APCs with highest levels found on B cells (Bauquet et al. 2009; Yong et al. 2009). ICOS-L on B cells is down-regulated following interaction with ICOS, thus suggesting tight regulation of ICOS:ICOSL interactions (Crotty 2011; Yong et al. 2009).

While CD28:B7/CD40L:CD40 interactions facilitate DC-priming of pre-Tfh cells, ICOS:ICOS-L interactions are central to B cell mediated Tfh cell formation and the maintenance of GCs (Deenick et al. 2011; Yong et al. 2009). However, recent data also suggest the requirement of ICOS for Tfh differentiation during DC priming (Crotty 2011).

ICOS is predominantly expressed by Tfh cells residing in the light zone of GCs, sites where Tfh cells interact with GC B cells (Allen et al. 2007; Yong et al. 2009). Sustained ICOS expression is required for GC formation. B cell signalling through ICOS-L is important for isotype class switching, affinity maturation and the development of memory B cells (Crotty 2011; King et al. 2008; Suh et al. 2004; Yong et al. 2009).

On the T cell side, signalling through ICOS in the presence of TCR stimulation results in strong activation of the phosphatidylinositol 3-kinase (PI3K) signalling pathway. The exact signalling mechanism employed by ICOS remains poorly understood, however, data have suggested that a series of signalling cascades may be involved (Deenick et al. 2011; Simpson et al. 2010; Yong et al. 2009). Ligation of ICOS does have multiple downstream consequences such as Th cell survival, up-regulation of cell surface molecules (i.e. CD40L), production of cytokines such as IL-4, IL-10, IL-21 and, to a lesser extent, the production of IL-2, IFN-γ and TNF-α (Bauquet et al. 2009; King 2009; King et al. 2008; Vu et al. 2008). IL-4 and IL-10 assist in T:B interactions, promote B cell proliferation and plasma cell differentiation (King et al. 2008; Yong et al. 2009). IL-10 also supports antibody production and the survival of GC B cells (Yong et al. 2009). The amount of IL-10 and IL-21 produced by Tfh cells is relative to the levels of ICOS expressed (King et al. 2008; Vogelzang et al. 2008). In the absence of ICOS, IL-10 production is impaired (King et al. 2008).

Both human and murine studies have shown the importance of ICOS in the development and function of Tfh cells. Patients with human common variable immunodeficiency (CVID) have a homozygous loss of ICOS (Rasheed et al. 2006). CVID patients lack GCs in their lymph nodes and consequently have reduced number of memory B cells, low IL-10 and reduced serum IgG and IgA levels (Bauquet et al. 2009; King et al. 2008; Yong et al. 2009). Such defects result from inadequate B cell help due to the absence of ICOS:ICOS-L interactions (Yong et al. 2009). Furthermore, circulating $CXCR5^{+}CD4^{+}$ T cells were reduced in CVID patients. Thus, ICOS deficiency also had an effect on Tfh development but whether this is direct or secondary to the failure in GC formation remains unresolved

(Haynes 2008; King et al. 2008). In connection to the above, one *in vitro* study showed that only those Tfh cells that expressed high levels of ICOS were potent inducers of IgG production and CXCL13 secretion (Rasheed et al. 2006). Furthermore, data from recent studies suggest that ICOS:ICOS-L interactions may mediate Tfh development by regulating IL-21 production, possibly through c-Maf, which is 'downstream' of ICOS (Bauquet et al. 2009; Vogelzang et al. 2008). IL-21 produced in response to ICOS stimulation may support Tfh development and drive ICOS expression in an autocrine manner (King et al. 2008; Yong et al. 2009).

#### 1.4.4.4 CD70

CD70, another TNF family member, is only transiently expressed on activated T cells, B cells and DCs. CD70 binds to its receptor CD27, which is constitutively expressed on T cells and induced through antigen receptor triggering on B cells. CD27 is maintained throughout B cell differentiation with highest expression found on GC B cells, which is progressively lost after the centroblast stage. CD27:CD70 interactions between T cells and DCs come into play during the T cell priming phase. CD27 signalling appears critical for T cell expansion and survival (Denoeud and Moser 2011). Ligation of CD27 on B cells by CD70 on Tfh cells stimulates B cell activation and antibody production by promoting plasma cell differentiation (Borst et al. 2005; Elgueta et al. 2010; Jacquot 2000; Nolte et al. 2009). *In vitro* studies using human B cells have shown triggering of CD27 to enhance IgM, IgG, IgA, and IgE production (Xiao et al. 2004). In contrast, triggering of CD70 on B cells was shown to inhibit IgG production, thus has the opposite effects to CD27 (Arens et al. 2004; Borst et al. 2005; Kobata et al. 1995). However, in CD27 knockout mice the absence of CD27 on B cells had no effect on isotype switching, somatic hypermutation, or antibody production (Nolte et al. 2009; Xiao et al. 2004).

#### 1.4.4.5 PD-1

In addition to the high expression of co-stimulatory molecules, GC Tfh cells also express extremely high levels of the co-inhibitory receptor PD-1 (CD279) (Crotty 2011). PD-1 is

not restricted to T cells and can also be up-regulated on activated B cells (Good-Jacobson and Shlomchik 2010). Ligands for PD-1 include PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC) which are expressed at high levels on GC B cells, but are also found on activated T cells, macrophages and DCs (Haynes 2008; Ribot et al. 2011). Within GCs, engagement of PD-1 delivers negative signals that counteract positive signals provided by ICOS (Yong et al. 2009). This limits T cell responses such as proliferation and cytokine production (Fife and Pauken 2011; Leitner et al. 2010).

### 1.4.5 IL-21

IL-21 is a potent immunomodulatory four-α-helical-bundle type I cytokine that shows significant homology to IL-2, IL-4, and IL-15. IL-21 is produced by activated $CD4^+$ T cells and NKT cells. The highest expression of IL-21 is found in Tfh and Th17 cells. IL-21 is known as a 'helper' Tfh cell associated cytokine because it is the major cytokine abundantly produced by GC Tfh cells (Crotty 2011; King et al. 2008; Spolski and Leonard 2008) **(Figure 1.6)**. *In vitro* studies have shown several cytokines such as IL-6, IL-12, IL-21 and IL-23 to be inducers of IL-21 expression in naïve $CD4^+$ T cells. During initial DC priming of naïve $CD4^+$ T cells, IL-12 produced by activated DCs is thought to induce both Tfh cell development and production of IL-21 (Deenick et al. 2011). In B cell follicles, Tfh cell derived IL-21 has several very important functions on both GC B cells and Tfh cells (Spolski and Leonard 2008).

IL-21 is a key determinant of B cell proliferation, isotype switching and differentiation of B cells into antibody-forming cells. In comparison to other Tfh-secreted cytokines (IL-2, IL-4 and IL-10) IL-21 is the most potent at inducing plasma cell differentiation and regulating CSR. IL-21 alone or in combination with various other cytokines regulates class switching to IgG, IgA, or IgE isotypes. This has been extensively shown using *in vivo* mouse models and *in vitro* culture systems with human B cells. However, some differences where found between the cytokines controlling CSR in mice compared to those in humans. *In vitro*

studies using CD40L-stimulated human B cells showed the following: IL-21 predominantly induced CSR to IgG (particularly IgG1 and IgG3), IL-10 in the presence of IL-21 induced switch to IgA whereas the addition of IL-4 had an inhibitory effect, TGF-β1 also induced IgA production, IL-4 promoted production of IgE and finally IFN-γ inhibited switching of certain isotypes such as IgE (Konforte et al. 2009; Pistoia and Cocco 2009). The type of cytokines present at the time of B cell stimulation is associated with the type of defence required against the pathogen involved. For example an IL-4 induced switch to IgE is important for a response to parasites, whereas isotype switching in response to IFN-γ is vital for antiviral immunity (King et al. 2008).

A unique role of IL-21 is controlling the fate of activated B cells. IL-21 stimulation only promotes the survival of those B cells that are antigen specific and have received appropriate signals (via the BCR and CD40) from cognate T cells. B cells that have been activated non-specifically through the BCR alone (without CD40 co-stimulation) or by TLR ligands (i.e. LPS and CpG) are instructed to undergo apoptosis (King et al. 2008; Konforte et al. 2009). IL-21 also acts as an autocrine growth factor for Tfh cell development and long-term survival. In contrast to IL-2 and IL-15, IL-21 only induces proliferation of T cells that have been stimulated via the TCR. Thus, IL-21 functions as a T cell co-stimulator with the ability of possibly enhancing TCR signalling (King et al. 2008; Rolf et al. 2010; Spolski and Leonard 2008).

### 1.4.6 IL-21 receptor

The effects of IL-21 are mediated by binding to the transmembrane IL-21R, which is predominantly found on αβ T cells and B cells (King et al. 2008). The level of IL-21R expressed on peripheral blood naïve $CD4^+$ and $CD8^+$ T cells is low. Expression of IL-21R is regulated biphasically in T cells. An early up-regulation of IL-21R is seen following TCR stimulation with subsequent decline, a secondary spontaneous response is induced upon stimulation provided by cytokines such as IL-21. In contrast, other signals such as

IFN-α can decrease expression of IL-21R mRNA in T cells (Leonard et al. 2008). In regards to B cell subsets, IL-21R is found on naïve and GCs B cells, but not on memory B cells and plasma cells. IL-21R can also be found on NK cells, NKT cells, macrophages, and DCs and a variety of non-immune cells such as fibroblasts, keratinocytes, epithelial and endothelial cells (Davis et al. 2007; Konforte et al. 2009; Monteleone et al. 2009; Spolski and Leonard 2008).

The IL-21R is a heterodimer, composed of a common γ-chain subunit (γc; CD132) and an IL-21 specific α chain. The γc subunit is a component shared by the cytokine receptors for IL-2, IL-4, IL-7, IL-9, and IL-15. Like other γc-dependent cytokines, IL-21:IL21R binding initiates intracellular signalling through Jak1 and Jak3, which activate STAT3 and STAT1 and to a weaker extent STAT4 and STAT5 proteins (Brandt et al. 2007; Davis et al. 2007; Konforte et al. 2009; Leonard et al. 2008; Schmitt et al. 2009). In $CD4^+$ T cells and B cells, STAT3 is the most sustained and important STAT protein for IL-21 signalling and cell proliferation (Crotty 2011; Spolski and Leonard 2008). IL-21 ligand binding can also trigger activation of mitogen-activated protein kinase (MAPK) and PI3K pathways, which is important for IL-21-mediated cell proliferation (Davis et al. 2007; Leonard et al. 2008; Spolski and Leonard 2008).

In B cells, IL-21 signalling can result in the induction of the proapoptotic factor Bim, Bcl6 and Blimp1, depending on the co-stimulatory signals present. High expression of Bcl6 in the centroblast subset of GC B cells promotes cell cycle progression but inhibits B cell differentiation. In contrast, IL-21 induced expression of Blimp1 through activation of STAT3 and to a lesser extent STAT5 promotes formation of plasma cells and isotype-switched memory B cells (Davis et al. 2007; Schmidlin et al. 2009; Tarlinton et al. 2008) (**Figure 1.7**).

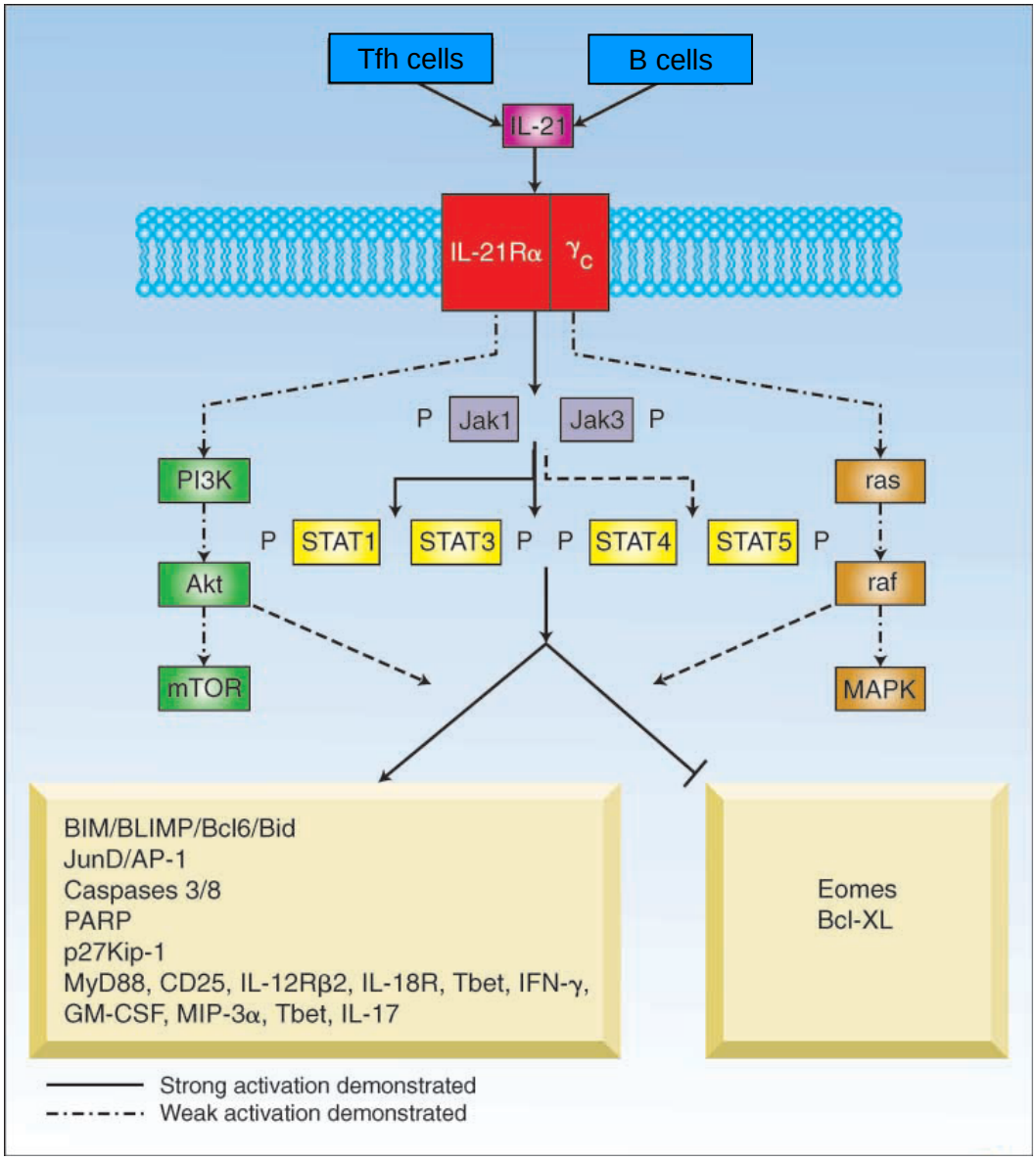


**Figure 1.7. IL-21R signalling pathways.**
IL-21 mainly signals via the Janus-activated kinase (Jak)/STAT pathway with STAT1 and STAT3 as primary targets. Signalling through the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways may also occur in certain physiologic or pathologic conditions. Adapted from (Davis et al. 2007).

## 1.5 Vγ9Vδ2 T cells and role in immune responses

While the crucial role of Tfh cells in providing B cell help is undisputed other T cell subsets including $CD8^+$ T cells, NKT cells and γδ T cells contribute to the outcome of humoral immune responses (Brandes et al. 2003; Ebert et al. 2004; Galli et al. 2003; Quigley et al. 2007). This chapter will focus primarily on the role of human γδ T cells in immune responses to microbial infections and their involvement in humoral immune responses.

### 1.5.1 γδ T cells in humans

γδ T cells were first described in the mid 1980s as a small but unique subset of T cells expressing a TCR composed of γ and δ chains (Allison et al. 2001; Brenner et al. 1986). Similar to αβ T cells, γδ T cells express a rearranged TCR complexed with CD3 (γ, δ, ε) and ζ signalling chains, which are associated with cytoplasmic cell signalling proteins (Allison et al. 2001). However the majority of γδ T cells do not express CD4 or CD8 membrane proteins (Hayday 2000). γδ T cells differ from conventional αβ T cells in that they have a unique pattern of antigen recognition, which does not require processing by professional APCs or presentation by MHC or MHC-related molecules (Eberl and Moser 2009; Holtmeier and Kabelitz 2005).

Furthermore, the majority of human γδ T cells do not respond to peptide antigens but instead use their TCR in a pattern recognition receptor-like way in recognising relatively conserved structures (i.e. non-peptide metabolites) on a diverse array of pathogens (Carding and Egan 2002; Hayday 2000; Holtmeier and Kabelitz 2005; Morita et al. 2007). γδ T cells also express several TLRs (TLR1, 2, 3, 5, 6, and 7) (Beetz et al. 2007) and NK receptors (i.e. NKG2D and CD94) (Battistini et al. 1997; Beetz et al. 2007). Expression of these innate cell surface receptors allows γδ T cells to respond to other non-peptide microbial

components or to self-antigens that have been up-regulated upon stress, e.g., infection, cell death, or cellular transformation such as MHC class I-related chains A/B (MICA and MICB) and UL16-binding proteins (ULBPs) (Beetz et al. 2007; Holtmeier and Kabelitz 2005; Morita et al. 2007). Due to the combination of innate like features and characteristics of T cells, γδ T cells have been termed unconventional, or transitional T cells thought to be involved in bridging the gap between innate and adaptive immune response (Bonneville et al. 2010; Pennington et al. 2005).

In humans, γδ T cells are divided into subsets based on TCR δ chain usage. There is estimated to be eight to ten distinct Vδ γδ T cell subsets in total (Hayday 2000). Vδ1 and Vδ2 are the two prominent subsets existing in humans, the others Vδ subsets exist in much lower frequencies and on which data is very limited. The Vδ1 (associated with different Vγ chains) subset is predominantly found resident within epithelial tissues and the Vδ2 (predominantly associated with the Vγ9 chain) subset represent the majority (>80%) of γδ T cell in peripheral blood (Beetz et al. 2007; Fagioli et al. 1990; Holtmeier and Kabelitz 2005). This thesis focuses on the major subset of human γδ T cells expressing the Vγ9Vδ2 TCR.

## 1.5.2 Activators of Vγ9Vδ2 γδ T cells

### 1.5.2.1 (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate

In healthy individuals Vγ9Vδ2 γδ T cells (also known as Vγ2Vδ2 T cells according to an alternative nomenclature) normally constitute 0.5–5% of circulating T cells (Caccamo et al. 2006b). However, some healthy individuals present with consistently high (up to 15–40% of circulating T cells) Vγ9Vδ2 T cell counts (Eberl and Moser 2009). Vγ9Vδ2 T cells expand rapidly both *in vivo* and *in vitro,* in response to infections with microbes producing the low molecular weight metabolite (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) (Davey et al. 2011; Eberl and Moser 2009; Morita et al. 2007). HMB-PP is an

invariant intermediate of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (also known as the non-mevalonate pathway) of isoprenoid biosynthesis, a pathway essentially required for microbial physiology (**Figure 1.8**).

HMB-PP is produced by numerous potentially harmful Gram-positive and Gram-negative bacteria, as well as by malaria parasites but not by higher eukaryotes, thus HMB-PP is unique to micro-organisms (Bonneville et al. 2010; Eberl and Moser 2009; Morita et al. 2007). Many of these HMB-PP producing pathogens are the causative agents of lethal infectious diseases such as cholera, diphtheria, plague, tuberculosis and typhoid (Davey et al. 2011). Activated Vγ9Vδ2 T cells are found to be elevated in patients suffering from acute infections with HMB-PP$^+$ bacteria; this has been well documented in patients undergoing peritoneal dialysis and in *Mycobacterium tuberculosis* infected patients (Davey et al. 2011; Meraviglia et al. 2011; Morita et al. 2007).

*In vitro*, Vγ9Vδ2 T cells can be activated with synthetic HMB-PP at concentrations as low as 0.1 nM, which displays identical stimulatory activities to the *E.coli* derived natural HMB-PP (Eberl et al. 2003; Moser and Eberl 2007). Thus, since its discovery, HMB-PP has been identified as the most potent, pathogen specific and natural activator of Vγ9Vδ2 T cells (Eberl et al. 2003; Hintz et al. 2001). For reasons that are not yet understood, Vγ9Vδ2 T cells and the responsiveness to HMB-PP are only found in higher primates including humans but are absent in all other vertebrates including rodents (Bonneville et al. 2010; Eberl and Moser 2009).

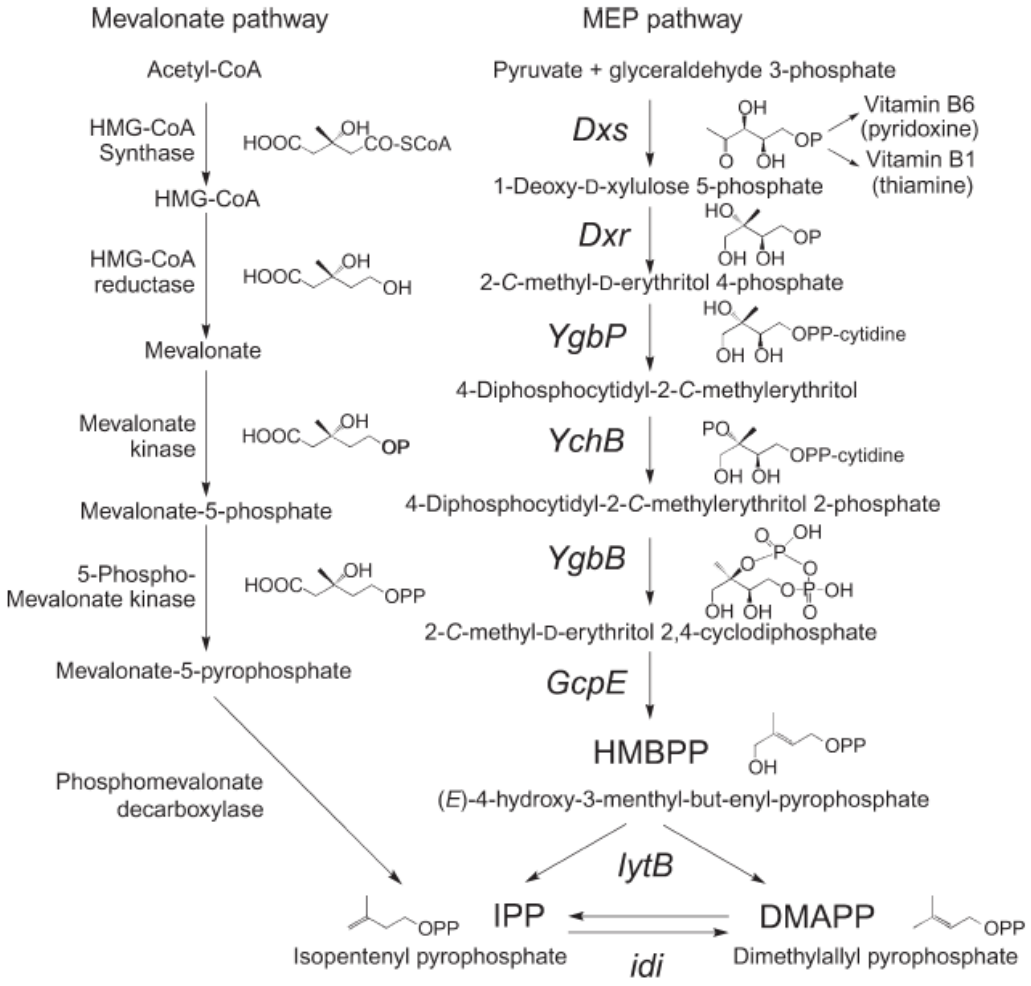


**Figure 1.8. MEP and mevalonate pathways for isoprenoid biosynthesis.**
Isopentenyl pyrophosphate (IPP) is a product of the MEP (in many prokaryotes) and mevalonate pathway (in eukaryotes and in some prokaryotes) whereas (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) is specifically produced via the MEP pathway in prokaryotes and apicomplexan parasites (and in the chloroplasts of all green plants). Taken from (Morita et al. 2007).

### 1.5.2.2 Isopentenyl pyrophosphate

Molecules structurally related to HMB-PP (with a 10,000-fold lower potency) such as isopentenyl pyrophosphate (IPP) are also capable of activating Vγ9Vδ2 T cells. IPP is a central intermediate of isoprenoid biosynthesis, synthesised by the mevalonate and or non-mevalonate pathways, which are utilised by all living eukaryotic and prokaryotic organisms (**Figure 1.8**). The mevalonate pathway is essential for cell survival. However, IPP is much less potent than HMB-PP and under normal physiological conditions, levels of IPP are not sufficient enough to induce significant Vγ9Vδ2 T cell activation (Holtmeier and Kabelitz 2005; Jomaa et al. 1999; Kistowska et al. 2008; Morita et al. 2007; Moser and Eberl 2011; Urban et al. 2010).

### 1.5.2.3 Alkylamines

Other natural compounds capable of activating Vγ9Vδ2 T cells are alkylamines, which are ubiquitously present in human body fluids of healthy individuals, plant foods such as tea, apples and wine and are also produced by certain commensal and pathogenic bacteria (Bukowski et al. 1999; Holtmeier and Kabelitz 2005; Morita et al. 2007). However their physiological relevance remains questionable as millimolar concentrations are required for them to have any stimulatory effect on Vγ9Vδ2 T cells (Eberl and Moser 2009; Moser and Eberl 2007).

### 1.5.2.4 Aminobisphosphonates

Recently, synthetic aminobisphosphonates (i.e. pamidronate and zoledronate), a class of drugs widely used to treat osteoporosis and bone metastasis, have demonstrated to be potent (*in vitro* and *in vivo*) activators of Vγ9Vδ2 T cells. Recognition of aminobisphosphonates by Vγ9Vδ2 T cells is TCR dependent and requires antigen presentation by APCs (Morita et al. 2007). Zoledronate induces Vγ9Vδ2 T cell secretion of high levels of proinflammatory cytokines and enhances cytotoxicity (required for tumour clearance), as demonstrated by several preclinical *in vitro* and *in vivo* tumour models (Benzaïd et al. 2011; Morita et al.

2007; Thedrez et al. 2007). *In vitro* studies have shown highly activated Vγ9Vδ2 T cells to kill a variety of tumour cells in a TCR-independent and dependent manner using natural killer cell-like activity and/or CD8 T cell activity, respectively (Ferrarini et al. 2002; Tanaka 2006). Furthermore, in studies involving treatment of patients with lymphoid malignancies, breast or prostate cancer, clinical evidence suggested treatment with pamidronate or zoledronate (plus IL-2), to have anticancer effects in the presence of Vγ9Vδ2 T cells (Dieli et al. 2007; Meraviglia et al. 2010; Wilhelm et al. 2003).

The effects of aminobisphosphonates is thought to be due to an indirect mechanism, whereby aminobisphosphonates function intracellularly by blocking farnesyl pyrophosphate synthase (FPP), this leads to accumulation of intracellular IPP which in turn, stimulates Vγ9Vδ2 T cells (Benzaïd et al. 2011; Holtmeier and Kabelitz 2005; Morita et al. 2007). A current novel strategy for cancer immunotherapy is to sensitise tumour cells for target killing by Vγ9Vδ2 T cells by inducing intracellular accumulation of IPP by aminobisphosphonates (Dieli et al. 2007).

### 1.5.3 Recognition and pathogen release of HMB-PP

The exact mechanism of how Vγ9Vδ2 T cells recognise and become activated by non-peptide antigens remains unclear. Attempts to show direct γδ TCR:ligand binding by crystallisation studies have failed so far. However, studies involving antibody blocking of TCR and gene transfer approaches showed that recognition of non-peptide antigens and cell activation was dependent on the Vγ9Vδ2 TCR (Bukowski et al. 1995; Morita et al. 2007; Tanaka 2006; Thedrez et al. 2007).

The Vγ9Vδ2 T cell response to non-peptide antigens is not dependent on any known antigen presenting molecule, as demonstrated by mutant APCs that lacked MHC class I, β2-microglobulin, CD1, or MHC class II molecules, but were still able to present antigens to

Vγ9Vδ2 T cells, possibly by unknown surface molecules (Eberl and Moser 2009; Morita et al. 2007). HMB-PP induced robust Vγ9Vδ2 T cell activation and proliferation requires cell-cell contact with accessory (or feeder) cells of human origin that do not need to be APCs, such as human monocytes/DCs, αβ T cells or B cells (Eberl et al. 2009; Moser and Eberl 2007, 2011).

What remains to be determined is exactly how an intracellular metabolite such as HMB-PP gets released by invading bacteria or becomes visible to the immune system. Findings have suggested uptake of whole bacteria by monocytes, macrophages, or DCs and the killing of these infected cells by Vγ9Vδ2 T cells (Dieli et al. 2001; Eberl and Moser 2009). Vγ9Vδ2 T cells have been shown to kill both extracellular and intracellular bacteria such as *Mycobacterium tuberculosis* through the release of perforin and granulysin (Dieli et al. 2001). A recent study showed Vγ9Vδ2 T cell specific response to neutrophils harbouring phagocytosed HMB-PP producing live bacteria, which was amplified in the presence of monocytes. This indicated direct communication and interplay between the three cell types in response to pathogen. From these findings it was concluded that under physiological conditions HMB-PP is unlikely to be released into the microenvironment by live micro-organisms, however, biologically relevant traces may escape after phagocytosis of extracellular bacteria by neutrophils (Davey et al. 2011).

### 1.5.4 Vγ9Vδ2 T cell migratory profile

Unlike αβ T cells, the majority of circulating Vγ9Vδ2 T cell in human adults are effector memory cells, which lack the lymph node homing receptor CCR7 but instead express multiple inflammatory chemokine receptors, such as CXCR3, CCR1, CCR2 and CCR5 (Brandes et al. 2003). Such characteristics (also shared by granulocytes, monocytes, immature DCs, and NK cells) indicate a prerequisite for the rapid recruitment of Vγ9Vδ2 T cells to tissue sites of inflammation and infection, where they may encounter recently entered pathogens (Eberl and Moser 2009). At tissue sites Vγ9Vδ2 T cells would be

activated upon exposure to HMB-PP, either released after phagocytosis by neutrophils and presented by neighbouring monocytes or presented on the surface of infected monocytes or DCs. As an innate immune response activated Vγ9Vδ2 T cells may perform immediate effector functions such as secretion of cytokines and chemokines, lysis of infected cells and processing of bacterial antigen (Hayday 2000; Moser and Eberl 2007). TCR triggering of resting Vγ9Vδ2 T cells has been shown to induce a switch from inflammatory profile to a lymph node homing phenotype (Brandes et al. 2003). A rapid and transient up-regulation of CCR7 (and other lymph node homing receptors) allows pathogen activated Vγ9Vδ2 T cells to migrate to secondary lymphoid tissue (Moser and Brandes 2006; Moser and Eberl 2007, 2011).

## 1.5.5 Vγ9Vδ2 T cell memory subsets

In freshly isolated peripheral Vγ9Vδ2 T cells, Dieli et al. (2003) identified four different functional subsets of Vγ9Vδ2 T cells based on the co-expression of surface CD45RA and CD27, as follows: $CD45RA^{+}$ $CD27^{+}$ as naïve, $CD45RA^{-}$ $CD27^{+}$ as central memory, $CD45RA^{-}CD27^{-}$ as effector memory and $CD45RA^{+}CD27^{-}$ as terminally differentiated cells. Lymph node homing receptors CCR7 and CD62L were found to be expressed on naïve and central memory Vγ9Vδ2 T cells but not on effector memory cells. Furthermore, Vγ9Vδ2 T cells found in lymph nodes expressed CCR7 and CD62L, but not the inflammatory chemokines CCR5 and CXCR3. Conversely, Vγ9Vδ2 T cells found at inflammatory sites expressed CCR5 and CXCR3 but not CCR7 and CD62L. Thus, central memory Vγ9Vδ2 T cells home to lymph nodes and upon antigen re-encounter provide a secondary immune responses with a proportion of cells differentiating into effector memory cells. On the other hand, effector memory Vγ9Vδ2 T cells migrate to peripheral tissues where they provide immediate effector functions and can further differentiate into terminally differentiated cells (Craig et al. 2012; Dieli et al. 2003).

## 1.5.6 Vγ9Vδ2 T cell effector functions

Freshly isolated resting human Vγ9Vδ2 T cells are characterised by a largely pro-inflammatory cytokine profile comprising IFN-γ, TNF-α and GM-CSF although co-expression of IL-4 is observed (Eberl et al. 2009; Sireci et al. 1997; Vermijlen et al. 2007). Importantly however, they can be skewed toward distinct effector functions depending on the microenvironment, in analogy to, and even beyond, the emerging plasticity of $CD4^+$ αβ T cells. As such, under appropriate culture conditions, Vγ9Vδ2 T cells divert from the 'default' Th1/CTL-like phenotype and assume features reminiscent of Th2 cells (Vermijlen et al. 2007; Wesch et al. 2001), Th17 cells (Caccamo et al. 2011; Ness-Schwickerath et al. 2010), Treg cells (Casetti et al. 2009) and even professional APCs (Brandes et al. 2005). This illustrates the multi-functional nature of Vγ9Vδ2 T cells, which reflects their involvement in diverse pathophysiological processes (Bonneville et al. 2010; Moser and Eberl 2011).

## 1.5.7 APC function of Vγ9Vδ2 T cells

As reported by several studies, activated human Vγ9Vδ2 T cells have the potential to act as professional APCs (Brandes et al. 2009; Brandes et al. 2005; Himoudi et al. 2012; Landmeier et al. 2009; Meuter et al. 2010; Moser and Eberl 2011; Wu et al. 2009). APC markers are found to be absent on freshly isolated resting peripheral Vγ9Vδ2 T cells, however *in vitro* stimulation with IPP or HMB-PP results in rapid up-regulation of molecules required for APC function such as, antigen presenting molecules (MHC class I and II), co-stimulatory (CD80 and CD86) and adhesion receptors (CD11a, CD18, CD54) (Brandes et al. 2005). Activated Vγ9Vδ2 T cells not only express phenotypical APC features but also function as potent and professional APCs (Brandes et al. 2009; Brandes et al. 2005; Meuter et al. 2010). As professional APCs Vγ9Vδ2 T cells have the ability to induce differentiation of naïve $CD4^+$ and $CD8^+$ T cells into cytokine secreting effector cells. Features of Vγ9Vδ2 T cell APC function are maintained over long periods of tissue culture,

which is in clear contrast to the transient APC nature of monocyte-derived DCs (Brandes et al. 2009; Brandes et al. 2005; Moser and Brandes 2006; Moser and Eberl 2011). In support to the above, a large proportion of tonsillar γδ T cells express activation markers (CD69) and high levels of MHC class II and CD80 and CD86 (Brandes et al. 2005). Also, Vγ9Vδ2 T cells were recently shown to phagocytose opsonised bacteria (Wu et al. 2009).

Furthermore, human Vγ9Vδ2 T cells have been shown to perform robust cross-presentation functions, more efficient than monocyte-derived DCs. Activated Vγ9Vδ2 T cells displaying APC features have been shown to process and cross-present antigens such as: purified influenza matrix protein M1, inactivated influenza particles and extracts from influenza-infected cells (Brandes et al. 2009; Meuter et al. 2010). Most viruses and tumours do not arise in APCs, thus elicitation of $CD8^+$ CTL response to exogenous tumour antigens (such as mutant proteins) by APCs relies on cross presentation routed via the MHC class I pathway (Meuter et al. 2010; Moser and Eberl 2011). Cross presentation of a range of mutant proteins from tumours by γδ T cell APCs could provide a novel cellular vaccine for induction of a more broader and effective tumour specific CTL response in cancer patients (Moser and Eberl 2011).

### 1.5.8 Vγ9Vδ2 T cell involvement in B cell responses

γδ T cells have been demonstrated to support antibody production in immunised and infected mice (Huang et al. 2009; McMenamin et al. 1994; Wen et al. 1996). GCs are present in TCRα-/- and TCRβ-/- mice and develop in SCID mice upon adoptive transfer of γδ T cells and B cells, demonstrating that γδ T cells are sufficient to orchestrate follicular responses (Dianda et al. 1996; Pao et al. 1996; Wen et al. 1996) (**Figure 1.9**). In humans, γδ T cells can be found in secondary lymphoid tissues (Brandes et al. 2003; Dieli et al. 2003; Groh et al. 1989), where they are scattered throughout the T zone and clustered within reactive GCs (**Figure 1.10**), but not in primary follicles lacking GCs.

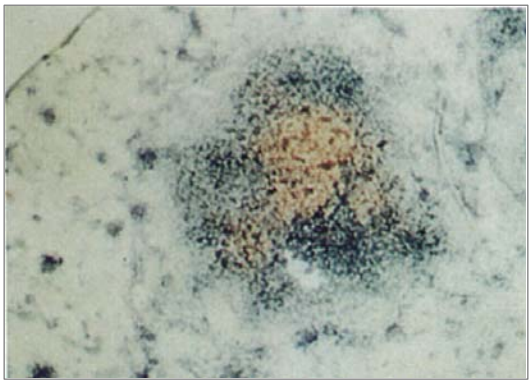

**Figure 1.9. Murine γδ T cells support the formation of germinal centres in severe combined immunodeficiency (SCID) mice.**
SCID mice are a strain of mice lacking in T and B lymphocytes and immunoglobulins. Adoptive transfer of splenocytes from mice congenitally deficient in αβ T cells resulted in the formation of GCs in SCID mice. GCs were probed with peanut agglutinin (PNA); clusters of cells are shown in brown. Picture taken from (Wen et al. 1996).

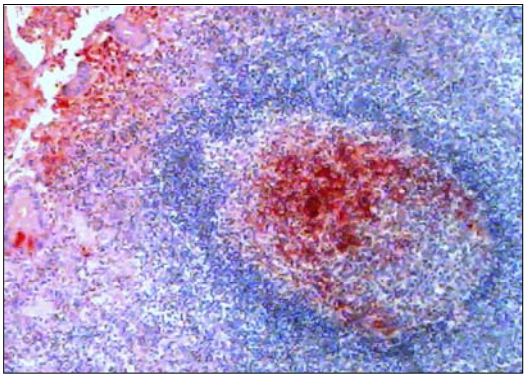

**Figure 1.10. Clusters of human γδ T cells found within a reactive germinal centre.**
A tissue section of an active gut follicle from a patient with *Yersinia* ileitis, immunohistochemical staining for γδ T cells shown in red. Picture taken from (Vermijlen et al. 2007).

Furthermore, a higher number of γδ T cells were found in disease-associated lymph nodes in comparison to non-involved lymph nodes (Brandes et al. 2003).

Early studies in lupus patients led to the isolation of human γδ T cell lines capable of inducing autoantibody production by autologous B cells (Rajagopalan et al. 1990). Subsequent investigations demonstrated that *in vitro* activated human γδ T cells, stimulated with IPP, or the T cell specific mitogen phytohaemagglutinin (PHA), or with the synthetic compound bromohydrin pyrophosphate (BrHPP), can readily provide help to B cells via contact-dependent mechanisms, involving CD40L and ICOS and soluble factors including IL-4 and IL-10 (Brandes et al. 2003; Caccamo et al. 2006a). Activated human γδ T cells may also express additional markers of relevance for a possible interaction with B cells including OX40, PD-1, CD27 and CD70 (Brandes et al. 2003; DeBarros et al. 2011; Iwasaki et al. 2011). CXCR5 expression is required for follicular homing and GC localisation, which has been detected on peripheral human Vγ9Vδ2 T cells by some (Caccamo et al. 2006a) but not by others (Brandes et al. 2003), therefore the regulation of CXCR5 expression on peripheral human Vγ9Vδ2 T cells is still a subject of debate. More recent data from microarray studies revealed that Vγ9Vδ2 T cells express a range of Tfh cell associated molecules when stimulated with HMB-PP in the presence of IL-21, including the B cell attracting chemokine CXCL13, thus suggesting a potential role in the GC reaction (Vermijlen et al. 2007).

Collectively, all the above findings strongly suggest γδ T cells to possess Tfh-like B cell helper qualities, however a number of questions still remain, such as, exactly how Vγ9Vδ2 T cells acquire the ability to provide B cell help in secondary lymphoid tissue and what the possible implications are for humoral immune responses in infection, autoimmunity and in immunotherapy.

## 1.6 Hypothesis and aims

The overall aim of this study was to gain a better understanding of Vγ9Vδ2 T cell role in humoral immune response and to provide a functional validation of previous microarray data/findings (Vermijlen et al. 2007). I here hypothesised that in response to HMB-PP during microbial infection activated γδ T cells respond to IL-21 produced by Tfh cells. This induces or modulates γδ T cell phenotypes associated with B cell help, thus allowing γδ T cells to interact with B cells leading to antibody production and class switching.

Specific aims of this study were to investigate the following:

1. The effects of IL-21 in regulating expression of IL-21R, CXCL13, CXCR5, co-stimulatory molecules and APC markers on *in vitro* HMB-PP stimulated peripheral Vγ9Vδ2 T cells.

2. Expression of IL-21R, CXCL13, CXCR5, co-stimulatory molecules and APC markers by Vγ9Vδ2 T cells in secondary lymphoid tissue and by *ex vivo* IL-21 and HMB-PP stimulated tonsillar Vγ9Vδ2 T cells.

3. The ability and mechanism of *in vitro* activated Vγ9Vδ2 T cells to provide B cell help.

# Chapter 2:

# Materials and Methods

# 2.1 Cell isolation

## 2.1.1 Isolation of peripheral blood mononuclear cells

Peripheral blood obtained from healthy adult donors by venous puncture was collected into a tube containing anti-coagulation buffer (PBS containing 20 U/ml Heparin and 15 mM EDTA), a 1:5 dilution of anti-coagulation buffer to venous blood was used. Peripheral blood mononuclear cells (PBMCs) were isolated from donor blood by density gradient centrifugation using Lymphoprep$^{TM}$ (Axis-Shield). Lymphoprep$^{TM}$ is a ready made sterile and endotoxin tested solution for the isolation of pure mononuclear cells, which contains sodium diatrizoate 9.1% (w/v) and polysaccharide 5.7% (w/v). Peripheral blood was diluted with an equal volume of sterile phosphate buffered saline (PBS) solution (deionised $H_2O$ containing 1 X PBS). 20 mls of Lymphoprep$^{TM}$ was added to a 50 ml Falcon tube and 30 mls of diluted blood was carefully layered on top, avoiding any disruption of the two layers. Tubes were centrifuged in a swing-out rotor, at room temperature (approximately 20°C) for 25 minutes at 1500 rpm (450 x g) with no brake. After centrifugation the cloudy layer of mononuclear cells formed at the sample/ Lymphoprep$^{TM}$ interface were carefully harvested using a Pasteur pipette. The harvested mononuclear fraction was washed twice by diluting cells in either PBMC wash buffer (PBS containing 2% foetal calf serum (FCS) and 2 mM EDTA) or in RPMI-1640 medium and centrifuged at 1300 rpm (300 x g, 4°C, for 10 minutes). Supernatants from wash steps were discarded and cells were re-suspended in complete RPMI-medium for culturing or in PBMC wash buffer for antibody staining.

## 2.1.2 Isolation of mononuclear cells from lymphoid tissue

In order to study cells from secondary lymphoid tissues, mononuclear cells were freshly isolated from the following tissues: fresh tonsils (palatine tonsils) of patients undergoing tonsillectomy; fresh appendix tissue of patients undergoing appendectomy; or from biopsy specimens of the gastrointestinal tract of patients undergoing diagnostic colonoscopy. Performed under sterile conditions, simple mechanical disaggregation was used to obtain single cell suspensions from primary tissue such as tonsils, appendix and colon biopsies. To preserve maximum cell viability and avoid any enzymatic manipulation/destruction of cell surface proteins I chose not to use any enzymatic disaggregation methods. To remove any blood, tissue samples were washed with PBS before being placed in a dish containing RPMI-1640 medium (without supplements). Tissue was cut with scissors into small pieces and then mashed through a stainless steel mesh cell filter. To separate the mechanically released cells from any tissue fragments homogenised tissue was re-suspended in RPMI-1640 medium and passed through a 70 μM cell strainer followed by a 40 μM cell strainer (BD Biosciences). The resulting single cell suspension was washed twice by pelleting the cells by centrifugation (300 x g, 4°C, 10 minutes) and re-suspending in RPMI-1640 medium. Mononuclear cells were isolated from tissue derived cell suspensions by density gradient centrifugation using Lymphoprep$^{TM}$ (as described above, in section 2.1.1).

## 2.1.3 Isolation of mononuclear cells from peritoneal dialysis fluid

Patients on continuous ambulatory peritoneal dialysis (CAPD) for ≤5 years were recruited from the Peritoneal Dialysis Unit, University Hospital of Wales, Cardiff. Diagnosis of CAPD-associated peritonitis was based on the presence of abdominal pain, a cloudy peritoneal effluent with a leukocyte count $>10^5$ per ml, and a positive microbiological culture (data not shown). Two litres of overnight dwell effluent bags were collected on ice from patients with acute peritonitis. Peritoneal effluent cells were harvested from chilled

peritoneal effluents by centrifugation at 1,200 *g* and 4°C for 25-30 minutes by Chan-Yu Lin (as described by Eberl et al. 2009 and Davey et al. 2011).

## 2.2 Cell sorting

All cell sorting was carried out under aseptic conditions in a Class II laminar airflow unit, cells and reagents were kept on ice throughout the cell sorting procedure.

Single cell suspensions were sorted by magnetic activated cell sorting (MACS) using MACS® column technology (Miltenyi Biotech). MACS® column technology is based on the use of MACS microbeads, MACS columns and MACS separators. Cells are directly or indirectly labelled with magnetic beads and are separated over a MACS column placed in the magnetic field of a MACS separator (a strong permanent magnet). Magnetically labelled cells are retained on the column, while unlabelled cells pass through. The retained cells are eluted from the MACS column after removal from the magnet.

In cases where high purities could not be achieved by magnetic cell sorting using MACS®, cells were further sorted to higher purities using a MoFlo cell sorter (Dako cytomation), which was operated by a core facility (Central Biotechnology Services; CBS) technician. The MoFlo cell sorter is a high-speed cell sorter flow cytometer (8-color, 3-laser system) which can concurrently sort up to four cell populations.

### 2.2.1 Cell sorting for co-culture experiments

For co-culture experiments, cells were isolated by magnetic cell sorting using MACS® column technology. Peripheral Vγ9Vδ2 T cells were positively isolated (>97%) using Vδ2-

PE (B6.1; BD Biosciences) or Vγ9-PE-Cy5 (Immu360; Beckman-Coulter) antibodies and anti-PE microbeads, followed by isolation of peripheral B cells or monocytes (as feeder cells) by positive selection using anti-CD19 microbeads (>98%) or anti-CD14 microbeads (>96%), respectively, as described below.

**Sample preparation for γδ T cell isolation**. Pellet from PBMC isolation was re-suspended in 1 ml MACS buffer (PBS containing 5 mM EDTA and 2% FCS) in a 15 ml falcon tube. Before staining, Fc receptors on cells were blocked for 10 minutes at 4°C (on ice) using human Ig serum (1:100; Kiovig, Baxter). For γδ surface labelling, Vδ2-PE or Vγ9-PE-Cy5 antibodies were added to cell suspension at appropriate concentrations (see Table 2.1) and mixed by pipetting, then incubated in the dark at 4°C (on ice) for 30 minutes. To remove excess unbound antibody, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes), the supernatant was discarded. Cells were magnetically labelled with microbeads by re-suspending pellet in 1 part anti-PE microbeads to 4 parts MACS buffer (i.e. 10 µl beads + 40 µl MACS buffer was added per $10^7$ total cells) and incubated in the dark at 4-8°C (in the fridge) for 15 minutes. To remove excess unbound microbeads, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). The supernatant was discarded and cells were re-suspended in 2 ml MACS buffer.

**Magnetic separation of γδ T cells**. A LS MACS column was placed in the magnetic field of a MACS separator. The column was rinsed by adding 2 ml of MACS buffer before proceeding to magnetic separation. The cell suspension was applied to the column and the unlabelled fraction of cells that passed through the column (flow through) was collected into a 15 ml Falcon tube. The column was washed with 2 ml MACS buffer; this was also collected into the same 15 ml Falcon tube. The flow through containing the unlabelled fraction of cells was kept aside on ice for B cell or monocyte isolation. To get rid of any unlabelled cells, the column was further washed by adding 2 ml of MACS buffer. Wash step was repeated three times and the buffer collected from wash steps was discarded. The column was removed from the magnetic separator and placed on a 15 ml Falcon collection

tube. 5 ml of MACS buffer was pipetted onto the column and the magnetically labelled γδ T cells were flushed out by firmly pushing the plunger into the column.

**Sample preparation for B cell or monocyte isolation.** For feeder cells, B cells or monocytes were positively isolated from the γδ T cell depleted population. The unlabelled fraction of cells were centrifuged (300 x g, 4°C, 10 minutes), the supernatant was discarded and the cell pellet was re-suspended in 1 part anti-CD19 microbeads (for B cell isolation) or in 1 part anti-CD14 microbeads (for monocyte isolation) to 4 parts MACS buffer (100 µl beads + 400 µl MACS buffer), cells were incubated in the dark at 4-8°C (in the fridge) for 15 minutes. To remove excess unbound microbeads, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). The supernatant was discarded and cells were re-suspended in 2 ml MACS buffer.

**Magnetic separation of B cells or monocytes.** A LS MACS column was placed in the magnetic field of a MACS separator. The column was rinsed by adding 2 ml of MACS buffer before proceeding to magnetic separation. The cell suspension was applied to the column and the unlabelled fraction of cells that passed through the column was collected into a 15 ml Falcon tube. The column was washed with 2 ml MACS buffer; this was also collected into the same 15 ml Falcon tube. To get rid of any unlabelled cells, the column was further washed by adding 2 ml of MACS buffer. Wash step was repeated three times and the buffer collected from wash steps was discarded. The column was removed from the magnetic separator and placed on a 15 ml Falcon collection tube. 5 ml of MACS buffer was pipetted onto the column and the magnetically labelled cells were flushed out by firmly pushing the plunger into the column. For use as feeder cells in co-culture experiments, isolated peripheral monocytes or B cells were irradiated at 30 Gy (3000 rad). Purities of cell populations obtained were determined by flow cytometry and total cell numbers obtained were determined by manual cell counting using a haemocytometer.

## 2.2.2 Cell sorting for functional assays

Functional assays for investigating B cell help were carried out using cells purified from either freshly isolated PBMCs or tonsillar mononuclear cells or both. Using MACS® technology peripheral Vγ9Vδ2 T cells (>97%) were isolated using Vδ2-PE antibodies and anti-PE microbeads, as feeder cells peripheral B cells (>98%) were purified from Vγ9Vδ2 T cell depleted PBMC using anti-CD19 microbeads (as described above) or for antibody production by using the untouched B cell isolation kit (>95%) (as described below).

Tonsillar Tfh cells ($CXCR5^+$ $CD4^+$ T cells; >85%) and tonsillar $CXCR5^+$ B cells (>95%) were purified by magnetic cell sorting, tonsillar Vγ9Vδ2 T cells (>95%) were purified by MACS® technology followed by a MoFlo cell sorter, as follows:

**Tonsillar Tfh cell isolation.** Pellet from PBMC isolation was re-suspended in 1 ml MACS buffer (PBS containing 5 mM EDTA and 2% FCS) in a 15 ml falcon tube. Before staining, Fc receptors on cells were blocked for 10 minutes at 4°C (on ice) using human Ig serum (1:100). For CD4 T cell surface labelling, anti-CD4-FITC labelled antibodies were added to cell suspension at appropriate concentrations (see Table 2.1) and mixed by pipetting, then incubated in the dark at 4°C (on ice) for 30 minutes. To remove excess unbound antibody, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). FITC labelled $CD4^+$ T cells were magnetically labelled by re-suspending pellet in 90 µl of buffer and 10 µl of anti-FITC multisort microbeads per $10^7$ total cells and incubated in the dark at 4-8°C (in the fridge) for 15 minutes. To remove excess unbound microbeads, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). The supernatant was discarded and cells were re-suspended in 2 ml MACS buffer. $CD4^+$ T cells were magnetically separated by positive selection using an LS column. A LS MACS column was placed in the magnetic field of a MACS separator. The column was rinsed by adding 2 ml of MACS buffer before proceeding to magnetic separation. The cell suspension was applied to the column and the unlabelled fraction of cells that passed through the column was collected into a 15 ml

Falcon tube. The column was washed with 2 ml MACS buffer; this was also collected into the same 15 ml Falcon tube. The flow through containing the unlabelled fraction of cells was kept aside on ice for γδ and B cell isolation. To get rid of any unlabelled cells, the column was further washed by adding 2 ml of MACS buffer. Wash step was repeated three times and the buffer collected from wash steps was discarded. The column was removed from the magnetic separator and placed on a 15 ml Falcon collection tube. 5 ml of MACS buffer was pipetted onto the column and the magnetically labelled $CD4^+$ T cells were flushed out by firmly pushing the plunger into the column. Following positive $CD4^+$ T cell selection (>98 %) the magnetic particles were removed from the cells by adding 20 µl of multisort Release Reagent per 1 ml cell suspension, cells were incubated for 10 minutes at 4-8°C (in the fridge) . To remove any residual magnetically labelled cells, magnetic separation was repeated over a new column.

To isolate tonsillar Tfh cells, purified tonsillar $CD4^+$ T cells were stained with anti-CXCR5-PE labelled antibodies (incubated in the dark at 4°C on ice for 30 minutes). To remove excess unbound antibody, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). PE labelled $CXCR5^+$ $CD4^+$ T cells were magnetically labelled by re-suspending pellet in 1 part anti-PE microbeads to 4 parts MACS buffer (100 µl beads + 400 µl MACS buffer) and incubated in the dark at 4-8°C (in the fridge) for 15 minutes. To remove excess unbound microbeads, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). The supernatant was discarded and cells were re-suspended in 2 ml MACS buffer. $CXCR5^+$ $CD4^+$ T cells (Tfh cells) were magnetically separated by positive selection using an LS column. A LS MACS column was placed in the magnetic field of a MACS separator. The column was rinsed by adding 2 ml of MACS buffer before proceeding to magnetic separation. The cell suspension was applied to the column and the unlabelled fraction of cells that passed through the column was collected into a 15 ml Falcon tube. The column was washed with 2 ml MACS buffer; this was also collected into the same 15 ml Falcon tube. To get rid of any unlabelled cells, the column was further washed by adding 2 ml of MACS buffer. Wash step was repeated three times and the buffer collected from wash steps was discarded. The column was removed from the magnetic separator and placed on a 15 ml Falcon collection tube. 5 ml of MACS buffer was pipetted onto the column and the

magnetically labelled $CXCR5^+$ $CD4^+$ T cells were flushed out by firmly pushing the plunger into the column.

**Tonsillar Vγ9Vδ2 T cell isolation**. Tonsillar Vγ9Vδ2 T cells were positively isolated from the CD4 T cell depleted population by magnetic cell sorting using Vδ2-PE antibodies and anti-PE microbeads (as described above for peripheral Vγ9Vδ2 T cell isolation), resulting in 20-24% purity. For a higher purity Vγ9Vδ2 positive cells were further purified by flow cytometry using a MoFlo cell sorter as follows. For selection of $CD3^+$ Vγ9Vδ2 T cells and exclusion of B cells (and any remaining CD4-FITC labelled cells) the enriched Vδ2 population (already stained with anti-Vδ2-PE) was stained with anti-CD3-APC, Vg9-PeCy5 and CD19-FITC (incubated in the dark on 4°C for 30 minutes). To remove excess unbound antibody, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). Cells were re-suspended in MACs buffer to a concentration of 10 million/ml for moflo sorting. Vγ9Vδ2 T cells were sorted as $V\gamma9^+$ $V\delta2^+$ $CD3^+$ $CD4^-$ $CD19^-$ lymphocytes, unstained and single stained controls were used for compensation. Sorted Vγ9Vδ2 T cells were collected in a tube containing 1 ml of FCS.

**Tonsillar $CXCR5^+$ B cell isolation.** Tonsillar B cells were isolated from the CD4 and Vγ9Vδ2 T cell depleted population using the untouched B cell isolation kit from Miltenyi Biotech. The B cell isolation kit consists of two components, a biotin-antibody cocktail (containing biotin-conjugated monoclonal antibodies against CD2, CD14, CD16, CD36, CD43 and CD235a) and anti-biotin microbeads. Cells were centrifuged (300 x g, 4°C, 10 minutes), the supernatant was discarded and the cell pellet was re-suspended in 200 µl of MACS buffer and 50 µl of biotin-antibody cocktail per $10^7$ cells. After 10 minutes of incubation in the dark at 4-8°C (in the fridge), 30 µl of MACS buffer and 20 µl of anti-biotin microbeads per $10^7$ cells, were added and left to incubate for a further 15 minutes in the dark at 4-8°C (in the fridge). To remove excess unbound microbeads, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). The supernatant was discarded and cells were re-suspended in 2 ml MACS buffer. A LS MACS column was placed in the magnetic field of a MACS separator. The column was rinsed by adding 2 ml of MACS buffer before

proceeding to magnetic separation. The cell suspension was applied to the column and the unlabelled fraction of cells (purified CD19 positive B cells) that passed through the column was collected into a 15 ml Falcon tube. The column was washed with 2 ml MACS buffer, this was also collected into the same 15 ml Falcon tube. The column was removed from the magnetic separator and placed on a 15 ml Falcon collection tube. 5 ml of MACS buffer was pipetted onto the column and the magnetically labelled cells (non-B cells) were flushed out by firmly pushing the plunger into the column.

To isolate tonsillar $CXCR5^+$ B cells, purified unlabelled $CD19^+$ B cells were stained with anti-CXCR5-PE labelled antibodies (incubated in the dark at 4°C on ice for 30 minutes). To remove excess unbound antibody, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). PE labelled $CXCR5^+$ $CD19^+$ B cells were magnetically labelled by re-suspending pellet in 1 part anti-PE microbeads to 4 parts MACS buffer (100 µl beads + 400 µl MACS buffer) and incubated in the dark at 4-8°C (in the fridge) for 15 minutes. To remove excess unbound microbeads, cells were washed once in MACS buffer (300 x g, 4°C, 10 minutes). The supernatant was discarded and cells were re-suspended in 2 ml MACS buffer. $CXCR5^+$ $CD19^+$ B cells were magnetically separated by positive selection using an LS column. A LS MACS column was placed in the magnetic field of a MACS separator. The column was rinsed by adding 2 ml of MACS buffer before proceeding to magnetic separation. The cell suspension was applied to the column and the unlabelled fraction of cells that passed through the column was collected into a 15 ml Falcon tube. The column was washed with 2 ml MACS buffer; this was also collected into the same 15 ml Falcon tube. To get rid of any unlabelled cells, the column was further washed by adding 2 ml of MACS buffer. Wash step was repeated three times and the buffer collected from wash steps was discarded. The column was removed from the magnetic separator and placed on a 15 ml Falcon collection tube. 5 ml of MACS buffer was pipetted onto the column and the magnetically labelled $CXCR5^+$ $CD19^+$ B cells were flushed out by firmly pushing the plunger into the column. Purities of cell populations obtained were determined by flow cytometry and total cell numbers obtained were determined by manual cell counting using a haemocytometer.

## 2.3 Cell counting

To determine the number of cells per ml, manual cells counts were performed using a haemocytometer. A 15 µl aliquot of evenly suspended cells was applied to the haemocytometer chamber and allowed to settle for 20 seconds. The main divisions separate the grid into 9 large squares; each large square has surface area of 1 mm-squared and a depth of 0.1 mm, giving it a volume of 0.1 mm-cubed. Each large corner square is divided into 16 squares, the number of cells in one set of 16 corner squares is equivalent to the number of cells x $10^4$/ml (Experimental Biosciences) (**Figure 2.1**). Cells were counted in each of the four large corner squares by focusing on the grid lines of the haemocytometer using the 10X objective of the microscope. Cell count was determined using the following formula:

$$\text{Number of cells/ml} = \text{average cell count per square} \times 10^4$$

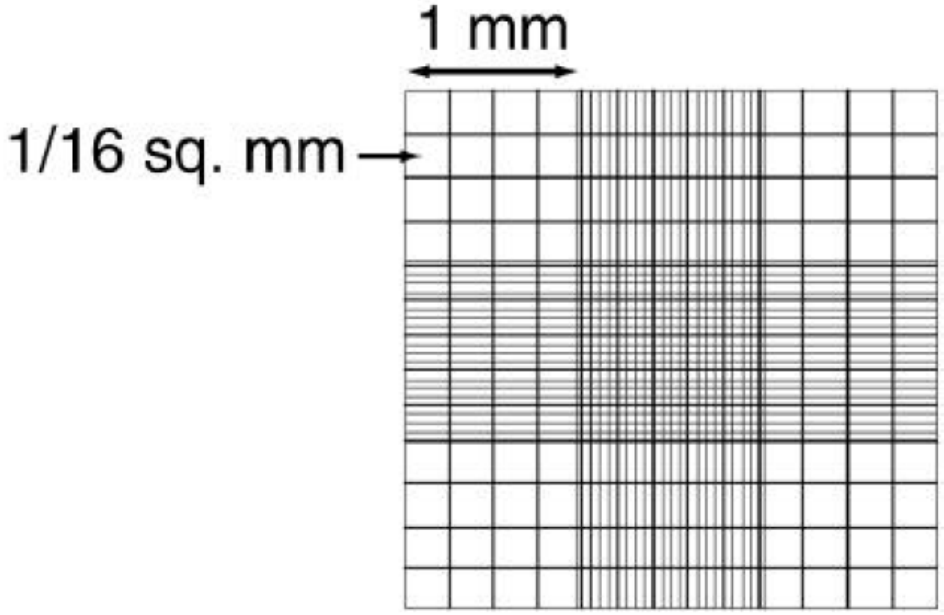


**Figure 2.1. Schematic diagram showing gridlines on a haemocytometer grid.** Taken from (Experimental Biosciences).

## 2.4 Cell culture

The cell culture medium used throughout this study was RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 50 μg/ml penicillin/streptomycin, 50 μM β-mercaptoethanol, and 10% FCS (Invitrogen). FCS added to medium was first heat inactivated (56°C for 30 minutes) in order to inactivate complement components and aliquated (50 ml) for storage at -20°C. All cells were cultured in U-round bottomed 96 well plates (Sigma-Aldrich, Kent, UK) at 37°C in a humidified incubator with 5% CO2. All cultures procedures were carried out under aseptic conditions in a Class II laminar airflow unit, using tissue culture grade plasticware and reagents.

### 2.4.1 Phenotypic assays

Surface marker expression on freshly isolated and stimulated Vγ9Vδ2 T cells, $CD4^+$ T cells and B cells was studied as follows. Total PBMC or tonsillar mononuclear cells or sorted γδ T cells (co-cultured with sorted B cells) were plated at 0.1 to $0.3x10^6$ cells per well of a 96-well round bottom plate and cultured for up to 7 days in the presence of 0.1-100 nM HMB-PP (synthetic, LPS-free; kindly provided by Dr Hassan Jomaa, University Hospital Giessen and Marburg, Giessen, Germany) with or without 0.1-100 U/ml IL-2 or 0.1-100 ng/ml IL-21 (kindly provided by Dr Donald Foster, Zymogenetics, Seattle, USA). As controls, cells were stimulated with 0.1-100 U/ml IL-2 or 0.1-100 ng/ml IL-21 alone, or with 1 μg/ml phytohemagglutinin (PHA; Sigma) or with 10 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma) and 1 μg/ml ionomycin (Sigma) or in medium alone. After 0, 1, 2, 3, 4, 5, 6, and 7 days in culture cells were harvested and stained with fluorochrome-conjugated antibodies targeted towards specific markers and analysed by flow cytometry.

## 2.4.2 CXCL13 and cytokine production assays

For analysis of CXCL13 and cytokine production by peripheral γδ T cells, purified peripheral γδ T cells were co-cultured with feeder cells (purified peripheral irradiated monocytes or irradiated B cells). Cells were plated at $10^5$ cells of each T and feeder cell, per well of a 96-well round bottom plate and cultured in medium alone, or in the presence of 0.1-100 nM HMB-PP with or without 0.1-100 U/ml IL-2 or 0.1-100 ng/ml IL-21. After 2-3 days culture supernatants were harvested and concentration of CXCL13 and IL-17 was measurement by enzyme-linked immunosorbent assay (ELISA). Cells were stained and analysed for intracellular detection of CXCL13 (by flow cytometry and immunofluorescence microscopy) and cytokines (by flow cytometry).

For analysis of intracellular CXCL13 and cytokine expression in tonsillar γδ and $CD4^+$ T cells, isolated tonsillar mononuclear cells were plated at $0.3x10^6$ cells per well of a 96-well round bottom plate. To induce robust cell activation and stimulate cytokine production cells were treated with PMA (10 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (20 ug/ml). After 3 hours, cells were harvested and stained with fluorochrome-conjugated antibodies and analysed by flow cytometry.

## 2.4.3 Functional assays

**Tonsillar B cell help (Part i).** The ability of pre-stimulated peripheral Vγ9Vδ2 T cells to induce antibody production by tonsillar B cells was studied using a non-autologous system as follows. To induce acquisition of a Tfh-like phenotype purified peripheral blood Vγ9Vδ2 T cells were pre-activated *in vitro* with 10 nM HMB-PP or in medium alone in the presence of purified irradiated autologous peripheral B cells. After 42 hours, HMB-PP stimulated cultures were treated with or without 50 ng/ml IL-21 or 20 U/ml IL-2. Non-stimulated Vγ9Vδ2 T cells cultured in medium alone served as negative control. After a

further 24 hours, stimulated and non-stimulated γδ T cells were harvested and washed several times to ensure removal of any residual HMB-PP, IL-21 and IL-2, thus minimising direct action of IL-21 or IL-2 on B cells. For antibody production $CXCR5^+$ B cells and Tfh cells were purified from tonsillar mononuclear cells of the same donor. Non-stimulated (medium), HMB-PP, IL-2+HMB-PP or IL-21+HMB-PP pre-stimulated peripheral γδ T cells were co-cultured with an equal number of purified tonsillar $CXCR5^+$ B cells, unless otherwise indicated. Cells were plated at $10^5$ cells of each γδ T and B cell per well of a 96-well round bottom plate in the absence or presence of either 10 nM HMB-PP or with an equal number of tonsillar Tfh cells to B cells. As controls, tonsillar $CXCR5^+$ B cells ($10^5$ cells/well) were cultured in medium alone, or with an equal number of Tfh cells or in the presence of 0.1-50 ng/ml of IL-21 alone. After 10 days of culture, total IgM, IgG and IgA levels in culture supernatants were measured by ELISA.

**Tonsillar B cell help (Part ii).** The ability of tonsillar Vγ9Vδ2 T cells to induce antibody production by tonsillar B cells was studied using an autologous system as follows. Mononuclear cells were freshly isolated from inflamed tonsils. All three cell types, Vγ9Vδ2 T cells, $CXCR5^+$ B cells and Tfh cells were purified from tonsillar mononuclear cells of the same donor. In co-culture experiments B cells were cultured with an equal number of γδ T cells or Tfh cells. In triple cultures Vγ9Vδ2 T cells, B cells and Tfh cells were cultured at a ratio of 1:1:1, unless otherwise indicated, i.e. in experiments involving titration of γδ T cells the number of Vγ9Vδ2 T cells to B cells was decreased, but the number of Tfh cells to B cells remained equal. Cells were plated at $5 \times 10^4$ cells of each T and B cell per well of a 96-well round bottom plate in medium alone, or in the presence of 10 nM HMB-PP with or without 20 U/ml IL-2 or 50 ng/ml IL-21, or with 50 ng/ml IL-21 alone. As controls tonsillar B cells ($5 \times 10^4$ cells/well) were cultured in medium alone, or stimulated with 0.01% *Staphylococcus aureus* Cowan 1 (SAC) (PANSORBIN® cells, Calbiochem) in the presence of 100 U/ml IL-2, or with 10 nM HMB-PP in the presence or absence of 20 U/ml IL-2 or 50 ng/ml IL-21, or with 50 ng/ml IL-21 alone. To prevent occurrence and carry over of fungal contamination from tonsillar tissue, all cultures and medium used throughout was treated with 2.5 μl/ml of fungizone® an anti-fungal antibiotic

(Invitrogen). After 10 days of culture, total IgM, IgG, IgA and IgE levels in culture supernatants were measured by ELISA.

**Peripheral B cell help.** The ability of peripheral Vγ9Vδ2 T cells to induce peripheral B cell activation, APC marker expression, differentiation and antibody production was studied using an autologous system as follows. Purified peripheral B cells were co-cultured with an equal number of purified peripheral γδ T cells (obtained from the same donor), unless otherwise indicated. In experiments involving titration of γδ T cells the number of Vγ9Vδ2 T cells to B cells was decreased 4-fold from a 1:1 ratio to a 1:64 ratio. Cells were plated at $10^5$ or 8 x $10^4$ cells of each γδ T and B cell per well of a 96-well round bottom plate, in medium alone, or in the presence of 10 nM HMB-PP with or without 20 U/ml IL-2 or 50 ng/ml IL-21, or with 20 U/ml IL-2 alone or 50 ng/ml IL-21 alone. As controls B cells ($10^5$ or 8 x $10^4$ cells) were cultured in medium alone or with 0.01% SAC and 100 U/ml IL-2, in the presence of 10 nM HMB-PP with or without 20 U/ml IL-2 or 50 ng/ml IL-21, or with 20 U/ml IL-2 alone or 50 ng/ml IL-21 alone. After 3 days, cells were harvested and stained for activation (CD69 and CD25), APC (CD86, CD40 and HLA-DR) and naïve/memory (IgD and CD27) markers in combination with CD3, CD19 and Vδ2 or Vγ9 surface markers before being analysed by flow cytometry. After 10 days of culture, total IgM, IgG, IgA and IgE levels in culture supernatants were measured by ELISA.

# 2.5 Flow cytometry

## 2.5.1 Antibodies and cell staining

Anti-human antibodies together with appropriate isotype controls and secondary reagents used for flow cytometric analysis are described in the table below (**Table 2.1**). The correct dilutions of the antibodies to give efficient staining were established by titration of the antibodies by different members of our lab group. All cell staining was performed in U-round bottomed 96 well plates (Sigma-Aldrich, Kent,UK) and all wash steps were carried out by re-suspending cells in FACS buffer and by centrifugation at 300 x g, (4°C, 3 minutes). For antibody staining, depending on the total number of cells per well of a 96-well plate, antibody cocktails were made up in a final volume of 30-50 µl of staining buffer per well/sample. For example, a total volume of 50 µl was used per 1 x $10^6$ cells/well.

**Cell staining for surface phenotyping.** To eliminate dead cells from flow cytometric analysis a fixable Live/Dead Aqua viability stain (LIVE/DEAD® Aqua; Invitrogen) was used prior to antibody staining. For this, cells were washed twice in PBS and 3 to 6 µl of diluted (1:10; in PBS) live/dead aqua was added directly to the cell pellet. Cells were left to stain at room temperature for 15 minutes in the dark then washed twice in FACS buffer. Before antibody staining, Fc receptors on cells were blocked to reduce non-specific background staining using 10 mg/ml of purified human Igs diluted in FACS buffer (PBS containing 5 mM EDTA and 2% FCS) for 10 minutes at 4°C. For staining of surface markers, 30 µl to 50 µl of diluted (in FACS buffer) antibody along with isotype-matched controls were added to the cells and incubated for 20 minutes at 4°C in the dark. To remove unbound antibodies cells were washed twice in FACS buffer. Cells stained with unlabelled antibodies were further incubated for 20 minutes in 30 to 50 µl of diluted (in FACS buffer) biotinylated or fluorochrome labelled secondary antibody and washed twice in FACS buffer to remove unbound secondary antibodies. For biotinylated antibodies, cells were

additionally incubated for 20 minutes in 30 to 50 µl of conjugated streptavidin diluted in FACS buffer and washed twice in FACS buffer to remove unbound streptavidin. Cells were re-suspended in 200 to 400 µl of FACS buffer and immediately acquired/analysed by flow cytometery.

**Intracellular staining for CXCL13 and cytokines.** For intracellular detection of CXCL13 and cytokines brefeldin A (Sigma) was added to cultures at 10 µg/ml 4 hours prior to harvesting. To remove brefeldin A, cells were washed twice in PBS (300 x g, 4°C, 3 minutes) and then stained with LIVE/DEAD® fixable aqua stain. Cells were washed twice in PBS before being fixed and permeabilized using the Fix&Perm kit (eBioscience). Subsequent washes and incubations were performed using permeabilization buffer (eBioscience). Prior to staining cells were washed once and Fc receptors were blocked for 10 minutes at 4°C in the dark using 10 mg/ml of purified human Igs diluted in permeabilization buffer. Cells were incubated with mouse anti-CXCL13 or with an isotype control antibody (mouse IgG1; MR9) for 30 minutes at room temperature. Cells were washed twice and incubated (in dark, 20 minutes at RT) with an Alexa fluor488-conjugated goat anti-mouse secondary antibody (Dako). Cells were washed twice and incubated for 15 minutes at RT in the dark with 10 mg/ml of purified mouse Igs diluted in Permeabilization buffer. Cells were further incubated with antibodies targeting specific surface markers or intracellular cytokines, along with isotype-matched controls and left for 20 minutes at 4°C in the dark. Cells were re-suspended in 200 to 400 µl of FACS buffer and immediately acquired/analysed by flow cytometery.

| Antibody | Fluorochrome | Clone | Species and Isotype | Company | Final Dilution |
|---|---|---|---|---|---|
| ***Primary surface*** | | | | | |
| Vδ1 | FITC | TS8.2 | mouse IgG1 | Endogen | 1:10 |
| Vδ2 | PE | B6.1 | mouse IgG1 | BD Biosciences | 1:60 |
| Vδ2 | FITC | B6.1 | mouse IgG1 | BD Biosciences | 1:60 |
| Vγ9 | PE-Cy5 | Immu360 | mouse IgG1 | Beckman Coulter | 1:400 |
| CD3 | APC | HIT3a | mouse IgG2a | BD Biosciences | 1:20 |
| CD3 | APC-H7 | SK7 | mouse IgG1 | BD Biosciences | 1:80 |
| CD3 | Pacific Blue | UCHT1 | mouse IgG1 | BD Biosciences | 1:100 |
| CD4 | FITC | RPA-T4 | mouse IgG1 | BD Biosciences | 1:20 |
| CD4 | ECD | SFCI12T4D11 | mouse IgG1 | Beckman Coulter | 1:100 |
| CD8 | PeCy7 | SK1 | mouse IgG1 | BD Biosciences | 1:600 |
| CD14 | FITC | MOP9 | mouse IgG2b | BD Biosciences | 1:20 |
| CD14 | Pacific Blue | M5E2 | mouse IgG1 | BD Biosciences | 1:40 |
| CD19 | FITC | HD37 | mouse IgG1 | DAKO | 1:50 |
| CD19 | APC | SJ25C1 | mouse IgG1 | eBioscience | 1:800 |
| CD25 | APC-H7 | M-A251 | mouse IgG1 | BD Biosciences | 1:50 |
| CD25 | PE-Cy5 | M-A251 | mouse IgG1 | BD Biosciences | 1:40 |
| CD25 | Pe-Cy7 | M-A251 | mouse IgG1 | BD Biosciences | 1:40 |
| CD27 | FITC | M-T271 | mouse IgG1 | BD Biosciences | 1:40 |
| CD27 | PE-Cy5 | 1A4CD27 | mouse IgG1 | Beckman Coulter | 1:40 |
| CD28 | PE-Cy5 | CD28.2 | mouse IgG1 | BD Biosciences | 1:20 |
| CD40 | PE | mAB89 | mouse IgG1 | Beckman Coulter | 1:20 |
| CD45RA | APC | HI100 | mouse IgG2b | eBioscience | 1:40 |
| CD45RA | FITC | HI100 | mouse IgG2b | BD Biosciences | 1:20 |
| CD69 | FITC | FN50 | mouse IgG1 | BD Biosciences | 1:20 |
| CD70 | FITC | Ki-24 | mouse IgG3 | BD Biosciences | 1:5 |
| CD86 | FITC | 2331 | mouse IgG1 | BD Biosciences | 1:40 |
| CD134/OX40 | FITC | ACT35 | mouse IgG1 | BD Biosciences | 1:5 |
| CD154/CD40L | Pacific Blue | 24-31 | mouse IgG1 | BD Biosciences | 1:100 |
| CD278/ICOS | PE | DX29 | mouse IgG1 | BD Biosciences | 1:4 |
| CXCR5 | PE | 51505.111 | mouse IgG2b | R&D Systems | 1:16 |
| CXCR5 | Biotin | 51505.111 | mouse IgG2b | R&D Systems | |
| CCR7 | unlabelled | 3D12 | Rat IgG2a | kindly provided by Dr Martin Lipp, Max Delbrück Center for Molecular Medicine, Berlin, Germany | 1:10 |
| HLA-DR | APC-H7 | L243 | mouse IgG2a | BD Biosciences | 1:40 |
| IgD | PE | IA6-2 | mouse IgG2a | BD Biosciences | 1:75 |
| IL-21R | unlabelled | 9N1.1 | | kindly provided by Dr Charles Mackay, Monash University, Victoria, Australia | 1.15 µg/ml |

**Table 2.1 Antibodies used for flow cytometry.**

**Table 2.1 continued…..**

| Antibody | Fluorochrome | Clone | Species and Isotype | Company | Final Dilution |
|---|---|---|---|---|---|
| ***Primary intracellular*** | | | | | |
| CXCL13 (BCA-1) | unlabelled | 53610.11 | mouse IgG1 | R&D Systems | 1:25 |
| IL-4 | PE | 8D4 | mouse IgG1 | BD Biosciences | 1:10 |
| IFN-γ | Pacific Blue | 4S.B3 | mouse IgG1 | eBioscience | 1:5 |
| TNF-α | APC | 6401.1111 | mouse IgG1 | BD Biosciences | 1:20 |
| | | | | | |
| ***Isotypes*** | | | | | |
| mouse IgG1 | FITC | — | — | BD Biosciences | — |
| mouse IgG1 | PE | — | — | BD Biosciences | — |
| mouse IgG1 | PeCy5 | — | — | BD Biosciences | — |
| mouse IgG1 | PeCy7 | — | — | BD Biosciences | — |
| mouse IgG1 | Pacific Blue | — | — | BD Biosciences | — |
| mouse IgG1 | APC | — | — | BD Biosciences | — |
| mouse IgG1 | APC-H7 | — | — | BD Biosciences | — |
| mouse IgG2a | PE | — | — | eBiosciences | — |
| mouse IgG2a | FITC | — | — | BD Biosciences | — |
| mouse IgG2a | APC-H7 | G155-178 | — | BD Biosciences | — |
| mouse IgG2b | FITC | — | — | BD Biosciences | — |
| mouse IgG2b | PE | — | — | BD Biosciences | — |
| mouse IgG2b | APC | — | — | eBiosciences | — |
| mouse IgG2b | Biotin | — | — | BD Biosciences | — |
| mouse IgG1 | unlabelled | MR9 | — | A gift from Patrick | — |
| Rat IgG2a | unlabelled | — | — | BD Biosciences | — |
| | | | | | |
| ***Secondary*** | | | | | |
| α-mouse Ig | PE | — | goat Ig | DAKO | 1:80 |
| α-mouse Ig | FITC | — | goat Ig | DAKO | 1:100 |
| α-mouse-IgG1 | Alexa fluor488 | — | goat Ig | Molecular Probes | 1:1000 |
| α-rat IgG | Biotin | — | Rabbit Ig | DAKO | 1:100 |
| | | | | | |
| ***Conjugates*** | | | | | |
| Strepavidin | PeCy7 | — | — | eBioscience | 1:1000 |
| Strepavidin | APC | — | — | DAKO | 1:80 |
| Strepavidin | PE | — | — | DAKO | 1:80 |
| Strepavidin | FITC | — | — | DAKO | 1:80 |

**Table 2.1 Antibodies used for flow cytometry.**
A list of antibodies used in this study including fluorescence label, species and clone, isotype, company and final dilution.

## 2.5.2 Flow cytometry analysis

For flow cytometric analysis cells were acquired using either a four-color FACSCalibur (BD Biosciences) or a nine-colour CyAn ADP (Beckman Coulter) flow cytometer and were analysed using FloJo 7.5 software (TreeStar). To correct multiple parametric flow cytometry data for spectral overlap, compensation was performed for each individual experiment using a series of single-colour stained cells, each stained with a single fluorescent marker for every colour used in the experiment.

## 2.5.3 Gating strategy

For analysis of each sample the same gating strategy was adopted. Firstly, the lymphocyte population was gated based on typical lymphocyte forward scatter (FSC) and side scatter (SSC) patterns (**Figure 2.2 step A**). Next, dead cells were gated out as these can bind antibodies non-specifically, a fixable dye (LIVE/DEAD® Aqua) was used for this purpose. Cells with compromised membranes readily stain with the dye and therefore have higher fluorescence intensity than cells with intact cell membranes (**Figure 2.2 step B**). To exclude doublets or cell aggregates (which would appear as a distinct population above the single cells) a gate was set on the single population of cells (singlets) (**Figure 2.2 step C**). The $V\gamma9^+$ or $V\delta2^+$ T cell populations were gated from the live lymphocyte, singlets population based on fluorescent intensity levels from Vγ9 or Vδ2 and CD3 specific antibody staining (**Figure 2.2 step D**). The percentages Vγ9 or Vδ2, CD3 positive cells expressing the different surface or intracellular markers of interest were determined by comparison with the appropriate isotype controls, as shown in the example for CD25 and OX40 expressing $V\gamma9^+CD3^+$ cells (**Figure 2.2 E and F**).

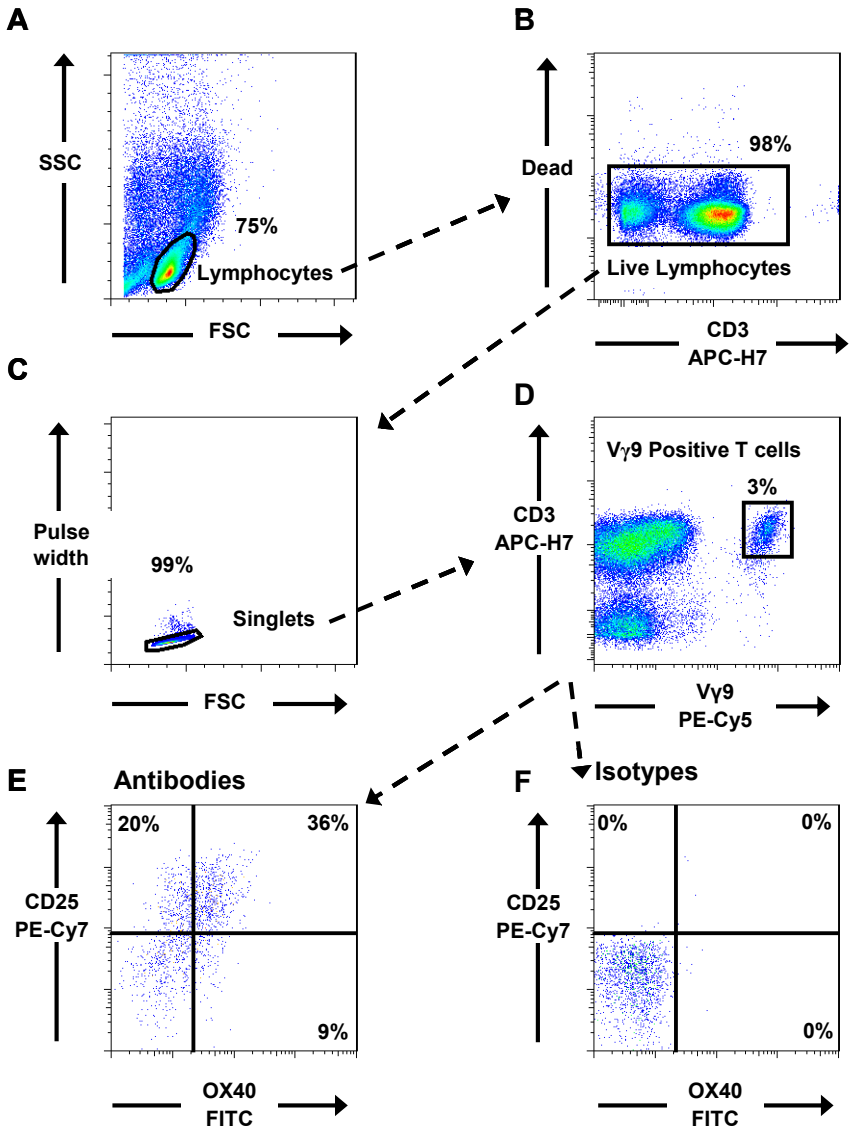


**Figure 2.2. Example of gating strategy for identifying CD25 and OX40 positive Vγ9Vδ2 T cell populations.**
Following 3 days in culture, HMB-PP stimulated PBMCs were harvested for phenotypic analysis. Cells were stained with Live/Dead Aqua viability stain and subsequently with flow cytometry with anti-CD3-APC-H7, anti-Vγ9-PE-Cy5, anti-CD25- PE-Cy7 and anti-OX40-FITC. Lymphocytes were defined according to their FSC and SSC (step A). Live lymphocytes were identified as those with low fluorescence levels of Live/Dead (step B). Doublets were gated out by gating on single cells (C) γδ T cells were identified by gating on $CD3^+$ lymphocytes and $V\gamma9^+$ (or $V\delta2^+$) lymphocytes (step D). The percentage of $CD3^+V\gamma9^+$ T cells expressing CD25 and OX40 (E) was determined by setting quadrants in dot plots using isotype-matched control antibodies (F). Numbers in dot plots represent the percentage of positive cells.

## 2.6 Immunofluorescence microscopy

Cells were prepared for detection of intracellular CXCL13 by immunofluorescence microscopy as follows. Following 3 days of culture, cells were treated with brefeldin A (10 μg/ml) 4 hours prior to harvesting. Staining for CXCL13 was carried out in U-round bottomed 96 well plates as described for flow cytometry, briefly; cells were fixed, permeabilized, blocked and stained with mouse anti-CXCL13, followed by Alexa Fluor488-conjugated goat anti-mouse IgG1 (green) and anti-Vδ2-PE (red) antibodies. Cells were counterstained with 0.5 μg/ml of DAPI (a blue fluorescent nuclear stain; Sigma) for 10 minutes at RT in the dark. Around 40,000 cells re-suspended in PBS were transferred onto poly-Lysine coated slides and allowed to settle for 30 minutes in the dark before being coversliped with prolong® gold anti-fade reagent (Invitrogen). Slides were analysed by Immunofluorescence microscopy using a Nikon microscope (Eclipse 80i, Nikon). Images were captured with a Nikon digital camera (DXM1200F) controlled by ACT-1 2.70 imaging software, and processed with Adobe Photoshop 6.0.

## 2.7 ELISA

Cell culture supernatants were analyzed using ELISA kits for CXCL13 and IL-17 according to the manufacturer's protocols (R&D Systems). Antibody levels were determined using the human IgM, IgG, IgA, and IgE quantitation kits (Universal Biologicals). All samples were measured in duplicate or triplicates on a Dynex MRX II reader.

## 2.8 Health and safety

All practical work was undertaken in the laboratories of Cardiff University. All chemicals and reagents used were COSHH assessed. Reagents were prepared, stored and disposed correctly according to the departmental policy and appropriate protective clothing was worn.

Ethical approval for this study was obtained from the South East Wales Local Ethics Committee (04WSE04/27 and 08/WSE04/17) and conducted according to the principles expressed in the Declaration of Helsinki. All patients and volunteers provided written informed consent.

## 2.9 Statistical analysis

All statistical analysis was carried out using GraphPad Prism 4.0 for Windows, GraphPad Software, San Diego California USA. Firstly, data were analysed using the normality test to assess if distribution of data points was normal. To analyse the significance of differences between two groups with normal distribution the two-tailed Student's *t* tests was used. In cases where normality of data could not be determined (i.e. sample size too small to determine normality) or if data failed to pass the normality test, two groups of data were compared using the non-parametric Mann-Whitney two-tailed test. Differences considered significant were indicated with *P* values in the figures and text and significance was defined as $P<0.05$.

# Chapter 3:

## IL-21 co-stimulates expression of key molecules involved in B cell help on peripheral HMB-PP activated Vγ9Vδ2 T cells

## 3.1 Chapter specific hypothesis and aims

The overall objective of this chapter was to induce and examine markers associated with follicular B cell help on peripheral blood Vγ9Vδ2 T cells. I hypothesised that IL-21 regulates expression of IL-21 receptor (IL-21R), CXCL13, CXCR5 and co-stimulatory molecules on *in vitro* HMB-PP activated peripheral Vγ9Vδ2 T cells.

Aims of this chapter were to investigate the effects of IL-21 and HMB-PP stimulation on peripheral Vγ9Vδ2 T cell expression of:

1. IL-21R
2. CXCL13
3. CXCR5
4. Co-stimulatory molecules involved in B cell help
5. APC markers

## 3.2 Results

### 3.2.1. Freshly isolated peripheral Vγ9Vδ2 T cells express the receptor for IL-21

In order to examine the effects of IL-21 stimulation on peripheral Vγ9Vδ2 T cells, surface expression of a functional IL-21R would be essential. Previous studies have found expression of the IL-21R on Vγ9Vδ2 T cells at the mRNA level (Vermijlen et al. 2007) whereas expression at the protein level has not been documented to date. Therefore I began by addressing the question whether freshly isolated peripheral Vγ9Vδ2 T cells would express surface IL-21R.

PBMCs were freshly isolated from normal healthy donors. Cells were stained with an unconjugated mouse anti-human monoclonal antibody against IL-21R, followed by a secondary α-mouse Ig-PE antibody. Cells were also stained with fluorochrome-conjugated antibodies specific for CD4, CD19, CD14, Vδ2 or Vγ9 together with CD3. Cells were analysed by flow cytometry and assessed for IL21R expression by gating on live cells and on $V\gamma9^+CD3^+$ T cells, $CD4^+CD3^+$ T cells, $CD19^+CD3^-$ B cells or $CD14^+CD3^-$ monocytes. IL-21R expression was detected on freshly isolated peripheral Vγ9Vδ2 T cells (**Figure 3.1A**), $CD4^+$ T cells **(Figure 3.1B)** $CD19^+$ B cells (**Figure 3.1C**) but not on $CD14^+$ monocytes (**Figure 3.1D**). Highest expression was found on B cells (geometric mean fluorescence intensity (GMFI) fold increase of 5.8 ± 2.16), followed by $CD4^+$ T cells (GMFI fold increase of 2.9 ± 0.85) and Vγ9Vδ2 T cells (GMFI fold increase of 1.8 ± 0.16). Monocytes served as a negative control and consistent with published data expression of IL-21R was not found on resting monocytes (Parrish-Novak et al. 2000; Strengell et al. 2006; Ueda et al. 2005).

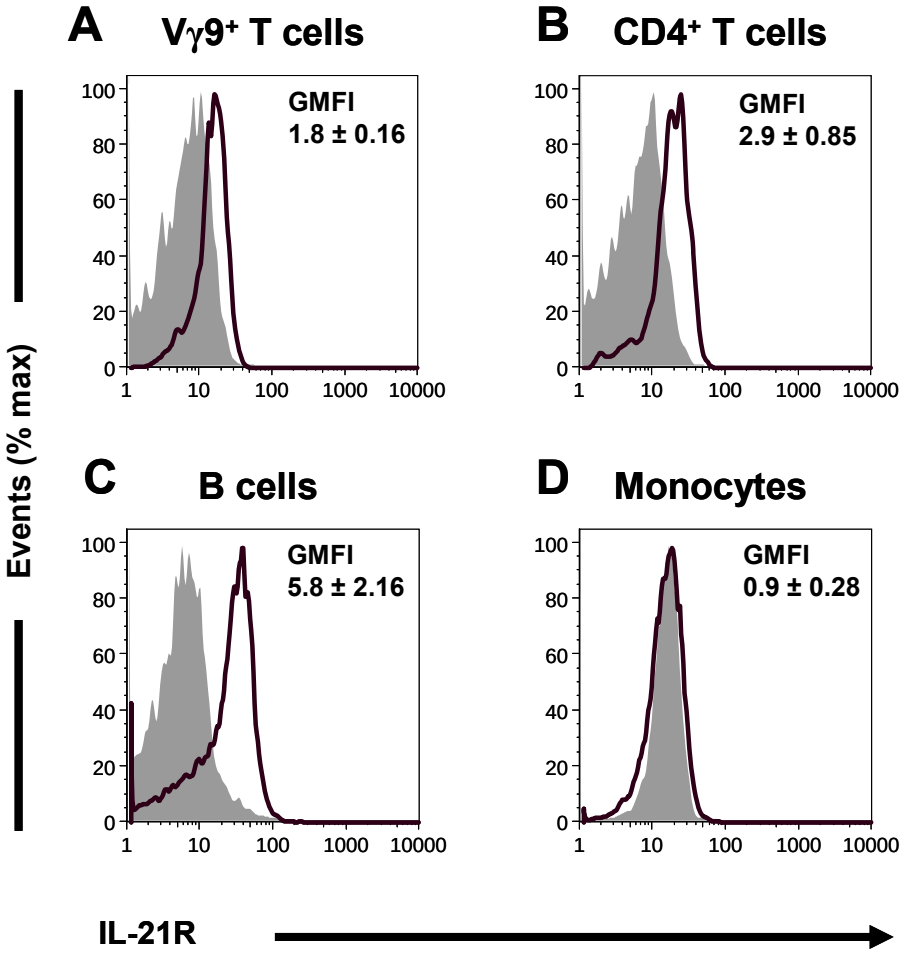


**Figure 3.1. Freshly isolated peripheral blood Vγ9Vδ2 T cells express the receptor for IL-21.**

PBMCs were freshly isolated and stained with unlabelled mouse anti-human mAb against IL-21R (black-outlined histogram) or with an isotype matched control mAb (solid gray histogram) followed by detection with anti-mouse Ig-PE. IL-21R expression was measured by flow cytometry. Cells were analysed by gating on live cells and on (A) $V\gamma9^{+}CD3^{+}$ T cells, (B) $CD4^{+}CD3^{+}$ T cells (C) $CD19^{+}CD3^{-}$ B cells or (D) $CD14^{+}$ monocytes. Flow cytometry histograms represent one of 4 independent experiments. The values shown within histograms represent mean fold change in GMFI, calculated as (geometric MFI of IL-21R)/(geometric MFI of isotype control). Results are expressed as mean ± standard deviation (SD) from four individual donors.

## 3.2.2. IL-21R is significantly up-regulated on HMB-PP activated peripheral Vγ9Vδ2 T cells

The microbial metabolite HMB-PP has been described as the most potent and specific activator of Vγ9Vδ2 T cells. Recognition of HMB-PP during many pathogenic infections leads to rapid activation and expansion of Vγ9Vδ2 T cells to very high numbers. Previous *in vitro* studies have demonstrated HMB-PP to stimulate uniform activation of human Vγ9Vδ2 T cells (Brandes et al. 2009; Davey et al. 2011; Eberl et al. 2009; Vermijlen et al. 2007). Whilst others have shown enhanced expression of activation markers on *in vitro* stimulated peripheral Vγ9Vδ2 T cells in the presence of cytokines (Eberl et al. 2002a; Eberl et al. 2002b; Vermijlen et al. 2007).

IL-21 is a Tfh signatory cytokine which plays a central role in B cell help. Here I examined the effects of IL-21 and HMB-PP stimulation on peripheral Vγ9Vδ2 T cell IL-21R surface expression. Freshly isolated PBMCs were treated with medium alone, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Cells were kept in culture for 1, 2, 3, 4 and 5 days. Every 24 hours cells were harvested and assessed for IL-21R expression by flow cytometry.

Cumulative IL-21R data from 4 different healthy donors, taken on day 0 (freshly isolated) and over a 5 day time course are shown in **Figure 3.2**. Here, as a measurement of IL-21R surface expression, the geometric MFI of IL-21R fluorescence is displayed on the y-axis. IL-21R surface expression was analysed on Vγ9Vδ2 T cells, $CD4^+$ T cells and B cells in response to each culture condition (**Figure 3.2**).

The most striking observations where made on day 2, were IL-21R expression on HMB-PP stimulated Vγ9Vδ2 T cells had peaked and was significantly ($P$=0.028, Mann-Whitney two-tailed test) up-regulated 3-fold (GMFI 15.5 ± 3.2) in comparison to base line IL-21R

expression (cells cultured in medium alone; GMFI 5 ± 0.58). This up-regulation of IL-21R observed in response to HMB-PP stimulation was short-lived, as on day 3 IL-21R expression levels had dramatically decreased (GMFI 9 ± 1.9). On day 5, IL-21R expression levels were similar to those observed on day 1 (GMFI 6.6 ± 1.3) (**Figure 3.2A**). This effect was specific to Vγ9Vδ2 T cells as HMB-PP stimulation of PBMCs had no effect on IL-21R expression on $CD4^+$ T cells (**Figure 3.2B**) or on B cells (**Figure 3.2C**).

In contrast, after 1 day of culturing PBMCs with HMB-PP in the presence of IL-21 co-stimulation, an initial down-regulation of IL-21R on Vγ9Vδ2 T cells was observed, which returned to baseline expression on days 2 and 3. More interestingly after long-term culture of 5 days, IL-21R expression levels had increased (GMFI 8 ± 0.7) to levels which were higher to those at baseline expression (GMFI 5.3 ± 0.3). Again, this pattern of IL-21R expression and response to co-stimulation with IL-21 in the presence of HMB-PP was unique to Vγ9Vδ2 T cells **(Figure 3.2A**), as it was not observed on $CD4^+$ T cells (**Figure 3.2B**).

Stimulation of PBMCs with IL-21 alone resulted in a more long-lived down-regulation of the IL-21R on both Vγ9Vδ2 and $CD4^+$ T cells. Throughout the 5 day culture, IL-21R expression levels had been down-regulated to levels lower than those seen in the medium control. In contrast to cells stimulated with both HMB-PP and IL-21, stimulation with IL-21 alone did not allow recovery of IL-21R expression on both Vγ9Vδ2 and $CD4^+$ T cells (**Figure 3.2A and B**). Similarly, IL-21R expression on B cells was also significantly down-regulated after 1 day with IL-21 stimulation (with or without HMB-PP) ($P$=0.028, Mann-Whitney two-tailed test), except here a gradual recovery of IL-21R expression levels was seen after just 2 days of culture (**Figure 3.2C**).

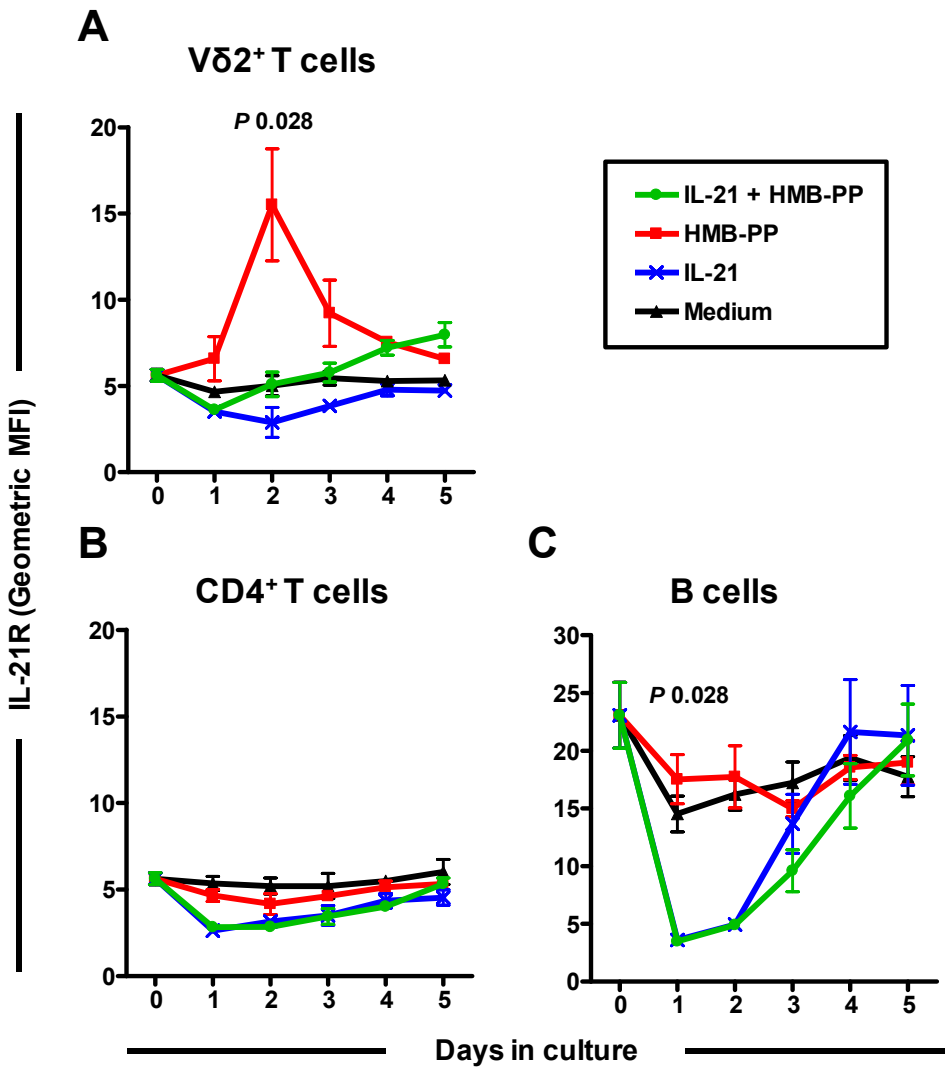


**Figure 3.2. HMB-PP induced up-regulation of IL-21R expression on peripheral blood Vγ9Vδ2 T cells.**

Freshly isolated PBMCs were cultured in medium (control, represents baseline IL-21R expression), or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated PBMCs (Day 0) and PBMCs after 1, 2, 3, 4 and 5 days in culture were stained for flow cytometry (as described in Figure 3.1) Cells were analysed for IL-21R expression by gating on live cells, lymphocytes and on (A) $V\delta2^{+}CD3^{+}$ cells, (B) $CD4^{+}CD3^{+}$ T cells or (C) $CD19^{+}CD3^{-}$ B cells. IL-21R expression is shown as GMFI. Results are expressed as mean ± SEM from four independently assessed donors. A statistically significant difference on $V\delta2^{+}$ T cell day 2 data between medium and HMB-PP treated cells/cultures was found ($P$=0.028). A statistically significant difference on B cells day 1 data between medium and IL-21 treated cells/cultures was found ($P$=0.028).

Furthermore, after one day, HMB-PP stimulation induced the early activation marker CD25, which was significantly ($P$=0.028, Mann-Whitney two-tailed test) up-regulated on 25% of the Vγ9Vδ2 T cells and was further enhanced to 58% with co-treatment with IL-21. In response to HMB-PP stimulation, activated Vγ9Vδ2 T cells continuously maintained surface expression of CD25 throughout the 5 days of culture (**Figure 3.3A**).

Vγ9Vδ2 T cells cultured with IL-21 alone and in medium alone served as negative controls, under these conditions CD25 expression was absent on Vγ9Vδ2 T cells (**Figure 3.3A**). Similarly, CD25 was not up-regulated on $CD4^+$ T cells (**Figure 3.3B**) under any of the indicated culture conditions, thus indicating a γδ T cell specific response to HMB-PP. A small and short-lived up-regulation of CD25 after 1 day, was seen on B cells in response to IL-21 alone (**Figure 3.3C**). Notably, co-staining with CD25 or CD69 revealed that IL-21R expression was primarily up-regulated on HMB-PP activated CD25 positive (**Figure 3.4**) and CD69 positive (data not shown) Vγ9Vδ2 T cells.

Taken together, these data indicated that not only do γδ T cells become highly activated after 2 days with HMB-PP stimulation but also up-regulate surface IL-21R expression to peak levels, thus allowing Vγ9Vδ2 T cells to become highly responsive *in vitro* to exogenously added IL-21 and *in vivo* to IL-21 released from neighbouring cells in the microenvironment, such as Tfh cells in secondary lymphoid tissues.

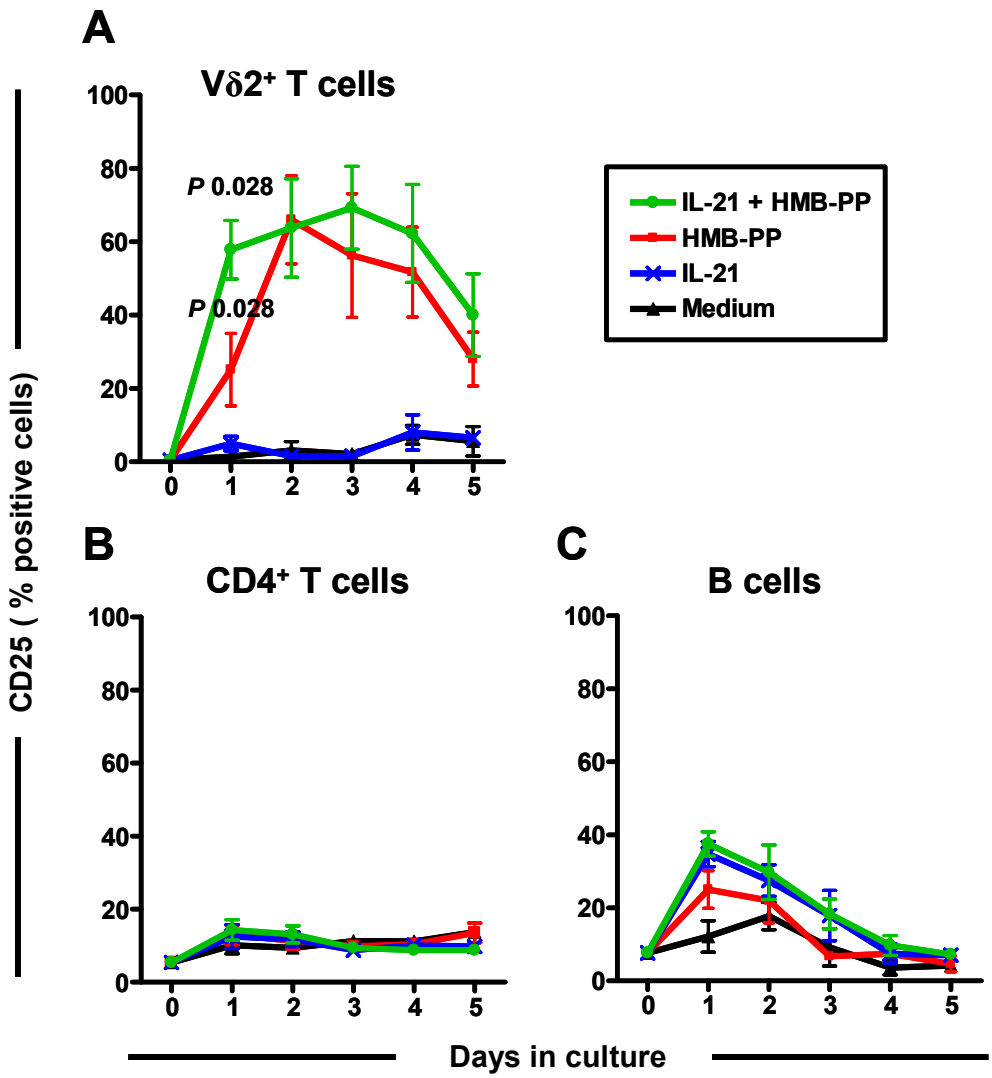


**Figure 3.3. Sustained CD25 expression on HMB-PP activated peripheral blood Vγ9Vδ2 T cells.**

Freshly isolated PBMCs were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated PBMCs (Day 0) and PBMCs after 1, 2, 3, 4 and 5 days in culture were stained for flow cytometry with anti-CD25-PE-Cy5, anti-CD3-APC and anti-Vδ2-FITC or anti-CD4-FITC or anti-CD19-FITC. Cells were analysed for CD25 expression, by gating on live cells lymphocytes and on (A) $V\delta2^{+}CD3^{+}$ cells, (B) $CD4^{+}CD3^{+}$ T cells or (C) $CD19^{+}CD3^{-}$ B cells. Results are expressed as mean percentage ± SEM from four independently assessed donors. A statistically significant difference on $V\delta2^{+}$ T cell day 1 data between medium and HMB-PP treated; and between medium and IL-21+HMB-PP treated cells/cultures was found (*P*=0.028).

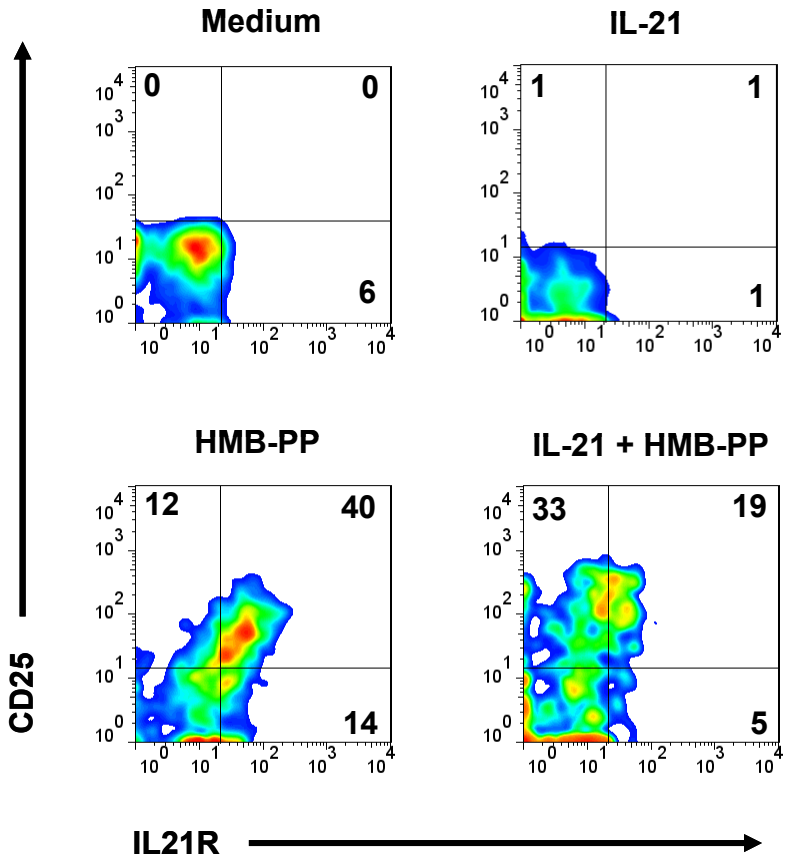


**Figure 3.4. IL-21R expression is up-regulated on HMB-PP activated CD25$^+$ Vγ9Vδ2 T cells.**
Freshly isolated PBMCs were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). After 2 days, cells were harvested and stained for flow cytometry, with unlabelled mouse anti-IL-21R followed by detection with anti-mouse Ig-PE, anti-CD25-PE-Cy5, anti-Vδ2-FITC and anti-CD3-APC. Cells were analysed for CD25 and IL-21R expression, by gating on live cells, lymphocytes and on Vδ2$^+$CD3$^+$ cells, quadrants were set using isotype-matched control antibodies. Numbers in dot plots represent the percentage of Vδ2$^+$ T cells positive for CD25 and IL-21R. Results are representative of four independently assessed donors.

### 3.2.3. IL-21 enhances expression of CD25 and CD69 activation markers on HMB-PP stimulated peripheral Vγ9Vδ2 T cells

I and others have demonstrated that Vγ9Vδ2 T cells in PBMCs can be successfully activated in response to HMB-PP and cytokine co-stimulation (Vermijlen et al. 2007). Here the aim was to establish optimal conditions for γδ T cell activation in both PBMCS and in γδ T-B cell co-cultures. Thus, I here examined the effects of IL-21 and HMB-PP stimulation on peripheral Vγ9Vδ2 T cells in PBMCs alongside Vγ9Vδ2 T cells in γδ T-B cell co-cultures.

Vγ9Vδ2 T cells were positively isolated from freshly isolated PBMCs using the magnetic cell sorting system from Miltenyi Biotec, resulting in >97% purity (**Figure 3.5A**). For optimum in *vitro* Vγ9Vδ2 T cell stimulation with HMB-PP, the presence of accessory (feeder) cells is required. As feeder cells, peripheral B cells or (**Figure 3.5B**) peripheral monocytes (**Figure 3.5C**) were isolated from Vγ9Vδ2 T cell depleted PBMC using anti-CD19 or anti-CD14 microbeads, resulting in >98% and >96% purity, respectively.

For supporting HMB-PP activation of purified γδ T cells, monocytes have already been well established as 'feeder' cells (Eberl et al. 2009), thus as initial control experiments, HMB-PP treated co-cultures were set-up using irradiated purified monocytes. For comparison, at the same time, purified Vγ9Vδ2 T cells were co-cultured with purified irradiated B cells, in the presence of HMB-PP stimulation. Flow cytometry analysis of activation markers showed that in comparison to monocytes, irradiated peripheral B cells were equally as effective at providing 'feeder' qualities. Here, irradiated B cells had successfully supported HMB-PP activation of purified γδ T cells. Furthermore, reducing the number of B cells co-cultured with γδ T cells from a 1:1 to a 1:2 ratio, did not affect the ability of irradiated B cells to provide effective 'feeder' qualities for γδ T cell activation (data not shown).

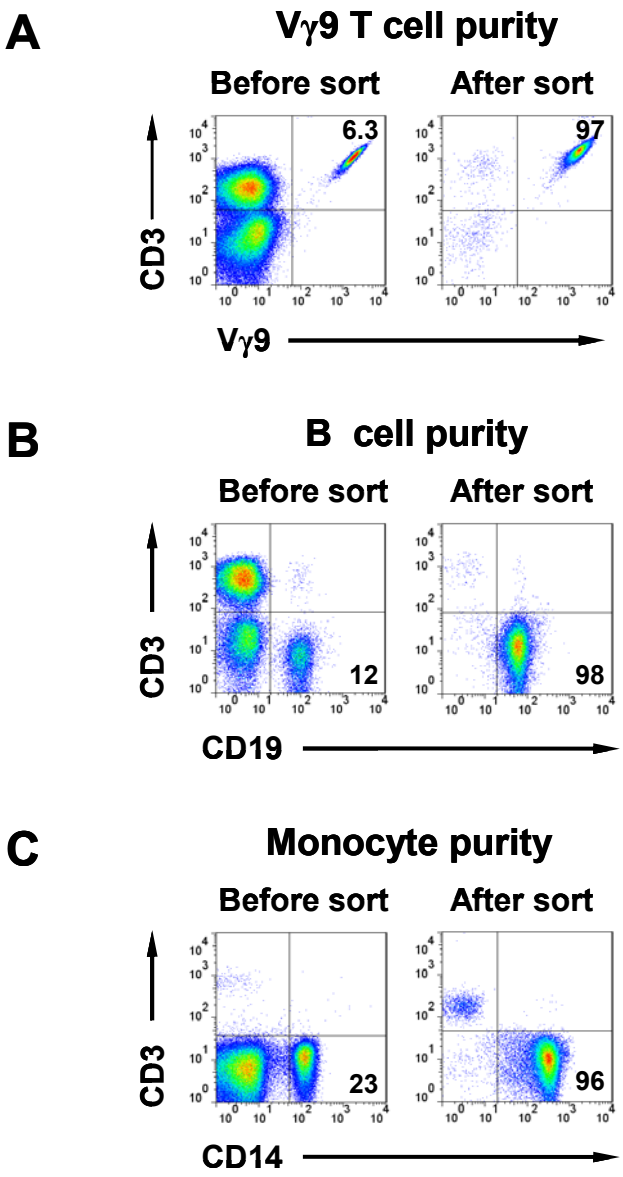


**Figure 3.5. Flow cytometric purity analysis of freshly isolated Vγ9Vδ2 T cells, B cells and monocytes.**
Freshly isolated PBMCs were blocked with human Igs and stained with anti-Vγ9-PE-Cy5 antibodies followed with anti-PE microbeads. (A) Vγ9Vδ2 T cells were positively isolated from PBMCs using the magnetic cell sorting system from Miltenyi Biotec, resulting in >97% purity. (B) B cells or (C) Monocytes were isolated from Vγ9Vδ2 T cell depleted PBMC using anti-CD19 or anti-CD14 microbeads, resulting in >98% and >96% purity, respectively. Results are representative of 10 or more experiments performed.

Next, I established optimal conditions for γδ T cell activation in the presence of IL-21 and HMB-PP stimulation, in PBMCs and in γδ T-B cell co-cultures. PBMCs alongside purified Vγ9Vδ2 T cells co-cultured with purified irradiated B cells (at a ratio of 1:1), were cultured with medium alone, with HMB-PP (10 nM) and in the presence of IL-21 (50 ng/ml). After 24 hours cells were harvested, stained with fluorochrome-conjugated antibodies and analysed by flow cytometry for CD25 and CD69 expression.

Representative flow cytometry dotplots of CD25 and CD69 expression on Vγ9Vδ2 T cells in both PBMCS and in co-cultures is shown in **Figure 3.6**. In PBMCs, stimulation of Vγ9Vδ2 T cells with HMB-PP alone induced co-expression of CD25 and CD69 in 38% of Vγ9Vδ2 T cells, which was further enhanced (48%) with co-treatment with IL-21. In comparison, stimulation of Vγ9Vδ2 T cells in co-cultures with HMB-PP alone induced co-expression of CD25 and CD69 in only 18% of Vγ9Vδ2 T cells, which was further enhanced (25%) with co-treatment with IL-21 (**Figure 3.6**). Thus, in agreement with previous studies (Vermijlen et al. 2007), the presence of cytokines, such as IL-21 does enhance expression of activation markers on *in vitro* HMB-PP stimulated peripheral Vγ9Vδ2 T cells.

Furthermore, in PBMCs the percentage of Vγ9Vδ2 T cells expressing CD25 or CD69 was dose-dependent on the concentration of HMB-PP. As the concentration of HMB-PP was decreased 10 fold from 10 nM to 1 and 0.1 nM, the percentage of Vγ9Vδ2 T cells expressing either CD25 or CD69 had also decreased accordingly. CD25 or CD69 expression induced on Vγ9Vδ2 T cells was not influenced by the high concentrations of IL-21 (50-100 ng/ml) used here (**Figure 3.7**). However, as previously reported, much lower concentrations of IL-21 (0.1-10 ng/ml), become less potent at providing co-stimulation for Vγ9Vδ2 T cell activation (Eberl et al. 2002a).

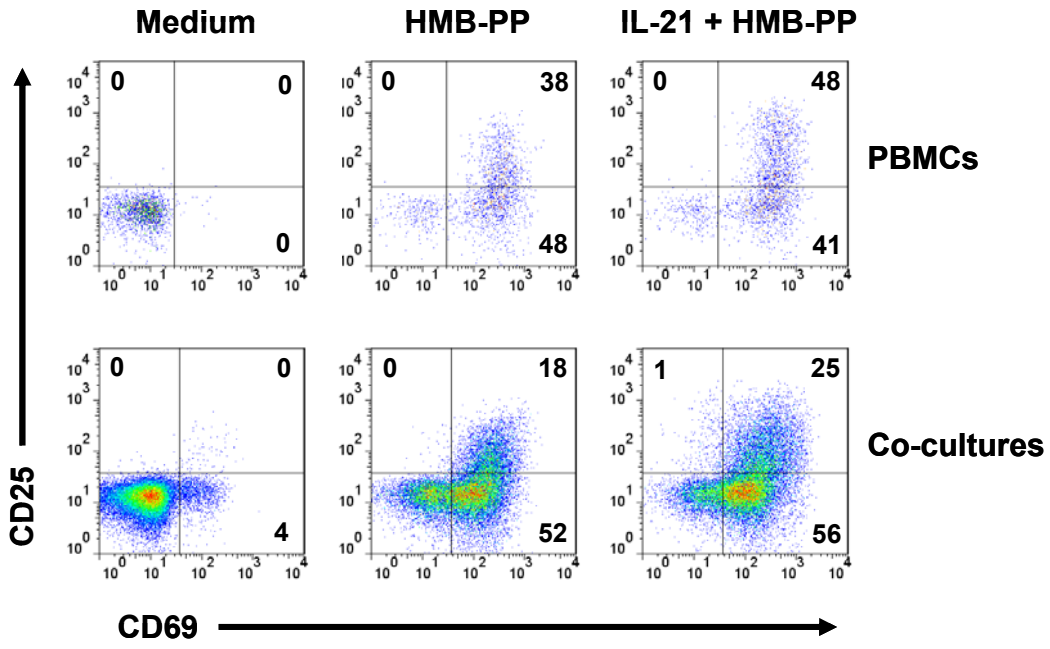


**Figure 3.6. HMB-PP activated Vγ9Vδ2 T cells in both PBMCs and in co-cultures co-express CD25 and CD69.**
Freshly isolated PBMCs (top three dot plots) and co-cultures of γδ T cells with B cells at a 1:1 ratio (bottom three dot plots) were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml). After 24 hours, cells were harvested and stained for flow cytometry, with anti-Vγ9-PE-Cy5, anti-CD3-APC and anti-CD25-PE and anti-CD69-FITC. Cells were analysed for CD25 and CD69 expression, gating on live cells, lymphocytes and on $V\gamma9^{+}CD3^{+}$ T cells, quadrants were set using isotype-matched control antibodies. Numbers in dot plots represent the percentage of $V\gamma9^{+}$ T cells positive for CD25 and CD69. Results are representative of four independently assessed donors.

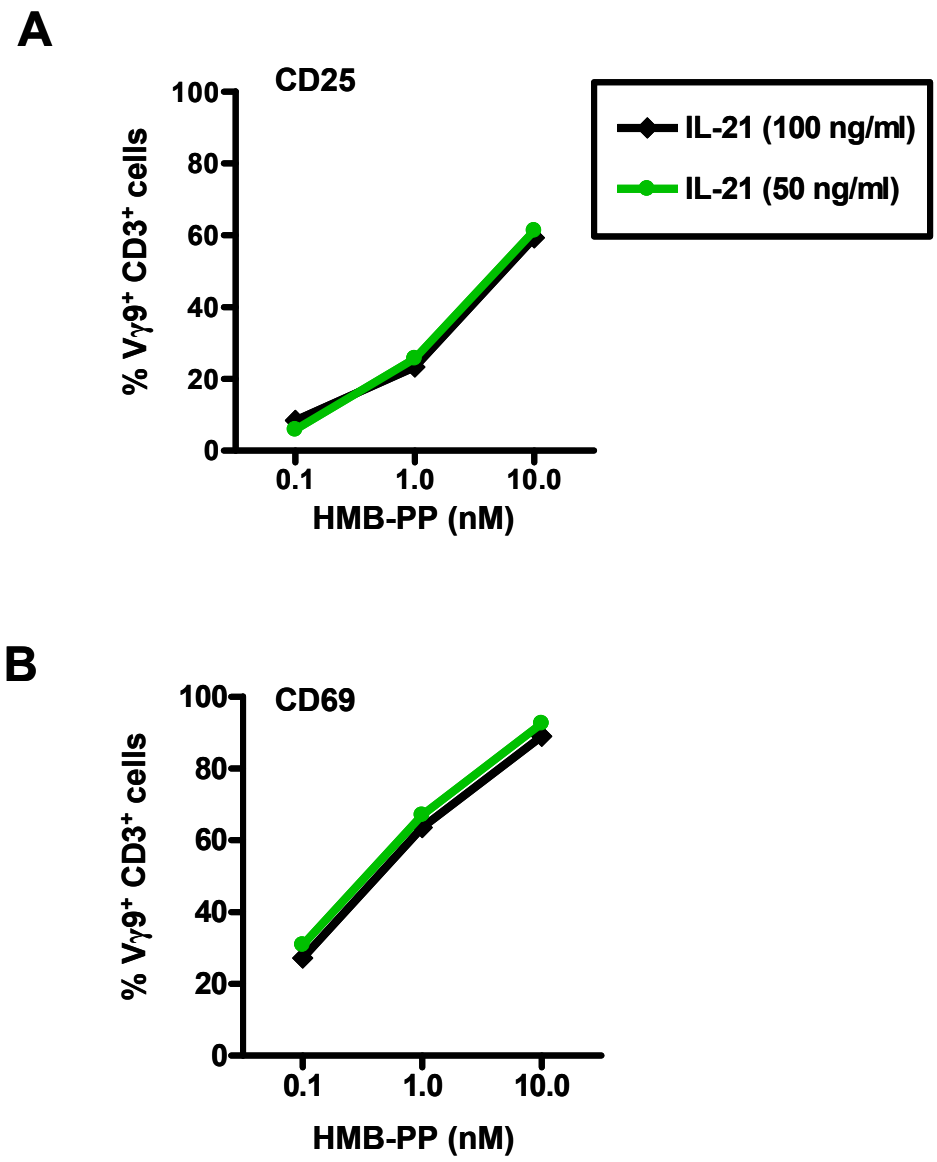


**Figure 3.7. CD25 and CD69 expression on Vγ9Vδ2 T cells is dependent on the concentration of HMB-PP.**
Freshly isolated PBMCs were cultured in medium with 0.1, 1 or 10 nM of HMB-PP and in the presence of 50 or 100 ng/ml of IL-21. After 24 hours, cells were harvested and stained for flow cytometry with anti-Vγ9-PE-Cy5, anti-CD3-APC and anti-CD25-PE and anti-CD69-FITC. Cells were analysed for CD25 and CD69 expression, by gating on live cells, lymphocytes and on $V\gamma9^{+}CD3^{+}$ cells, isotype-matched antibodies were used as controls. Graphs show percentage of (A) CD25 and (B) CD69 positive, $V\gamma9^{+}CD3^{+}$ T cells. Results are representative of two experiments performed.

### 3.2.4. IL-21 in the presence of HMB-PP induces expression of the B cell-attracting chemokine CXCL13 by peripheral Vγ9Vδ2 T cells

Migration and positioning of B and Tfh cells to B cell follicles are tightly controlled by the chemokine CXCL13 and its receptor CXCR5 (King 2009; King et al. 2008). CXCL13 is constitutively secreted by FDCs and Tfh cells in GCs, which attracts B and T cells bearing the CXCR5 receptor (Allen and Cyster 2008; Elgueta et al. 2010; Förster et al. 1994; Kim et al. 2004; Moser and Loetscher 2001; Moser et al. 2002; Rasheed et al. 2006). In order for γδ T cells to induce B cell activation and provide potent B cell help direct cell-to-cell contact between the two cell types would be required. Therefore, cell proximity would be a prerequisite for such cell-to-cell contact to occur. Here, I propose IL-21 induced secretion of CXCL13 by HMB-PP activated γδ T cells, as a possible mechanism of attracting circulating $CXCR5^+$ B cells and Tfh cells towards CXCL13 producing γδ T cells (Vermijlen et al. 2007).

It has previously been shown that Vγ9Vδ2 T cells stimulated with HMB-PP in the presence of IL-21 but not in the presence of IL-2 or IL-4 expressed mRNA levels of CXCL13. Furthermore, secretion of CXCL13 protein into the supernatants of PBMC stimulated with HMB-PP and IL-21 depended on the presence of Vγ9Vδ2 T cells (Vermijlen et al. 2007). Here, the aim was to establish optimal *in vitro* conditions for detection of CXCL13 in Vγ9Vδ2 T cells stimulated with HMB-PP in the presence of IL-21 and to directly confirm γδ T cells as the source of CXCL13 on the protein level.

In order to achieve this, preliminary experiments were designed to establish optimal conditions for detection of CXCL13, which began by examining the effects of using various HMB-PP and IL-21 concentrations on Vγ9Vδ2 T cell secretion of CXCL13. For a more precise analysis of CXCL13 expression, I utilised purified peripheral Vγ9Vδ2 T cells instead of PBMCs, this is because γδ T cells represent only a small population of cells

within PBMCs. Peripheral blood Vγ9Vδ2 T cells and B cells (as feeder cells) were isolated to high purities as previously described in **Figure 3.5**. Purified peripheral γδ T cells were co-cultured with purified irradiated peripheral B cells (at a ratio of 1:1), with various concentrations of HMB-PP (1 and 10 nM), IL-21 (0.1, 1, 10, and 100 ng/ml) or IL-2 (0.1, 1, 10, and 100 units/ml). After, 3 days total CXCL13 levels in culture supernatants were measured by using a well established ELISA method.

In co-cultures stimulated with 10 nM of HMB-PP, IL-21 induced a clear dose-dependent effect on CXCL13 secretion, in contrast IL-2 was dose-dependent in inhibiting CXCL13 secretion (**Figure 3.8A**). In comparison, the effects of IL-21 co-stimulation in the presence of a lower concentration of HMB-PP (1 nM) was less prominent on CXCL13 secretion (**Figure 3.8A**). IL-21 alone, in the absence of HMB-PP stimulation did not induce secretion of CXCL13 into cultures supernatants (data not shown). These results identified 10 nM of HMP-PP along with 10-100 ng/ml of IL-21 as optimum concentrations for inducing CXCL13 secretion by Vγ9Vδ2 T cells in co-cultures. Furthermore, noticeably higher levels of CXCL13 protein were detected in culture supernatants harvested following 3 days of incubation in comparison to supernatants harvested after 2 days (data not shown). Thus, indicating a 3 day culture period to be optimal for detecting higher levels of secreted CXCL13.

After having established the above optimum culture conditions, experiments were repeated using peripheral blood γδ and B cells from four different donors. Vγ9Vδ2 T cells and irradiated B cells were co-cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (100 ng/ml), or with IL-21 alone (100 ng/ml). After 3 days culture supernatants were harvested and concentration of secreted CXCL13 was measurement by ELISA (**Figure 3.8B**). Here, HMB-PP alone had induced production of CXCL13 (460 pg/ml), this was substantially increased four-fold (1950 pg/ml) with co-treatment with IL-21(**Figure 3.8A and B**). Secretion of CXCL13, induced by HMB-PP+IL-21 stimulation was significantly ($P$=0.016, Mann-Whitney two-tailed test) higher when compared to the medium control (**Figure 3.8B**). In addition some of these co-cultures were also treated with

blocking antibodies, to block of any IL-4 and IFN-γ which may be inhibiting γδ T cell response to IL-21 and in turn inhibiting CXCL13 expression, blocking of these cytokines did not effect CXCL13 expression. The possibility of IL-21 inducing production of IL-17 by γδ T cells was also ruled out, as IL-17 in culture supernatants was not detected by ELISA (data not shown).

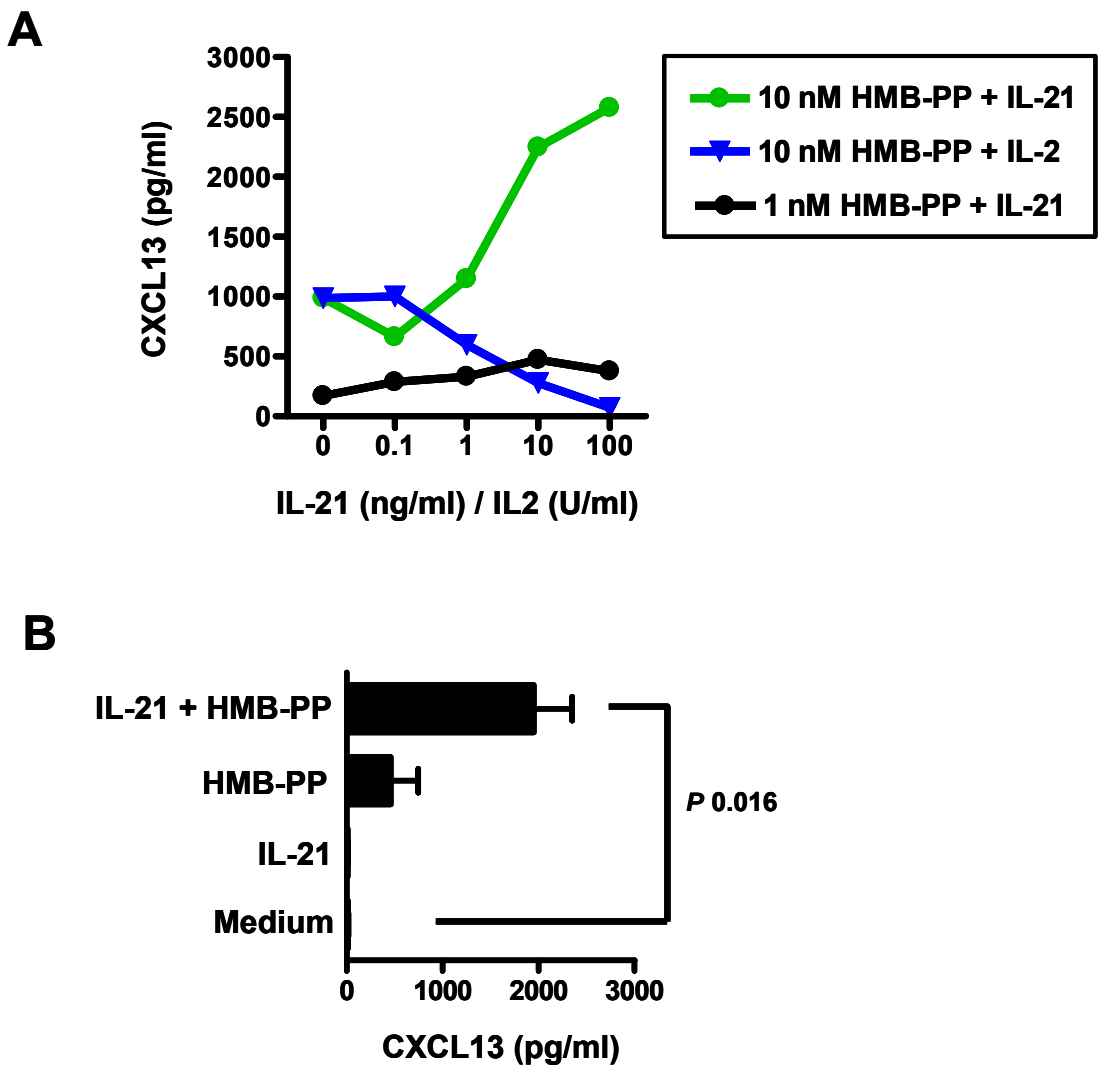


**Figure 3.8. IL-21 induces secretion of CXCL13 by HMB-PP activated Vγ9Vδ2 T cells.** Peripheral Vγ9Vδ2 T cells and peripheral B cells were freshly isolated (as described in Figure 3.5) and co-cultured at a ratio of 1:1 under the indicated conditions, after 3 days culture supernatants were harvested and concentration of CXCL13 was measurement by ELISA. (A) Vγ9Vδ2 T cells and irradiated B cells were co-cultured in medium with 1 nM or 10 nM HMB-PP and in the absence or presence of various concentrations (0.1, 1, 10, and 100) of IL-21 (ng/ml) or IL-2 (U/ml). Results are representative of two experiments performed. (B) Vγ9Vδ2 T cells and irradiated B cells were co-cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (100 ng/ml), or with IL-21 alone (100 ng/ml). After 3 days culture supernatants were harvested and concentration of CXCL13 was measurement by ELISA. Results are expressed as mean + SEM of three or four experiments. Statistical difference between medium and IL-21 + HMB-PP treated was found to be significant ($P$= 0.016).

To confirm γδ T cells as source of CXCL13 on the protein level, I established a unique and novel method to detect intracellular CXCL13. Vγ9Vδ2 T cells and irradiated B cells were co-cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (100 ng/ml), or with IL-21 alone (100 ng/ml). After 2 days, to block the secretion of intracellular CXCL13 cells were treated with brefeldin A. After 4 hours, cells were harvested, fixed and permeabilised. Cells were stained with fluorochrome-conjugated antibodies specific for CXCL13, CD25, CD3 and Vγ9. Flow cytometry analysis revealed that intracellular CXCL13 was expressed by 0.8% of HMB-PP stimulated $CD25^+$ Vγ9Vδ2 T cells, which was further enhanced (2%) with co-treatment with IL-21 (**Figure 3.9**). This clear shift in CXCL13 positive population was evident only on gated Vγ9Vδ2 T cells but not on gated B cells (data not shown).

These findings were further confirmed by immunofluorescence microscopy. Co-cultures were set up as above. After 3 days cells were treated with brefeldin and after 4 hours, cells were harvested, fixed and permeabilised. Cells were stained for intracellular CXCL13 (conjugated to a green- fluorescent dye), surface Vδ2 (conjugated to a red- fluorescent dye) and counterstained with a blue fluorescent nuclear stain called DAPI. Cells were mounted on to microscope slides and viewed under a fluorescence microscope. Intracellular CXCL13 was clearly observed in Vδ2 positive cells stimulated with IL-21+HMB-PP (**Figure 3.10D**) but not in cells incubated in medium alone (**Figure 3.10A**). Vδ2 negative cells identified as B cells, stained negative for intracellular CXCL13 (**Figure 3.10C**). An isotope-matched antibody was used as control staining for CXCL13 which was also negative for intracellular CXCL13 (**Figure 3.10B**).

Cytokines such as IL-4 and IFN-γ are also known to regulate B cell differentiation and antibody production (Crotty 2011; King 2009). Previous studies have shown activated human γδ T cells to provide help to B cells *via* soluble factors such as IL-4 and IL-10 (Caccamo et al. 2006a). Thus, Vγ9Vδ2 T cells in co-cultures were also simultaneously stained for intracellular IL-4 and IFN-γ and TNF-α. HMB-PP stimulation induced intracellular expression of IL-4, IFN-γ and TNF-α in 1.8%, 4.7% and 3% of Vγ9Vδ2 T

cells, respectively. The percentage of IL-4 (12.5%) positive and IFN-γ (18%) positive Vγ9Vδ2 T cells, was further increased with co-treatment with IL-21 (**Figure. 3.11**), there was no change in TNF-α positive Vγ9Vδ2 T cells (data not shown). Intracellular cytokine expression of IL-4, IFN-γ and TNF-α was not found in Vγ9Vδ2 T cells treated with medium or with IL-21 alone (data not shown). Noticeably, $CXCL13^{+}$ $CD25^{+}$ Vγ9Vδ2 T cells also expressed intracellular IL-4 and IFN-γ (**Figure 3.11**).

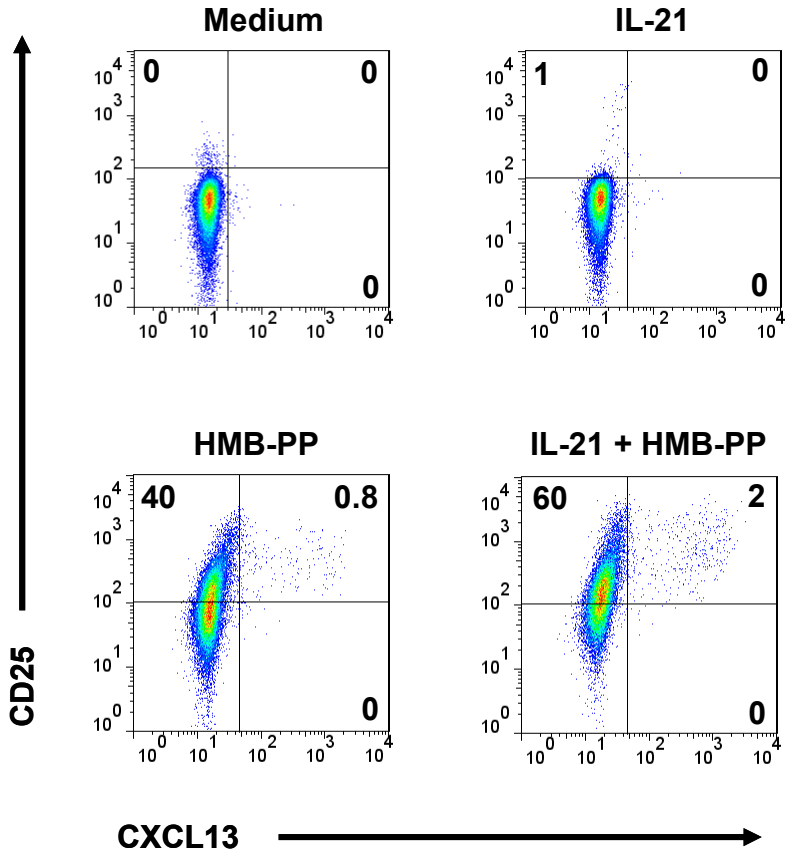


**Figure 3.9. Detection of intracellular CXCL13 in Vγ9Vδ2 T cells by flow cytometry.** Vγ9Vδ2 T cells and B cells were freshly isolated (as described in Figure 3.5) and co-cultured at a ratio of 1:1 in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (100 ng/ml). After 2 days, cultures were treated with brefeldin A (20 ug/ml). After 4 hours, cells were harvested, fixed and permeabilised. Cells were stained for flow cytometry with mouse anti-CXCL13, followed by detection with an Alexa fluor488-conjugated goat anti-mouse-secondary antibody, anti-Vγ9-PE-Cy5, anti-CD3-APC and anti-CD25-PE-Cy7. Quadrants in dot plots were set using isotype-matched control antibodies. Numbers in dot plots represent the percentages of $V\gamma9^{+}CD3^{+}$ T cells positive for CXCL13 and CD25. Data are representative of three independently assessed donors.

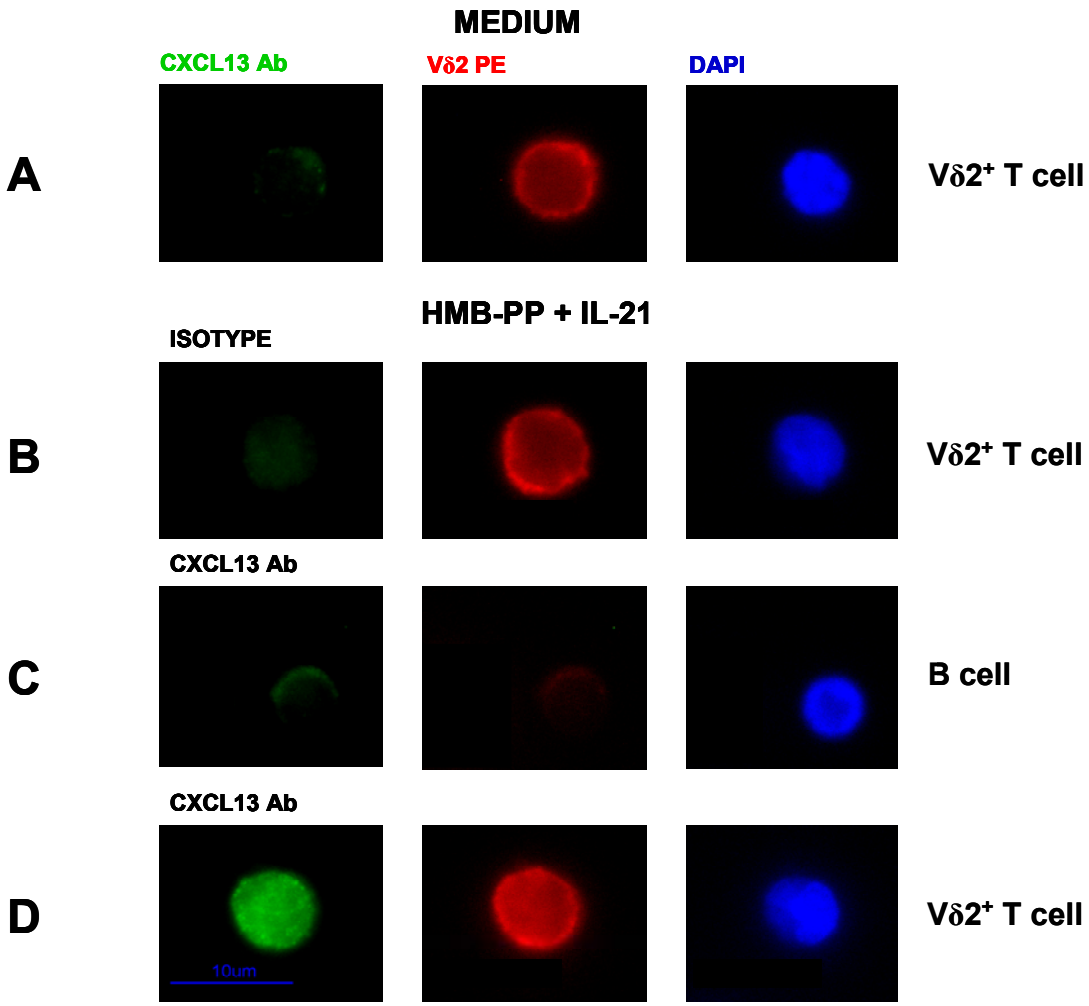


**Figure 3.10. Detection of intracellular CXCL13 in Vγ9Vδ2 T cells by immunofluorescence microscopy.**
Vγ9Vδ2 T cells and B cells were freshly isolated (as described in Figure 3.5) and co-cultured at a ratio of 1:1 in (A) medium, or (B-D) with HMB-PP (10 nM) in the presence of IL-21 (100 ng/ml). After 3 days cultures were treated with brefeldin A (20 ug/ml). After 4 hours, cells were harvested, fixed and permeabilised. Cells were stained with mouse anti-CXCL13, followed by detection with an Alexa fluor488-conjugated goat anti-mouse IgG1-secondary antibody (green), anti-Vδ2-PE (red), and counterstaining with DAPI for nuclear staining (blue). (B) Isotope-matched antibody was used as control staining for CXCL13 antibody staining. Cells were analysed by immunofluorescence microscopy. Data are representative of two independently assessed donors.

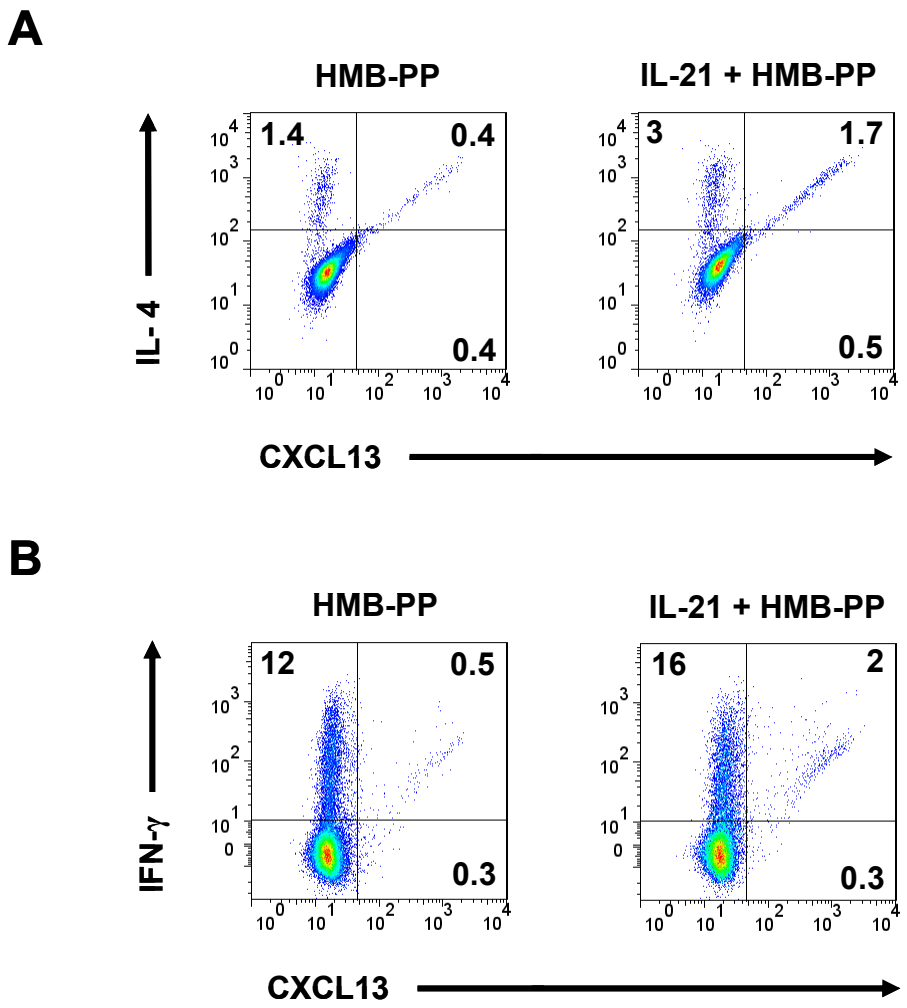


**Figure 3.11. CXCL13 positive Vγ9Vδ2 T cells express IL-4 and IFN-γ.**
Vγ9Vδ2 T cells and B cells were freshly isolated (as described in Figure 3.5) and co-cultured at a ratio of 1:1 in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (100 ng/ml). After 2 days, cultures were treated with brefeldin A (20 ug/ml). After 4 hours, cells were harvested, fixed and permeabilised. Cells were stained for flow cytometry for intracellular CXCL13, IL-4 and IFN-γ. Dot plots show data from HMB-PP treated cultures, in the absence and presence of IL-21. Numbers in dot plots represent the percentages of $V\gamma9^{+}CD3^{+}$ T cells positive for (A) IL-4 and CXCL13 and (B) IFN-γ and CXCL13. Quadrants in dot plots were set using isotype-matched control antibodies. Data are representative of three independently assessed donors.

### 3.2.5. IL-21 in the presence of HMB-PP stimulation does not induce expression of CXCR5 on peripheral Vγ9Vδ2 T cells.

Expression of the chemokine receptor CXCR5 allows activated T cells to migrate into CXCL13-rich B cell follicles of secondary lymphoid tissues, where they provide instructions for B cell differentiation into plasma cells (King 2009; King et al. 2008). Here, I examined surface expression of CXCR5 on freshly isolated and *in vitro* HMB-PP and IL-21 activated peripheral blood Vγ9Vδ2 T cells. CXCR5 was absent on freshly isolated peripheral Vγ9Vδ2 T cells (**Figure 3.12A**) but present on 7% of freshly isolated peripheral $CD4^+$ T cells (**Figure 3.12B**).

To study the expression of CXCR5 on HMB-PP and IL-21 *in vitro* stimulated peripheral Vγ9Vδ2 T cells, freshly isolated PBMCs from normal healthy donors were cultured in medium, with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml) or with IL-21 alone. After 2 days cells were harvested and stained with fluorochrome-conjugated antibodies specific for CXCR5. Cells were analysed by flow cytometry and assessed for CXCR5 expression by gating on lymphocytes, gating out dead cells and doublets, and gating on $Vγ9^+CD3^+$ T cells or $CD4^+$ $CD3^+$ T cells.

Representative flow cytometry dotplots of CXCR5 expression on HMB-PP and IL-21 *in vitro* stimulated $Vγ9^+CD3^+$ T cells is shown in **Figure 3.13A**. Surface expression of CXCR5 was not detected on HMB-PP stimulated Vγ9Vδ2 T cells and the addition of IL-21 did not induce expression of CXCR5. Similarly, stimulation with IL-21 alone (100 and 50 ng/ml) did not induce expression of CXCR5 on Vγ9Vδ2 T cells (data not shown).

Expression of CXCR5 is known to be rapid and transient, therefore I further examined for CXCR5 expression on peripheral Vγ9Vδ2 T cells, harvested at earlier (6 hours) and later

timepoints (day 1, 3, 4, 5, 6). Pre-stimulation of Vγ9Vδ2 T cells with HMB-PP for 42 hours before the addition of IL-21, or co-treatment with higher concentrations of IL-21 (100ng/ml) did not induce CXCR5 expression (data not shown). In addition, experiments where repeated using peripheral blood from several individual donors. Here, CXCR5 was found to be absent and not detected on any of the timepoints or donors tested (data not shown).

Previously Schaerli et al. (2001) showed induction of CXCR5 on peripheral blood naïve and CXCR5-negative memory $CD4^+$ T cells with PHA stimulation. Therefore similarly, to induce CXCR5 surface expression, I cultured PBMCs with PHA (1µg/ml). After 2 days cells were harvested, stained for CXCR5 and analysed by flow cytometry. Although PHA stimulation did not induce CXCR5 expression on peripheral Vγ9Vδ2 T cells (**Figure 3.13B**), however, the percentage of $CD4^+$ T cells expressing CXCR5 had increased from 7% (day 0) to 22% (day 2) (**Figure 3.12B and 3.13B**).

As a source of obtaining and stimulating naïve γδ T cells, human cord blood mononuclear cells were stimulated with HMB-PP in the presence of various concentrations of IL-21 or IL-2. CXCR5 was not detected on the very small population of γδ T cells present in cord blood (data not shown).

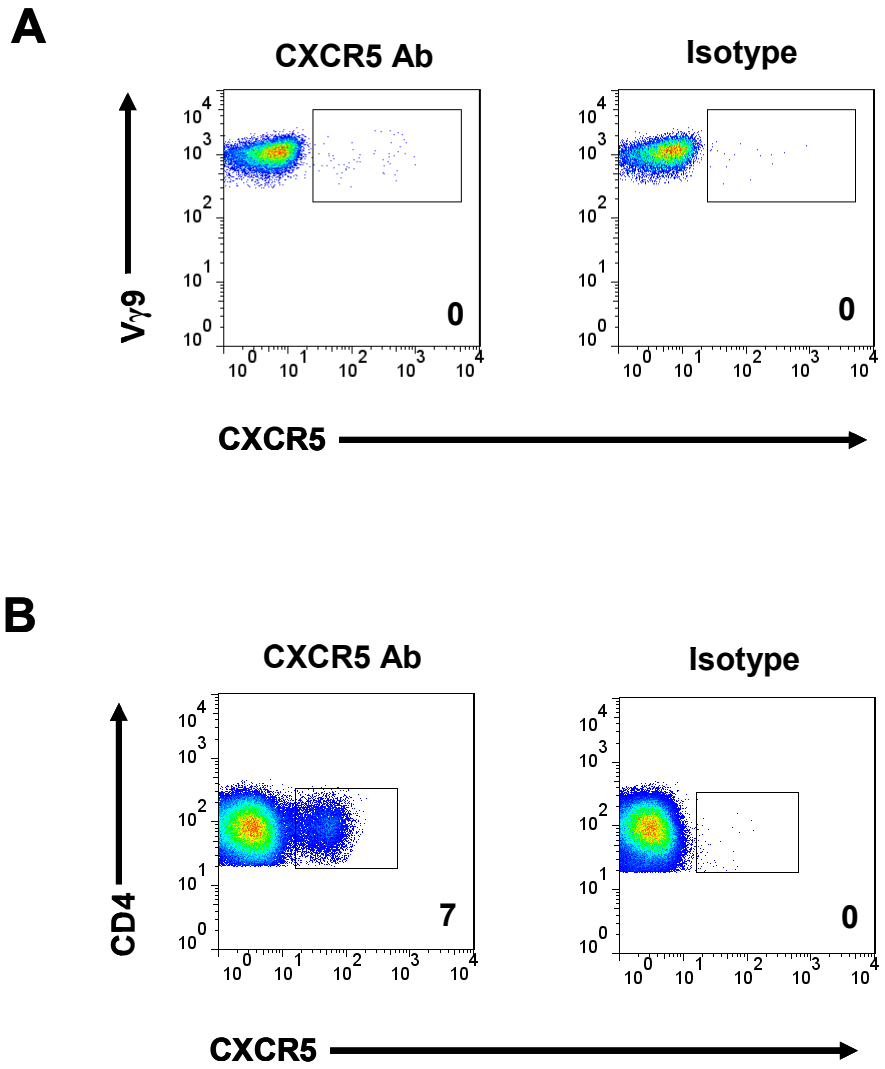


**Figure 3.12. Freshly isolated peripheral Vγ9Vδ2 T cells do not express CXCR5.** Freshly isolated PBMCs from a single donor were stained with anti-CXCR5-PE, anti-Vγ9-E-Cy5, anti-CD4-FITC and anti-CD3-APC. Cells were analysed by flow cytometry for CXCR5 expression by gating on lymphocytes, gating out dead cells and doublets, and gating on, (A) $V\gamma9^{+}CD3^{+}$ cells or (B) $CD4^{+}CD3^{+}$ T cells (positive control). Gates were set using isotype-matched control antibodies. Numbers in dot plots represent the percentage of CXCR5 positive cells. Data are representative of seven independently assessed donors.

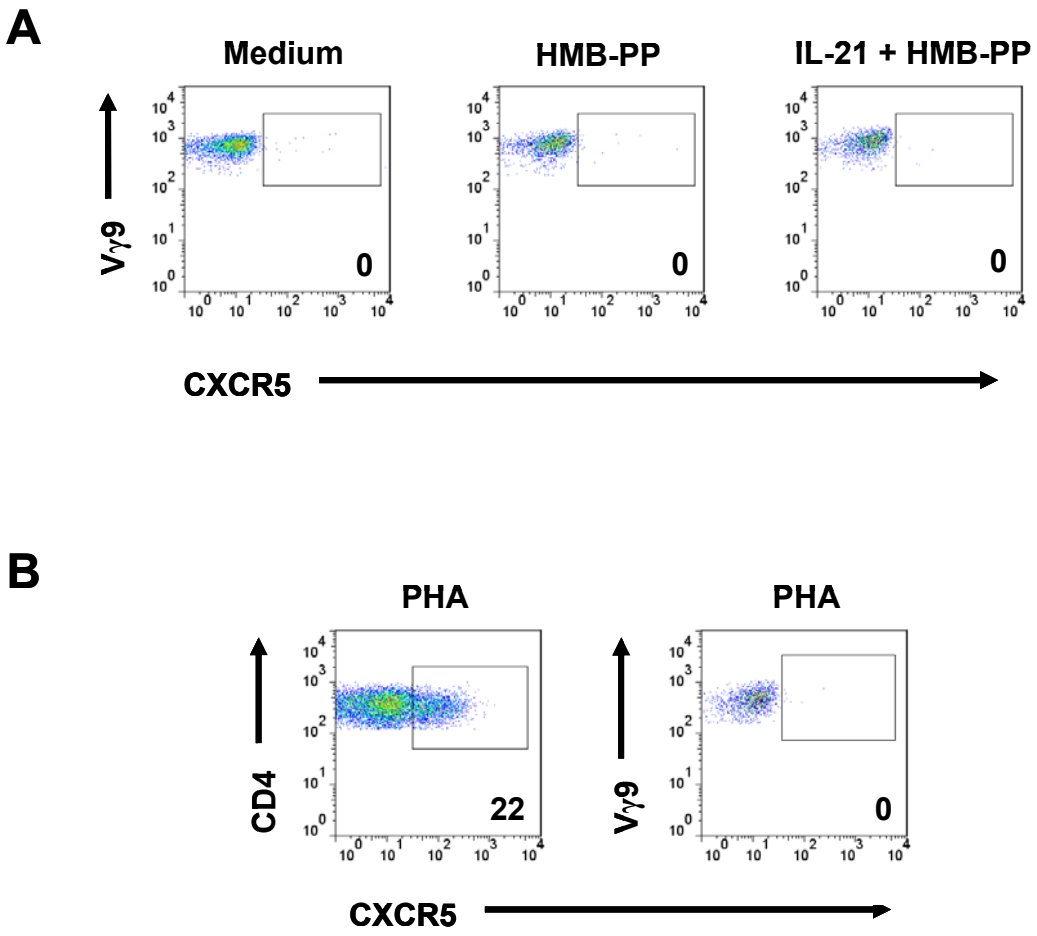


**Figure 3.13. IL-21, HMB-PP or PHA stimulation does not induces CXCR5 expression in peripheral Vγ9Vδ2 T cells.**
Freshly isolated PBMCs from a single donor were cultured in medium, with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml) or with PHA (1μg/ml). After 2 days in culture, stimulated PBMCs were stained with anti-CXCR5-PE, anti-Vγ9-PE-Cy5, anti-CD4-FITC and anti-CD3-APC. Cells were analysed by flow cytometry for CXCR5 expression, by gating on lymphocytes, gating out dead cells and doublets, and gating on $V\gamma9^{+}CD3^{+}$ cells or $CD4^{+}CD3^{+}$ T cells (positive control). Gates were set using isotype-matched control antibodies. Numbers in dot plots represent the percentage of CXCR5 positive cells. Data are representative of seven (A) and four (B) independently assessed donors.

### 3.2.6. IL-21 and HMB-PP stimulation induces expression of ICOS, OX40 and CD70 co-stimulatory molecules on peripheral Vγ9Vδ2 T cells

Numerous co-stimulatory molecules have been identified playing a role in the initiation of T-dependent B cell responses. Successful γδ-induced B cell activation and response would require physical interaction via presentation of co-stimulatory molecules. Earlier studies involving the use of a weaker Vγ9Vδ2 T cell activator (IPP) in the absence of cytokine stimulation have shown up-regulation of some co-stimulatory molecules (CD40L, OX40, ICOS and CD70) involved in B cell help (Brandes et al. 2003). Here, by using IL-21 in the presence of HMB-PP, I use a more physiologically relevant and novel way of inducing Vγ9Vδ2 T cell expression of co-stimulatory molecules. Furthermore, I utilise 9-colour flow cytometry, which not only allows gating out of dead cells and doublets but also allows several markers to be studied at the same time. This approach not only allows expression of several markers to be studied on Vγ9Vδ2 T cells but also simultaneously (as a control) on $CD4^+$ T cells. Here, I examined surface expression of co-stimulatory molecules CD40L, ICOS, OX40, CD70, CD28 and CD27 on freshly isolated and *in vitro* HMB-PP and IL-21 stimulated peripheral blood Vγ9Vδ2 T cells.

Surface expression of the early co-stimulatory molecule CD40L is transient and is usually only expressed by activated T cells (Daoussis et al. 2004). Here, surface CD40L was not detected on freshly isolated peripheral Vγ9Vδ2 T cells or on $CD4^+$ T cells (**Figure 3.14**). Similarly, the late co-stimulatory molecules ICOS and OX40 were both found to be absent on freshly isolated peripheral Vγ9Vδ2 T cells (**Figure 3.15A**). Although, ICOS (6.6%) and OX40 (7.2%) was detected on a small percentage of freshly isolated peripheral $CD4^+$ T cells (**Figure 3.15B**). CD70, another important co-stimulatory molecule involved in B cell help was also found to be absent on freshly isolated peripheral Vγ9Vδ2 T cells (data not shown). Whereas co-stimulatory molecules CD28 and CD27 were found to be expressed by 67%

and 92% of freshly isolated Vγ9Vδ2 T cells, respectively (**Figure 3.16**). Similarly, the majority (94%) of freshly isolated peripheral $CD4^+$ T cells also expressed CD27 (data not shown).

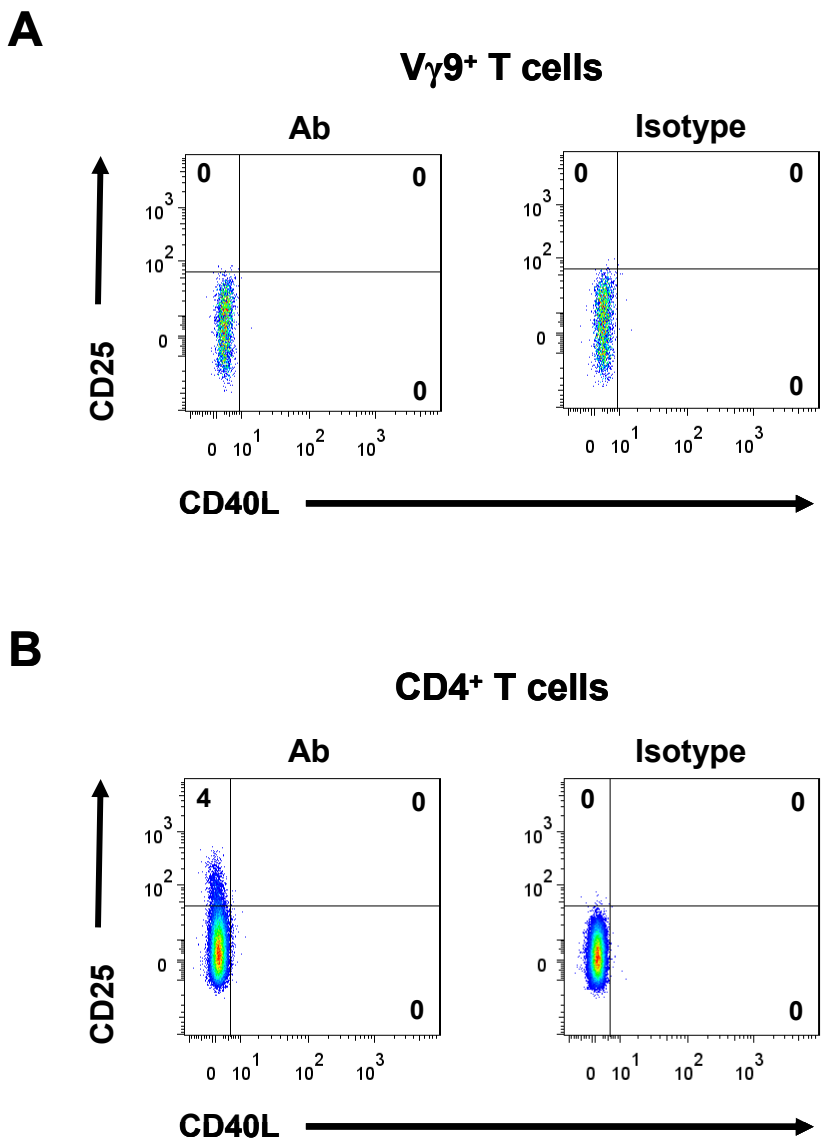


**Figure 3.14. Freshly isolated peripheral Vγ9Vδ2 T cells do not express CD40L.** Freshly isolated PBMCs from a single donor were stained with anti-CD40L-Pacific Blue, anti-CD25- Pe-Cy7, anti-Vγ9-PE-Cy5, anti-CD4-ECD and anti-CD3-APC-H7. Cells were analysed by flow cytometry by gating on lymphocytes, gating out dead cells and doublets, and gating on (A) $V\gamma9^{+}CD3^{+}$ cells or (B) $CD4^{+}CD3^{+}$ T cells. Gates were set using isotype-matched control antibodies. Numbers in dot plots represent the percentage of CD25 and CD40L positive cells. Data are representative of three independently assessed donors.

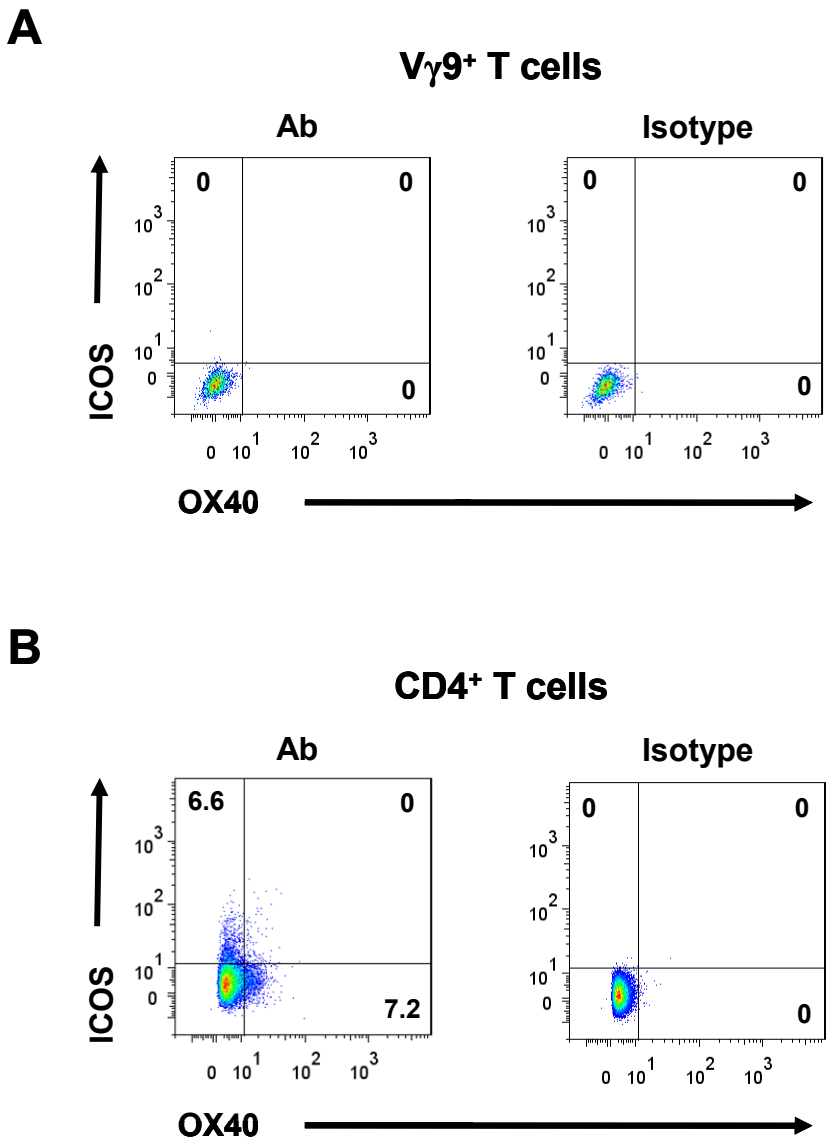


**Figure 3.15. Freshly isolated peripheral Vγ9Vδ2 T cells do not express ICOS or OX40.** Freshly isolated PBMCs from a single donor were stained with anti-ICOS-PE, anti-OX40-FITC, anti-Vγ9-PE-Cy5, anti-CD4-ECD and anti-CD3-APC. Cells were analysed by flow cytometry by gating on lymphocytes, gating out dead cells and doublets, and gating on (A) $V\gamma9^{+}CD3^{+}$ cells or (B) $CD4^{+}CD3^{+}$ T cells (positive control). Gates were set using isotype-matched control antibodies. Numbers in dot plots represent the percentage of ICOS and OX40 positive cells. Data are representative of three independently assessed donors.

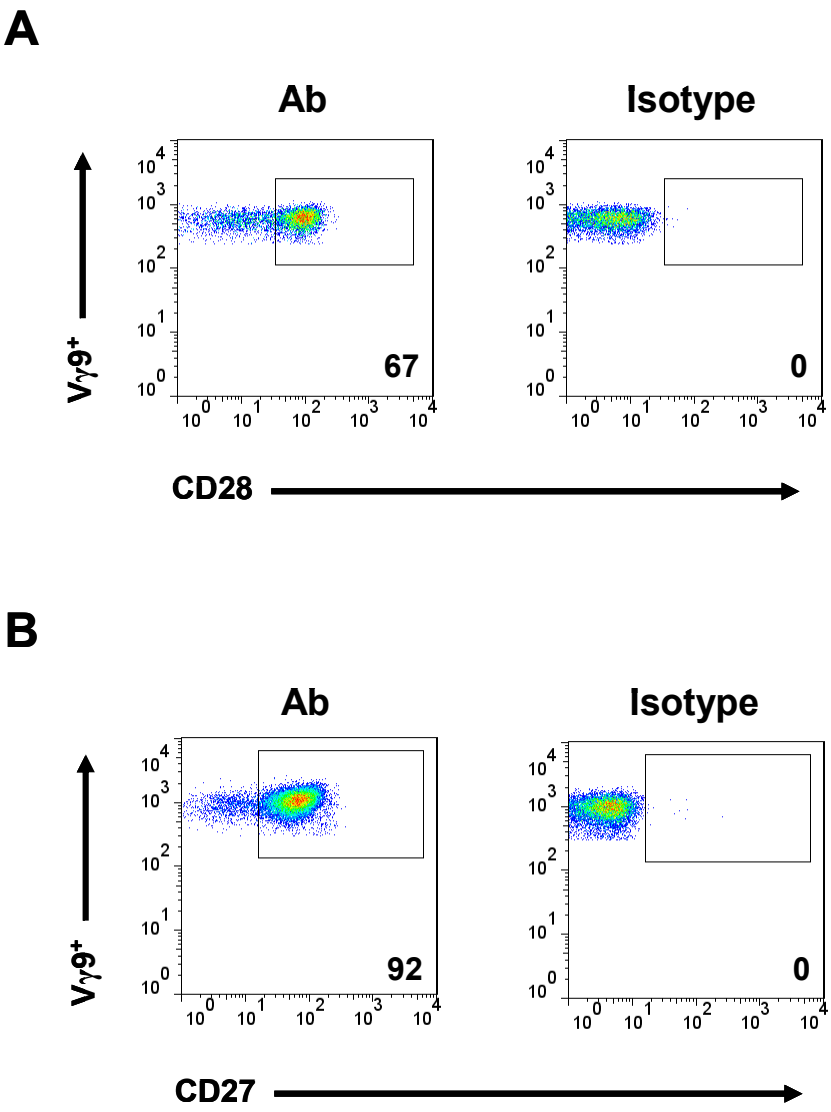


**Figure 3.16. Freshly isolated peripheral Vγ9Vδ2 T cells express CD28 and CD27.** Freshly isolated PBMCs from a single donor were stained with anti-CD28-PE-Cy5, anti-CD27-FITC, anti-Vγ9-PE-Cy5 or FITC conjugated and anti-CD3-APC. Cells were analysed by flow cytometry by gating on lymphocytes, gating out dead cells and doublets, and gating on $V\gamma9^{+}CD3^{+}$ T cells. Gates were set using isotype-matched control antibodies. Numbers in dot plots represent the percentage of (A) CD28 and (B) CD27 positive cells. Data are representative of (A) one and (B) three independently assessed donors.

To study surface expression of CD40L, ICOS and OX40 co-stimulatory molecules on HMB-PP and IL-21 stimulated peripheral Vγ9Vδ2 T cells, freshly isolated PBMCs were cultured in medium with or without HMB-PP. After 42 hours of HMB-PP pre-stimulation, HMB-PP stimulated and non HMB-PP stimulated cultures (medium alone) were treated with or without IL-21. After 2, 3, 4, 5 and 6 days in culture, cells were harvested and stained with fluorochrome-conjugated antibodies specific for CD40L, ICOS, OX40 and CD25. Cells were analysed by flow cytometry and assessed for CD40L, ICOS, OX40 and CD25 surface expression by gating on lymphocytes, gating out dead cells and doublets, and gating on $V\gamma9^{+}CD3^{+}$ or $CD4^{+}CD3^{+}$ T cells.

Representative flow cytometry dotplots of CD40L expression on $V\gamma9^{+}CD3^{+}$ and $CD4^{+}CD3^{+}$ T cells is shown in **Figure 3.17**. Expression of the early co-stimulatory marker CD40L was not detected on HMB-PP-stimulated or on non-stimulated (medium control) Vγ9Vδ2 T cells (**Figure 3.17A**). $CD4^{+}$ T cells are known to rapidly up-regulated CD40L when cultured for 6 hours with PMA and ionomycin. To test methodology used and the binding potential of the CD40L antibody, I examined induction of CD40L expression on $CD4^{+}$ T cells. Here, PMA and ionomycin stimulation induced CD40L on 51% of $CD4^{+}$ T cells (**Figure 3.17B**).

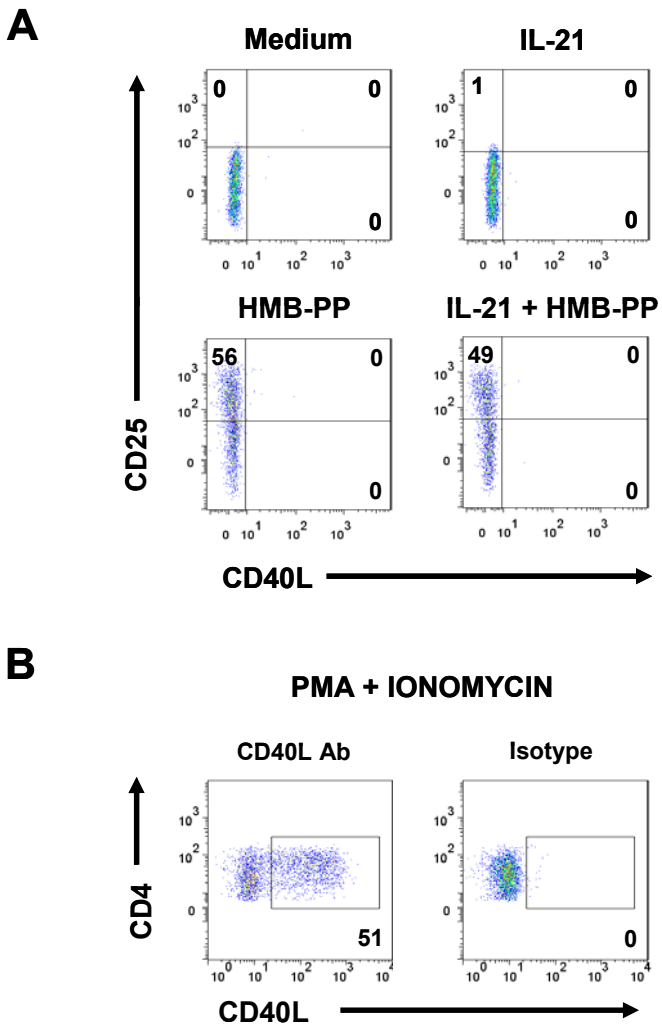


**Figure 3.17. IL-21 does not induce CD40L expression by HMB-PP activated peripheral Vγ9Vδ2 T cells.**
(A) Freshly isolated PBMCs were cultured in medium with or without HMB-PP (10 nM). After 42 hours, HMB-PP stimulated and non HMB-PP stimulated cultures (medium alone) were treated with or without IL-21 (50 ng/ml). After 2 days (6 hours post IL-21 stimulation) cells were harvested and stained with anti-CD40L-Pacific Blue, anti-CD25- Pe-Cy7, anti-Vγ9-PE-Cy5 and anti-CD3-APC-H7. Cells were analysed by flow cytometry by gating on lymphocytes, gating out dead cells and doublets, and gating on $V\gamma9^{+}CD3^{+}$ cells. Gates were set using isotype-matched control antibodies. Numbers in dot plots represent the percentage of CD25 and CD40L positive cells. (B) As a positive control PBMCs were also cultured with PMA (10 ng/ml) and ionomycin (1 µg/ml). After 6 hours, cells were harvested and analysed by flow cytometry by gating on lymphocytes, live single cells and $CD4^{+}CD3^{+}$ cells. Numbers in dot plots represent the percentage of CD40L positive cells. Data are representative of (A) three and (B) one independently assessed donor(s).

Vγ9Vδ2 T cell surface expression of the late co-stimulatory molecules OX40 and ICOS was analysed over a 6 day culture period. Cumulative data from 3 different healthy donors is shown in **Figure 3.18A**. On day 2, in the presence of HMB-PP stimulation (with and without IL-21 co-stimulation), a substantial up-regulation of OX40 was observed, with 43% of Vγ9Vδ2 T cells expressing OX40. Similar levels of OX40 expression was maintained for a further 1 day in response to HMB-PP, and a further 2 days with co-stimulation with IL-21. OX40 expression on Vγ9Vδ2 T cells induced by HMB-PP alone declined considerably after 3 days in culture and after 4 days in the presence of IL-21 co-stimulation (**Figure 3.18A**).

In contrast, up-regulation of ICOS expression on Vγ9Vδ2 T cells was more gradual. On day 2, in response to HMB-PP stimulation alone 12.7% (± 3.8%) of Vγ9Vδ2 T cells expressed ICOS increasing to 21% (± 7.7%) on day 3. Percentage of ICOS positive Vγ9Vδ2 T cells did not increase further with HMB-PP stimulation alone. Strikingly, the addition of IL-21 co-stimulation induced and maintained a higher percentage of ICOS positive Vγ9Vδ2 T cells. Over the 6 day culture period, levels of ICOS expression remained constant in response to HMB-PP stimulation, whereas co-treatment with IL-21 resulted in a steady increase in percentage of ICOS positive Vγ9Vδ2 T cells. On day 3, 26% (± 6%) of Vγ9Vδ2 T cells expressed ICOS this was increased to 40% (± 8.5%) on day 4, and similar levels were maintained after 5 and 6 days (44% ± 3%) in culture. Thus, revealing an IL-21 dependent response in maintenance of a higher percentage of ICOS positive Vγ9Vδ2 T cells (**Figure 3.18A**). Stimulation of PBMCs with HMB-PP and IL-21 had no effect on ICOS or OX40 expression on $CD4^+$ T cells, indicating a Vγ9Vδ2 T cell specific response (**Figure 3.18B**). Representative flow cytometry dotplots of ICOS and OX40 expressingVγ9Vδ2 T cells, after 4 days in culture is shown in **Figure 3.19**. Notably, co-stimulation with IL-21 resulted in 16% of Vγ9Vδ2 T cells co-expressing ICOS and OX40 compared to only 3% with HMB-PP alone.

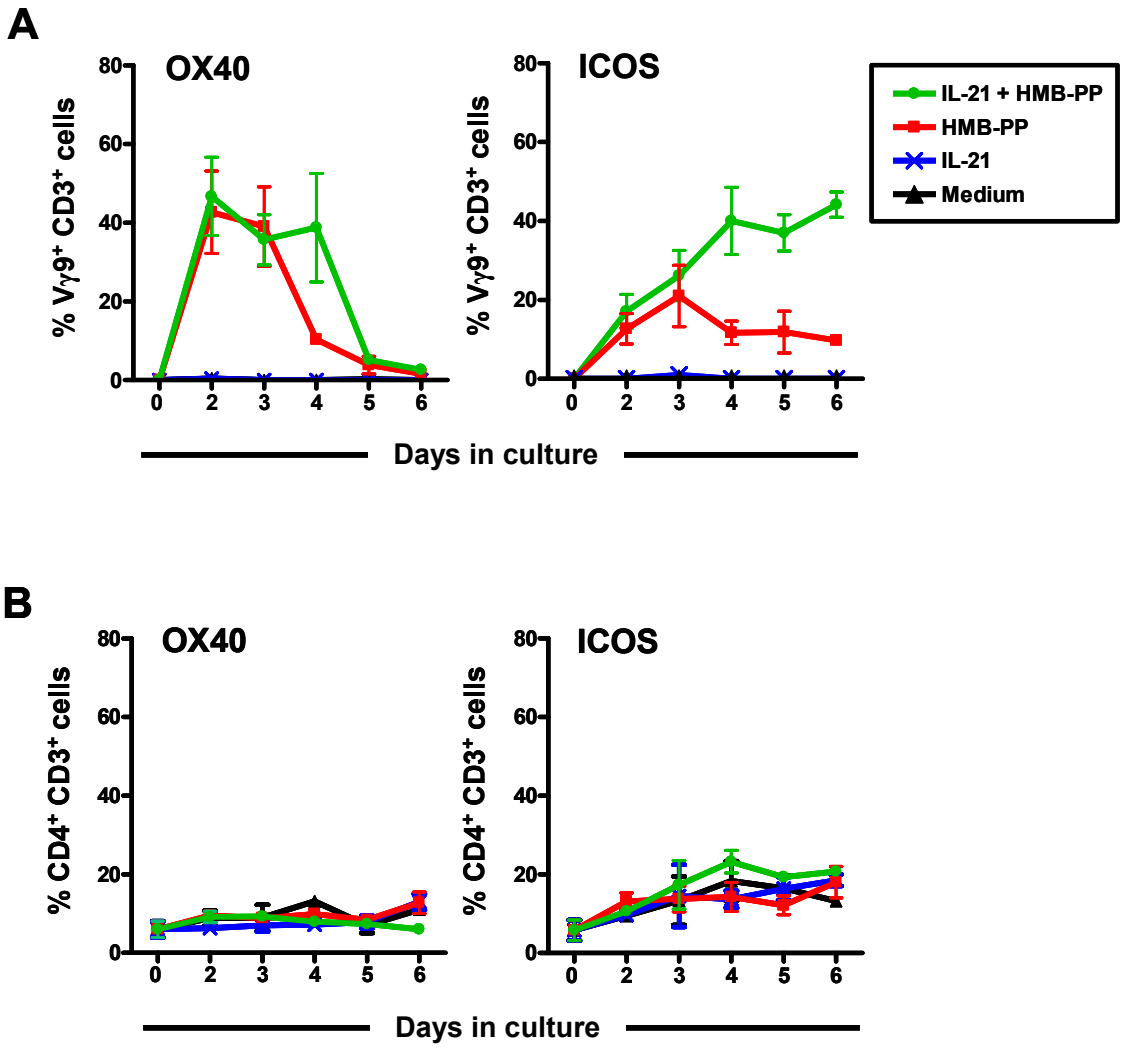


**Figure 3.18. IL-21 maintains higher expression of OX40 and ICOS on HMB-PP activated peripheral Vγ9Vδ2 T cells.**
Freshly isolated PBMCs were cultured in medium with or without HMB-PP (10 nM). After 42 hours, HMB-PP stimulated and non HMB-PP stimulated cultures (medium alone) were treated with or without IL-21 (50 ng/ml). Freshly isolated PBMCs (Day 0) and PBMCs after 2, 3, 4, 5 and 6 days in culture, were stained for flow cytometry with anti-ICOS-PE, anti-OX40-FITC, anti-Vγ9-PE-Cy5, anti-CD4-ECD and anti-CD3-APC. Isotope-matched antibodies were used as control staining. Cells were analysed for percentage of OX40 and ICOS positive (A) $V\gamma9^{+}CD3^{+}$cells, and (B) $CD4^{+}CD3^{+}$ cells. Results are expressed as mean ± SEM of three independently assessed donors.

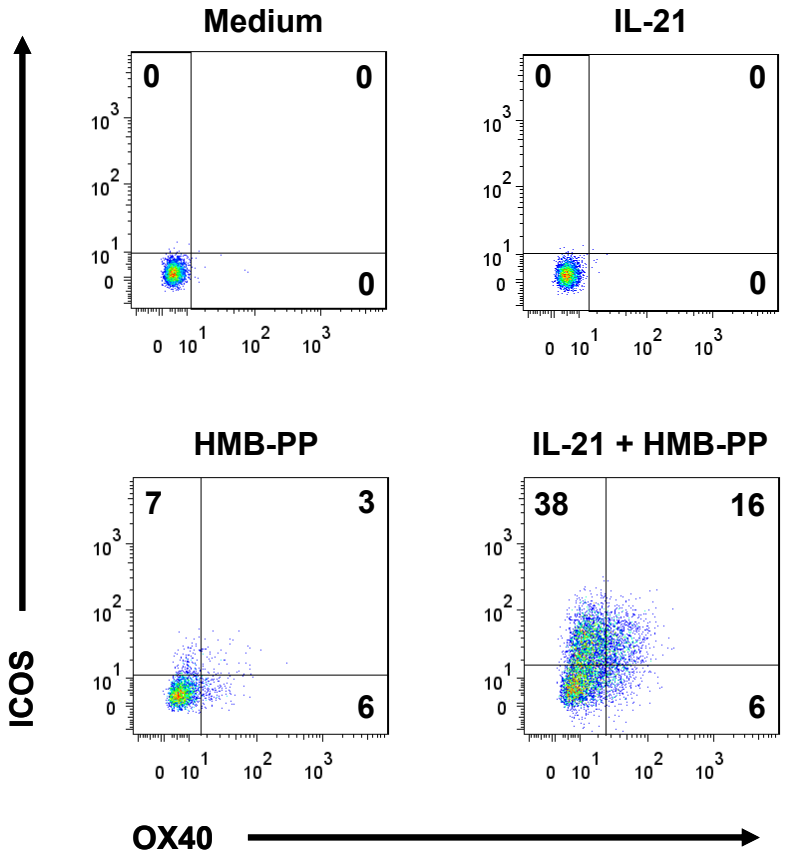


**Figure 3.19. IL-21 induces co-expression of ICOS and OX40 on HMB-PP activated peripheral Vγ9Vδ2 T cells.**
Freshly isolated PBMCs were cultured in medium with or without HMB-PP (10 nM). After 42 hours, HMB-PP stimulated and non HMB-PP stimulated cultures (medium alone) were treated with or without IL-21 (50 ng/ml). After 4 days cells were harvested and stained with anti-ICOS-PE, anti-OX40-FITC, anti-Vγ9-PE-Cy5 and anti-CD3-APC. Cells were analysed by flow cytometry by gating on lymphocytes, gating out dead cells and doublets, and gating on $V\gamma9^{+}CD3^{+}$ cells. Gates were set using isotype-matched control antibodies. Numbers in dot plots represent the percentage of ICOS and OX40 positive cells. Data are representative of three independently assessed donors.

Surface expression of other co-stimulatory molecules such as CD70, CD28 and CD27 was also studied on HMB-PP and IL-21 stimulated peripheral Vγ9Vδ2 T cells. Here, freshly isolated PBMCs were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml). Freshly isolated PBMCs (Day 0) and PBMCs after 1, 2 3 and 4 days in culture were harvested and stained with fluorochrome-conjugated antibodies specific for CD70, CD28 and CD27. Cells were analysed by flow cytometry and assessed for CD70, CD28 and CD27 surface expression by gating on live cells lymphocytes and on $V\delta2^{+}CD3^{+}$ or $V\gamma9^{+}CD3^{+}$ cells. Data from 1 to 4 different healthy donors is shown in **Figure 3.20**.

On day 2, in the presence of HMB-PP stimulation expression of CD70 was detected on 14% (± 3.5%) of Vγ9Vδ2 T cells, which peaked to 39% (± 4%) on day 3, expression was not maintained long-term, as a dramatic decrease was observed on day 4. The addition of IL-21 to HMB-PP stimulated Vγ9Vδ2 T cells resulted in suppression of surface CD70, as only 9% (± 3.7%) and 14% (± 5%) of Vγ9Vδ2 T cells expressed CD70 on day 2 and day 3, respectively (**Figure 3.20A**).

The majority of freshly isolated peripheral Vγ9Vδ2 T cells expressed both CD28 and CD27 (**Figure 3.20**). Culturing of PBMCs over 3 days in medium alone (regardless of culture condition) resulted in a remarkable loss of CD28 expressing Vγ9Vδ2 T cells (**Figure 3.20B**) and a gradual but minor decrease in CD27 expressing cells (**Figure 3.20C**).

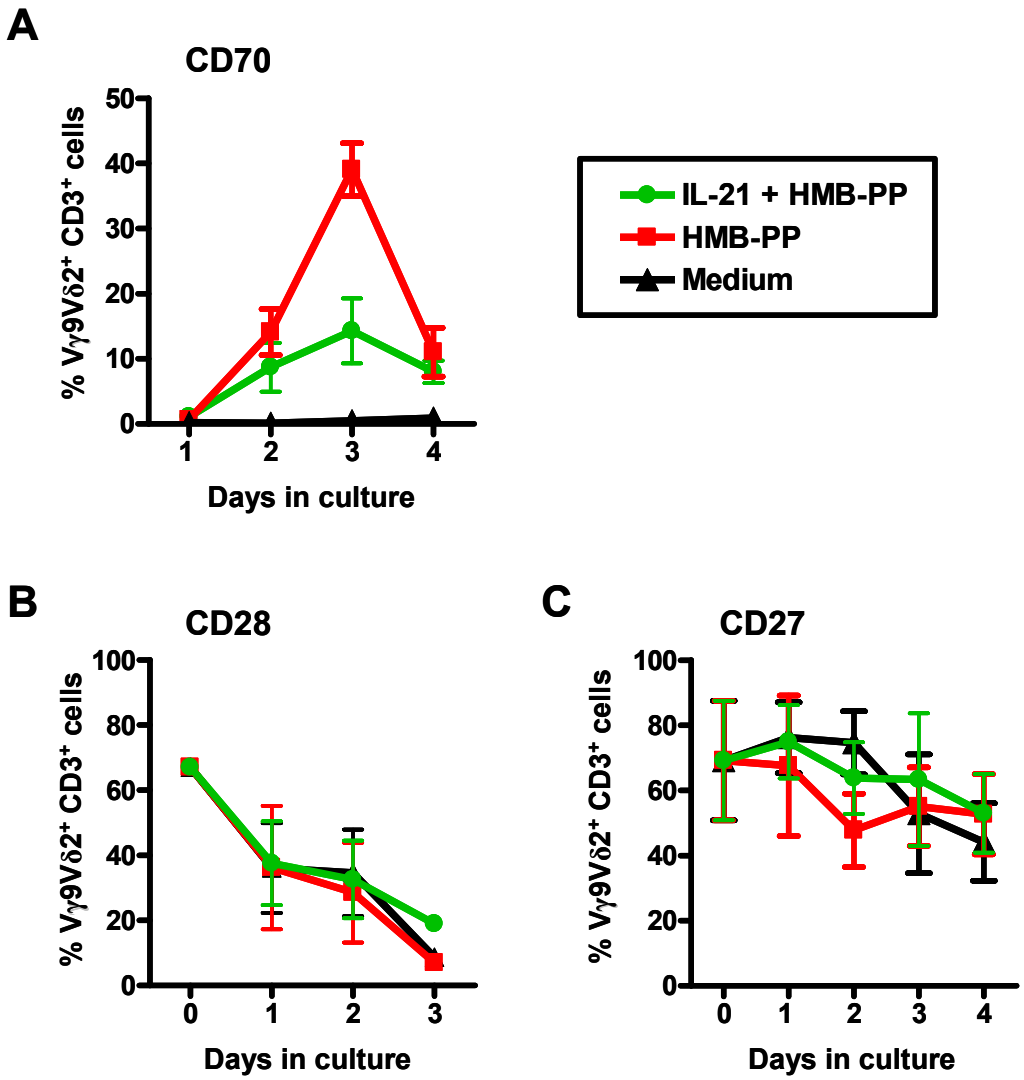


**Figure 3.20. CD70, CD28 and CD27 co-stimulatory molecule expression on non-activated and HMB-PP activated peripheral Vγ9Vδ2 T cells.**
Freshly isolated PBMCs were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml). Freshly isolated PBMCs (Day 0) and PBMCs after 1, 2, 3 and 4 days in culture were stained and analysed by flow cytometry for (A) CD70 (B) CD28 and (C) CD27 expression, by gating on live cells, lymphocytes and on $V\delta2^{+}CD3^{+}$ or $V\gamma9^{+}CD3^{+}$ cells. Gates were set using isotype-matched control antibodies. CD28 day 0 and day 3 data representative of one donor, all other results are expressed as mean percentage ± SEM of two or four donors.

### 3.2.7. HMB-PP stimulation induces expression of CCR7, CD40, CD86 and HLA-DR on peripheral Vγ9Vδ2 T cells

After infection, antigen-presenting cells (APCs) initiate T cell immunity by the capture and presentation of foreign antigens to circulating T cells within secondary lymphoid organs. Expression of the chemokine receptor CCR7 allows migration and continuous recirculation of peripheral T and B cells into secondary lymphoid tissues. Circulating T cells become activated by APCs after presentation of cognate antigen and APC markers (Moser and Eberl 2007).

As shown by previous studies, activated γδ T cells have the ability to differentiate into professional antigen presenting cells (γδ T-APCs) and present antigen, thus enabling γδ T cells to induce $CD4^+$ and $CD8^+$ T cell responses (Brandes et al. 2009; Brandes et al. 2005; Meuter et al. 2010). As an additional and alternative mechanism of γδ T cell in providing B cell help, I propose HMB-PP activated γδ T cells to differentiate into γδ T-APCs allowing interaction of γδ T cells as APCs with $CD4^+$ T cells in secondary lymphoid tissue. In this working model, expression of CCR7 would allow migration of peripheral γδ T cells into secondary lymphoid tissues, where γδ T cell secretion of CXCL13 would attracts CXCR5-positive $CD4^+$ T and B cells. $CD4^+$ T cells become activated via co-localization and presentation of microbial antigen and APC markers by γδ T-APCs. In turn, activated $CD4^+$ T cells (as Tfh cells) along with activated γδ T cells work in synergy to provide B cell help (Moser and Eberl 2007; Vermijlen et al. 2007). Thus, here I examined the expression of APC markers on freshly isolated and *in vitro* HMB-PP and IL-21 stimulated peripheral blood Vγ9Vδ2 T cells.

To study the expression of APC markers on Vγ9Vδ2 T cells, freshly isolated PBMCs were cultured in medium with or without HMB-PP (10 nM). After 42 hours, HMB-PP stimulated and non HMB-PP stimulated cultures (medium alone) were treated with or without IL-21 (50 ng/ml). Freshly isolated PBMCs (Day 0) and PBMCs after 1, 2, and 3 days in culture

were stained with fluorochrome-conjugated antibodies specific for CCR7, CD40, CD86 and HLA-DR APC markers. Cells were analysed by flow cytometry and assessed for surface APC marker expression by gating on lymphocytes, gating out dead cells and doublets, and gating on $V\gamma9^{+}CD3^{+}$ T cells.

Cumulative data from 2 and 3 different healthy donors over a 6 day culture period is shown in **Figure 3.21**. Freshly isolated peripheral blood Vγ9Vδ2 T cells lack surface expression of CCR7, CD86 and HLA-DR, however around 20% express surface CD40. Culturing of PBMCs with HMB-PP alone, for 2 days (in the presence or absence of IL-21) induced Vγ9Vδ2 T cell surface expression of CCR7 (7.8 % ± 1.3%), CD86 (6.5 % ± 1.5%) and HLA-DR (26 % ± 5.6%) and further enhanced expression of CD40 (70 % ± 3.7%). In response to HMB-PP, the percentage of CCR7 and HLA-DR expressing cells increased from day 2 to day 3 in culure whereas the percentage of CD40 and CD86 expressing cells remained the same.

Co-treatment with IL-21 did not further enhance expression of CD40, CD86 or HLA-DR. However, 3 day with IL-21 co-stimulation (in the presence of HMB-PP) did somewhat increase the percentage of CCR7 positive Vγ9Vδ2 T cells (18% ± 4.6%) compared to culturing with HMB-PP alone (14% ± 4.2%), however, this was not found to be significant (**Figure 3.21**).

Furthermore, HMB-PP stimulated Vγ9Vδ2 T cells co-expressed CCR7, CD40 and HLA-DR. Representative flow cytometry dotplots from a single donor display co-expression of CD40 and HLA-DR (**Figure 3.22A**), CD40 and CCR7 (**Figure 3.22B**), HLA-DR and CCR7 (**Figure 3.22C**) on HMB-PP stimulated Vγ9Vδ2 T cells.

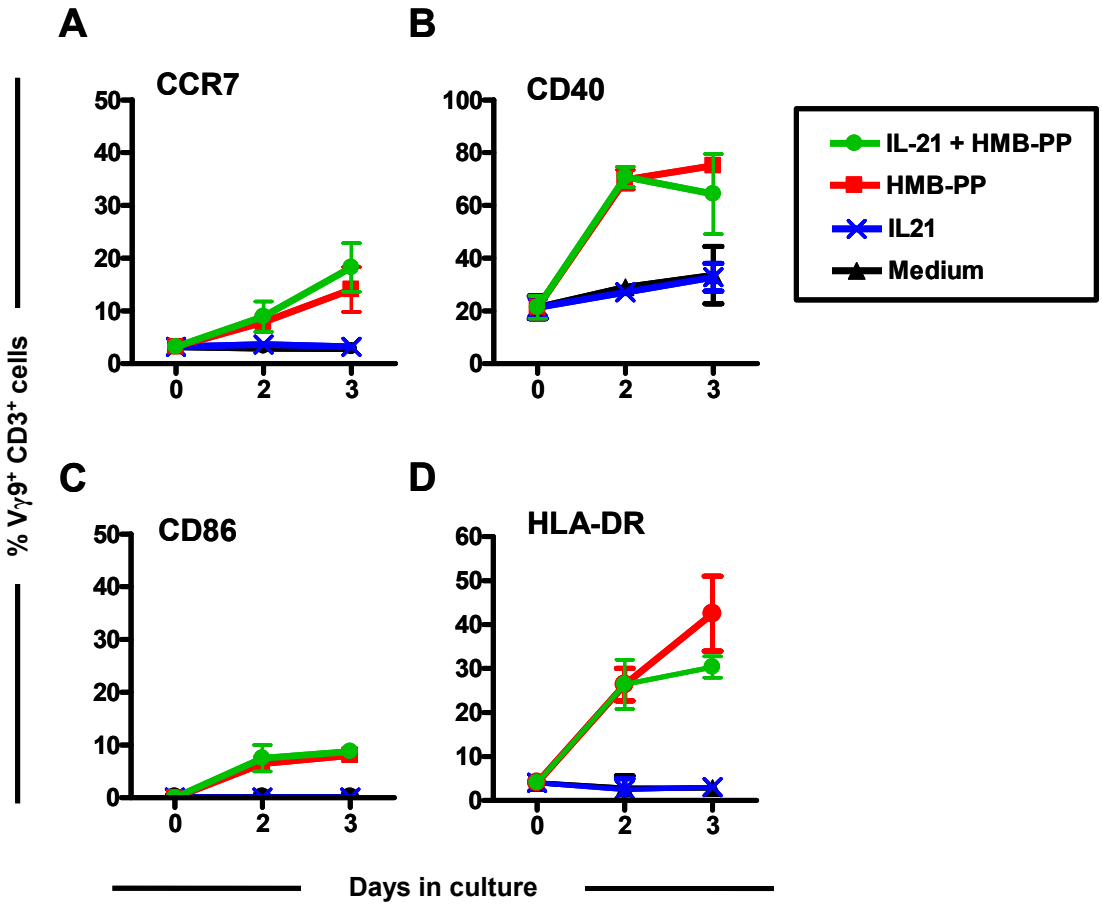


**Figure 3.21. HMB-PP stimulation up-regulates CCR7, CD40, CD86 and HLA-DR expression on peripheral Vγ9Vδ2 T cells.**
Freshly isolated PBMCs were cultured in medium with or without HMB-PP (10 nM). After 42 hours, HMB-PP stimulated and non HMB-PP stimulated cultures (medium alone) were treated with or without IL-21 (50 ng/ml). Freshly isolated PBMCs (Day 0) and PBMCs after 2 and 3 days in culture were stained with anti-CCR7, anti-CD40-PE, anti-CD86-FITC, HLA-DR-APC-H7, anti-CD3-APC-H7 and anti-Vγ9-Pe-Cy5. Cells were analysed by flow cytometry for (A) CCR7, (B) CD40, (C) CD86 and (D) HLA-DR expression by gating on lymphocytes, gating out dead cells and doublets, and gating on $V\gamma9^{+}CD3^{+}$ cells. Gates were set using isotype-matched control antibodies. Results are expressed as mean percentage ± SEM of (C) two and (A, B and D) three donors.

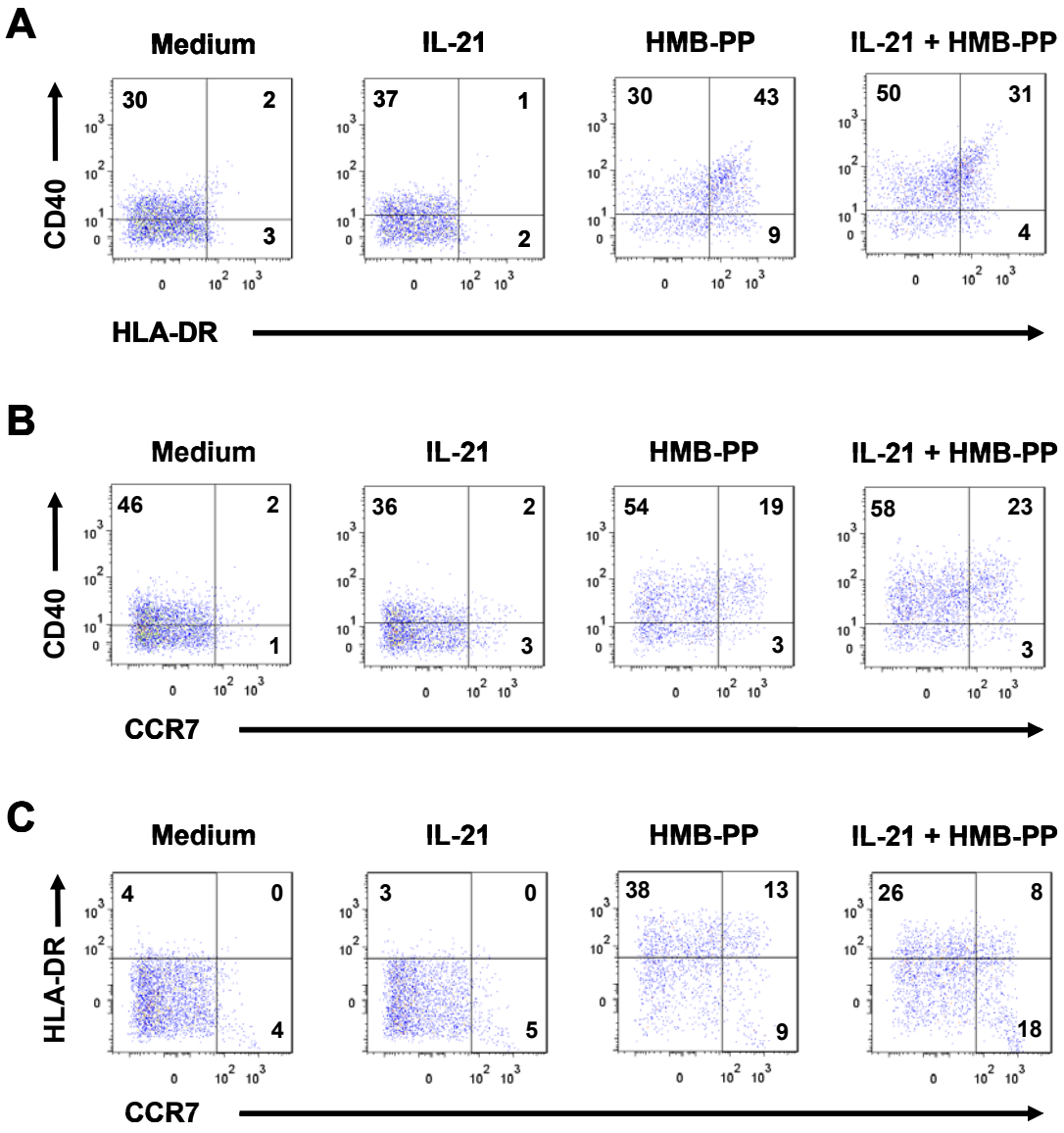


**Figure 3.22. HMB-PP stimulation induces co-expression of CCR7, CD40 and HLA-DR on peripheral Vγ9Vδ2 T cells.**
PBMCs were cultured in medium with or without HMB-PP (10 nM). After 42 hours, HMB-PP stimulated and non HMB-PP stimulated cultures (medium alone) were treated with or without IL-21 (50 ng/ml). After 3 days cells were harvested and stained for flow cytometry as described in Figure 3.21. Cells were analysed by gating on live, single, $CD3^+$ $V\gamma9^+$ cells. Numbers in dot plots represent the percentage of (A) CD40 and HLA-DR, (B) CD40 and CCR7, (C) HLA-DR and CCR7 positive Vγ9Vδ2 T cells. Gates were set using isotype-matched control antibodies. Data are from a single donor and is representative of two donors.

## 3.2.8. Proliferation and differentiation of Vγ9Vδ2 T cell subsets in response to IL-21 and HMB-PP stimulation

Similar to circulating peripheral $CD4^+$ T cells, peripheral Vγ9Vδ2 T cells are heterogeneous and comprise of distinct memory populations, that can be distinguished on the basis of either CD45RA/CCR7 or CD45RA/CD27 surface marker expression (Dieli et al. 2003; Moser and Eberl 2007; Sallusto et al. 2004). Here, using these two different methods of identifying Vγ9Vδ2 T cell memory populations, I investigated the differentiation and proliferation of Vγ9Vδ2 T cell naïve/memory subsets in response to *in vitro* IL-21 and HMB-PP stimulation.

Freshly isolated PBMCs were cultured in medium or with HMB-PP, in the absence or presence of IL-21 or with IL-21 alone. Freshly isolated PBMCs and PBMCs after 1, 2, and 3 days in culture were harvested and stained with fluorochrome-conjugated antibodies specific for memory markers CD45RA and CCR7 or CD27, in combination with Vδ2 or Vγ9 and CD3 surface markers. Cells were analysed by flow cytometry by gating on lymphocytes, gating out dead cells and doublets, and gating on $Vγ9^+CD3^+$ or $Vδ2^+CD3^+$ T cells. On the gated Vγ9Vδ2 T cells a dot plot with CCR7 or CD27 along the y-axis and CD45RA on the x-axis was created. Quadrants in dot plots were set using isotype-matched control antibodies. Vγ9Vδ2 T cell memory subsets were identified based on CD45RA/CCR7 expression as follows: $CD45RA^+CCR7^+$ (Naïve), $CD45RA^-CCR7^+$ (T central memory; $T_{CM}$), $CD45RA^-CCR7^-$ (T effector memory; $T_{EM}$) and $CD45RA^+CCR7^-$ (terminally differentiated effector memory T cell; $T_{EMRA}$) (**Figure 3.23E**). Here, in freshly isolated PBMCs 1% of total Vγ9Vδ2 T cells were naïve (± 0.6 [SEM] n = 3), 1% $T_{CM}$ (± 1.3%), 61% $T_{EM}$ (± 9%) and 35% $T_{EMRA}$ (± 9%). Thus majority of peripheral Vγ9Vδ2 T cells were $T_{EM}$, whilst the naïve and the $T_{CM}$ subset represented a minor or absent population (**Figure 3.23**). The distribution of Vγ9Vδ2 T cell naïve/memory subsets differed form donor to donor.

Culturing of PBMCs over 3 days in medium alone had not maintained Vγ9Vδ2 T cell naïve/memory pools. Pattern of responsiveness of Vγ9Vδ2 T cell $T_{EM}$ and $T_{EMRA}$ subsets cultured in medium alone was comparable to cultures stimulated with HMB-PP and IL21. Hence, HMB-PP or IL-21 stimulation had no major additional effects on Vγ9Vδ2 T cell $T_{EM}$ and $T_{EMRA}$ populations. After 1 day of culturing in medium alone, the percentage of Vγ9Vδ2 T cells in the $T_{EM}$ pool increased from 60 to 88% (**Figure 3.23C**), reciprocally the percentage of cell in the $T_{EMRA}$ pool decreased from 38% to 6% (**Figure 3.23D**). After 3 days the percentage of cells in the $T_{EM}$ pool had gradually returned back to similar levels as that seen on day 0 (**Figure 3.23C**).

However, stimulation with HMB-PP and IL-21 did have additional effects on Vγ9Vδ2 T naïve and $T_{CM}$ populations. Culturing of PBMCs in medium alone resulted in a loss of naïve Vγ9Vδ2 T cells. Whereas in the presence of HMB-PP stimulation loss of naïve Vγ9Vδ2 T cells was somewhat rescued and levels were maintained throughout the 3 day culture (**Figure 3.23 A**). Furthermore, an increase in the $T_{CM}$ population (9% ± 3%) was observed after 3 days in response to HMB-PP stimulation, which was further enhanced in the presence of IL-21 stimulation (13% ± 6%) (**Figure 3.23 B**).

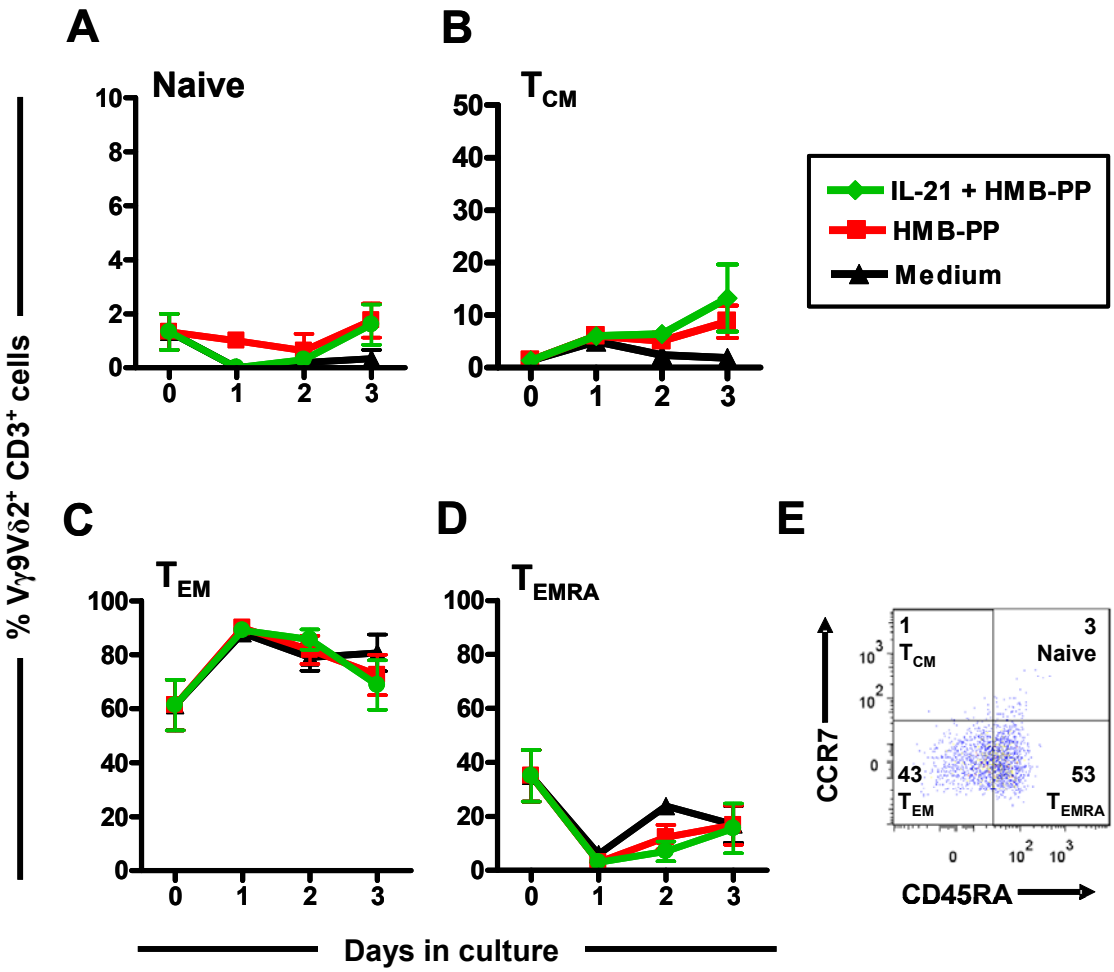


**Figure 3.23. The effects of IL-21 and HMB-PP stimulation on Vγ9Vδ2 T cell memory subsets, based on CCR7 expression.**
Freshly isolated PBMCs were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml). Freshly isolated PBMCs (0 hours) and PBMCs after 1, 2, and 3 days in culture were harvested and stained for memory markers CCR7 and CD45RA in combination with Vδ2 or Vγ9 and CD3 surface markers. Cells were analysed by flow cytometry by gating on lymphocytes, gating out dead cells and doublets, and gating on $Vγ9^+CD3^+$ or $Vδ2^+CD3^+$ T cells. Memory subsets were classified as follows: (A) Naïve = $CD45RA^+CCR7^+$, (B) T central memory ($T_{CM}$) = $CD45RA^-CCR7^+$, (C) T effector memory ($T_{EM}$) = $CD45RA^-CCR7^-$ and (D) terminally differentiated effector memory T cell ($T_{EMRA}$) = $CD45RA^+CCR7^-$. (E) A representative dot plot showing percentage of freshly isolated $Vδ2^+$ T cells expressing CCR7 and CD45RA. Quadrants were set using isotype-matched control antibodies. Results are expressed as mean percentage ± SEM of three (day 0, 2 and 3 data) and one (day 1 data) healthy donor(s).

Vγ9Vδ2 T cell memory subsets were also identified based on CD45RA/CD27 expression as follows: $CD45RA^{+}CD27^{+}$ (Naïve), $CD45RA^{-}CD27^{+}$ ($T_{CM}$), $CD45RA^{-}/CD27^{-}$ ($T_{EM}$) and $CD45RA^{+}CD27^{-}$ ($T_{EMRA}$) (**Figure 3.24E**). Cumulative data from 3 different healthy donors over a 3 day culture period is shown in **Figure 3.24.** Here, approximately 50% of freshly isolated peripheral Vγ9Vδ2 T cells were $T_{CM}$, 25% $T_{EM}$, 16% $T_{EMRA}$ and 8% naïve. Noticeably, Vγ9Vδ2 T cell memory subsets identified based on CD45RA/CD27 expression differed to those identified based on CD45RA/CCR7 expression (**Figure 3.24 and Figure 3.23**).

Similar to the above findings based on CD45RA/CCR7 expression, culturing in medium alone had not maintained naïve/memory pools. Over the three day culture period and regardless of culture condition, there was a gradual decrease in the percentage of naïve (**Figure 3.24A**) and $T_{CM}$ (**Figure 3.24B**) Vγ9Vδ2 T cells, whilst a gradual increase in the percentage of $T_{EM}$ (**Figure 3.24C**) and $T_{EMRA}$ (**Figure 3.24D**) Vγ9Vδ2 T cells. Here, HMB-PP or IL-21 stimulation had no major additional effect on the percentage of naïve/memory Vγ9Vδ2 T cells in short term cultures.

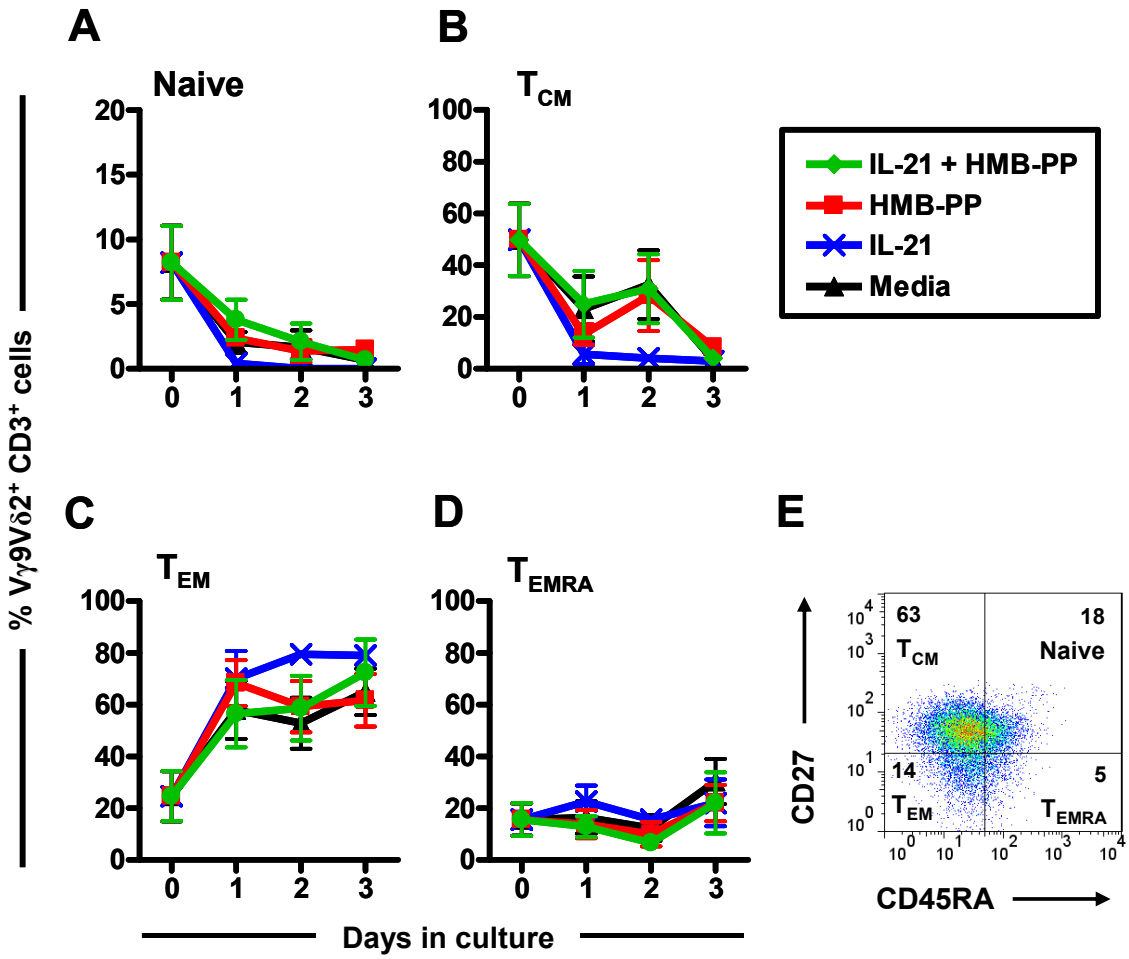


**Figure 3.24. The effects of IL-21 and HMB-PP stimulation on Vγ9Vδ2 T cell memory subsets, based on CD27 expression.**
Freshly isolated PBMCs were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated PBMCs (0 hours) and PBMCs after 1, 2, and 3 days in culture were harvested and stained for memory markers CD27 and CD45RA in combination with Vδ2 or Vγ9 and CD3 surface markers. Cells were analysed by flow cytometry by gating on lymphocytes, gating out dead cells and doublets, and gating on $V\gamma9^{+}CD3^{+}$ or $V\delta2^{+}CD3^{+}$ T cells. Memory subsets were classified as follows: (A) Naïve = $CD45RA^{+}CD27^{+}$, (B) $T_{CM}$ = $CD45RA^{-}CD27^{+}$, (C) $T_{EM}$ = $CD45RA^{-}CD27^{-}$ and (D) $T_{EMRA}$ = $CD45RA^{+}CD27^{-}$ (E) A representative dot plot showing percentage of freshly isolated $V\delta2^{+}$ T cells expressing CD27 and CD45RA. Quadrants were set using isotype-matched control antibodies. Results are expressed as mean percentage ± SEM of two to six healthy donors.

Finally, the proliferative capacity of Vγ9Vδ2 T cell in PBMCs in response to IL-21 and HMB-PP stimulation was also examined. Cumulative data from 3 different healthy donors, over a 7 day culture period is shown in **Figure 3.25**. In freshly isolated PBMCs, Vγ9Vδ2 T cells represented 4% (± 0.2) of total CD3 positive cells. In response to HMB-PP stimulation, Vγ9Vδ2 T cells began proliferating after 5 days in culture, increasing to 13.5% (± 4.8) after 7 days. The addition of IL-21 in the presence of HMB-PP enhanced proliferation of Vγ9Vδ2 T cell. After 7 days of culture Vγ9Vδ2 T cells represented 28.5% (± 8.6) of total CD3 positive cells. The error bars displayed in **Figure 3.25**, account for donor-to-donor variability between the three donors, in the expansion potential of peripheral Vγ9Vδ2 T cells (**Figure 3.25**).

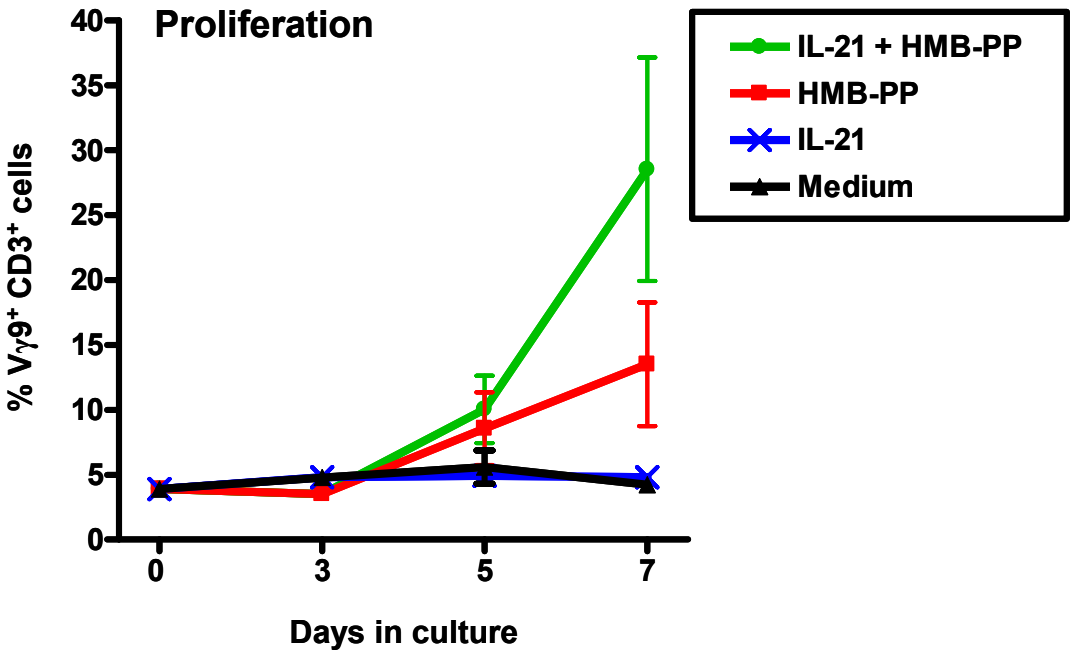


**Figure 3.25. Peripheral blood Vγ9Vδ2 T cells proliferate in response to IL-21+HMB-PP stimulation.**
Freshly isolated PBMCs were cultured in medium with or without HMB-PP (10 nM). After 42 hours, HMB-PP stimulated and non HMB-PP stimulated cultures (medium alone) were treated with or without IL-21 (50 ng/ml). Freshly isolated PBMCs (Day 0) and PBMCs after 3, 5 and 7 days in culture were stained for flow cytometry with anti-CD3-APC-H7 and anti-Vγ9-PE-Cy5. The percentage of $V\gamma9^+$ $CD3^+$ cells was determined by gating on lymphocytes, gating out dead cells and doublets, and gating on $V\gamma9^+$ $CD3^+$ cells. Data are shown as mean ± SEM from 3 individual donors.

# Chapter 4:

## Up-regulation of key molecules involved in B cell help on tonsillar Vγ9Vδ2 T cells

## 4.1 Chapter specific hypothesis and aims

After having established conditions that favour the acquisition of a B helper-Tfh-like phenotype on peripheral blood Vγ9Vδ2 T cells, the objective of this chapter was to similarly examine for markers associated with follicular B cell help on Vγ9Vδ2 T cells from secondary lymphoid tissue. I hypothesised that Vγ9Vδ2 T cells found in inflamed tonsils readily express markers associated with follicular B cell help, which can be further up-regulated *in vitro* by HMB-PP and IL-21 stimulation.

Aims of this chapter were to investigate expression of the following markers on freshly isolated and on *in vitro* IL-21 and HMB-PP stimulated tonsillar Vγ9Vδ2 T cells:

1. IL-21R
2. CXCL13
3. CXCR5
4. Co-stimulatory molecules involved in B cell help
5. APC markers

## 4.2 Results

### 4.2.1 Presence of activated Vγ9Vδ2 T cells in lymphoid tissue

Although Vγ9Vδ2 T cells represent only a minor subset of the peripheral lymphocyte population (0.5 to 5%), Vγ9Vδ2 T cells can expand dramatically following infection by pathogens that produce the microbial metabolite HMB-PP (Eberl et al. 2003; Holtmeier and Kabelitz 2005; Vermijlen et al. 2007). Resting peripheral blood Vγ9Vδ2 T cells exhibit an inflammatory migration profile, which guides Vγ9Vδ2 T cells to sites of microbial infection. Activation of Vγ9Vδ2 T cells in response to certain pathogens induces a rapid switch from inflammatory to lymph node homing phenotype, whereby expression of CCR7 permits migration to secondary lymphoid tissue (Brandes et al. 2003).

Increasing evidence shows γδ T cells to be present in several secondary lymphoid organs such as the draining lymph node, spleen, Peyer's patches, tonsils, inflamed appendix and gastrointestinal mucosa (Brandes et al. 2003; Dieli et al. 2003; Groh et al. 1989). One particular study showed increased number of γδ T cells, to be found in secondary follicles of infection related lymph nodes compared to lymph nodes from healthy individuals. More so, γδ T cells in infection related lymph nodes were detected within GCs of B cell follicles co-localised with CXCL13-producing cells (Brandes et al. 2003). Collectively, these findings strongly indicate an involvement of γδ T cells in humoral immune responses (Brandes et al. 2003; Caccamo et al. 2006a; Soo et al. 1995; Vermijlen et al. 2007).

In my aim to investigate B helper-Tfh-like phenotype on Vγ9Vδ2 T cells in secondary lymphoid tissue, primarily I set out to identify the most easily available source of inflamed/infection related human lymphoid tissue that would provide a good source and yield of Vγ9Vδ2 T cells. Here, I proposed the presence of activated/pre-activated Vγ9Vδ2 T cells in infection related inflamed lymphoid tissue.

Tonsillectomies, appendectomies and biopsies of the lower gastrointestinal tract, are routinely performed surgical procedures, which result in removal of inflamed lymphoid tissue. Surgical removal of tonsils is not a treatment for the acute condition, but it is aimed at reducing the incidence of recurrent infections (Callery 2011; Crayford 2007). Thus, a tonsillectomy is not performed during an episode of tonsillitis but after a response to infection is over. However, tonsils similar to lymph nodes have large active GCs, that have been developed due to the constant exposure to antigens from the throat and upper respiratory tract, which makes tonsils an ideal organ for studying B cell responses (Crotty 2011).

In contrast, appendectomies are performed immediately after the onset of an acute infection. Hence, because the inflamed appendix is completely removed during the infection (Humes and Simpson 2006), this makes the appendix more ideal for examining an inflammatory immune response to a recent local acute infection.

Mucosal surfaces of the gastrointestinal tract (i.e. intestines and colon) are colonised by lymphocytes that can be found in distinct aggregations (Peyer's patches) or in loosely organized lymphoid follicles. These lymphocytes are constantly exposed to extreme amounts of potentially pathogenic ingested and inhaled microorganisms (Nagler-Anderson 2001; Pistoia and Cocco 2009). Clusters of γδ T cells have been found to be present in gastrointestinal lymphoid tissues (Brandes et al. 2003). Thus, mucosal biopsy specimens of the gastrointestinal tract, taken from patients undergoing diagnostic colonoscopy (i.e. for inflammatory bowel disease), could provide as an ideal source of γδ T cells from both inflamed and non-inflamed lymphoid tissue.

Acute peritonitis is a sudden localised inflammation of the peritoneum, which usually occurs due to bacterial infection (Johnson et al. 1997). For early diagnosis, cloudy peritoneal effluents containing inflammatory infiltrates are routinely collected from

peritoneal dialysis patients suffering from an episode of acute bacterial infection. Thus, cloudy peritoneal effluents provide a ready source of immune cells including γδ T cells which have recently been involved in an immune response to acute infection (Davey et al. 2011).

Here, mononuclear cells were isolated from inflamed tonsils, inflamed appendices, from colon biopsies (inflamed and non-inflamed) taken from various parts of the large intestine (caecum, colon, sigmoid and rectum), and from peritoneal effluents taken from patients suffering from acute peritonitis. Mononuclear cells were analysed for the percentage of $V\delta2^+$ T cells amongst the $CD3^+$ T cell population. Cumulative data from several independent donors showed that $V\delta2^+$ T cells represented 0.7% (± 0.1), 1% (± 0.6), 1% (± 0.7) and 1% (± 0.2) of the total $CD3^+$ T cell population, isolated from inflamed tonsils, inflamed appendices, colon biopsies and peritoneal effluents, respectively (**Figure 4.1A**).

For comparison, mononuclear cells isolated from inflamed tonsils, inflamed appendices, colon biopsies were also analysed for Vδ1, CD4, and CD8 T cell populations. $CD4^+$ T cells represented the majority of total $CD3^+$ T cells isolated from inflamed tonsils, inflamed appendices and colon biopsies, followed by a lower percentage of $CD8^+$ T cells and a minor population of $V\delta1^+$ T cells. $V\delta1^+$ T cells represented 7% (± 2.2%) and 1.3 % (± 0.3%) of total $CD3^+$ T cells in colon biopsies and tonsils, respectively, which was higher than the percentage of $V\delta2^+$ T cells. Here, percentages of $V\delta1^+$ T cells isolated from the appendix could not be confirmed, this was mainly due to the unavailability of a reliable clone of anti-Vδ1 antibody, the original clone of antibody used was no longer commercially available (**Figure 4.1B-D**).

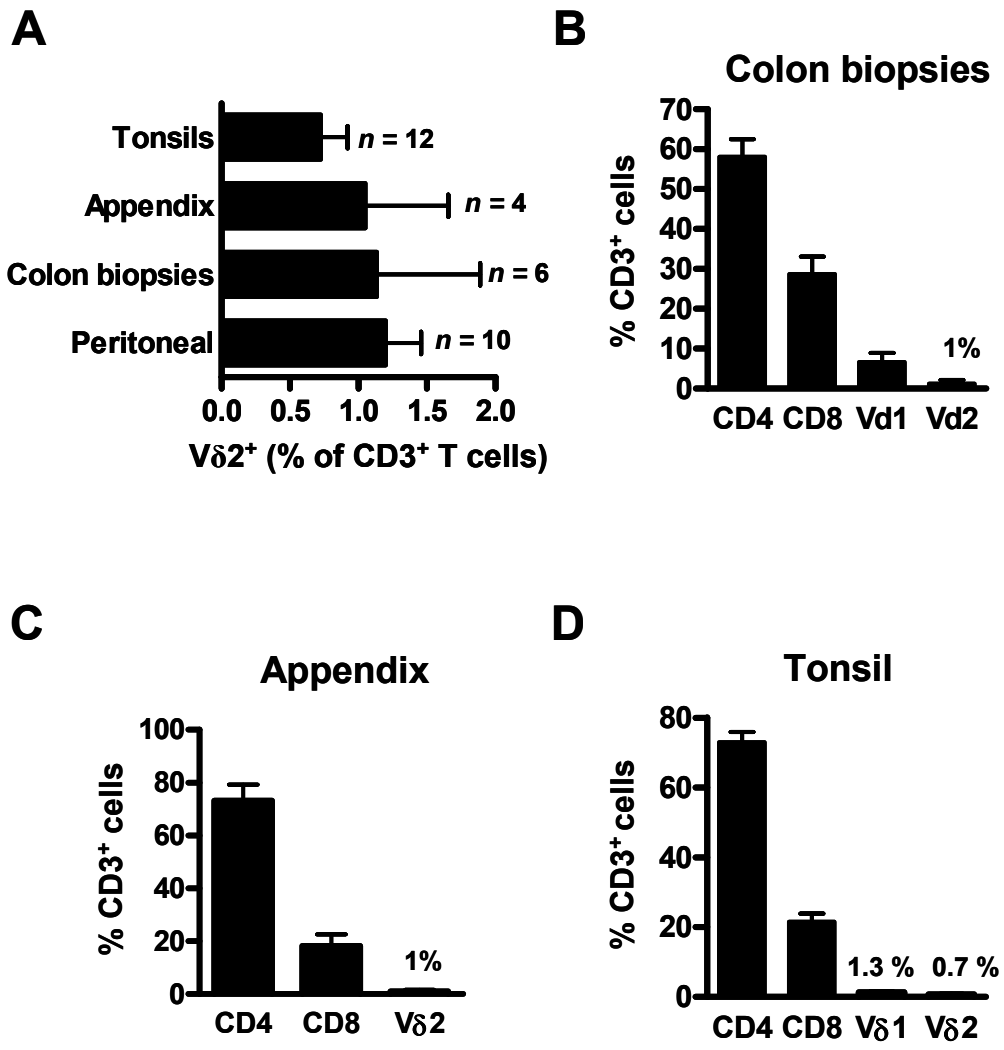


**Figure 4.1. Vγ9Vδ2 T cells are detectable in a variety of human lymphoid tissue.** Mononuclear cells were isolated from inflamed tonsils, inflamed appendices and colon biopsies. Peritoneal cells were harvested from peritoneal effluents obtained from peritoneal dialysis patients suffering from an episode of acute bacterial infection. Cells were stained for surface CD3, CD4, CD8, Vδ1 and Vδ2 expression. Cells were analysed by flow cytometry by gating on lymphocytes, live cells, singlets, and CD3 positive cells. (A) Percentage of $V\delta2^+$ T cells in various human tissues expressed as a percentage of CD3 positive cells. Percentage of $CD4^+$, $CD8^+$ and γδ T cell expressed as a percentage of CD3 positive cells in human (B) colon biopsies, (C) appendix and (D) tonsils. Data are shown as mean percentage + SEM from three to twelve independently assessed donors. Peritoneal data was kindly provided by Chan-Yu Lin, Institute of Infection & Immunity, School of Medicine, Cardiff University (Davey et al. 2011).

To examine for *in vivo* activation of Vγ9Vδ2 T cells, mononuclear cells isolated from colon biopsies, inflamed appendices and inflamed tonsils were analysed for the early activation markers CD69 and CD25. Interestingly, in colon biopsies a similar percentage (around 60 %) of $V\delta2^+$, $V\delta1^+$, $CD8^+$, and $CD4^+$ T cells expressed the activation marker CD69 (**Figure 4.2A**). Similarly CD69 was also expressed on a large proportion of $V\delta2^+$ T cells (44% ± 9%), $CD8^+$ (19% ± 5%) and $CD4^+$ T cells (37% ± 4%) isolated from the appendix (**Figure 4.2B**), whereas in tonsils CD69 was expressed on a smaller percentage of $V\delta2^+$ T cells (5% ± 1.8%), $CD8^+$ T cells (12% ± 2.5%) and $CD4^+$ T cells (28% ± 4%) (**Figure 4.2C**).

The activation marker CD25 was consistently found to be expressed by a small population of $CD4^+$ T cells (10-20%), but only by < 5% of $V\delta2^+$ and $CD8^+$ T cells in colon biopsies, appendices and tonsils (**Figure 4.2**). Thus, in contrast to the absent expression of surface CD69 and CD25 on peripheral blood $V\delta2^+$, $CD8^+$, and $CD4^+$ T cells, both CD69 and to a lesser extent CD25, was clearly found to be expressed on a distinct population of tissue derived $V\delta2^+$, $CD8^+$ and $CD4^+$ T cells.

Notably, although CD69 and CD25 were only expressed by a minority of tonsillar Vγ9Vδ2 T cells, this differed to expressions found on peripheral blood Vγ9Vδ2 T cells (**Figure 4.3**). The presence of CD69/CD25 activated $CD4^+$, $CD8^+$ and Vγ9Vδ2 T cells within the same pool of tonsillar mononuclear cells strongly suggests *in vivo* activation of all three subsets of T lymphocytes, possibly in response to recent or chronic infection.

Next, I wanted to find out if pre-activated tonsillar Vγ9Vδ2 T cells could be re-activated upon *in vitro* re-stimulation. Here, to induce activation of tonsillar Vγ9Vδ2 and $CD4^+$ T cells, mononuclear cells isolated from tonsils were stimulated with phorbol myristate acetate (PMA; 10ng/ml) and ionomycin (1 μg/ml). Expression of CD25 was significantly (35% (± 4.7%) vs 1.8% (± 0.5%), $P$=0.0028, Mann-Whitney two-tailed test) up-regulated on tonsillar Vδ2 T cells upon 3 hours of *in vitro* PMA and ionomycin stimulation. Similarly CD25 expression on freshly isolated $CD4^+$ T cells was also up-regulated (54% (± 9.4%) vs 8% (± 1.4%)), but not found to be significant (**Figure 4.4**). Thus demonstrating, previously

pre-activated Vγ9Vδ2 T cells and $CD4^+$ T cells, from inflamed tonsils, can be rapidly re-activated upon *in vitro* re-stimulation, thus have the capacity to respond to antigen re-challenge.

In conclusion, activated Vγ9Vδ2 T cells along side activated $CD4^+$ and $CD8^+$ T cells are detectable in infection related lymphoid tissues such as the inflamed tonsils and inflamed appendix. Moreover, for several reasons, I found the inflamed tonsil as the most reliable and most accessible source of human secondary lymphoid tissue. Tonsils, in comparison to colon biopsies and the appendix, provided a much better yield of not only Vγ9Vδ2 T cells but of total mononuclear cells (data not shown). Therefore, from this point on, the rest of my study was carried out using cells from inflamed human tonsils.

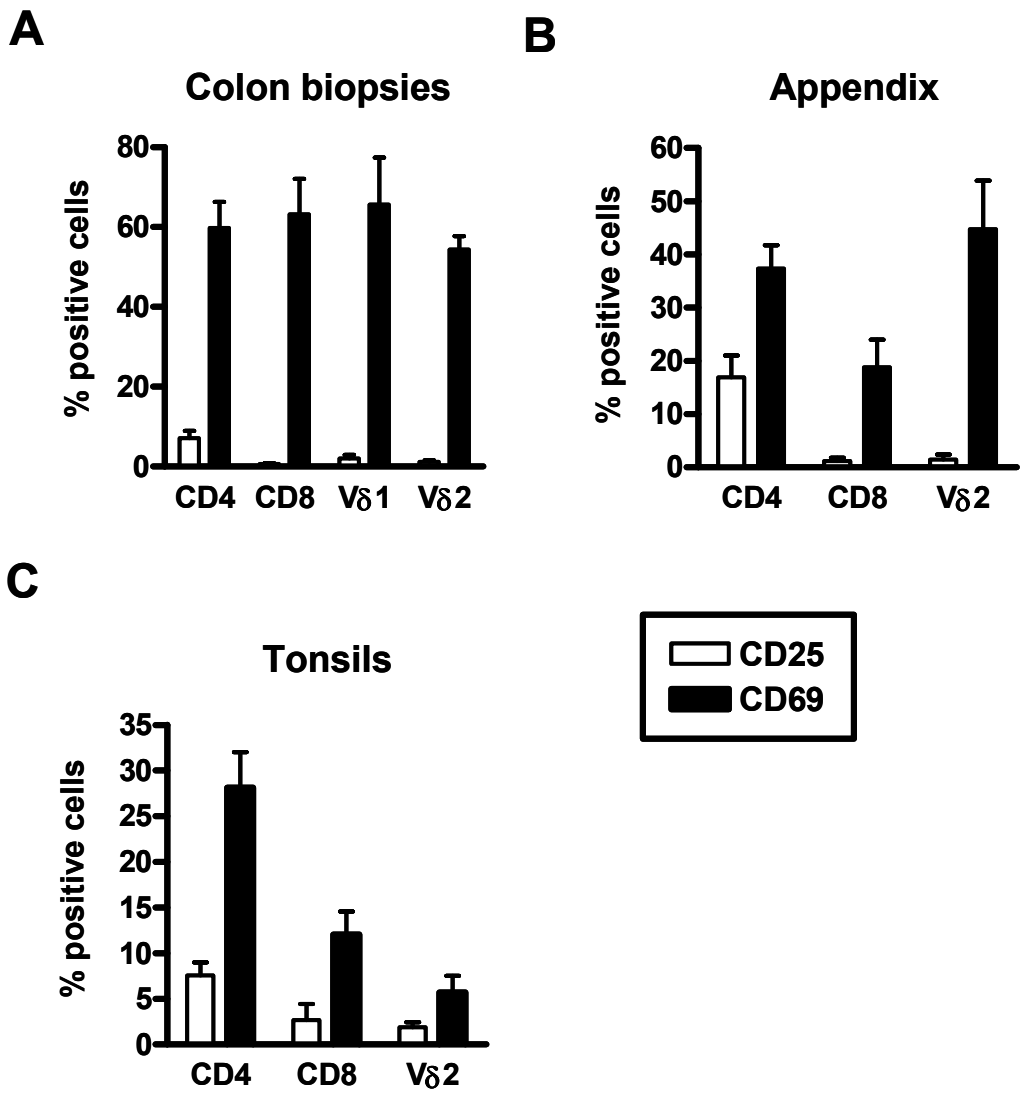


**Figure 4.2. Vγ9Vδ2 T cells from colon biopsies, appendix and tonsils express surface CD69.**

Mononuclear cells were isolated from human tonsils, appendix tissue and colon biopsies. Peritoneal cells were harvested from peritoneal effluents obtained from individuals on continuous ambulatory peritoneal dialysis. Cells were stained for surface CD3, CD4, CD8, Vδ1, Vδ2, CD25 and CD69 expression. Cells were analysed by flow cytometry by gating on lymphocytes, live cells, singlets and CD3 positive cells. CD25 (white bars) and CD69 (black bars) surface expression was analysed on $CD4^+$, $CD8^+$ and γδ T cell subsets from human (A) colon biopsies, (B) appendix and (C) tonsils. Gates were set using isotype-matched control antibodies. Data are shown as mean percentage + SEM from three to twelve independently assessed donors.

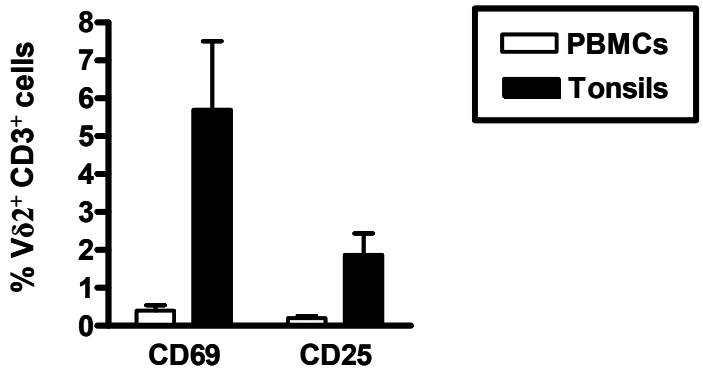


**Figure 4.3. Comparison of CD69 and CD25 expression on peripheral blood and tonsillar Vγ9Vδ2 T cells.**
Freshly isolated PBMCs and tonsillar mononuclear cells were stained for surface CD25 and CD69 expression. Cells were analysed by flow cytometry by gating on lymphocytes, live cells, singlets, $V\delta2^+CD3^+$ cells. Gates were set using isotype-matched control antibodies. Data are shown as mean percentage + SEM from three (PBMCs) and nine (tonsils) independent donors.

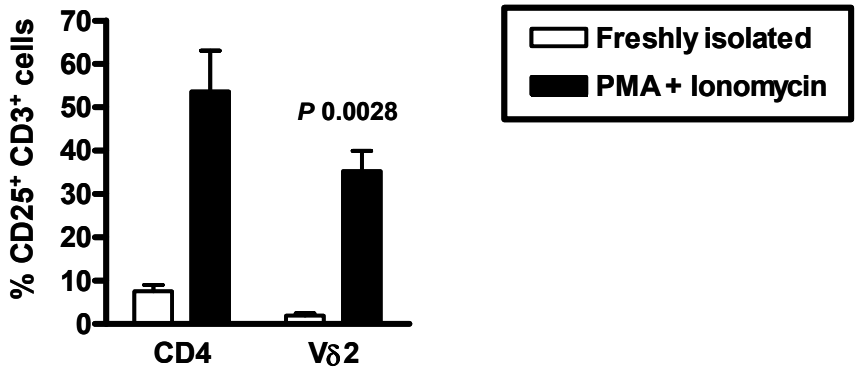


**Figure 4.4. CD25 expression on tonsillar Vγ9Vδ2 T cells is up-regulated upon *in vitro* PMA and ionomycin stimulation.**
Mononuclear cells isolated from human tonsils were cultured in medium. After 1 hour PMA (10ng/ml), ionomycin (1 μg/ml) and brefeldin A (20 ug/ml) was added to the cultured cells. After 3 hours cultured cells and freshly isolated tonsillar mononuclear were stained for surface CD3, CD4, Vδ2 and CD25 expression. Cells were analysed for CD25 expression by gating on lymphocytes, live cells, singlets, $CD4^+CD3^+$ cells and $V\delta2^+CD3^+$ cells. Gates were set using isotype-matched control antibodies. Data are shown as mean percentage + SEM from four to nine independent donors. A statistically significant difference on $V\delta2^+$ T cells between freshly isolated and PMA/ionomycin treated cells/cultures was found ($P$=0.0028).

### 4.2.2 Freshly isolated tonsillar Vγ9Vδ2 T cells do not express CXCL13 but express IL-4, TNF-α and IFN-γ

CXCL13 is a homeostatic chemokine constitutively expressed in follicles of secondary lymphoid tissue, selectively attracts B and Tfh cells via binding to its receptor CXCR5. Tfh cells provide B cell help via presentation of a range of co-stimulatory molecules and by secretion of cytokines (King et al. 2008). Tfh cells express helper cytokines such as IL-21, IL-2, IL-4 and IL-10 and limited amounts of IFN-γ, that influence B cell differentiation and antibody production (Crotty 2011; King 2009; King et al. 2008). Some of these cytokines are also important in mediating function of Tfh cells (Crotty 2011; Reinhardt et al. 2009).

Here, I proposed secretion of CXCL13 by tonsillar γδ T cells, as a possible mechanism of attracting circulating $CXCR5^+$ B cells towards CXCL13 producing γδ T cells in tonsils. γδ T cells have been well established as producers of pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10) (Caccamo et al. 2006a; Caccamo et al. 2011; Hayday 2000; Vermijlen et al. 2007). Secretion of cytokines by Vγ9Vδ2 T cells, within secondary lymphoid tissues, may modulate Tfh and B cell antibody responses. Thus, here I examined tonsillar Vγ9Vδ2 T cells alongside tonsillar $CD4^+$ T cells, for intracellular expression of CXCL13, IL-4, TNF-α and IFN-γ.

Non-stimulated T lymphocytes produce cytokines and chemokines at levels which are too low for detection by flow cytometry. Therefore, appropriate *in vitro* stimulation is required for detection of cytokines and chemokines. PMA a protein kinase C activator is a potent T cell mitogen commonly used in conjunction with ionomycin (a calcium ionophore) to stimulate intracellular production of cytokines (Baran et al. 2001). To inhibit secretion of cytokines from the cells, a protein transport inhibitor such as brefeldin A (BFA) is added to cell cultures (Nylander and Kalies 1999). Here, to induce robust cell activation and potent cytokine production, mononuclear cells isolated from inflamed tonsils were stimulated with

PMA (10ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (20 ug/ml). After 3 hours, cells were harvested, fixed, permeabilised and stained with fluorochrome conjugated antibodies specific for CXCL13, IL-4, IFN-γ TNF-α and CD3, CD4 and Vγ9, before being analysed by flow cytometry.

Expression of intracellular CXCL13 was not detected in tonsillar Vγ9Vδ2 T cells, but was clearly expressed by a distinct population of tonsillar $CD25^+$ $CD4^+$ T lymphocytes (9 % ± 1.4%) (**Figure 4.5**). Unlike $CD4^+$ T cells, Vγ9Vδ2 T cells represent a very small population amongst tonsillar mononuclear cells. Here, I failed to detect CXCL13 producing Vγ9Vδ2 T cells mainly due to the very small percentage of Vγ9Vδ2 T cells amongst mononuclear cells and also due to the methodology used. The lengthy stimulation and staining procedure for the detection of CXCL13, resulted in lose of the very few Vγ9Vδ2 cells present. This also made analysis and interpretation of flow cytometry data very difficult. For a more reliable analysis of CXCL13 in tonsillar Vγ9Vδ2 T cells, it would be more ideal to work with highly purified tonsillar Vγ9Vδ2 T cells co-cultured with feeder cells. I was unable to carry out such experiments, due to the restricted availability of fresh tonsils and also due to the challenges of isolating such a small population of Vγ9Vδ2 T cells at high purities.

In response to PMA and ionomycin stimulation a small percentage of tonsillar Vγ9Vδ2 T cells (6% ± 3%) and $CD4^+$ T cells (14% ± 0.5%) expressed IL-4. Similar percentages of Vγ9Vδ2 T cells (34 % ± 8%) and $CD4^+$ T cells (46% ± 5%) expressed the pro-inflammatory cytokine TNF-α. Expression of IFN-γ was significantly higher in Vγ9Vδ2 T cells (58% ± 10%) in comparison to $CD4^+$ T cells (8% ± 1.5%) (P=0.0286, Mann-Whitney two-tailed test) (**Figure 4.6**). IFN-γ producing Vγ9Vδ2 T cells and $CD4^+$ T cells did not co-express IL-4 but co-expressed TNF-α (data not shown). Levels of cytokines secreted by Tfh cells within GCs, is thought to be dependent on environmental conditions (Crotty 2011). Inflammatory conditions such as tonsillitis may favour secretion of IFN-γ by Vγ9Vδ2 T cells but not by $CD4^+$ T cells. Secretion of IFN-γ by Vγ9Vδ2 T cells may be a way of modulating function of Tfh cells and B cell antibody responses.

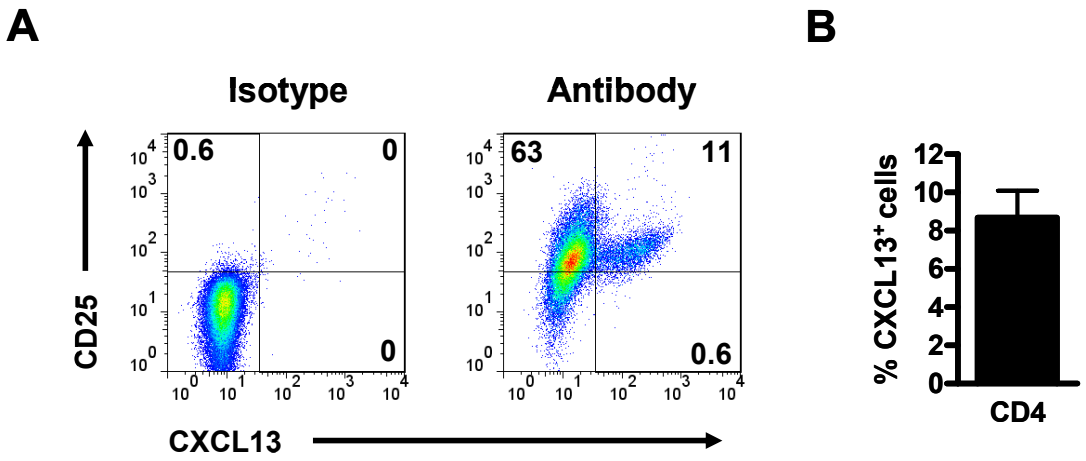


**Figure 4.5. Tonsillar $CD4^+$ T cells express intracellular CXCL13.**
Mononuclear cells isolated from human tonsils were cultured in medium. After 1 hour PMA (10ng/ml), ionomycin (1 μg/ml) and brefeldin A (20 ug/ml) was added to the cultured cells. After 3 hours cultured cells were harvested, fixed and permeabilized. Cells were stained for flow cytometry with mouse anti-CXCL13, followed by detection with an Alexa fluor488-conjugated goat anti-mouse-secondary antibody, anti-CD4-ECD, anti-CD3-APC and anti-CD25-PE-Cy7. Cells were analysed by gating on lymphocytes, live cells, singlets, $CD4^+CD3^+$ cells. (A) Numbers in dot plots represent the percentages of $CD4^+$ T cells positive for CXCL13 and CD25. Quadrants in dot plots were set using isotype-matched control antibodies. Data are representative of four independently assessed donors. (B) Data are shown as mean percentage + SEM from four independent donors.

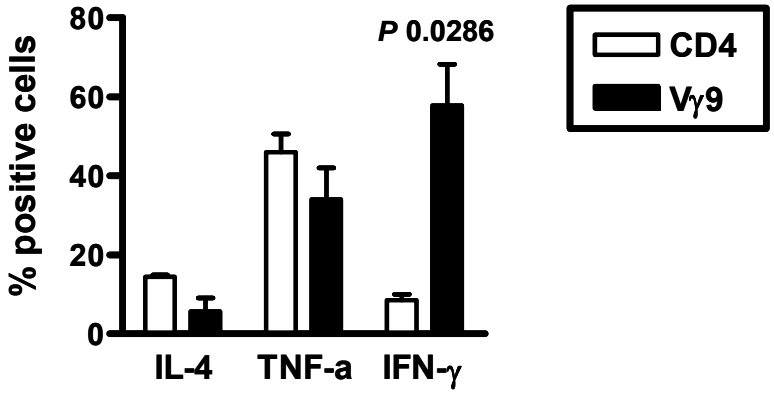


**Figure 4.6. Tonsillar γδ and CD4$^+$ T cells express IFN-γ, TNF-α and IL-4.**
Mononuclear cells isolated from human tonsils were cultured in medium. After 1 hour PMA (10ng/ml), ionomycin (1 μg/ml) and brefeldin A (20 ug/ml) was added to the cultured cells. After 3 hours cultured cells were harvested, fixed and permeabilized. Cells were stained for flow cytometry for intracellular IFN-γ, TNF-α and IL-4. Cells were analysed by gating on lymphocytes, live cells, singlets, CD4$^+$CD3$^+$ cells (white bars) and Vγ9$^+$CD3$^+$ cells (black bars). Gates were set using isotype-matched control antibodies. Data are shown as mean percentage + SEM from four independent donors. A statistically significant difference between the percentage of IFN-γ expressing Vγ9Vδ2 T cells and CD4$^+$ T cells was found (*P*=0.0286)

### 4.2.3 Freshly isolated tonsillar Vγ9Vδ2 T cells express CXCR5, ICOS, OX40 and CD40L

In chapter 3, I failed to detect expression of CXCR5 and CD40L on freshly isolated and on HMB-PP and IL-21 stimulated peripheral Vγ9Vδ2 T cells. However, expression of the co-stimulatory molecules ICOS, OX40 and CD70 were detected on HMB-PP and IL-21 stimulated peripheral Vγ9Vδ2 T cells. Here, I proposed expression of CXCR5 and co-stimulatory molecules on freshly isolated tonsillar γδ T cells. In my model, expression of CXCR5 on tonsillar Vγ9Vδ2 T cells would allow migration into CXCL13-rich B cell follicles, where they would provide instructions for B cell activation, differentiation and antibody production via presentation of co-stimulatory molecules and secretion of cytokines (Vermijlen et al. 2007).

To study the expression of CXCR5, and the co-stimulatory molecules ICOS, OX40 and CD40L on tonsillar Vγ9Vδ2 T cells, mononuclear cells freshly isolated from inflamed tonsils were stimulated with PMA (10ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (20 ug/ml). Freshly isolated and PMA and ionomycin stimulated mononuclear cells were stained with fluorochrome-conjugated antibodies specific for CXCR5, ICOS, OX40, CD40L, CD4, CD3 and Vγ9, before being analysed by flow cytometry. For comparison, expression of co-stimulatory molecules on Vγ9Vδ2 T cells was studied alongside $CD4^+$ T cells.

Here, a small population of freshly isolated tonsillar Vγ9Vδ2 T cells expressed CXCR5 (4 % ± 1%), ICOS (6 % ± 1%) and OX40 (3 % ± 1%). CD40L was found to be absent on freshly isolated tonsillar Vγ9Vδ2 T cells, but was significantly up-regulated (24% ± 5%) upon 3 hours with PMA/ionomycin stimulation (P=0.0167, Mann-Whitney two-tailed test). In contrast, stimulation with PMA/ionomycin did not further up-regulate expression of CXCR5, ICOS or OX40. Instead, the percentage of ICOS positive Vγ9Vδ2 T cells had

decreased and expression of OX40 was completely lost upon PMA/ionomycin stimulation (**Figure 4.7A**).

Freshly isolated tonsillar $CD4^+$ T cells expressed CXCR5 (22 % ± 4%), ICOS (59 % ± 7%) and OX40 (28 % ± 4%), at higher levels compared to freshly isolated tonsillar Vγ9Vδ2 T cells. Similar to tonsillar Vγ9Vδ2 T cells, CD40L was found to be absent on freshly isolated tonsillar $CD4^+$ T cells, but was significantly up-regulated (54% ± 6%) with PMA/ionomycin stimulation (P=0.0040, Mann-Whitney two-tailed test). In contrast, there was a drop in the percentage of CXCR5 and ICOS positive $CD4^+$ T cells. Similar to Vγ9Vδ2 T cells, expression of OX40 was also completely lost on $CD4^+$ T cells after PMA/ionomycin stimulation (**Figure 4.7B**).

Representative flow cytometry dot plots are shown demonstrating CD40L positive tonsillar Vγ9Vδ2 T cells (**Figure 4.7C**) and $CD4^+$ T cells (**Figure 4.7D**) after PMA/ionomycin stimulation. Interestingly, co-staining with intracellular CXCL13 revealed that within the $CD4^+$ T cell population all the CD40L positive cells co-expressed CXCL13 (**Figure 4.7D**).

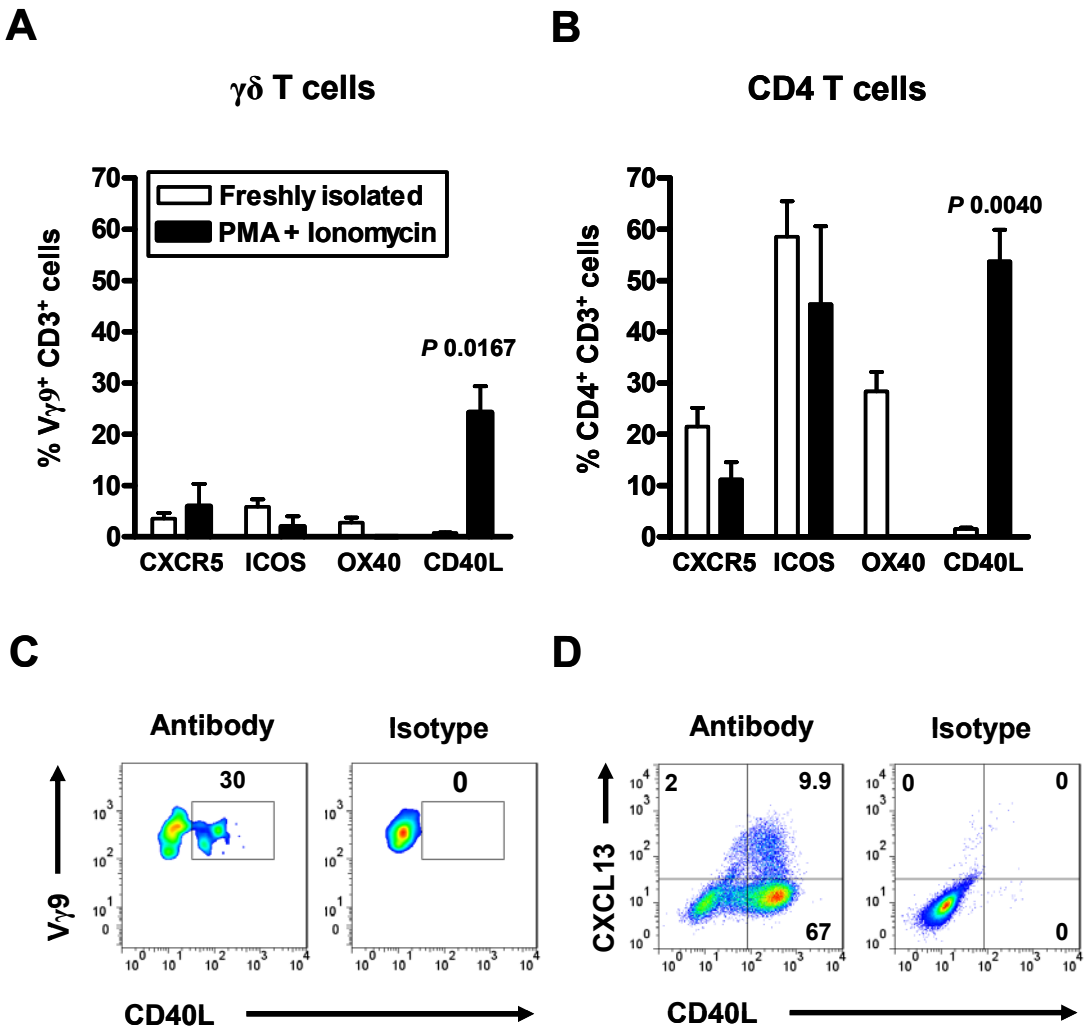


**Figure 4.7. CD40L expression on tonsillar Vγ9Vδ2 and CD4$^+$ T cells is up-regulated upon *in vitro* PMA and ionomycin stimulation.**
Mononuclear cells isolated from inflamed tonsils were cultured in medium. After 1 hour PMA (10ng/ml), ionomycin (1 μg/ml) and brefeldin A (20 ug/ml) was added to the cultured cells. After 3 hours cultured cells were harvested, fixed and permeabilized. Cultured cells and freshly isolated tonsillar mononuclear were stained for surface CD3, CD4, Vγ9, CXCR5, ICOS, OX40 CD40L and intracellular CXCL13. Cells were analysed by gating on lymphocytes, live cells, singlets, (A) Vγ9$^+$CD3$^+$ cells and (B) CD4$^+$CD3$^+$ cells. Data are shown as mean percentage + SEM from four to nine independent donors. Numbers in flow cytometry dot plot represent the percentages of (C) Vγ9$^+$CD3$^+$ cells positive for CD40L and (D) the percentage of CD4$^+$CD3$^+$ cells positive for CD40L and CXCL13. Gates were set using isotype-matched control antibodies. Data is representative of three to four independent donors. A statistically significant difference in the expression of CD40L between freshly isolated and PMA/ionomycin treated cells/cultures was found.

## 4.2.4. Freshly isolated tonsillar Vγ9Vδ2 T cells express CCR7, CD40 and HLA-DR

In Chapter 3, I proposed a possible interaction of γδ T cells as APCs with $CD4^+$ T cells and studied the expression of APC markers on freshly isolated peripheral Vγ9Vδ2 T cells. Here, I examined for APC marker expression on freshly isolated tonsillar γδ T cells.

To examine for APC marker expression on freshly isolated tonsillar Vγ9Vδ2 T cells, mononuclear cells freshly isolated from inflamed tonsils, were stained with fluorochrome-conjugated antibodies specific for CCR7, CD40, CD86 and HLA-DR. Cells were analysed by flow cytometry and assessed for surface marker expression by gating on $Vγ9^+CD3^+$ or $CD4^+$ $CD3^+$ T cells.

Freshly isolated tonsillar Vγ9Vδ2 T cells expressed CCR7 (8 % ± 2%), CD40 (34 % ± 6%) and HLA-DR (16 % ± 3%). Similarly, CCR7 (36 % ± 5%), CD40 (45 % ± 12%) and HLA-DR (51% ± 6%) was also expressed on freshly isolated tonsillar $CD4^+$ T cells. CD68 was found to be absent on both tonsillar Vγ9Vδ2 and $CD4^+$ T cells (**Figure 4.8**). Furthermore, in tonsils a higher percentage of Vγ9Vδ2 T cells expressed CCR7, CD40 and HLA-DR in comparison to Vγ9Vδ2 T cells in peripheral blood (**Figure 4.9**).

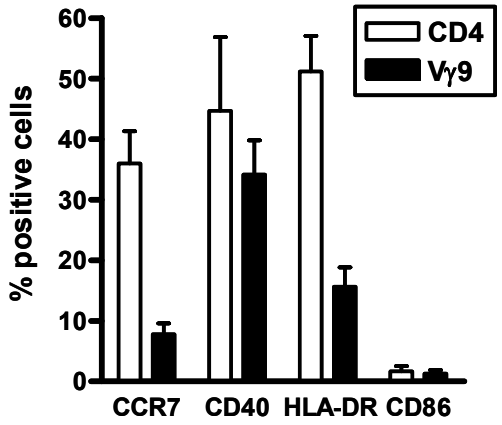


**Figure 4.8. Tonsillar Vγ9Vδ2 and $CD4^+$ T cells express CCR7, CD40 and HLA-DR.**
Mononuclear cells were freshly isolated from human tonsils. Cells were stained for surface CD3, CD4, Vγ9, CCR7, CD40, HLA-DR and CD86 expression. Cells were analysed by flow cytometry by gating on lymphocytes, live cells, singlets, and $CD4^+CD3^+$ cells (white bars) and $V\gamma9^+CD3^+$ cells (black bars). Gates were set using isotype-matched control antibodies. Data are shown as mean percentage + SEM from nine independent donors.

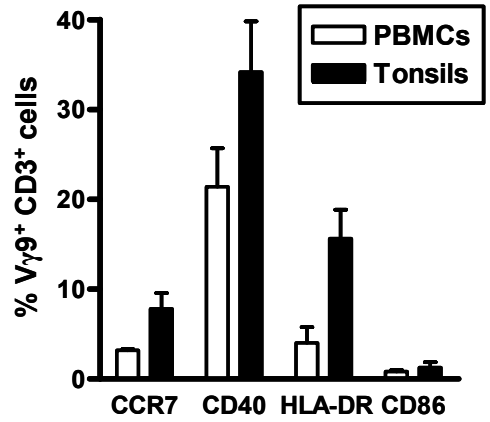


**Figure 4.9. Higher percentage of Vγ9Vδ2 T cells express CCR7, CD40 and HLA-DR in tonsils in comparison to peripheral blood.**
Freshly isolated PBMCs and tonsillar mononuclear cells were stained for surface CD3, Vγ9, CCR7, CD40, HLA-DR and CD86 expression. Cells were analysed by flow cytometry by gating on lymphocytes, live cells, singlets and $V\gamma9^+CD3^+$ cells. Gates were set using isotype-matched control antibodies. Data are shown as mean percentage + SEM from three (PBMCs) and nine (tonsils) independent donors.

## 4.2.5 Tonsillar Vγ9Vδ2 T cell memory subsets differ from peripheral blood

Expression of the chemokine receptor CCR7 allows migration and continuous recirculation of peripheral T and B cells into secondary lymphoid tissues. CCR7 is mainly found to be expressed by circulating naive and central memory T cells (Sallusto et al. 2004). Here, based on CD45RA/CCR7 surface marker expression I identified Vγ9Vδ2 T cell memory populations in tonsils.

Mononuclear cells were freshly isolated from inflamed tonsils and stained with fluorochrome-conjugated antibodies specific for memory markers CD45RA and CCR7 in combination with Vδ2 and CD3 surface markers. Cells were analysed by flow cytometry by gating on $V\delta2^+CD3^+$ T cells and memory subsets were identified based on CD45RA/CCR7 expression ($CD45RA^+CCR7^+$ (Naïve), $CD45RA^-CCR7^+$ ($T_{CM}$), $CD45RA^-CCR7^-$ ($T_{EM}$) and $CD45RA^+CCR7^-$ ($T_{EMRA}$)). In comparison, $CD4^+$ T cells from tonsils were also analysed for memory subsets.

In inflamed tonsils 6% (± 1.6%) of total Vγ9Vδ2 T cells were naïve, 2% (± 0.7%) $T_{CM}$, 34% (± 6%) $T_{EM}$ and the majority were $T_{EMRA}$ (57% ± 5%). In comparison, tonsillar $CD4^+$ T cells constituted of 18% (± 3.5%) naïve, 13% (± 3.4%) $T_{CM}$, 51% (± 7%) $T_{EM}$ and 18% (± 7%) $T_{EMRA}$. Thus the majority of Vγ9Vδ2 T cells found in tonsils belonged to the $T_{EM}$ and $T_{EMRA}$ pools, whilst the majority of $CD4^+$ T cells belonged to the $T_{CM}$ pool (**Figure 4.10**). Subpopulations of Vγ9Vδ2 T cells found in tonsils differed to those found in peripheral blood. In tonsils the majority of Vγ9Vδ2 T cells were $T_{EMRA}$ (57% ± 5%), whereas in peripheral blood the majority of Vγ9Vδ2 T cells were $T_{EM}$ (61% ± 9%). Furthermore, a noticeably higher percentage of naïve Vγ9Vδ2 T cells were found in tonsils in comparison to peripheral blood (6% (± 1.6%) vs 1% (± 0.6%)) (**Figure 4.11**).

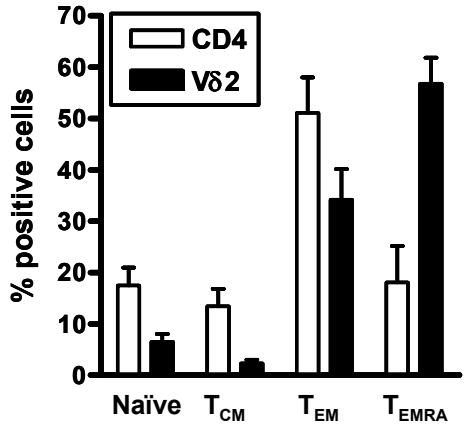


**Figure 4.10. Vγ9Vδ2 and $CD4^+$ T cell memory subsets in tonsils.**
Freshly isolated tonsillar mononuclear cells were stained for memory markers CCR7 and CD45RA in combination with CD3, CD4 and Vδ2 surface markers. Memory subsets were classified as follows: Naïve = $CD45RA^+CCR7^+$, T central memory ($T_{CM}$) = $CD45RA^-CCR7^+$, T effector memory ($T_{EM}$) = $CD45RA^-CCR7^-$ and terminally differentiated effector memory T cell ($T_{EMRA}$) = $CD45RA^+CCR7^-$. Cells were analysed by flow cytometry by gating on lymphocytes, live cells, singlets, $CD4^+CD3^+$ cells (white bars) and $V\delta2^+CD3^+$ cells (black bars)**.** Gates were set using isotype-matched control antibodies. Data are shown as mean percentage + SEM from seven independent donors.

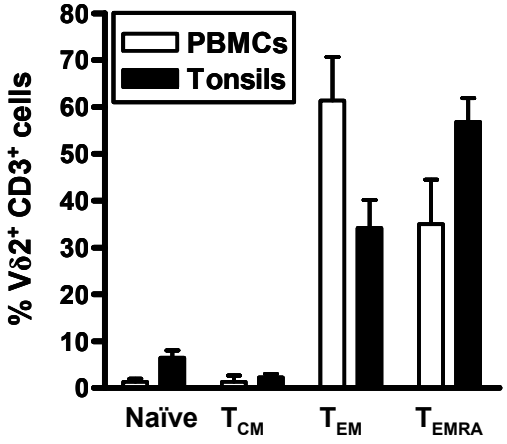


**Figure 4.11. Tonsillar memory Vγ9Vδ2 T cell populations differ from peripheral blood.**
Freshly isolated PBMCs and tonsillar mononuclear cells were stained and analysed for surface memory markers as described above in Figure 4.10. Cells were analysed by flow cytometry by gating on lymphocytes, live cells, singlets, and $V\delta2^+CD3^+$ cells. Gates were set using isotype-matched control antibodies. Data are shown as mean percentage + SEM from three (PBMCs) and nine (tonsils) independent donors.

## 4.2.6 Freshly isolated tonsillar Vγ9Vδ2 T cells express the receptor for IL-21

After having found expression of markers associated with follicular B cell help on only a small population of freshly isolated tonsillar Vγ9Vδ2 T cells, here, I proposed up-regulation of B cell helper markers on tonsillar Vγ9Vδ2 T cells upon *in vitro* stimulation with IL-21 and HMB-PP. Primarily, in order to examine the effects of IL-21 stimulation on tonsillar Vγ9Vδ2 T cells, surface expression of a functional IL-21R would be essential. In chapter 3, I showed expression of IL-21R on peripheral blood Vγ9Vδ2 T cells. Similarly, here I examined for surface expression of IL-21R on Vγ9Vδ2 and $CD4^+$ T cells from inflamed tonsils.

Mononuclear cells were freshly isolated from inflamed tonsils and stained with unconjugated mouse anti-human monoclonal antibody against IL-21R, followed by a secondary α-mouse Ig-PE antibody. Cells were also stained with fluorochrome-conjugated antibodies specific for Vγ9, CD4 and CD3. Cells were analysed by flow cytometry and assessed for IL-21R expression by gating on live single cells and on $V\gamma9^+CD3^+$ T cells or $CD4^+CD3^+$ T cells.

Surface IL-21R expression was found to be uniformly expressed on both freshly isolated tonsillar Vγ9Vδ2 T cells and $CD4^+$ T cells. However, the GMFI of IL-21R was found to be significantly lower on tonsillar Vγ9Vδ2 T cells in comparison to peripheral blood (GMFI 1.5 (± 0.08) vs GMFI 1.8 (± 0.16), $P$=0.0145 Mann-Whitney two-tailed test). Similarly the GMFI of IL-21R was also found to be lower on tonsillar $CD4^+$ T cells in comparison to peripheral blood, however this was found to be non-significant (GMFI 2.0 (± 1.5) vs GMFI 2.9 (± 0.85). These observations suggest a down-regulation of surface IL-21R within tonsils (**Figure 4.12**).

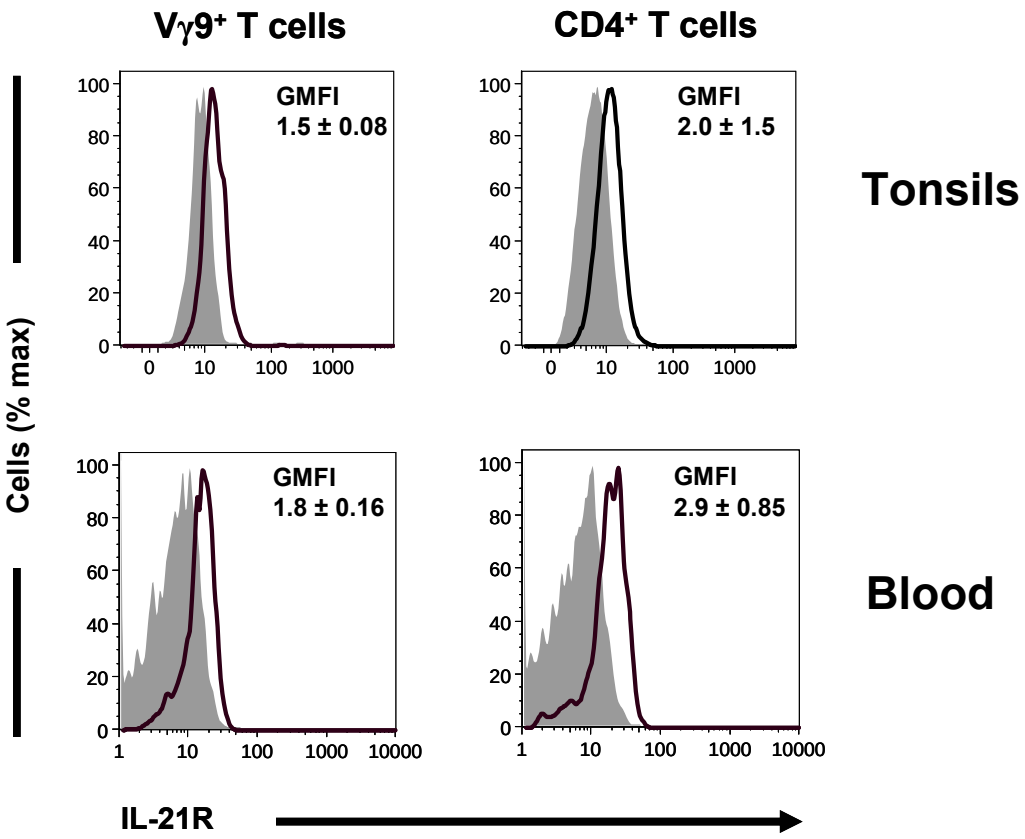


**Figure 4.12. Freshly isolated tonsillar Vγ9Vδ2 T cells express the receptor for IL-21.** Tonsillar mononuclear cells and PBMCs were freshly isolated and stained with mouse anti-human mAb against IL-21R (black-outlined histogram) or with an isotype matched control mAb (solid gray histogram) followed by detection with α-mouse Ig-PE. IL-21R expression was measured by flow cytometry. Cells were analysed for IL-21R expression by gating on live cells, singlets, $V\gamma9^{+}CD3^{+}$ T cells and on $CD4^{+}CD3^{+}$ T cells. Flow cytometry histograms represent one of 4 independent experiments. The values represent mean fold change in GMFI, calculated as (geometric MFI of IL-21R)/(geometric MFI of isotype control). Data are shown as mean ± SD from four individual donors. A statistically significant difference on $V\gamma9V\delta2_{+}$ T cells between tonsillar and peripheral blood was found ($P$=0.0145).

## 4.2.7. HMB-PP stimulation enhances expression of CD25 and CD69 activation markers on tonsillar Vγ9Vδ2 T cells

In the previous chapter, resting peripheral Vγ9Vδ2 T cells were rapidly activated upon *in vitro* HMB-PP stimulation. Here, I proposed up-regulation of activation markers CD25 and CD69 on tonsillar Vγ9Vδ2 T cells upon *in vitro* stimulation with IL-21 and HMB-PP.

Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained with fluorochrome-conjugated antibodies specific for CD25, CD69, CD4, CD3 and Vδ2, before being analysed by flow cytometry.

Here, a minority of freshly isolated tonsillar Vγ9Vδ2 T cells readily expressed CD25 (3% ± 1.5%) and CD69 (11% ± 2.9%). Following 1 day of culture, surprisingly, CD25 (22% ± 9.5%) and CD69 (54% ± 10%) was up-regulated on tonsillar Vγ9Vδ2 T cells cultured in medium alone. The addition of IL-21 alone had no effect on the expression of CD25 or CD69 on tonsillar Vγ9Vδ2 T cells (**Figure 4.13A**).

However, one day stimulation with HMB-PP alone, lead to enhanced expression of both CD25 and CD69 on tonsillar Vγ9Vδ2 T cells. In response to HMB-PP, CD69 expression peaked after just 1 day (86% ± 2.3%) but then declined considerably after 3 days (23% ± 6%). Similarly CD25 was also substantially up-regulated after one day with HMB-PP stimulation, but in contrast to CD69, the percentage of CD25 expressing tonsillar Vγ9Vδ2 T cells continued to increase from day 1 (66% ± 3%) to day 3 (97% ± 1%) in culture.

The addition of IL-21 to HMB-PP stimulated cultures did not further enhance expression of CD25 or CD69 on tonsillar Vγ9Vδ2 T cells (**Figure 4.13A**). Furthermore, the response to HMB-PP stimulation was specific to Vγ9Vδ2 T cells, as culturing of freshly isolated tonsillar mononuclear cells with HMB-PP in the absence or presence of IL-21, did not effect or further up-regulate CD25 expression on tonsillar $CD4^+$ T cells (**Figure 4.13B**).

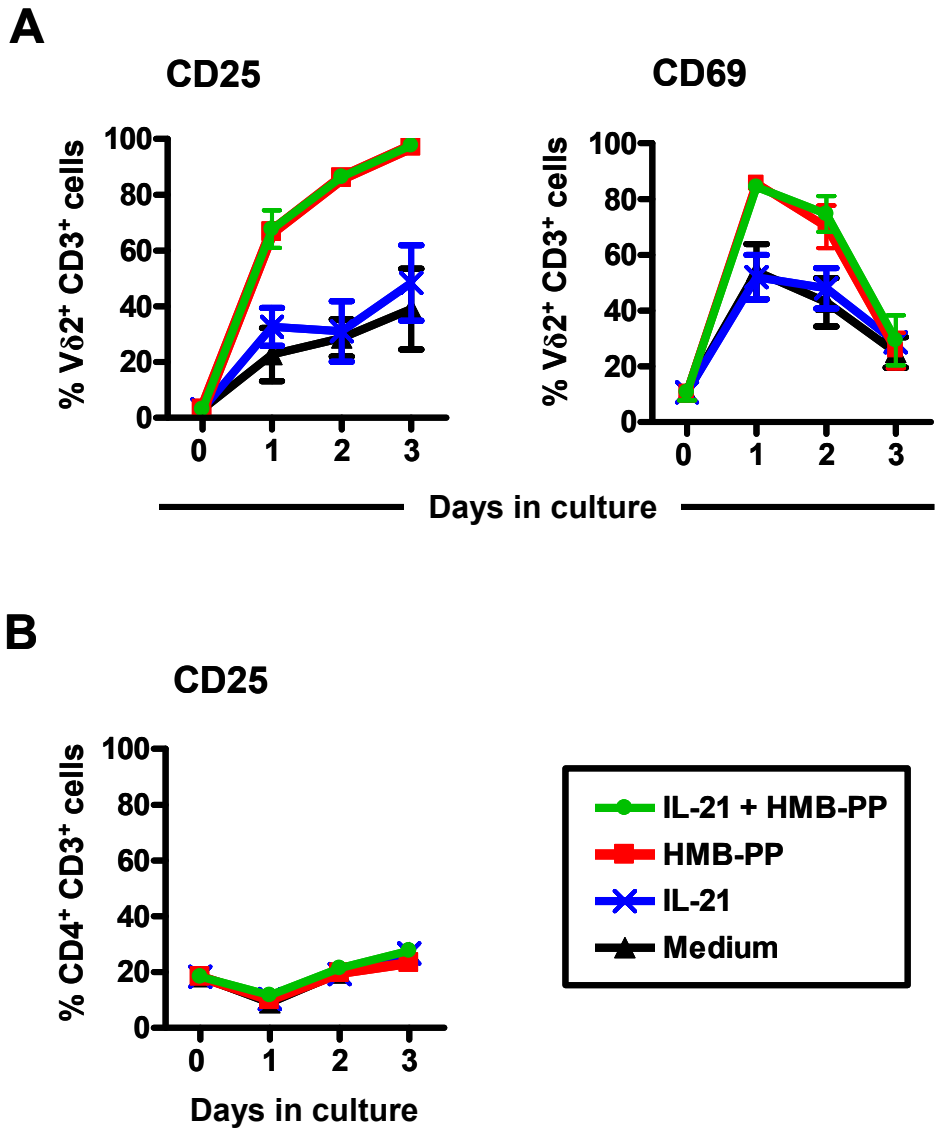


**Figure 4.13. HMB-PP induces up-regulation of CD25 and CD69 activation markers on tonsillar Vγ9Vδ2 T cells.**
Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained for flow cytometry with anti-CD25-PE-Cy7, anti-CD69-PE-Cy5, anti-CD3-APC-H7, anti-CD4-ECD and anti-Vδ2-FITC. Cells were analysed for CD25 and CD69 expression, by gating on lymphocytes, live cells, singlets, (A) $V\delta2^+CD3^+$ cells and (B) $CD4^+CD3^+$ cells. Gates were set using isotype-matched control antibodies. Data are shown as mean ± SEM from three individual donors.

## 4.2.8. IL-21 co-stimulation enhances expression of CXCR5 on HMB-PP stimulated tonsillar Vγ9Vδ2 T cells

In chapter 3, I failed to detect CXCR5 on resting or HMB-PP and IL-21 *in vitro* stimulated peripheral Vγ9Vδ2 T cells, but here showed expression of CXCR5 on a small percentage of tonsillar Vγ9Vδ2 T cells. Here, I proposed up-regulation of surface CXCR5 on tonsillar Vγ9Vδ2 T cells upon *in vitro* stimulation with IL-21 and HMB-PP.

Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained with fluorochrome-conjugated antibodies specific for CXCR5, CD25, CD3 and Vδ2, before being analysed by flow cytometry.

Culturing of tonsillar mononuclear cells in medium or IL21 alone, up-regulated expression of CD25 but not CXCR5 on tonsillar Vγ9Vδ2 T cells (**Figure 4.14A**). Surprisingly, one day stimulation with HMB-PP induced up-regulation of CXCR5 (13% ± 0.8%), which was further enhanced with IL-21 co-stimulation (20% ± 3.5%) (**Figure 4.14A and B**). CXCR5 was predominantly found on activated tonsillar Vγ9Vδ2 T cells that co-expressed CD25 (**Figure 4.14A**). Over the 3 day culture period, expression of CXCR5 reached peak levels after one day in culture and declined gradually between 2 and 3 days in culture. This pattern of expression was seen in cultures treated with HMB-PP alone and in cultures co-treated with both HMB-PP and IL-21. However, higher levels of CXCR5 were induced only in the presence of both HMB-PP and IL-21 stimulation, thus indicating a strong co-stimulatory effect of IL-21 on CXCR5 expression (**Figure 4.14B**).

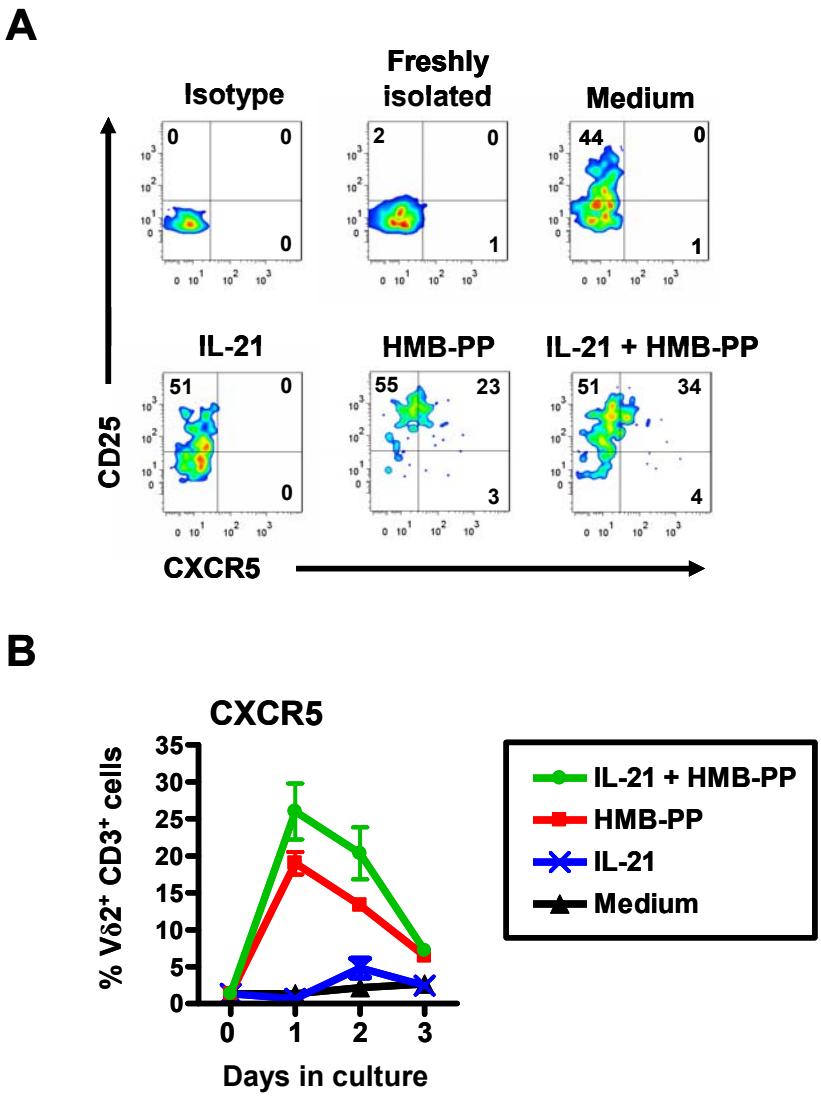


**Figure 4.14. IL-21 enhances expression of CXCR5 on HMB-PP stimulated tonsillar Vγ9Vδ2 T cells.**
Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained for flow cytometry with anti-CXCR5-PE, anti-CD25-PE-Cy7, anti-CD3-APC-H7 and anti-Vδ2-FITC. Cells were analysed for surface CXCR5 and CD25 expression, by gating on lymphocytes, live cells, singlets and $V\delta2^+CD3^+$ cells. Gates were set using isotype-matched control antibodies. (A) Flow cytometry FACs plots showing expression of CXCR5 and CD25 on freshly isolated tonsillar $V\delta2^+$ T cells and after 1 day in culture, under the indicated conditions, data representative of three donors. (B) Percentage of $V\delta2^+$ expressing CXCR5 over 3 days in culture. Data are shown as mean percentage ± SEM of three donors.

## 4.2.9. HMB-PP stimulation enhances expression of ICOS, OX40, CD70 and CD28 co-stimulatory molecules on tonsillar Vγ9Vδ2 T cells

In the previous chapter, I showed up-regulation of co-stimulatory molecules ICOS, OX40, CD70 but not CD40L on IL-21 and HMB-PP stimulated peripheral Vγ9Vδ2 T cells. Here, I proposed up-regulation of co-stimulatory molecules on tonsillar Vγ9Vδ2 T cells upon *in vitro* stimulation with IL-21 and HMB-PP. At the same time, $CD4^+$ T cells from tonsils were also analysed for the same co-stimulatory molecules.

Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained with fluorochrome-conjugated antibodies specific for ICOS, OX40, CD70, CD28, CD40L, CD4, CD3 and Vδ2 or Vγ9, before being analysed by flow cytometry.

Freshly isolated tonsillar Vγ9Vδ2 T cells did not express, ICOS, OX40, CD70, CD28 (**Figure 4.15**) or CD40L (data not shown). Surprisingly, culturing of tonsillar mononuclear cells over 3 days in medium alone somewhat up-regulated expression of ICOS, OX40, CD70 and CD28 (**Figure 4.15**) but not CD40L (data not shown). The addition of IL-21 alone did not further enhance expression of co-stimulatory molecules on tonsillar Vγ9Vδ2 T cells cultured in medium alone (**Figure 4.15**).

Stimulation with HMB-PP alone led to further up-regulation of ICOS, OX40, CD70, CD28 (**Figure 4.15**), but not CD40L (data not shown) on tonsillar Vγ9Vδ2 T cells. Over the 3 day culture period, highest expression of OX40 (17% ± 1.4%) and CD28 (21% ± 5%) was found on day 2 of culture. Expression of both OX40 and CD28 was transient, as on day 3 of culture, levels of OX40 had declined (10% ± 2% vs 17% ± 1.4%), whilst expression of

CD28 was completely lost. In contrast, HMB-PP induced a continuous up-regulation of both ICOS and CD70 expression, on tonsillar Vγ9Vδ2 T cells. Highest expression of ICOS (75% ± 5.5%) and CD70 (17% ± 3%) was found after 3 days in culture (**Figure 4.15**).

The addition of IL-21 to HMB-PP stimulated cultures had no effect on ICOS or CD28 expression. In contrast, co-stimulation with IL-21 resulted in a considerable but steady down-regulation of OX40 in >50 % of HMB-PP stimulated Vγ9Vδ2 T cells (7% ± 0.6% vs 17% ± 1.4%) and to a lesser extent down-regulation of CD70 (11% ± 1.7% vs 16% ± 2%) (**Figure 4.15**).

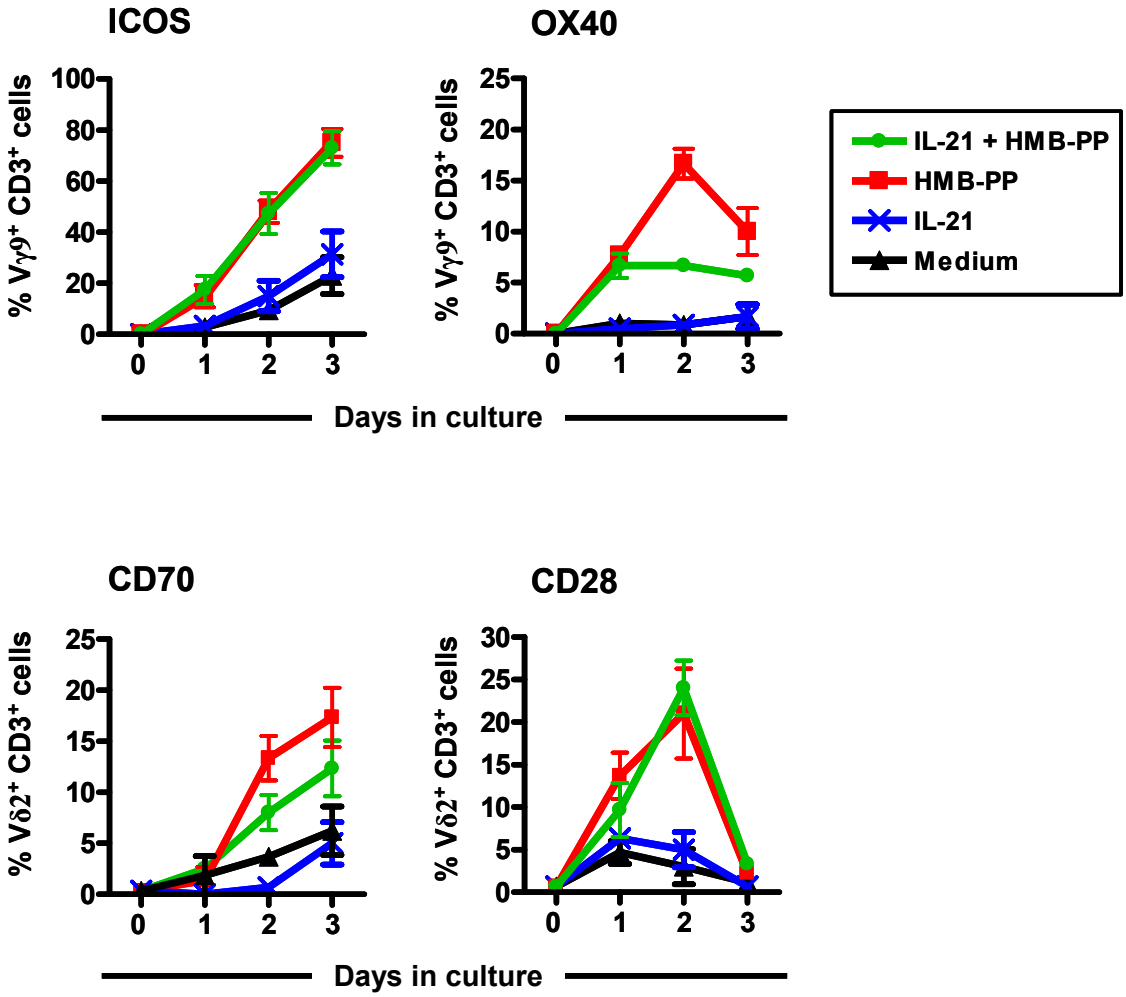


**Figure 4.15. HMB-PP induces up-regulation of ICOS, OX40, CD70 and CD28 co-stimulatory molecules on tonsillar Vγ9Vδ2 T cells.**
Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained for flow cytometry with anti-ICOS-PE, anti-OX40-FITC, anti-CD70-FITC, anti-CD28-PE-Cy5, anti-CD3-pacific blue ( or anti-CD3-APC-H7), and anti-Vγ9-PE-Cy5 or Vδ2-PE. Cells were analysed for ICOS, OX40, CD70 and CD28 expression, by gating on lymphocytes, live cells, singlets and $V\gamma9^{+}CD3^{+}$ or $V\delta2^{+}CD3^{+}$ cells. Gates were set using isotype-matched control antibodies. Data are shown as mean ± SEM from three individual donors.

In the same cultures, I also examined co-stimulatory molecule expression on tonsillar $CD4^+$ T cells. Here I found ICOS (64% ± 9%), OX40 (33% ± 4%), CD70 (8% ± 2%) and CD28 (17% ± 1.4%) to be expressed on freshly isolated tonsillar $CD4^+$ T cells (**Figure 4.16**). Similar to tonsillar Vγ9Vδ2 T cells, CD40L was not detected on freshly isolated $CD4^+$ T cells (data not shown).

After one day of culturing tonsillar mononuclear cells in medium alone, expression of ICOS (32% ± 10%), OX40 (15% ± 1.4%), CD70 (1% ± 0.6%) and CD28 (4% ± 0.3%) was considerably lost on 50% of $CD4^+$ T cells. OX40 and CD70 returned to baseline after 3 days in culture. In contrast, expression of ICOS did not return to baseline, but was continuously expressed by a lower percentage of $CD4^+$ T cells throughout the 3 day of culture. The percentage of CD28 positive $CD4^+$ T cells had declined over 2 days in culture and by day 3, CD28 was found to be absent on all tonsillar $CD4^+$ T cells (**Figure 4.16**). Furthermore, the addition of HMB-PP or IL-21 to tonsillar mononuclear cell cultures did not change expression of ICOS, OX40, CD70 or CD28 on tonsillar $CD4^+$ T cells. Thus, the response to HMB-PP and IL-21 stimulation was specific to Vγ9Vδ2 T cells (**Figure 4.16**).

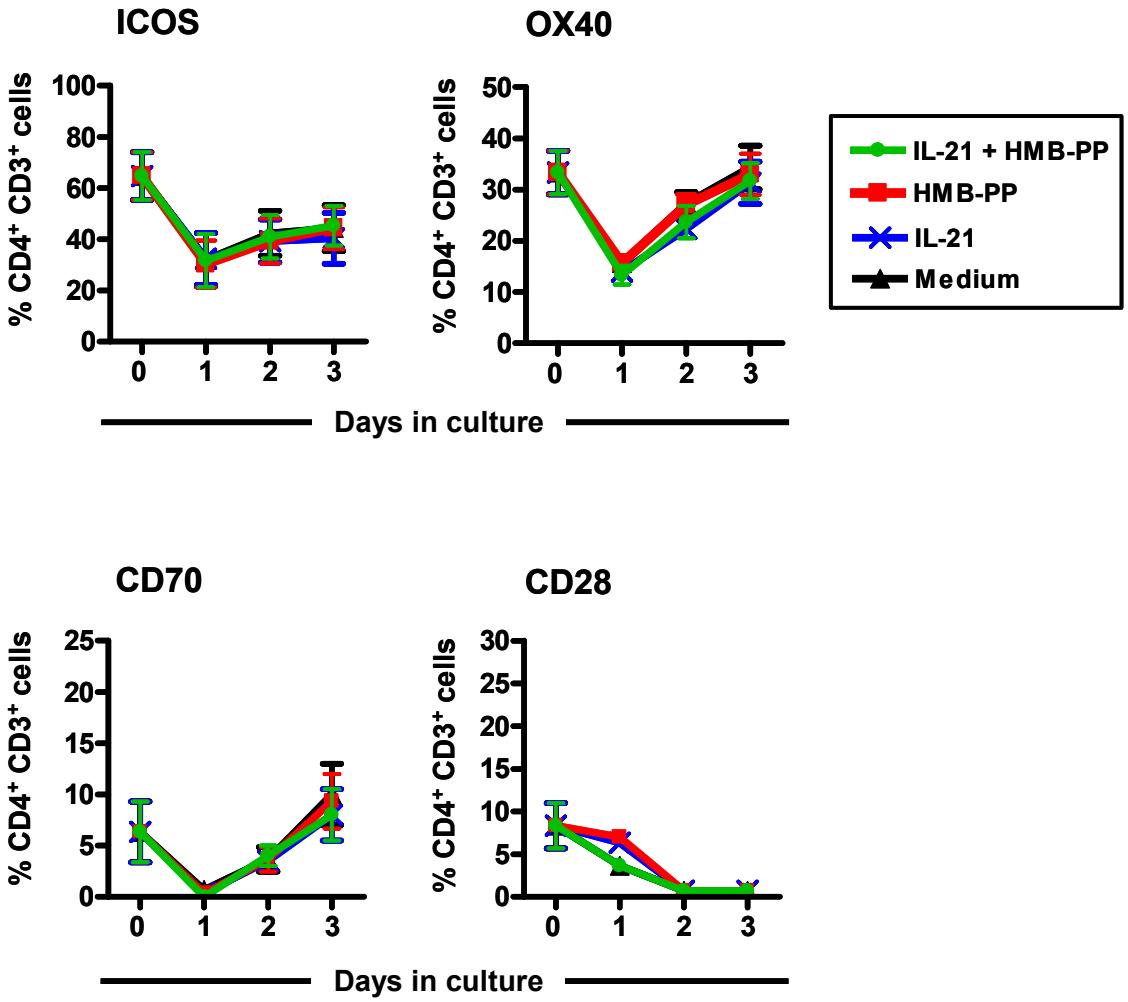


**Figure 4.16. HMB-PP or IL21 does not induce up-regulation of ICOS, OX40, CD70 or CD28 co-stimulatory molecules on tonsillar $CD4^+$ T cells.**
Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained for flow cytometry with anti-ICOS-PE, anti-OX40-FITC, anti-CD70-FITC, anti-CD28-PE-Cy5, anti-CD3-pacific blue ( or anti-CD3-APC-H7) and anti-CD4-ECD. Cells were analysed for ICOS, OX40, CD70 and CD28 expression, by gating on lymphocytes, live cells, singlets and $CD4^+CD3^+$ cells. Gates were set using isotype-matched control antibodies. Data are shown as mean ± SEM from three individual donors.

## 4.2.10. IL-21 and HMB-PP stimulation enhances expression of CCR7, CD40, CD86, and HLA-DR APC markers on tonsillar Vγ9Vδ2 T cells

In the previous chapter, I showed up-regulation of APC markers CCR7, CD40, CD86 and HLA-DR on HMB-PP stimulated peripheral Vγ9Vδ2 T cells. Here, I proposed up-regulation of APC markers on tonsillar Vγ9Vδ2 T cells upon *in vitro* stimulation with IL-21 and HMB-PP. In comparison, tonsillar $CD4^+$ T cells were also analysed for APC marker expression.

Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained with fluorochrome-conjugated antibodies specific for CCR7, CD40, CD86 and HLA-DR, CD3, CD4 and Vγ9, before being analysed by flow cytometry.

Expression of CCR7 was analysed on freshly isolated tonsillar mononuclear cells and cells after 1 day in culture. CCR7 was expressed on a minority of freshly isolated tonsillar Vγ9Vδ2 T cells (3% ± 0.2%), which was found to be up-regulated (19% ± 1.5%) after just 1 day of culturing tonsillar mononuclear cells in medium alone (**Figure 4.17**). This was not due to activation of cells but due to receptor recycling. T cells within secondary lymphoid tissues such as tonsils undergo receptor internalization of surface CCR7 as one way of rendering cells unresponsive to the ligands CCL19/CCL21. Removing cells from the CCL19/CCL21 rich tissue environment and culturing them in medium alone may allow receptors to recycle back to the plasma membrane (Otero et al. 2006). The addition of HMB-PP (25% ± 4.5%), or IL-21 in the absence (23% ± 1%) or presence of HMB-PP (24% ± 1%) had no major effects on CCR7 expression (**Figure 4.17**).

In contrast to CCR7, CD40 was found to be readily expressed by a large proportion of freshly isolated tonsillar Vγ9Vδ2 T cells (67% ± 5%), which was further up-regulated in response to HMB-PP stimulation, with highest expression found on day 3 (88% ± 3%). Furthermore, HMB-PP stimulation maintained expression of CD40, as in the absence of HMB-PP stimulation, the percentage of CD40 positive Vγ9Vδ2 T cells had decreased after 1 day in culture (54% ± 3%) (**Figure 4.17**).

Expression of CD86 was found to be absent on freshly isolated tonsillar Vγ9Vδ2 T cells. On day 3, in medium alone CD86 was up-regulated by a small percentage of Vγ9Vδ2 T cells (6% ± 1.5%), which was further up-regulated in response to HMB-PP stimulation (24% ± 1.4%). Similar to CCR7, expression of CD40 and CD86 was not affected by the presence of IL-21 stimulation (with or without HMB-PP stimulation) (**Figure 4.17**).

HLA-DR expressed on a minority of freshly isolated tonsillar Vγ9Vδ2 T cells (12% ± 1.8%) was found to be up-regulated in response to all four culture conditions. On day 3, HLA-DR was found to be up-regulated on Vγ9Vδ2 T cells cultured in medium alone (20% ± 5%), IL-21 alone (31% ± 7%), HMB-PP alone (58% ± 2%), with highest expression found in response to both HMB-PP and IL-21 stimulation (71% ± 2.4%). Hence the co-stimulatory effect of IL-21 did not enhance surface expression of CCR7, CD40 or CD86 but did somewhat enhance expression of HLA-DR on HMB-PP stimulated tonsillar Vγ9Vδ2 T cells (**Figure 4.17**).

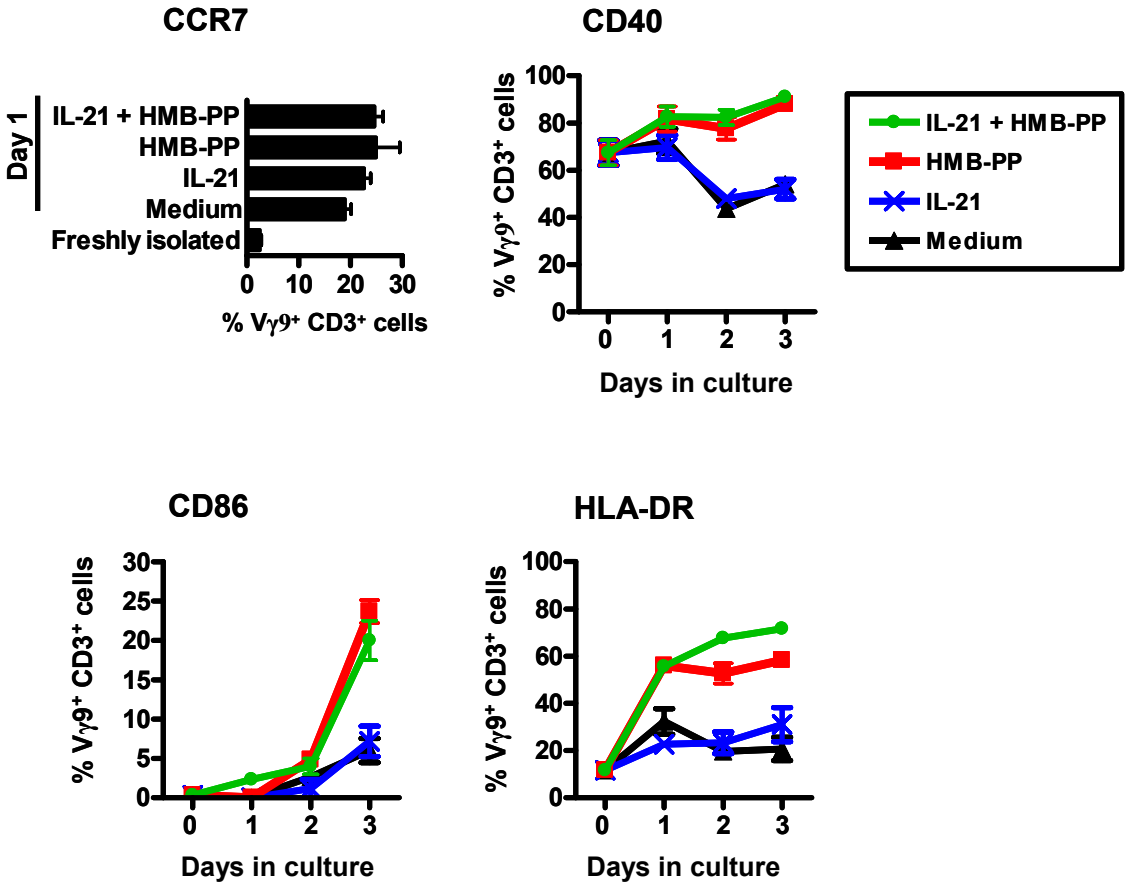


**Figure 4.17. HMB-PP induces up-regulation of CCR7, CD40, CD86 and HLA-DR on tonsillar Vγ9Vδ2 T cells.**
Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained for flow cytometry with unlabelled rat anti-CCR7, followed by anti-rat-biotin - secondary antibody and detection with streptavidin-PeCy7. Cells were also stained with anti-CD40-PE, anti-CD86-FITC, anti-HLA-DR-APC-H7, anti-CD3-pacific blue, anti-CD4-ECD and anti-Vγ9-PE-Cy5. $V\gamma9^{+}CD3^{+}$ cells were analysed for CCR7, CD40, CD86 and HLA-DR surface expression by gating on lymphocytes, live cells and singlets. Gates were set using isotype-matched control antibodies. Data are shown as mean ± SEM from three individual donors.

In the same cultures, I also examined expression of CCR7, CD40, CD86 and HLA-DR on tonsillar $CD4^+$ T cells. Similar to tonsillar Vγ9Vδ2 T cells, CCR7 on freshly isolated tonsillar $CD4^+$ T cells (18% ± 7.5%) was found to be up-regulated after 1 day of culturing tonsillar mononuclear cells in medium alone (63% ± 13%) (**Figure 4.18**).

CD40 (71% ± 4.5%) and HLA-DR (57% ± 6%) was readily expressed by a large proportion of freshly isolated tonsillar $CD4^+$ T cells. Both CD40 (41% ± 3%) and HLA-DR (23% ± 2.5%) were considerably down-regulated after 2 day of culturing in medium alone (**Figure 4.18**). In contrast, CD86 was expressed by a minority of freshly isolated tonsillar $CD4^+$ T cells (3% ± 6%). After one day of culturing tonsillar mononuclear cells in medium alone, expression of CD86 was lost but returned to levels above baseline after 3 days in culture (6% ± 0.7%) (**Figure 4.18**). Furthermore, the addition of HMB-PP or IL-21 to tonsillar mononuclear cell cultures did not change expression of CCR7, CD40, CD86 or HLA-DR on tonsillar $CD4^+$ T cells. Thus, the response to HMB-PP and IL-21 stimulation was specific to Vγ9Vδ2 T cells (**Figure 4.18**).

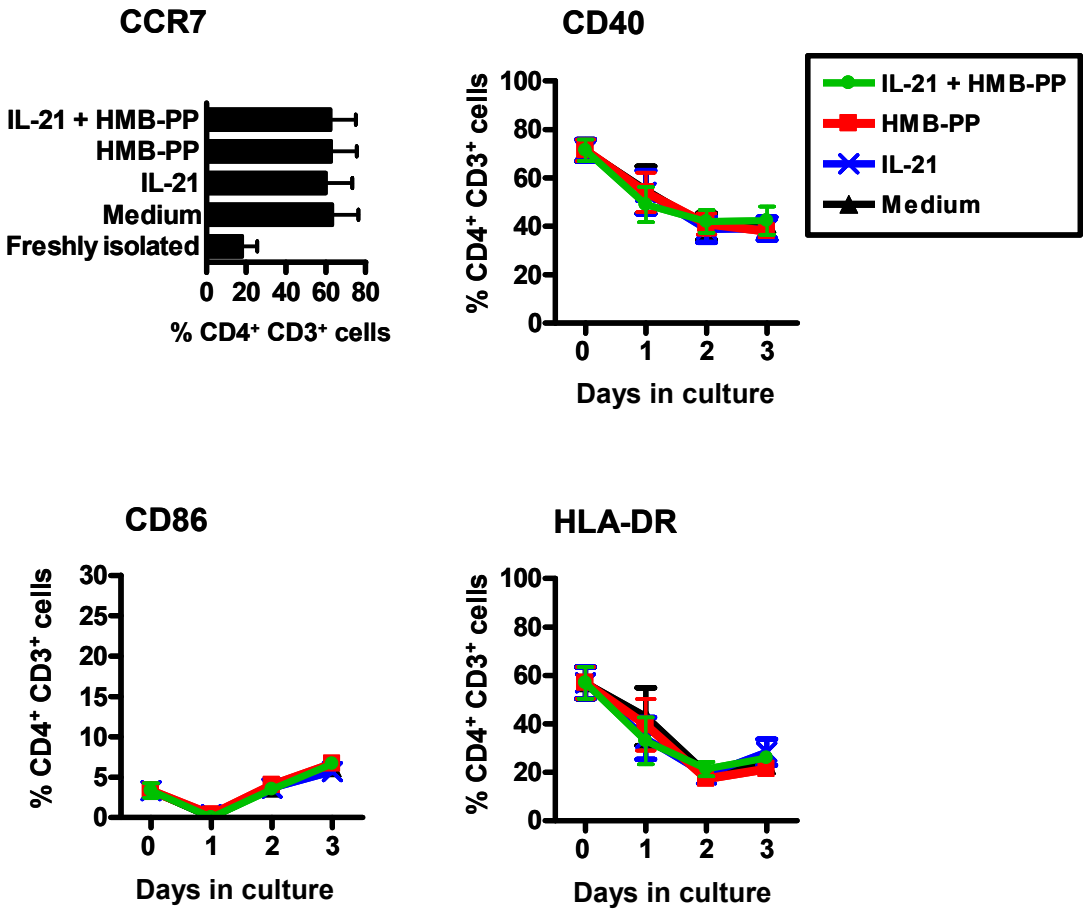


**Figure 4.18. HMB-PP and IL-21 does not induce up-regulation of CCR7, CD40, CD86 or HLA-DR on tonsillar $CD4^+$ T cells.**
Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 1, 2 and 3 days in culture were stained for flow cytometry with unlabelled rat anti-CCR7, followed by anti-rat-biotin - secondary antibody and detection with streptavidin- PeCy7. Cells were also stained with anti-CD40-PE, anti-CD86-FITC, anti-HLA-DR-APC-H7, anti-CD3-pacific blue, anti-CD4-ECD and anti-Vγ9- PE-Cy5. $CD4^+CD3^+$ cells were analysed for CCR7, CD40, CD86 and HLA-DR surface expression by gating on lymphocytes, live cells and singlets. Gates were set using isotype-matched control antibodies. Data are shown as mean ± SEM from three individual donors.

## 4.2.11. Selective proliferation of tonsillar Vγ9Vδ2 T cells in response to HMB-PP stimulation

In chapter 3, I showed HMB-PP and IL-21 induced proliferation of peripheral blood Vγ9Vδ2 T cells in PBMCs. Here, I examined the proliferative capacity of tonsillar Vγ9Vδ2 T cells alongside $CD4^+$ T cells in response to *in vitro* HMB-PP and IL-21 stimulation.

Freshly isolated tonsillar mononuclear cells taken from 3 different donors were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (day 0) and cells after 3, 5, 7 and 10 days in culture were stained with fluorochrome-conjugated antibodies specific for CD3, CD4 and Vδ2. Cells were analysed by flow cytometry for the total percentage of $Vδ2^+$ positive T cells amongst the CD3 positive population (**Figure 4.19A**).

In freshly isolated tonsillar mononuclear cells, Vγ9Vδ2 T cells represented 2 % (± 0.5%) of total CD3 positive cells. In response to HMB-PP stimulation, the percentage of Vγ9Vδ2 T cells amongst the CD3 positive population increased from day 3 (2 % ± 0.8%) to day 7 (23 % ± 5.7%) and declined on day 10 of culture (16% ± 8.6%). Co-stimulation with IL-21 suppressed proliferation of tonsillar Vγ9Vδ2 T cells to 15% (± 8%) on day 7 and to 12% on day 10 (± 6%). Culturing of tonsillar mononuclear cells in medium or IL-21 alone did not induce proliferation of tonsillar Vγ9Vδ2 T cells. Thus, stimulation with HMB-PP alone for up to 7 days provided the best conditions for *in vitro* proliferation of tonsillar Vγ9Vδ2 T cells **(Figure 4.19A)**. The error bars displayed in **Figure 4.19A**, account for donor-to-donor variability between the three donors, in the expansion potential of tonsillar Vγ9Vδ2 T cells.

In the same cultures, I also examined proliferation of tonsillar $CD4^+$ T cells. In freshly isolated tonsillar mononuclear cells, $CD4^+$ T cells represented 52 % (± 9.5%) of total CD3 positive cells. In response to culturing of tonsillar mononuclear cells in medium alone, the

percentage of $CD4^+$ T cells amongst the CD3 positive population increased from day 0 (52 % ± 9.5%) to day 7 (89 % ± 1.5%) and declined by day 10 (83 % ± 6%) in culture (**Figure 4.19B**). In contrast to Vγ9Vδ2 T cells, proliferation of tonsillar $CD4^+$ T cells was not specific to HMB-PP or IL-21 stimulation. Instead, the addition of HMB-PP or IL-21 to tonsillar mononuclear cell cultures, somewhat suppressed proliferation of tonsillar $CD4^+$ T cells (**Figure 4.19**).

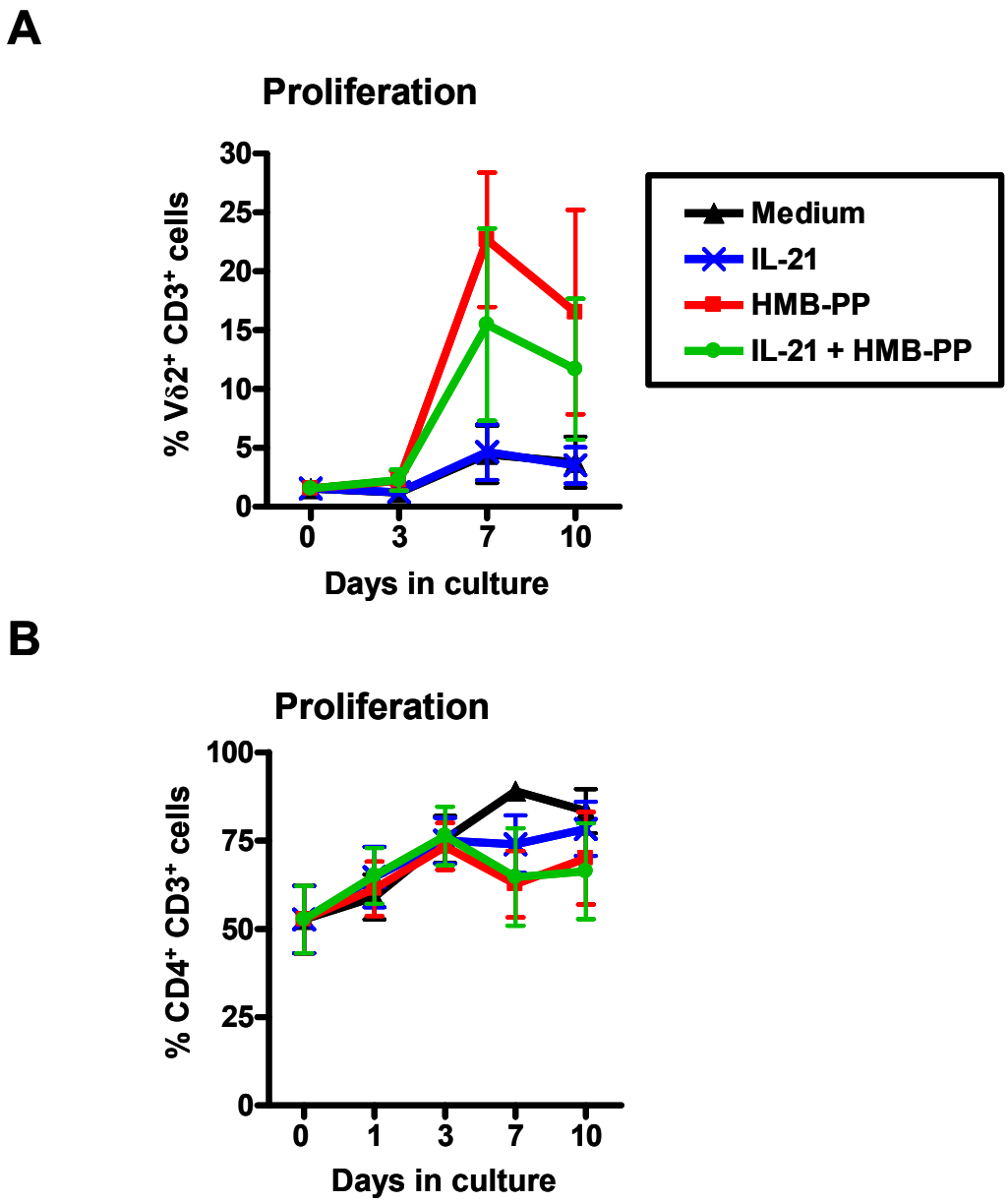


**Figure 4.19. Tonsillar Vγ9Vδ2 T cells proliferate in response to HMB-PP stimulation.** Freshly isolated tonsillar mononuclear cells were cultured in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). Freshly isolated tonsillar mononuclear cells (Day 0) and cells after 1, 3, 7 and 10 days in culture, cells were stained for flow cytometry with anti-CD3-pacific blue, anti-CD4-ECD and anti-Vδ2-PE. The percentage of (A) $V\delta2^+$ $CD3^+$ cells and (B) $CD4^+$ $CD3^+$ cells was determined by gating on lymphocytes, live cells and singlets. Data are shown as mean ± SEM from three individual donors.

# Chapter 5:

## IL-21 enhances the ability of HMB-PP activated Vγ9Vδ2 T cells to provide B cell help

## 5.1 Chapter specific hypothesis and aims

Human γδ T cells have previously been shown to induce antibody production during co-culture with B cells (Brandes et al. 2003; Caccamo et al. 2006a). Brandes et al. (2003) demonstrated peripheral blood γδ T cells stimulated with an anti-TCR antibody, in the absence of cytokines to induce tonsillar B cell IgM, IgA and IgG antibody production. Similarly, Caccamo et al. (2006) also showed peripheral blood and tonsillar Vγ9Vδ2 T cells to help autologous tonsillar B cells in antibody production, however, this was shown in the presence of a weak synthetic γδ T cell activator (bromohydrin pyrophosphate) (Morita et al. 2007) and also in the absence of cytokines. Here, by using the microbial metabolite HMB-PP and the Tfh-derived cytokine IL-21, my study uses a more physiologically relevant and novel method of investigating Vγ9Vδ2 T cell ability to induce antibody production during co-culture with B cells. Thus, after having established conditions that favour the acquisition of a B helper-Tfh-like phenotype on peripheral blood Vγ9Vδ2 T cells, as well as having identified Tfh-like phenotypes on tonsillar Vγ9Vδ2 T cells, the objective of this chapter was to investigate whether Vγ9Vδ2 T cells were able to provide B cell help using an *in vitro* co-culture model.

Here, I hypothesised that the IL-21 induced acquisition of a Tfh-like phenotype on HMB-PP activated Vγ9Vδ2 T cells equips Vγ9Vδ2 T cells to support B cell activation, differentiation, and antibody production.

Aims of this chapter were to:

1. Phenotype B cells from peripheral blood and tonsils.
2. Investigate the effects of HMB-PP and IL-21 on the ability of peripheral Vγ9Vδ2 T cells to induce antibody production by tonsillar B cells.
3. Investigate the effects of HMB-PP and IL-21 on the ability of tonsillar Vγ9Vδ2 T cells to induce antibody production by tonsillar B cells.
4. Investigate the effects of HMB-PP and IL-21 on the ability of peripheral Vγ9Vδ2 T cells to induce peripheral B cell activation, APC marker expression, differentiation and antibody production.

## 5.2. Results

### 5.2.1. Phenotypic analysis of freshly isolated tonsillar and peripheral blood B cells

In comparison to antigen-inexperienced naive B cells, memory B cells are antigen-experienced long-lived, quiescent B cells that express high-affinity surface antibodies. Memory B cells are capable of eliciting rapid and robust responses upon re-encounter with the pathogen (Good-Jacobson and Shlomchik 2010; Klein and Dalla-Favera 2008; Tangye and Tarlinton 2009). Furthermore, memory B cells are more sensitive to *in vitro* stimulation with the CD40 ligand (CD40L) plus IL-21, as well as with SAC plus IL-2, in differentiating into Ig-secreting plasma cells (Agematsu et al. 2000; Konforte et al. 2009; Tangye and Good 2007). Accordingly, memory B cells would be more sensitive to help provided by γδ T cells whereas naïve B cells would require priming with specific antigen or with a powerful surface Ig cross-linker such as SAC, before they can be used for co-culture experiments. Thus a good yield of memory B cells would be required for these co-culture experiments. To determine the better source for memory B cells, I here investigated naïve/memory subsets and phenotypes of B cells from tonsils in comparison to B cells from peripheral blood.

Percentages of total B cells and B cell subsets in peripheral blood and tonsils were evaluated in parallel. Freshly isolated PBMCs and tonsillar mononuclear cells were stained for CD19, CD3, IgD and CD27 surface markers and analysed by flow cytometry. B cells were identified by gating on lymphocytes, gating out dead cells and doublets and gating on $CD3^-$ $CD19^+$ cells.

Results showed that, in line with previous studies (Cernelc et al. 1979), the percentage of B cells amongst the lymphocyte population was significantly higher in the inflamed tonsils (73% ± 3%) than in the peripheral blood (10% ± 1%) ($p$<0.0001, paired t test) (**Figure 5.1A**).

In peripheral blood and tonsils, naïve and memory B cell subsets were identified on the basis of IgD and CD27 surface marker expression. On the gated $CD3^-$ $CD19^+$ B cell population a dot plot with IgD along the y-axis and CD27 on the x-axis was created. Quadrants in dot plots were set using isotype-matched control antibodies). B cell memory subsets were identified as follows: Naïve = $IgD^+CD27^-$, unswitched memory = $IgD^+CD27^+$, switched memory = $IgD^-CD27^+$ and double negative = $IgD^-CD27^-$ (**Figure 5.1B**) (Sanz et al. 2008). Evidently, the proportions of naïve and memory B cell subsets in peripheral blood differed to those found in tonsils. As expected, the percentage of naïve B cells amongst the B lymphocyte population was significantly higher in peripheral blood (64% ± 6.7%) than in the tonsils (34% ± 2.4%) ($P$=0.0238, Mann-Whitney two-tailed test) (Weill et al. 2009) (**Figure 5.1C**).

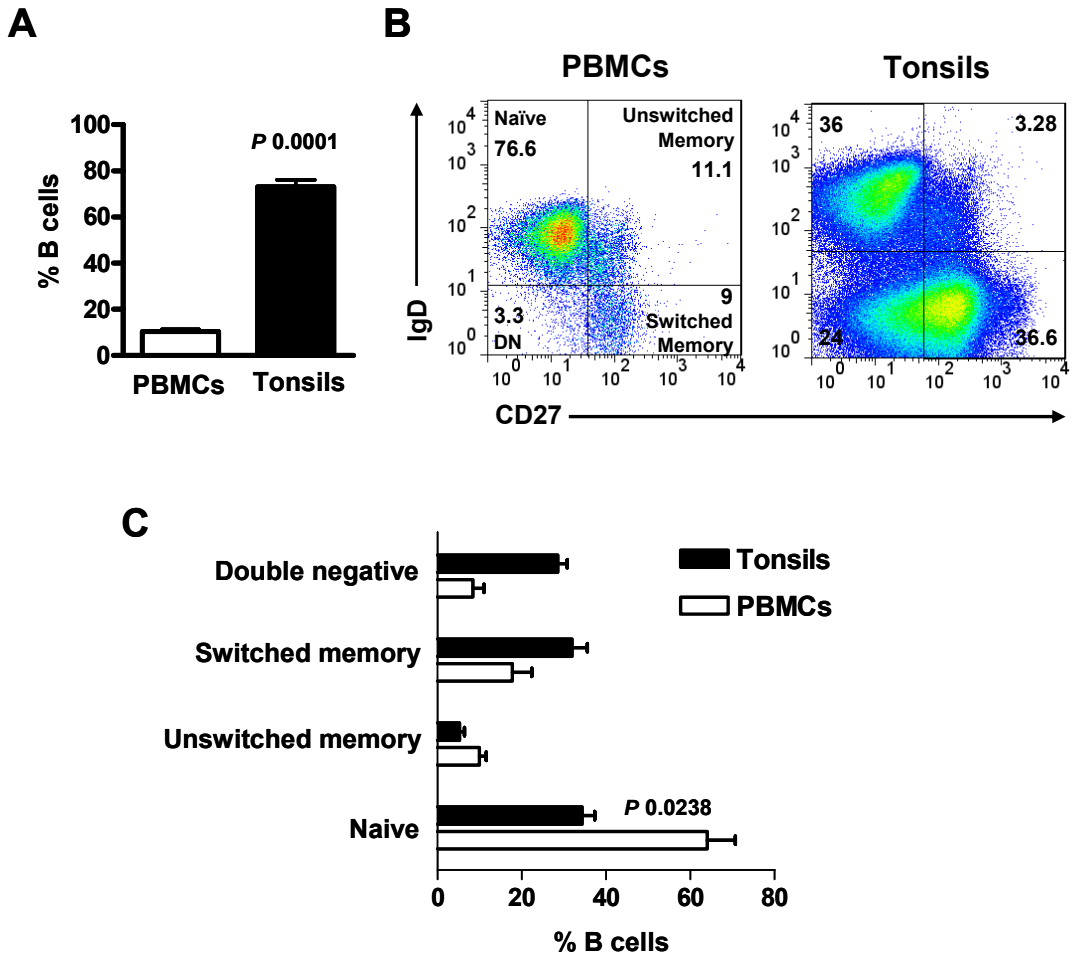


**Figure 5.1. Comparison of B cell subsets in tonsils and peripheral blood.**
PBMCs and tonsillar mononuclear cells were freshly isolated. (A) The percentage of total B cells and (B and C) B cell subsets were determined by staining cells for flow cytometry with CD19, CD3, IgD and CD27 surface markers. Cells were analysed for IgD and CD27 expression, by gating on lymphocytes, live cells and $CD3^-$ $CD19^+$ cells, quadrants were set using isotype-matched control antibodies. (B) Representative dot plots showing the percentage of peripheral blood and tonsillar B cells expressing IgD and CD27. (B and C) Naïve/memory subsets were classified as follows: Naïve = $IgD^+CD27^-$, unswitched memory = $IgD^+CD27^+$, switched memory = $IgD^-CD27^+$ and double negative = $IgD^-CD27^-$. Data are shown as mean percentage + SEM from nine (A) and three to five (C) independently assessed donors. In (A) the percentage of B cells amongst the lymphocyte population was statistically different in tonsils compared to peripheral blood. In (C) the percentage of naïve B cells amongst the B lymphocyte population was statistically different in tonsils compared to peripheral blood.

I next examined freshly isolated peripheral blood and tonsillar B cells for surface marker expression of APC markers (CD40, HLA-DR and CD86), activation markers (CD25 and CD69) and the follicular homing chemokine receptor CXCR5. Surface expression of CD40 HLA-DR and CD86 was comparable between tonsillar and peripheral blood B cells (**Figure 5.2A and B**). A minor percentage of tonsillar and peripheral blood B cells expressed the activation markers CD25 and CD69 (**Figure 5.2B**). The chemokine receptor CXCR5 was found to be uniformly expressed on both peripheral blood and tonsillar B cells (**Figure 5.2C**). However, in **Figure 5.2C** as indicated by the GMFI values and shift in fluorescence intensity of CXCR5 on B cells, as expected CXCR5 was much lower on tonsillar B cells (GMFI 4 ± 0.5) in comparison to peripheral blood B cells (GMFI 42 ± 11). Thus, indicating that B cells down-regulate CXCR5 expression levels when in tonsillar tissue (**Figure 5.2C**). These findings are in agreement with published findings and literature (Fairfax et al. 2008).

As expected, in comparison to peripheral blood, these studies confirmed tonsils to provide much higher yields of not only total but also switched memory B cells. These switched memory B cells represent antigen-activated B cells that have undergone CSR in the GC, thus are capable of secreting IgG, IgA and IgE antibodies upon re-stimulation. Concluding, tonsillar tissue would provide as a better source of B cells for use in co-culture experiments.

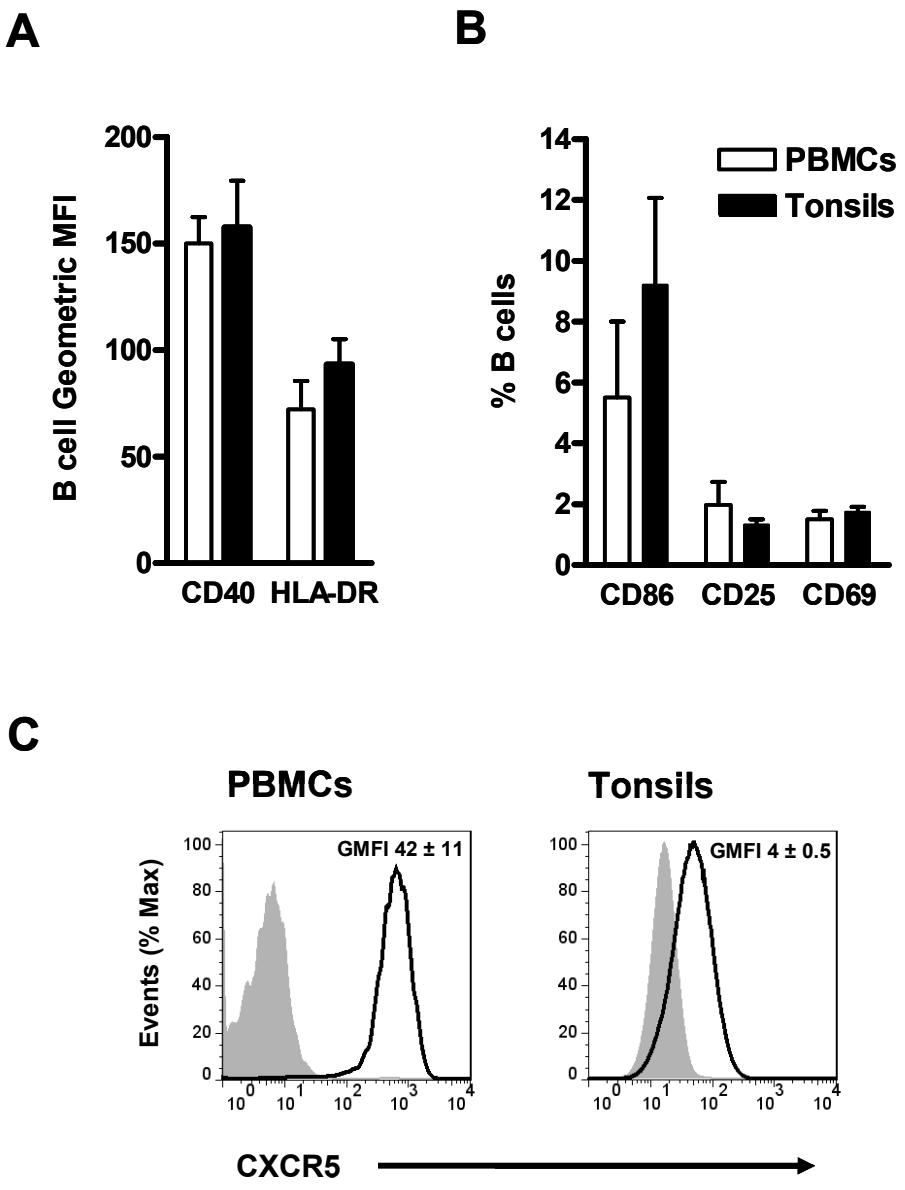


**Figure 5.2. Cell surface phenotype of freshly isolated B cells from peripheral blood and tonsils.**

PBMCs and tonsillar mononuclear cells were freshly isolated and stained with antibodies directed towards the indicated surface markers, along with isotype-matched control antibodies. Cells were analysed by flow cytometry for surface marker expression by gating on lymphocytes, live cells and $CD3^{-}$ $CD19^{+}$ cells. (A) CD40, HLA-DR and (C) CXCR5 data are shown as mean fold change (± SEM) in GMFI, calculated as (geometric MFI of antibody)/(geometric MFI of isotype control). (B) CD86, CD25 and CD69 data are shown as the mean percentage of $CD19^{+}$ B cells positive for each marker. Data are shown as mean ± SEM from three to nine individual donors.

## 5.2.2. Tfh cells and IL-21 induce B cell IgM, IgG and IgA antibody production

Humoral immune responses are initiated within B cell follicles of secondary lymphoid tissue, where naïve circulating B cells after recognition of their cognate antigen require help and support from T cells. Tfh cells, known as true helper cells for B cells, have been shown to provide direct support for B cell responses *in vivo, ex vivo* and *in vitro* (Ebert et al. 2004; King et al. 2008; Macaulay et al. 1998; Stockinger et al. 1996). IL-21, a major cytokine secreted by Tfh cells, plays a central role in stimulating B cells to undergo proliferation, isotype switching and differentiation (King 2009; King et al. 2008).

Secondary lymphoid tissues, such as the human tonsils, provide a good source of both B and Tfh cells, thus allowing both cell types to be isolated from the tonsil of the same donor for *in vitro* functional T cell-B cell co-culture assays. Unlike naïve resting peripheral blood B cells, the majority of tonsillar B cells are memory antigen experienced B cells (**Figure 5.1C**). Equally unlike the small percentage of resting Tfh cells found in peripheral blood (**Chapter 3, Figure 3.12B**), Tfh cells in tonsils are abundantly present and exist as activated cells (**Chapter 4, Figure 4.7B**).

Here, the aim was to establish and optimise an *in vitro* T cell-B cell co-culture system that was consistent in supporting B cell antibody production. I tested the methodology and conditions by examining the ability of tonsillar Tfh cells to induce antibody production by tonsillar B cells. Simultaneously I also investigated the direct *in vitro* effects of IL-21 on tonsillar B cell antibody production.

Tonsillar mononuclear cells were freshly isolated from tonsils. To isolate Tfh cells from tonsillar mononuclear cells, first $CD4^+$ T cells were positively isolated resulting in >98 % purity (**Figure 5.3A**) and from the $CD4^+$ T cell population, $CXCR5^+$ cells were isolated

resulting in 85% purity (**Figure 5.3B**). B cells were isolated from CD4 T cell depleted cells by negative selection, resulting in 97% purity (**Figure 5.3C**). $CXCR5^+$ B cells were isolated from the B cell population by positive selection, resulting in 96% purity (**Figure 5.3D**). In secondary lymphoid tissue such as the tonsils, as $CD19^+$ B cells differentiate into plasma cells surface expression of CXCR5 is down-regulated. Therefore here I specifically selected for CXCR5 positive tonsillar B cells in order to exclude $CD19^+$ $CXCR5^-$ plasma cells.

Isolated tonsillar $CXCR5^+$ B cells were cultured with either isolated tonsillar Tfh cells or with various concentrations of IL-21 (0.1, 0.5, 5, and 50 ng/ml) or neither. After 10 days of culture, total IgM, IgG and IgA levels in culture supernatants were measured by ELISA.

IL-21 induced a dose-dependent effect on IgM, IgG and IgA antibody production. Interestingly, stimulation of tonsillar B cells with 50 ng/ml of IL-21 induced IgM, IgG and IgA antibody levels that were comparable to those induced by tonsillar Tfh cells. These results identified concentrations 50 ng/ml of IL-21 as most potent at inducing antibody production (**Figure 5.4**).

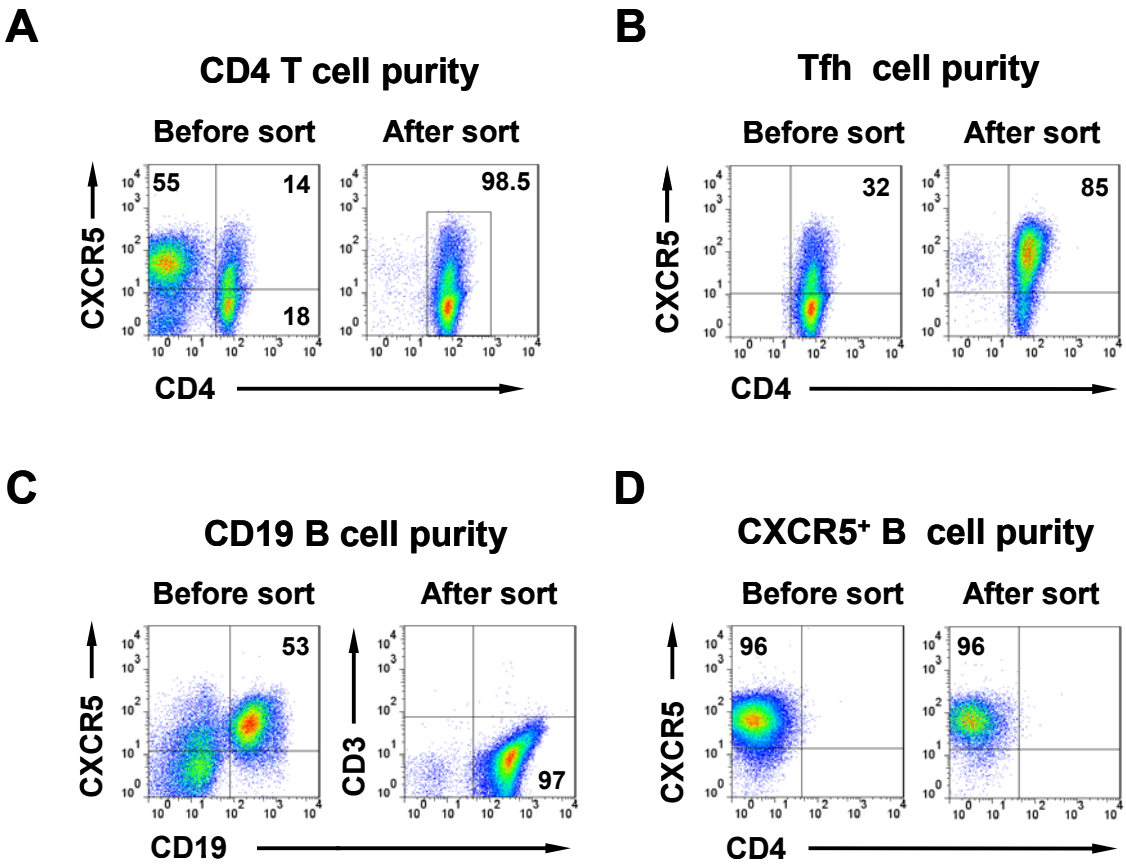


**Figure 5.3. Flow cytometric purity analysis of isolated tonsillar Tfh and CXCR5$^+$ B cells.**
Tonsillar mononuclear cells were isolated from fresh human tonsils. Tfh cells (CXCR5$^+$ CD4$^+$ T cells) and CXCR5$^+$ B cells were purified from either freshly isolated or from frozen tonsillar mononuclear cells using the Miltenyi Biotec magnetic cell sorting system. Cells were blocked with human Igs and stained with anti-CD4-FITC and CXCR5-PE. (A) CD4$^+$ T cells were isolated using the anti-FITC multiSort kit as follows: FITC labeled CD4$^+$ T cells were magnetically labeled with anti-FITC multiSort microbeads, following positive CD4$^+$ T cell selection (98.5% purity) the magnetic particles were removed from the cells by using the multisort release reagent. (B) CXCR5$^+$ CD4$^+$ T cells were isolated from the CD4$^+$ T cell population using anti-PE microbeads, resulting in 85% purity. (C) B cells were isolated from CD4 T cell depleted cells by negative selection, resulting in 97% purity. (D) CXCR5$^+$ B cells were isolated from the B cell population using anti-PE microbeads, resulting in 96% purity. Results are representative of ten experiments performed.

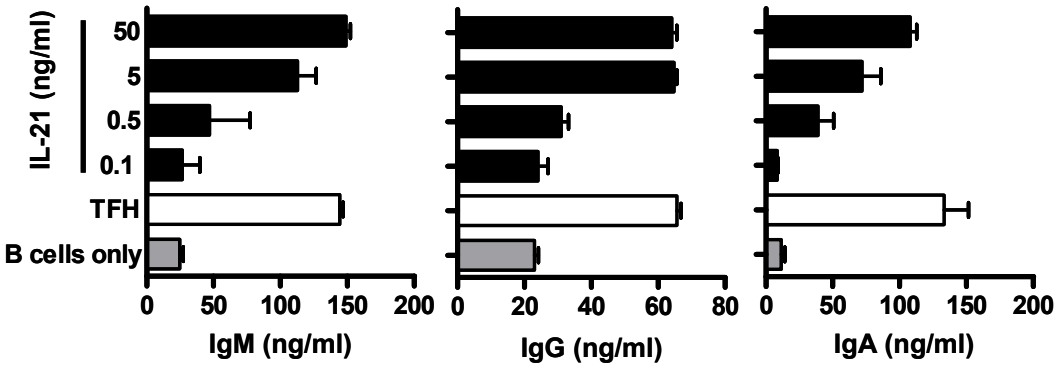


**Figure 5.4. IL-21 induces a dose-dependent effect on IgM, IgG and IgA antibody production.**
Tfh and CXCR5$^{+}$ B cells were purified from either freshly isolated or from frozen tonsillar mononuclear cells as described in Figure 5.3. Purified B cells were cultured with either purified Tfh cells (at a ratio of 1:1), or with various concentrations of IL-21 (0.1, 0.5, 5, and 50 ng/ml) or in medium alone (B cells only). After 10 days of culture, total IgM, IgG and IgA levels in culture supernatants were assessed by ELISA. Data are shown as mean + SEM from three to five individual wells and are representative of results from one of two similar experiments.

### 5.2.3. Peripheral Vγ9Vδ2 T cells provide help for tonsillar B cell antibody production

There is increasing evidence, showing an involvement of γδ T cells in humoral immune responses. γδ T cells have been found in several secondary lymphoid tissues such as the draining lymph node, Peyer's patches, inflamed tonsils and inflamed appendix (Brandes et al. 2009; Dieli et al. 2003; Groh et al. 1989; Soo et al. 1995). More specifically, γδ T cells have been found to be co-localised with CXCL13-producing cells within GCs of B cell follicles (Vermijlen et al. 2007). As shown here and by others, γδ T cell expression of IL-21R, CXCL13 and CXCR5 and long-term maintenance of co-stimulatory molecules strongly suggests a direct involvement in follicular B cell maturation (Brandes et al. 2003; Vermijlen et al. 2007). Here, I proposed a model involving interaction of activated γδ T cells, B cells and Tfh cells in secondary lymphoid tissue whereby HMB-PP activated peripheral γδ T cells would acquire a LN homing phenotype allowing migration to secondary lymphoid tissue. Within GC of B cell follicles, activated γδ T cells would respond to Tfh derived IL-21 equipping them to provide direct help for B cell activation, differentiation and production of antibodies (Vermijlen et al. 2007).

To study and mimic my model *in vitro*, I employed a γδ T cell-B cell co-culture system. To test whether or not γδ T cells were able to support B cells for antibody production initially I used a non-autologous system, whereby B cells were obtained from tonsils and Vγ9Vδ2 T cells from peripheral blood. This initial approach was taken not only because tonsils provided B and Tfh cells in a state of pre-activation, but also because higher yields of both cell types could be obtained from tonsils in comparison to peripheral blood. Equally, higher cell counts of Vγ9Vδ2 T cells were obtained from peripheral blood in comparison to tonsils. Here, obtaining practical number of all three B, Tfh and γδ T cells was important for initial optimisation of experiments, as it allowed simultaneous testing of various controls and culture conditions.

To begin with, the ability of peripheral blood γδ T cells to induce B cell antibody production was evaluated using a two-step culture system. Firstly, in order to rule out any direct effect on B cells, peripheral blood Vγ9Vδ2$^+$ T cells were pre-stimulated to induce acquisition of a Tfh-like phenotype before adding them to autologous B cells as follows: Purified blood Vγ9Vδ2$^+$ T cells were pre-stimulated with HMB-PP (10 nM) or in medium alone. After 42 hours, HMB-PP stimulated cultures were treated with or without IL-21 (50 ng/ml) or IL-2 (20 units/ml). Non-stimulated Vγ9Vδ2 T cells cultured in medium alone served as negative control. After a further 24 hours, stimulated and non-stimulated γδ T cells were harvested and washed several times to ensure removal of any residual HMB-PP, IL-21 and IL-2, thus minimising direct action of IL-21 or IL-2 on B cells.

For antibody production CXCR5$^+$ B and Tfh cells were freshly isolated from tonsils as described in **Figure 5.3**. Non-stimulated, HMB-PP, IL-2+HMB-PP or IL-21+HMB-PP pre-stimulated γδ T cells were co-cultured at a ratio of 1:1 with purified tonsillar CXCR5$^+$ B cells, in the absence or presence of HMB-PP (10 nM) or in the presence of tonsillar Tfh cells. As controls, tonsillar CXCR5$^+$ B cells were cultured alone (B cells only), or in the presence of Tfh cells or with a concentration of IL-21 (0.5 ng/ml) representing residual/contaminating IL-21 that may have been present after washing. After 10 days of culture, total IgM, IgG and IgA levels in culture supernatants were measured by ELISA.

Use of internal positive and negative controls demonstrated the reliability of my *in vitro* co-culture assay. As positive controls, co-culturing of Tfh cells with B cells induced significant amounts of IgM, IgA and IgG antibody production whilst, as negative controls, little or very low levels of antibody were detected when B cells were cultured with 0.5 ng/ml of IL-21 or in medium alone (**Figure 5.5**). Furthermore, no significant difference was found between B cells stimulated with 0.5 ng/ml of IL-21 in comparison to B cells cultured in medium alone (**Figure 5.5A**).

B cells cultured in the presence compared to the absence of non-stimulated Vγ9Vδ2 T cells (medium) resulted in a 2-fold increase in the production of IgM, 3-fold increase in the

production of IgA and a significant 4-fold increase in the production of IgG. Co-culturing B cells with HMB-PP, IL-2 or IL-21 pre-stimulated γδ T cells did not further enhance IgM, IgA or IgG antibody levels (**Figure 5.5A**). The addition of HMB-PP to co-cultures in the aim of re-stimulating γδ T cells did not improve the ability of γδ T cells to further enhance antibody production. In contrast, in response to HMB-PP re-stimulation, IgG antibody production was somewhat reduced to levels that were no longer found to be statistically significant (**Figure 5.5B**).

The synergistic effect of γδ T cells and Tfh cells on B cell antibody production was also examined. IgM antibody production increased almost 2-fold when Tfh and B cells were cultured in the presence compared to the absence of non-stimulated Vγ9Vδ2 T cells (medium). IgM antibody production increased to a lesser extent in the presence of HMB-PP stimulated Vγ9Vδ2 T cells whereas culturing of Tfh and B cells in the presence of IL-2+HMB-PP or IL-21+HMB-PP pre-stimulated γδ T cells had little or no effect on enhancing IgM antibody production induced by Tfh cells alone. Similarly, non-stimulated and HMB-PP, IL-2+HMB-PP or IL-21+HMB-PP pre-stimulated γδ T cells, had little or no effect on IgG or IgA antibody production already induced by Tfh cells alone (**Figure 5.5C**).

Notably, as indicated by the error bars significant donor-to-donor variation in the level of antibody production was observed, thus making interpretation of data difficult. In conclusion, cumulative data taken from 4-6 experiments indicated no clear effect of HMB-PP, IL-2, or IL-21 stimulation on the ability of γδ T cells to provide B cell help (**Figure 5.5**), although data from some (but not all) individual experiments did indicate an effect of IL-2, IL-21 and HMB-PP on γδ T cell induction of antibody production (data not shown).

Data from these initial experiments using pre-stimulated γδ T cells highlighted the challenges of overcoming variability and maintaining consistency between donors and experiments. Thus to this point, the idea of using pre-stimulated γδ T cells in investigating B-helper ability was re-thought and re-examined.

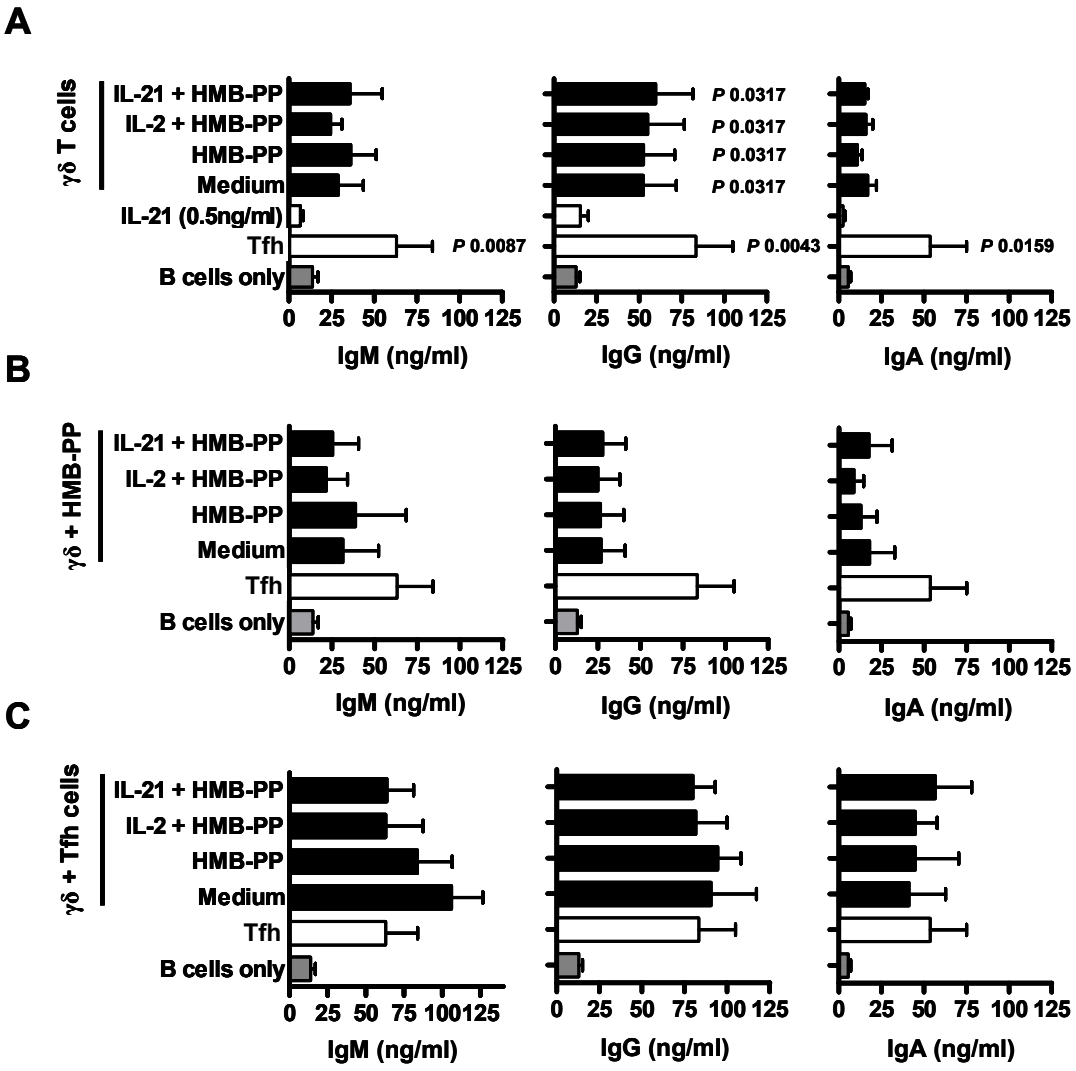


**Figure 5.5. Peripheral Vγ9Vδ2 T cells induce antibody production during co-culture with tonsillar B cells.**

Vδ2$^+$ T cells and B cells were positively isolated from PBMCs using magnetic cell sorting, resulting in >96 % purity. Peripheral Vδ2$^+$ T cells were co-cultured with irradiated peripheral B cells at a 2:1 ratio in medium, or with HMB-PP (10 nM). After 42 hours, HMB-PP stimulated cultures were treated with or without IL-21 (50 ng/ml) or IL-2 (20 units/ml). After 24 hours, stimulated and non-stimulated γδ T cells were harvested, washed and co-cultured at a ratio of 1:1 with purified tonsillar CXCR5$^+$ B cells, in the (A) absence or (B) presence of HMB-PP re-stimulation or in the presence of (C) tonsillar Tfh cells (ratio of 1:1:1). As controls, tonsillar CXCR5$^+$ B cells were cultured in medium alone (grey bars), or with Tfh cells (ratio of 1:1) (white bars), or with IL-21 (0.5 ng/ml) alone (white bars). After 10 days of culture, total IgM, IgG and IgA levels in culture supernatants were measured by ELISA. Data are shown as mean + SEM from four to six (A and C) and two (B) independent experiments. *P* values show statistically significant differences between B cells only and the indicated culture conditions.

## 5.2.4. IL-21 dependent tonsillar Vγ9Vδ2 T cells provide help for tonsillar B cell IgM antibody production

After having identified B helper-Tfh-like phenotypes on tonsillar Vγ9Vδ2 T cells, I addressed whether tonsillar Vγ9Vδ2 T cells were able to provide tonsillar B cell help. Here, by obtaining all three cells types (Vγ9Vδ2 T cells, B cells and Tfh cells) from inflamed tonsils taken from the same donor, my model was examined using a more physiologically relevant and autologous system.

The total percentage of Vγ9Vδ2 T cells found in human tonsils is very low (0.4 %) (**Figure 5.6A**) in contrast to $CD4^+$ T cells (32%) (**Figure 5.3A**) and B cells (73%) (**Figure 5.1A**). This made isolation of tonsillar γδ T cells more challenging and also limited the number of Vγ9Vδ2 T cells that could be isolated for use in co-culture experiments. Nonetheless, tonsillar Vγ9Vδ2 T cells were successfully isolated to high purities (95-99 %) using the magnetic cell sorting system followed by purification using the MoFlo cell sorter (**Figure 5.6**). At the same time and from the same tonsil, Tfh cells and $CXCR5^+$ B cells were also freshly isolated to high purities using the magnetic cell sorting system (as previously described in **Figure 5.6 and 5.3)**.

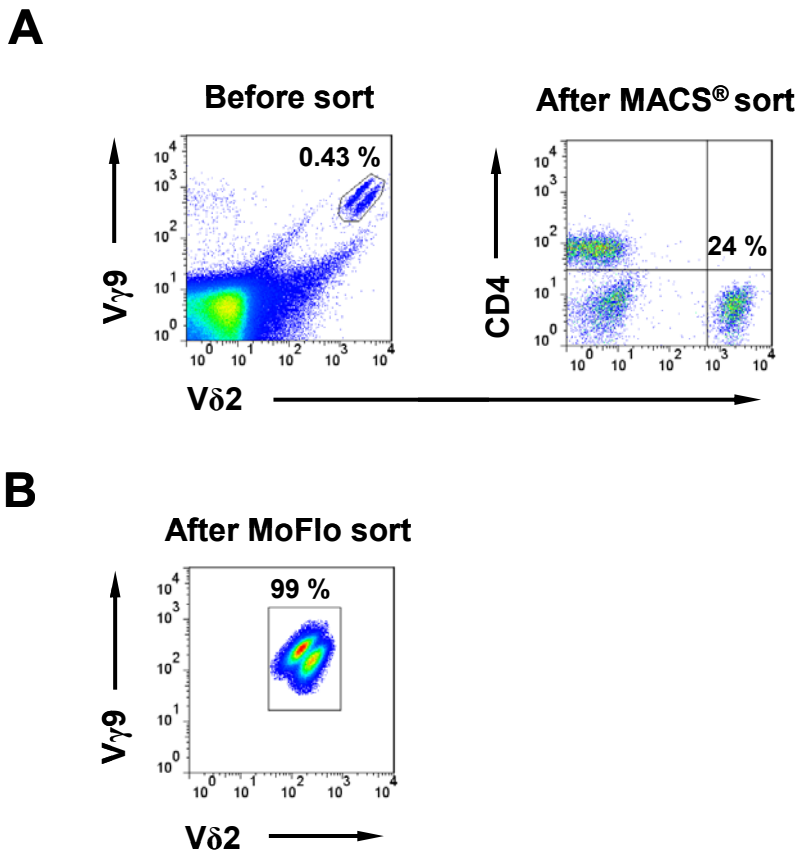


**Figure 5.6. Flow cytometric purity analysis of isolated tonsillar Vγ9Vδ2 positive T cells.**

Tonsillar mononuclear cells were isolated from fresh human tonsils. Tfh cells were isolated as described in Figure 5.3. (A) $V\delta2^+$ T cells were positively isolated from the CD4 T cell depleted population using the magnetic cell sorting system from Miltenyi Biotec, resulting in 20-24 % purity. (B) For a higher purity Vγ9Vδ2 positive cells were further purified by flow cytometry using a MoFlo cell sorter, resulting in 95-99 % purity. Tonsillar $CXCR5^+$ B cells were isolated from the Vγ9Vδ2 T cell depleted population as described in Figure 5.3. Results are representative of two experiments performed.

To test whether or not tonsillar γδ T cells were able to support tonsillar B cells for antibody production, tonsillar Vγ9Vδ2 T and B cells were co-cultured at a ratio of 1:1 in medium alone, or with HMB-PP (10 nM) in the presence or absence of IL-21 (50 ng/ml) or with IL-21 alone (50 ng/ml). To prevent occurrence of fungal contamination, all cultures were treated with an anti-fungal antibiotic (fungizone® antimycotic; 2.5 µl/ml). After 10 days of culture, total IgM, IgG, IgA and IgE levels in culture supernatants were measured by ELISA.

One of the major challenges of using tonsillar cells were obtaining inflamed tonsils from a donor that provided sufficient number of Vγ9Vδ2 T cells as well as B cells and Tfh cells for experimentation. Tonsils obtained from patients undergoing tonsillectomy are chronically infected organs, thus maintaining long term cultures from such a primary tissue can be challenging. Here, I encountered problems with maintaining long term cultures. This was not due to failure of maintaining sterile conditions but due to pre-existing fungal contamination of tonsillar tissue being carried over to culture. In some experiments, contamination of cultures was not eliminated after thoroughly washing tissue/cells several times in medium containing fungizone® and after culturing cells in the presence of fungizone®, which resulted in lose of cultures. Due to these challenges and due to donor-to-donor variation, results from two independent donors (donors 1 and 2) and experiments are shown, thus allowing for better interpretation of data (**Figure 5.7**).

As positive controls, tonsillar B cells were co-cultured with an equal number of Tfh cells or with the mitogenic B cell stimulator SAC, in the presence of IL-2 (as described in Ebert et al. (2004)). In comparison to B cells cultured in medium alone (B cells only), co-culturing of B cells with Tfh cells or with SAC in the presence of IL-2 induced considerable amounts of IgM, IgG and IgA antibody production **(Figure 5.7**).

To test and account for any direct action of HMB-PP, IL-21 or HMB-PP+IL-21 stimulation on B cell antibody production, tonsillar B cells were cultured in medium alone, with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml) or with IL-21 alone (50 ng/ml).

In comparison to B cells cultured in medium alone, HMB-PP stimulation had no direct effect on B cell IgM, IgG or IgA antibody production whereas stimulation of B cells with IL-21 alone (in the absence or presence of HMB-PP) induced considerable amounts of IgM, IgG and IgA antibody production, which in most cases was comparable to levels induced by Tfh cells, thus indicating a direct effect of IL-21 on tonsillar B cells (**Figure 5.7**).

Strikingly, co-culturing of tonsillar Vγ9Vδ2 T cells with B cells in the presence of both IL-21 and HMB-PP stimulation resulted in a substantial increase in the production of IgM. This was more striking in donor 1, where levels of IgM induced by Vγ9Vδ2 T cells were 2-fold higher than levels induced by Tfh cells or IL-21 alone. In contrast, tonsillar Vγ9Vδ2 T cells were unable to enhance IgM, antibody production in co-cultures of tonsillar B cells simulated with medium, HMB-PP or IL-21 alone thus indicating the requirement of both HMB-PP and IL-21 stimulation in equipping Vγ9Vδ2 T cells for providing support for tonsillar B cell IgM antibody production (**Figure 5.7A**).

Co-culturing of tonsillar Vγ9Vδ2 T cells with tonsillar B cells in the presence of both IL-21 and HMB-PP stimulation did not enhance IgG or IgA antibody production. Equally tonsillar Vγ9Vδ2 T cells were unable to enhance IgG or IgA, antibody production in co-cultures of tonsillar B cells simulated with medium, HMB-PP alone or IL-21 alone. Here, substantial amounts of both IgG and IgA antibody production were induced by direct action of IL-21 alone. Unlike IgM antibody production, IgG and IgA antibody production was more sensitive to the direct action of IL-21; this may have masked any additive effect/help provided by Vγ9Vδ2 T cells (**Figure 5.7B and C**). IgE antibody levels were not detected in any of the supernatants taken from B cell or Vγ9Vδ2 T-B cells co-cultures (data not shown). All the above findings were in close agreement with data from both donors and experiments (**Figure 5.7**).

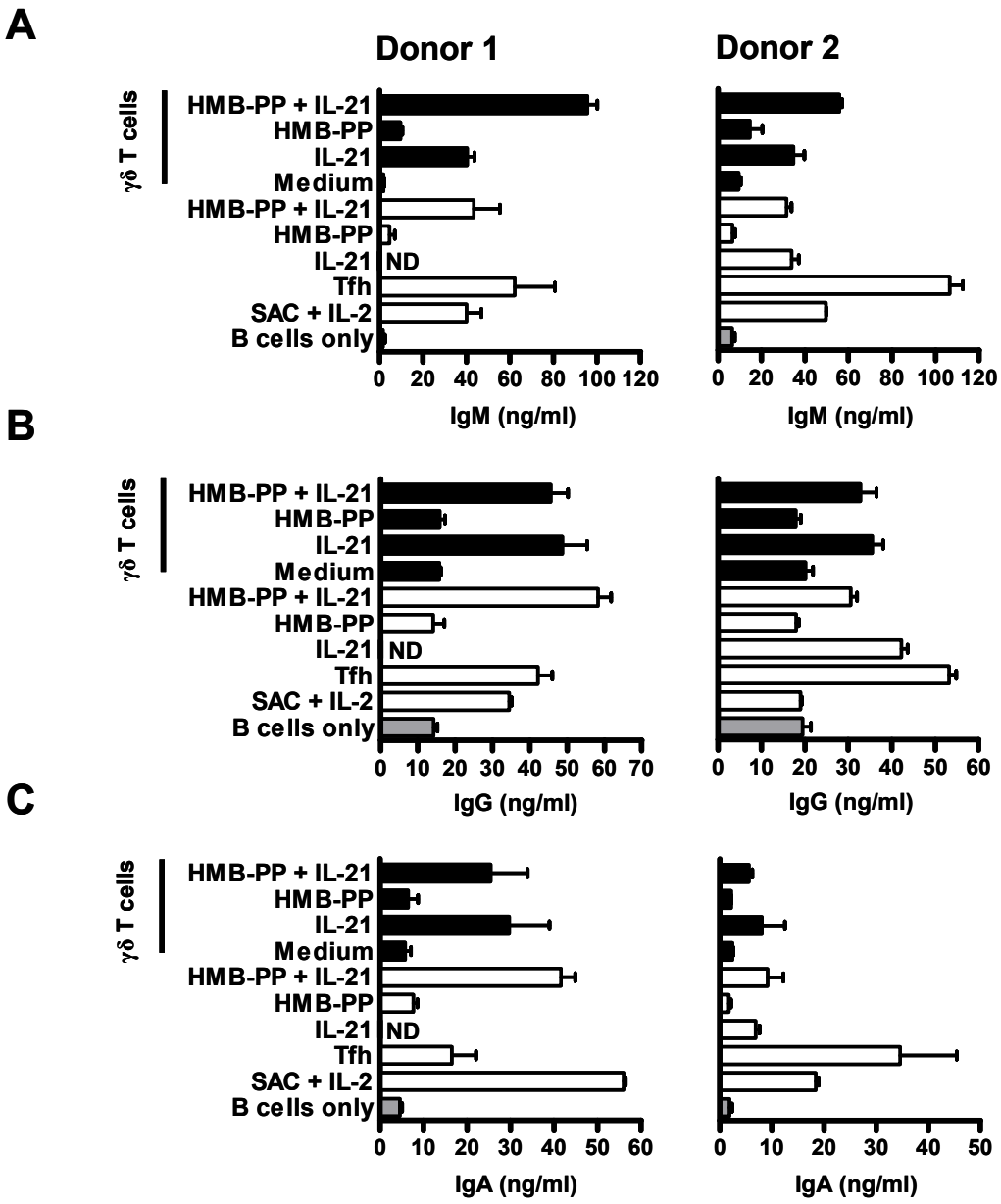


**Figure 5.7. HMB-PP and IL-21 dependent tonsillar Vγ9Vδ2 T cells induce potent IgM antibody production during co-culture with tonsillar B cells.**
Vγ9Vδ2 T cells, B cells and Tfh cells were freshly isolated from tonsillar mononuclear cells and co-cultured at a ratio of 1:1 in medium alone, or with HMB-PP (10 nM) in the presence or absence of IL-21 (50 ng/ml) or with IL-21 alone (50 ng/ml) (black bars). As controls tonsillar B cells were cultured in medium alone (grey bars), or with SAC and IL-2, or with Tfh cells, or with HMB-PP (10 nM) in the presence or absence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml) (white bars). After 10 days of culture, total (A) IgM, (B) IgG and (C) IgA levels in culture supernatants were measured by ELISA. Data are shown as mean + SEM from two to four individual wells and results from two individual donors (donor 1 and 2) and experiments is shown.

To further investigate the potent effects of IL-21+HMB-PP stimulation on the ability of tonsillar Vγ9Vδ2 T cells to provide support for tonsillar B cell antibody production, for comparison, co-cultures in the presence of HMB-PP were concurrently stimulated with IL-2 alongside IL-21. In order to account for any direct action of IL-2 and HMB-PP stimulation on B cell antibody production, tonsillar B cells were cultured in medium in the presence of IL-2 (20 units/ml) and HMB-PP (10 nM). In contrast to IL-21 (50 ng/ml), IL-2+HMB-PP stimulation had no direct effect on IgM antibody production (**Figure 5.8A**).

In response to IL-2+HMB-PP stimulation, IgM antibody production increased 30-fold when B cells were cultured in the presence compared to the absence of Vγ9Vδ2 T cells. Thus, similar to IL-21+HMB-PP stimulation, IL-2+HMB-PP stimulation, also equipped tonsillar Vγ9Vδ2 T cells for providing support for IgM antibody production. Nevertheless, total levels of IgM induced by IL-2+HMB-PP stimulation were 3-fold lower in comparison to those induced by IL-21+HMB-PP stimulation, hence making IL-21 a more potent stimulator for IgM antibody production (**Figure 5.8A**).

In contrast to IgM antibody production, IL-2+HMB-PP stimulation in the absence of Vγ9Vδ2 T cells, did have direct effects on IgG and IgA antibody production. This was due to the direct effects of IL-2, which was less potent to the direct effects of IL-21. The addition of Vγ9Vδ2 T cells to these cultures did not enhance the production of either IgG or IgA antibody production (**Figure 5.8B and C**).

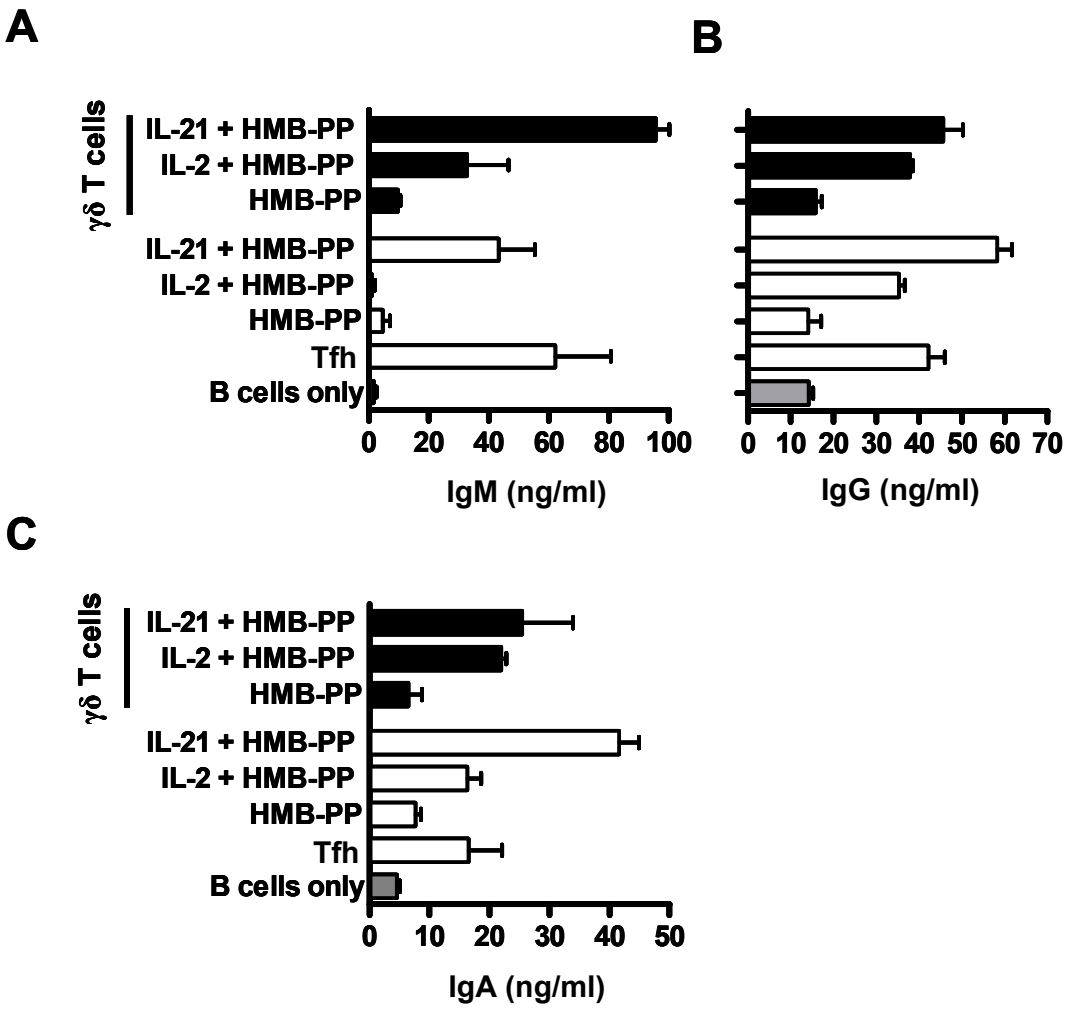


**Figure 5.8. Comparison of IL-21 and IL-2 stimulation on tonsillar Vγ9Vδ2 T cells help in antibody production.**
Vγ9Vδ2 T cells, B cells and Tfh cells were freshly isolated from tonsillar mononuclear cells and co-cultured at a ratio of 1:1 in medium with HMB-PP (10 nM) in the presence of IL-21 (50 ng/ml) or IL-2 (20 units/ml)(black bars). As controls tonsillar B cells were cultured in medium alone (grey bars), or with Tfh cells (at a ratio of 1:1), or with HMB-PP (10 nM) in the presence of IL-21 (50 ng/ml) or IL-2 (20 units/ml) (white bars). After 10 days of culture, total (A) IgM, (B) IgG and (C) IgA levels in culture supernatants were measured by ELISA. Data are shown as mean + SEM from two to three individual wells.

Next, I examined the synergistic effects of tonsillar γδ T cells and Tfh cells on B cell antibody production. All three cell types, Vγ9Vδ2 T cells, B cells and Tfh cells were freshly isolated from the tonsil of the same donor (as described in **Figure 5.6 and 5.3**) and cultured at a ratio of 1:1:1 in medium alone or with HMB-PP (10 nM). As controls B cells were cultured alone, or with Tfh cells in the absence or presence of HMB-PP (10 nM) (**Figure 5.9**).

IgM, IgG or IgA antibody production was not further enhanced when Tfh and B cells were cultured in the presence compared to the absence of non-stimulated Vγ9Vδ2 T cells (medium). The addition of HMB-PP to induce γδ T cell re-activation did not improve the ability of Vγ9Vδ2 T cells to further enhance antibody production already induced by Tfh cells (**Figure 5.9**). Furthermore, decreasing the number of Vγ9Vδ2 T cells to Tfh and B cells from a 1:1:1 to a 1:125:125 ratio did not effect or enhance antibody production induced by Tfh cells alone (**Figure 5.10**).

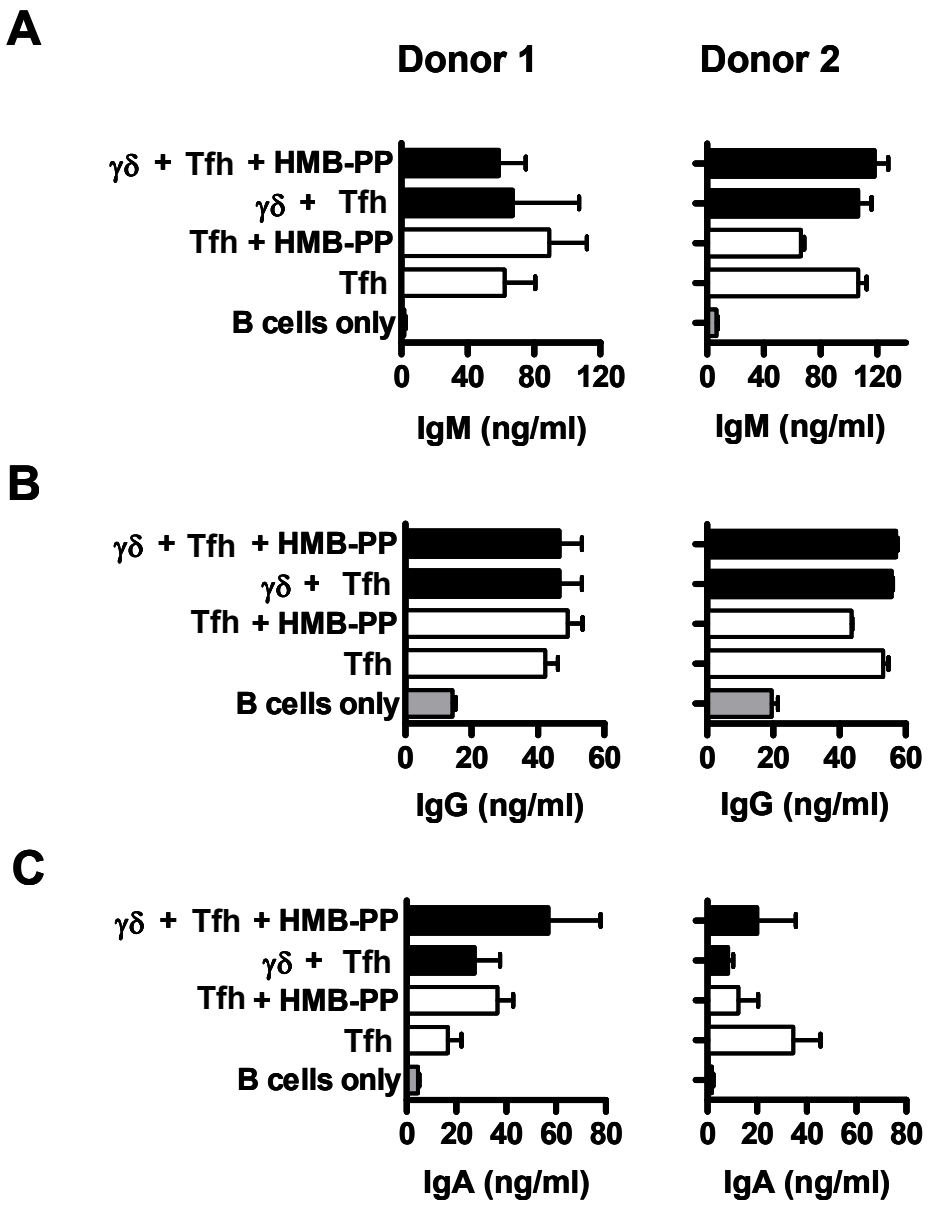


**Figure 5.9. Tonsillar Vγ9Vδ2 T cells do not further enhance antibody production induced by Tfh cells.**
Vγ9Vδ2 T cells, B cells and Tfh cells were freshly isolated from tonsillar mononuclear cells and co-cultured at a ratio of 1:1:1 in medium alone or with HMB-PP (10 nM) (black bars). As controls tonsillar B cells were cultured in medium alone (grey bars), or with Tfh cells (at a ratio of 1:1), with or without HMB-PP (10 nM) (white bars). After 10 days of culture, total (A) IgM, (B) IgG and (C) IgA levels in culture supernatants were measured by ELISA. Data are shown as mean + SEM from two to four individual wells and results from two individual donors (donor 1 and 2) and experiments are shown.

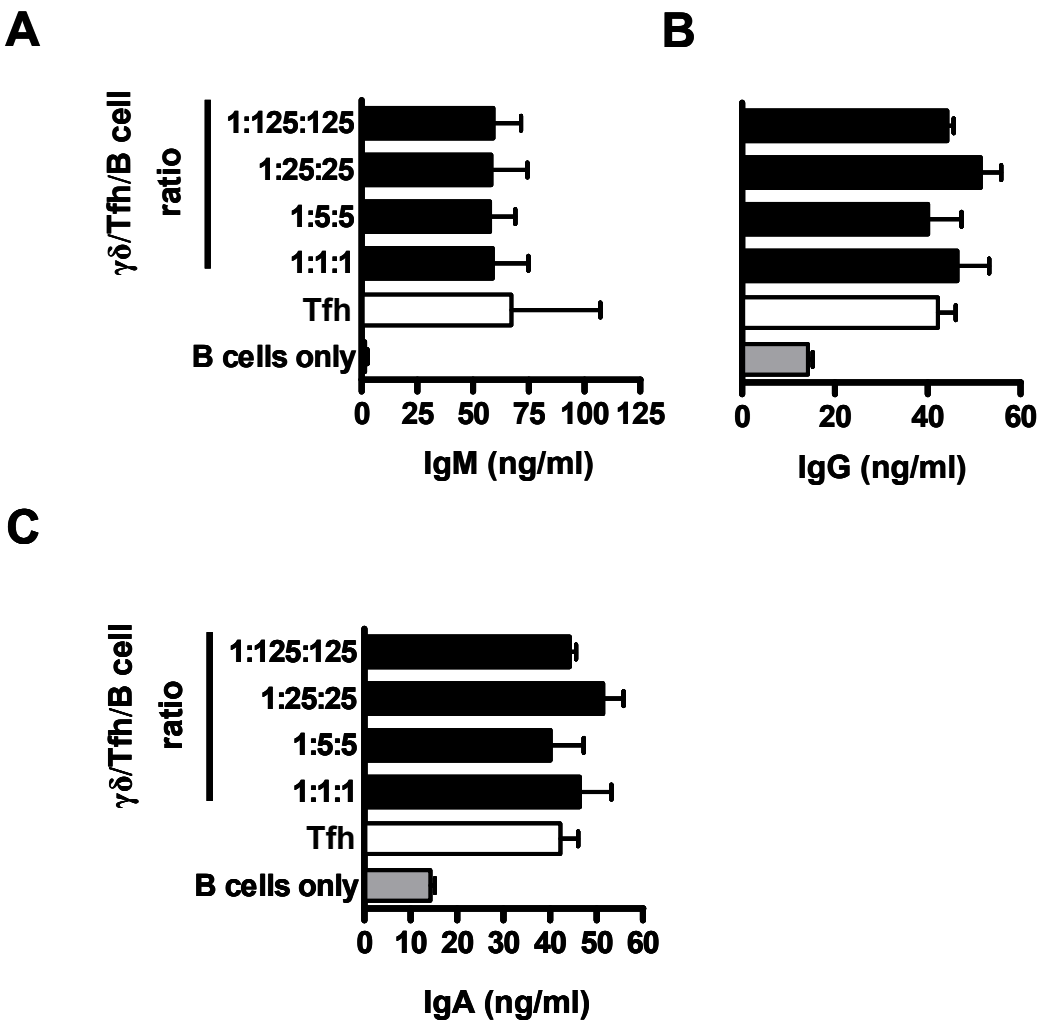


**Figure 5.10. Titration of tonsillar Vγ9Vδ2 T cells does not enhance antibody production induced by Tfh cells.**
Vγ9Vδ2 T cells, B cells and Tfh cells were freshly isolated from tonsillar mononuclear cells and co-cultured at the indicated ratios in medium alone (black bars). As controls tonsillar B cells were cultured in medium alone (grey bars) or with Tfh cells at a ratio of 1:1 (white bars). After 10 days of culture, total (A) IgM, (B) IgG and (C) IgA levels in culture supernatants were measured by ELISA. Data are shown as mean + SEM from three individual wells.

## 5.2.5. IL-21 and HMB-PP stimulated peripheral Vγ9Vδ2 T cells induce peripheral B cell activation, APC marker expression and antibody production

After having established conditions that favour the acquisition of a B helper-Tfh-like phenotype on peripheral blood Vγ9Vδ2 T cells, here I addressed whether HMB-PP and IL-21 stimulated peripheral Vγ9Vδ2 T cells were able to directly induce peripheral B cell activation, APC marker expression, differentiation and antibody production.

Highly purified peripheral Vγ9Vδ2 T cells (98-99% purity) were co-cultured with highly purified un-touched peripheral B cells (95-99% purity) at a ratio of 1:1, in medium alone, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). As controls B cells were cultured in medium alone (B cells only), or with SAC and IL-2 (100 units/ml), or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml).

In order to determine the ability of Vγ9Vδ2 T cells to induce B cell activation and APC marker expression, following 3 days of culture, B cells were examined for changes in expression of CD25 and CD69 activation markers, CD40 and CD86 and HLA-DR APC markers. Culturing of B cells with SAC (in the presence of IL-2) resulted in sufficient activation of B cells. SAC induced surface expression of CD69, a substantial increase in CD25 and CD86 expressing B cells, as well as an up-regulation of both CD40 and HLA-DR on B cells. In the absence of Vγ9Vδ2 T cells, IL-21 and HMB-PP did not have any direct effects on B cell CD25, CD69, CD40, CD86 or HLA-DR expression (**Figure 5.11**).

Co-culturing of Vγ9Vδ2 T cells with B cells in medium or IL-21 alone resulted in a minor increase in B cell CD69, CD25, CD40 and CD86 marker expression. In contrast co-culturing of Vγ9Vδ2 T cells with B cells in the presence of HMB-PP stimulation resulted in

an induction of CD69 (19 %), CD25 (7%), CD86 (28%), and an up-regulation of CD40 (GMFI of 98) and HLA-DR (GMFI of 91) on B cells (**Figure 5.11**).

Furthermore, co-culturing of Vγ9Vδ2 T cells with B cells in the presence of both HMB-PP and IL-21 in comparison to HMB-PP alone resulted in a higher proportion of B cells expressing CD69 (27%) and CD86 (31%) and higher expression levels of CD40 (GMFI of 105) on B cells (**Figure 5.11**). Thus, higher expression of some B cell markers was dependent on the presence of both HMB-PP and IL-21 stimulation

In Chapter 3, I investigated expression of activation markers, co-stimulatory molecules and APC markers on Vγ9Vδ2 T cells that were cultured in PBMCs or co-cultured with irradiated B cells. Here, following 3 days of culture I examined for surface CD25, CD69, CD27, CD86 and HLA-DR expression on purified Vγ9Vδ2 T cells cultured with purified un-touched peripheral B cells. HMB-PP stimulation alone induced surface expression of CD69, CD25, and HLA-DR on a large proportion of Vγ9Vδ2 T cells, and CD86 on a smaller proportion of Vγ9Vδ2 T cells. Furthermore, CD27 already expressed by 50 % of non-stimulated Vγ9Vδ2 T cells was up-regulated and found to be expressed by 70% of Vγ9Vδ2 T cells in response to HMB-PP stimulation alone. The addition of IL-21 co-stimulation did not further enhance Vγ9Vδ2 T cells surface expression of CD25, CD69, CD27, HLA-DR or CD86 (**Figure 5.12**).

Collectively, these results demonstrate that HMB-PP activated and IL21 co-stimulated Vγ9Vδ2 T cells not only themselves become highly activated APC marker expressing cells, but also at the same time become well equipped in inducing B cell activation and considerably modify expression of B cell APC markers.

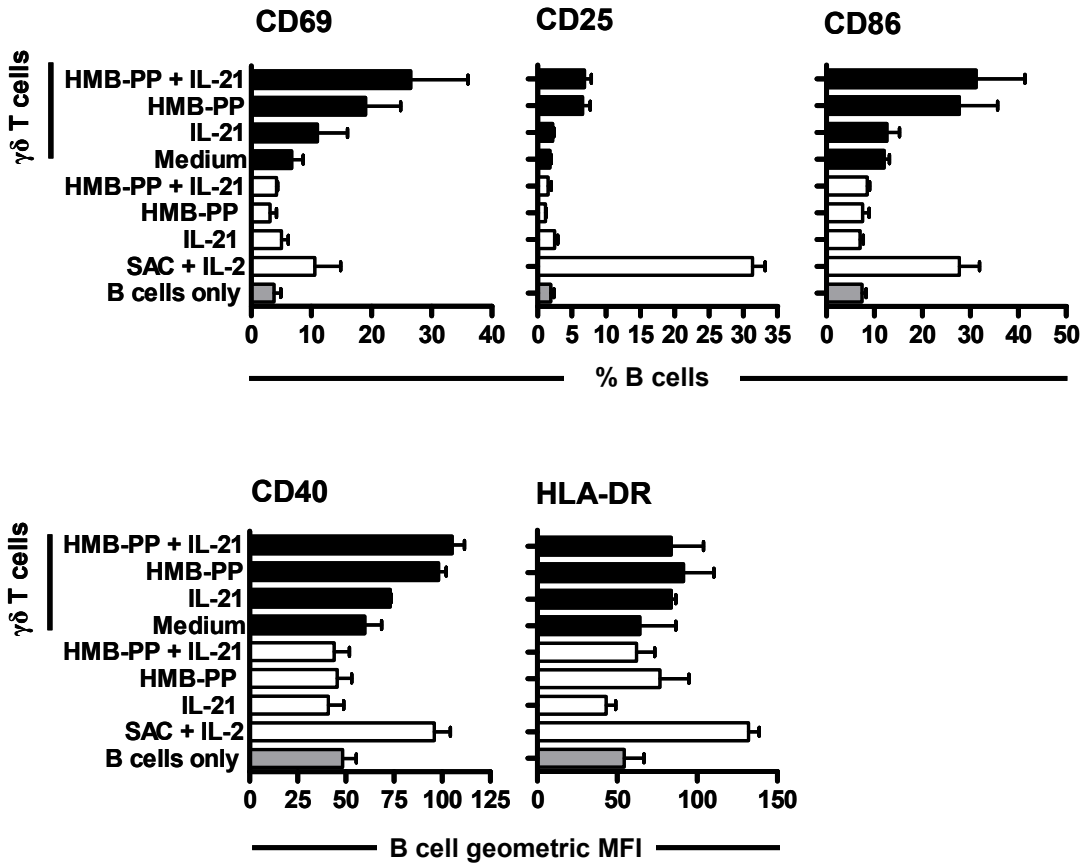


**Figure 5.11. HMB-PP activated peripheral γδ T cells in co-cultures up-regulate activation and APC marker expression on B cells.**
PBMCs were freshly isolated blocked with human Igs and stained with anti-Vδ2-PE antibodies followed with anti-PE microbeads. Vγ9Vδ2 T cells were positively isolated from PBMCs using the magnetic cell sorting system from Miltenyi Biotec, resulting in 98-99% purity. B cells were isolated from Vγ9Vδ2 T cell depleted PBMC by negative selection, resulting in 95-99% purity. Purified Vγ9Vδ2 T cells and B cells were co-cultured at a ratio of 1:1 in medium alone, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). As controls B cells were cultured in medium alone (grey bars), or with SAC and IL-2 (100 units/ml), or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml) (white bars). After 3 days, cells were harvested and stained for CD69, CD25, CD86, CD40 and HLA-DR in combination with CD19 and CD3 surface markers. Cells were analysed by flow cytometry for B cell surface marker expression by gating on lymphocytes, live cells and $CD3^{-}$ $CD19^{+}$ cells. CD69, CD25 and CD86 data are shown as the mean percentage of $CD19^{+}$ B cells positive for each marker. CD40 and HLA-DR expression data are shown as mean fold change in GMFI, calculated as (geometric MFI of antibody)/(geometric MFI of isotype control). Data are shown as mean + SEM from two to three independently assessed donors.

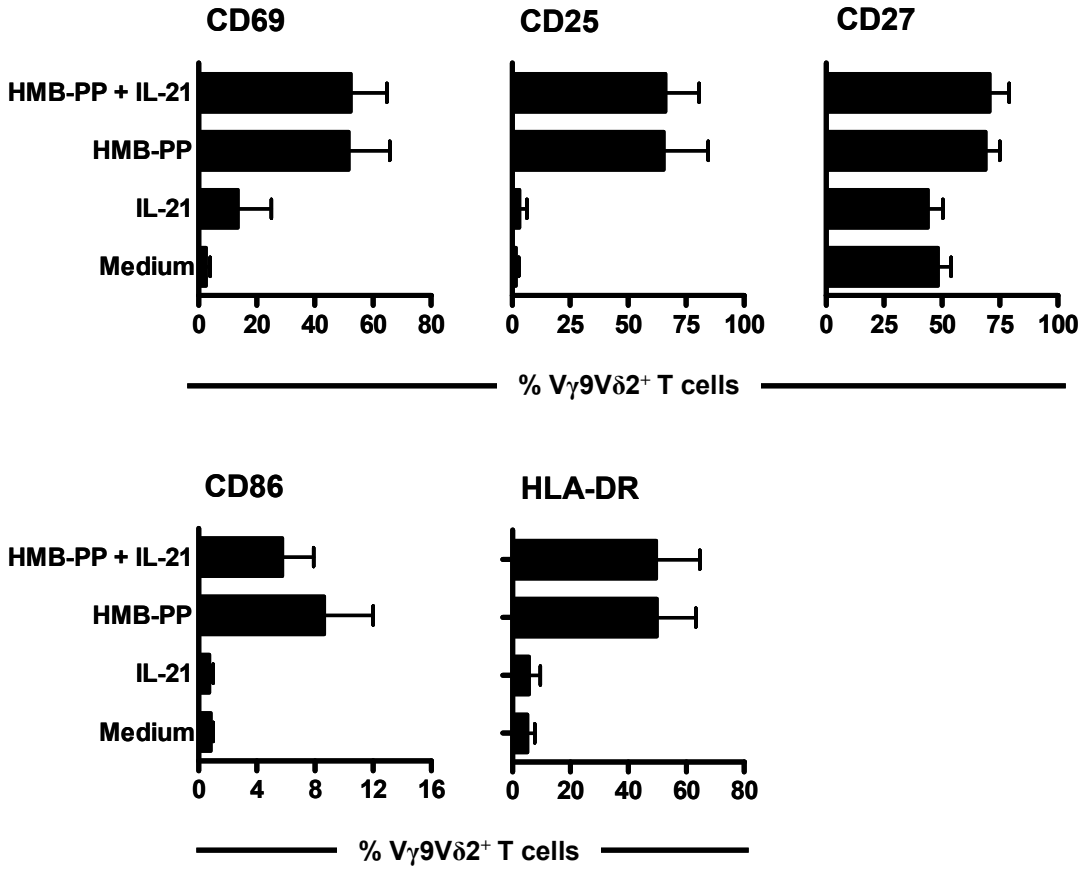


**Figure 5.12. HMB-PP activated peripheral γδ T cells in co-cultures up-regulate activation and APC marker expression.**
Peripheral blood Vγ9Vδ2 T and B cells were freshly isolated as described in Figure 5.11 and co-cultured at a ratio of 1:1 in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). After 3 days, cells were harvested and stained for CD69, CD25, CD27, CD86 and HLA-DR in combination with Vδ2 or Vγ9 and CD3 surface markers. Cells were analysed by flow cytometry for γδ T cell CD69, CD25, CD27, CD86 and HLA-DR surface expression by gating on lymphocytes, live cells and $CD3^+$ $V\delta2^+$ or $V\gamma9^+$ cells. Data are shown as mean percentage + SEM from two to three independently assessed donors.

Next I determined whether Vγ9Vδ2 T cells were able to induce B cell differentiation. Following 3 days of culture, B cell naïve/memory populations were identified based on IgD and CD27 surface expression and in response to various different culture conditions, changes in the percentage of B cell naïve/memory populations were examined.

Culturing of B cells in medium alone without any stimulation did not induce B cell differentiation, here 75% of the B cells remained naïve after 3 days of culture (**Figure 5.13**), which is similar to the proportion found in freshly isolated PBMCs (**Figure 5.1**). Whereas treatment with SAC (in the presence of IL-2) resulted in a dramatic loss of naïve (75% to 25%) and memory B cells and a corresponding increase in the pool of double negative B cells (10% to 65%), demonstrating SAC to be a potent stimulator of peripheral B cell differentiation (**Figure 5.13**).

Culturing of B cells with HMB-PP alone had no effect on naïve/memory B cell pools, whereas IL-21 stimulation alone resulted in a small decrease in the percentage of switched and unswitched memory B cells. Co-culturing of B cells with Vγ9Vδ2 T cells in the absence or presence of HMB-PP or IL-21 stimulation did not change the percentage of naïve/memory B cell subsets; instead the proportions of naïve/memory B cell subsets were maintained. Hence, during the relatively short 3 day culture period Vγ9Vδ2 T cells did not induce B cells to undergo differentiation (**Figure 5.13**).

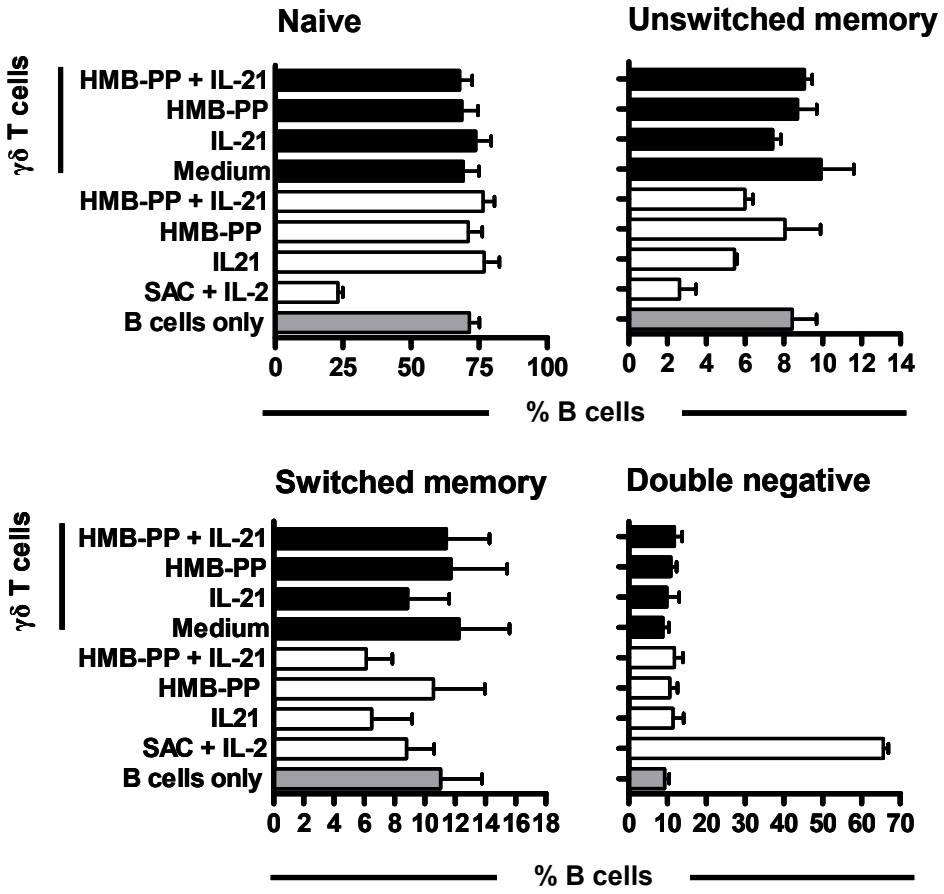


**Figure 5.13. HMB-PP and IL-21 stimulated Vγ9Vδ2 T cells do not induce B cell differentiation.**
Peripheral blood Vγ9Vδ2 T and B cells were freshly isolated as described in Figure 5.11 and co-cultured at a ratio of 1:1 in medium alone, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). As controls B cells were cultured in medium alone (grey bars), or with SAC and IL-2 (100 units/ml), or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml) (white bars). After 3 days, cells were harvested and stained for flow cytometry with CD19, CD3, IgD and CD27 surface markers. B cells were analysed for IgD and CD27 expression, by gating on lymphocytes, live cells and $CD3^-$ $CD19^+$ cells. Naïve/memory subsets were classified as follows: Naïve = $IgD^+/CD27^-$, unswitched memory = $IgD^+/CD27^+$, switched memory = $IgD^-/CD27^+$ and double negative = $IgD^-/CD27^-$. Data are shown as mean + SEM from two to three independently assessed donors.

To test whether or not peripheral γδ T cells were able to support peripheral B cells for antibody production, highly purified peripheral Vγ9Vδ2 T cells (98-99% purity) were co-cultured with highly purified un-touched peripheral B cells (95-99% purity) at a ratio of 1:1, in medium alone, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). As controls, B cells were cultured in medium alone (B cells only), or with SAC and IL-2 (20 units/ml), or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). After 10 days of culture, total IgM, IgG, IgA and IgE levels in culture supernatants were measured by ELISA.

Culturing of B cells in medium alone without any stimulation did not induce any B cell antibody production whereas treatment with SAC in the presence of IL-2 induced considerable amounts of IgM, IgG and IgA antibody production, demonstrating SAC to be a potent stimulator of peripheral B cell antibody production (**Figure 5.14**).

In the absence of Vγ9Vδ2 T cells, very low levels of IgM, IgA and IgG antibodies were detected in B cell cultures stimulated with IL-21, HMB-PP or both. Hence, IL-21 and HMB-PP stimulation did not have any major direct effects on peripheral B cell IgM, IgG or IgA antibody production. This is in contrast the strong direct effects of IL-21 on tonsillar B cells, previously shown in **Figure 5.4**. However, co-culture of B cells in the presence of Vγ9Vδ2 T cells and in the presence of both IL-21 and HMB-PP stimulation resulted in a 10-fold increase in the production of IgM and a 6-fold increase in the production of both IgG and IgA. Antibody production to a lesser extent was also induced in Vγ9Vδ2 T-B cell co-cultures stimulated with HMB-PP alone, whereby total IgM, IgG and IgA levels in culture supernatants were measured to be considerably lower in comparison to those induced in the presence of both IL-21 and HMB-PP stimulation. More strikingly, in comparison to SAC+IL-2 stimulation, HMB-PP+IL-21 co-stimulated Vγ9Vδ2 T cells were more potent at inducing IgM, IgG and IgA antibody production (**Figure 5.14**).

Here, HMB-PP+IL-21 co-stimulated Vγ9Vδ2 T cells did not induce differentiation of B cells over 3 days in culture (**Figure 5.13**) but did provide help for antibody production over 10 days in culture, thus indicating γδ T cell induction of B cell differentiation to plasma cells (**Figure 5.14**).

In contrast, co-culturing of peripheral Vγ9Vδ2 T cells with peripheral B cells in medium or with IL-21 alone resulted in very little IgM, IgG or IgA antibody production. Thus, indicating the requirement of both HMB-PP and IL-21 stimulation in equipping peripheral Vγ9Vδ2 T cells for providing potent support for peripheral B cell IgM, IgG and IgA antibody production (**Figure 5.14**). Here, IgE antibody levels were not detected in any of the supernatants taken from B cell or Vγ9Vδ2 T-B cells co-cultures (data not shown).

To further prove that antibody production induced in HMB-PP and IL-21 co-stimulated co-cultures was dependent on the presence of Vγ9Vδ2 T cells, in co-cultures the number of Vγ9Vδ2 T cells to B cells was decreased from a 1:1 ratio. Evidently, as the number of Vγ9Vδ2 T cells was decreased in HMB-PP stimulated co-cultures, equally the production of IgM, IgG and IgA antibody production also decreased accordingly (**Figure 5.15A**). This effect was more prominent in HMB-PP and IL-21 co-stimulated co-cultures (**Figure 5.15B**). Therefore it was evidently clear that peripheral Vγ9Vδ2 T cells provided dose dependent help on IgM, IgG and IgA antibody production (**Figure 5.15)**.

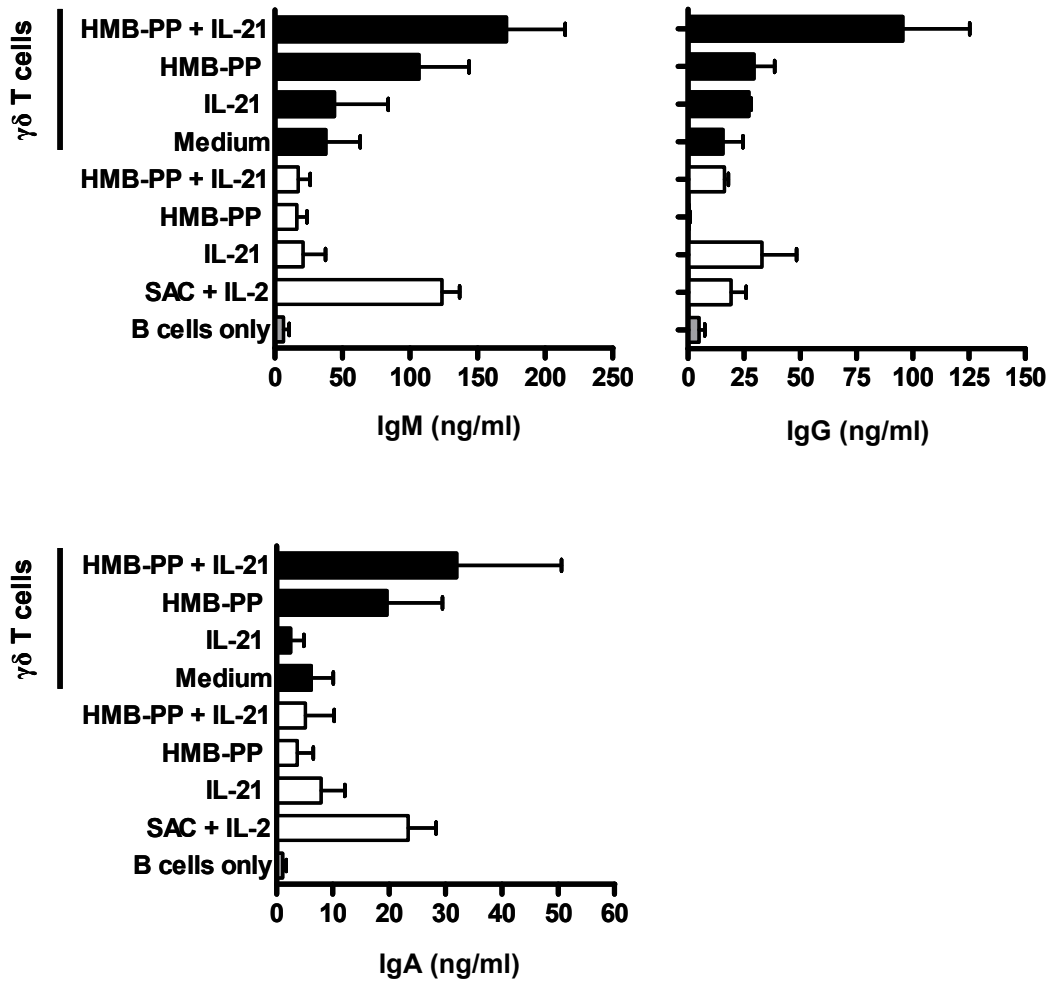


**Figure 5.14. HMB-PP and IL-21 stimulated peripheral Vγ9Vδ2 T cells induce potent antibody production during co-culture with peripheral B cells.**
Peripheral blood Vγ9Vδ2 T and B cells were freshly isolated as described in Figure 5.11 and co-cultured at a ratio of 1:1 in medium, or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml). As controls B cells were cultured in medium alone (grey bars), or with SAC and IL-2 (100 units/ml) or with HMB-PP (10 nM) in the absence or presence of IL-21 (50 ng/ml), or with IL-21 alone (50 ng/ml) (white bars). After 10 days of culture, total IgM, IgG and IgA levels in culture supernatants were measured by ELISA. Data are shown as mean + SEM from two to three independently assessed donors.

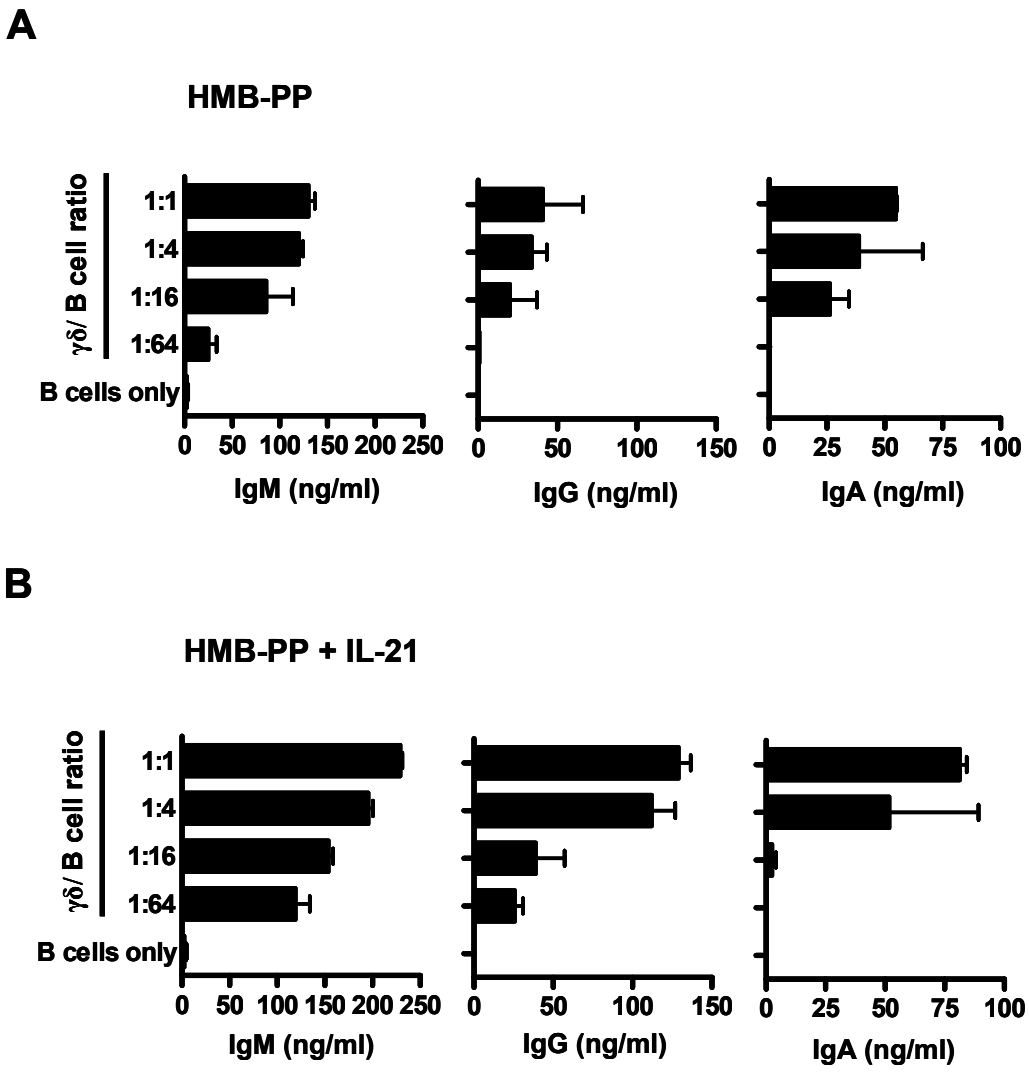


**Figure 5.15. HMB-PP and IL-21 stimulated peripheral Vγ9Vδ2 T cells provide dose dependent help on antibody production.**
Peripheral blood Vγ9Vδ2 T and B cells were freshly isolated as described in Figure 5.11 and co-cultured at the indicated ratios in medium with (A) HMB-PP alone (10 nM) or (B) in the presence of IL-21 (50 ng/ml). As controls B cells were cultured in medium alone (B cells only). After 10 days of culture, total IgM, IgG and IgA levels in culture supernatants were measured by ELISA. Data are shown as mean + SEM from two to three independent wells and is representative of results from one of two similar experiments.

# Chapter 6:

# Discussion

The establishment of long-term humoral immunity depends on the production of high affinity, class switched antibodies capable of neutralising and opsonising invading pathogens. These antibodies are generated through somatic hypermutation, class switch recombination and affinity maturation of activated B cells, processes that take place in the GCs of secondary lymphoid organs and depend on cognate help provided by $CD4^+$ Tfh cells through expression of several molecules (Breitfeld et al. 2000; King et al. 2008; Schaerli et al. 2000). The acquisition of Tfh-associated markers by Vγ9Vδ2 T cells in response to IL-21 was first suggested by recent microarray studies (Vermijlen et al. 2007). IL-21 turned out to have a similar capacity as the related cytokine IL-2 to support Vγ9Vδ2 T cell proliferation yet without promoting the supposedly 'signatory' molecules IFN-γ and TNF-α (Eberl et al. 2002a), thus highlighting a much greater plasticity of Vγ9Vδ2 T cell responses than previously appreciated. The main focus of this PhD was to gain a better understanding of Vγ9Vδ2 T cell role in humoral immune responses and to provide functional validation of previously published microarray studies (Vermijlen et al. 2007). Here, I investigated the effects of IL-21 co-stimulation on the ability of HMB-PP activated Vγ9Vδ2 T cells in providing B cell help.

In order to try to mimic the physiological conditions in the GC, an *in vitro* system of autologous Vγ9Vδ2 T cells and B cells from tonsils or blood, the microbial metabolite HMB-PP and the Tfh-derived cytokine IL-21 was used. The data presented in this thesis provide evidence that IL-21 contributes to the acquisition of B cell helper functions by human Vγ9Vδ2 T cells, thus suggesting a direct role for γδ T cells in the control of humoral immune responses. The main findings are discussed below.

Activation of Vγ9Vδ2 T cells by HMB-PP is dependent on the Vγ9Vδ2 TCR (Morita et al. 2007; Tanaka 2006; Thedrez et al. 2007) and the effects of IL-21 can only be mediated by binding to the IL-21R (Spolski and Leonard 2008). IL-21 is known to function as a T cell co-stimulator with the ability of possibly enhancing TCR signalling. Furthermore, as shown here and stated in literature, IL-21 only induces proliferation of T cells that have been stimulated via the TCR (King et al. 2008; Rolf et al. 2010; Spolski and Leonard 2008).

Binding of IL-21 to γδ T cells had so far only been described in mice (Ostiguy et al. 2007). In order to examine the effects of IL-21 stimulation on human Vγ9Vδ2 T cells surface expression of a functional IL-21R is essential. Expression of IL-21R mRNA by Vγ9Vδ2 T cells was reported recently (Vermijlen et al. 2007), yet no data were available to date on the protein level nor on the activation-dependent regulation of IL-21R expression by human γδ T cells. This study is the first to show that resting peripheral blood and tonsillar Vγ9Vδ2 T cells express IL-21R on their surface and that expression of IL-21R is greatly enhanced upon stimulation.

IL-21R was found on Vγ9Vδ2 T cells and $CD4^+$ T cells in freshly isolated PBMC and tonsils, as well as on peripheral B cells but not on monocytes. The IL-2Rα chain CD25 confers high-affinity responsiveness to IL-2, a cytokine closely related to IL-21, and is an early activation marker on T cells, including Vγ9Vδ2 T cells. I therefore examined whether expression of IL-21R is similarly activation-dependent. Indeed, incubation of PBMC in the presence of HMB-PP led to a significant up-regulation not only of CD25 but also of IL-21R on Vγ9Vδ2 T cells. In the same PBMC cultures, no effect was seen on CD25 or IL-21R expression by $CD4^+$ T cells, B cells and monocytes demonstrating the specificity of HMB-PP for Vγ9Vδ2 T cells. IL-21R was predominantly found on activated Vγ9Vδ2 T cells that co-expressed CD25 and CD69. Time-course experiments showed that stimulation with HMB-PP led to a transient up-regulation of IL-21R on Vγ9Vδ2 T cells, peaking at 48 hours and returning to baseline levels within 4-5 days. Furthermore, in response to HMB-PP and more so with IL-21 co-stimulation, high levels of surface CD25 remained detectable throughout the 5 day culture period, indicating the ability of Vγ9Vδ2 T cells to remain activated during long-term culture. In the presence of IL-21 co-stimulation a less-rapid but more sustained (not transient) up-regulation of IL-21R expression was observed on HMB-PP stimulated Vγ9Vδ2 T cells. Differences in the pattern of IL-21R expression observed here, in response to HMB-PP in comparison to IL-21 co-stimulation could be due to the biphasic regulation of IL-21R. In T cells, IL-21R is thought to be initially up-regulated following TCR stimulation with subsequent decline, a secondary spontaneous response requires stimulation via IL-21 (Leonard et al. 2008). In contrast, IL-21R was no longer detectable on Vγ9Vδ2 T cells, $CD4^+$ T cells and B cells shortly (<24 hours) after

stimulation of PBMCs with IL-21 alone (without HMB-PP). Binding of most chemokines to their receptors results in receptor down-regulation by internalization (Murdoch and Finn 2000). Similarly, IL-21R may have been undetectable due to receptor internalisation as a mechanism of down-regulating receptor signalling upon IL-21 ligation in the absence of TCR or BCR stimulation.

These important findings imply that resting Vγ9Vδ2 T cells are directly responsive to IL-21 in the microenvironment, and even more so shortly after activation with HMB-PP. Vγ9Vδ2 T cells are rapidly drawn to sites of infection where they will encounter invading pathogens and interact with other immune cells including neutrophils, monocytes and dendritic cells, thus defining an innate-like 'first line of defence' or sentinel function (Davey et al. 2011; Eberl and Moser 2009; Eberl et al. 2009). However, activation of both human and mouse γδ T cells triggers changes in their migratory pattern and induces up-regulation of CCR7 (Brandes et al. 2003; Shimura et al. 2010). CCR7 mediates recruitment of circulating lymphocytes from the blood and lymphatic system to secondary lymphoid organs such as the lymph nodes. Blood circulating lymphocytes enter lymph nodes via HEVs whilst lymphocytes within lymph fluid enter via afferent lymphatic vessels (Abbas et al. 2007; Förster et al. 2008). Once recruited to secondary lymphoid organs, pre-activated Vγ9Vδ2 T cells may thus encounter IL-21 produced by Tfh cells and as a consequence express a distinct set of molecules associated with providing B cell help. Lymph node-resident Vγ9Vδ2 T cells may also become stimulated by HMB-PP draining from the site of infection.

Within lymph nodes, expression of the CXCL13 receptor, CXCR5 would allow activated Vγ9Vδ2 T cells to migrate into the CXCL13-rich B cell follicles of secondary lymphoid tissues, where they would contribute to humoral immune responses by interacting with B cells and Tfh cells. Surface expression of CXCR5 on peripheral and tonsillar Vγ9Vδ2 T cells was therefore investigated. From this study, my data indicate that IL-21 also plays a role in supporting expression of CXCR5 by tonsillar Vγ9Vδ2 T cells. Of note, I was unable to induce significant levels of CXCR5 on peripheral Vγ9Vδ2 T cells, in line with previous

reports (Brandes et al. 2003) but at odds with others (Caccamo et al. 2006a). Control experiments confirmed expression of CXCR5 on peripheral $CD4^+$ T cells and B cells, and further up-regulation of CXCR5 on $CD4^+$ T cells stimulated with PHA, thus validating the reagents and methodology used. Also, lower CXCR5 expression values on tonsillar Vγ9Vδ2 T cells were observed in comparison to those reported by Caccamo *et al*. (2006a). However, here, stimulation of tonsillar Vγ9Vδ2 T cells with HMB-PP and IL-21 resulted in the rapid induction of CXCR5 expression within 24 hours. Thus, data presented in this thesis confirm that activated tonsillar Vγ9Vδ2 T cells can clearly express CXCR5 (Caccamo et al. 2006a), providing a molecular explanation for their clustering in GCs (Brandes et al. 2003; Vermijlen et al. 2007). Furthermore, peripheral blood γδ T cells express high levels of the inflammatory chemokine receptor CXCR3, which has been shown to act as an alternative receptor for CXCL13 in the absence of CXCR5. Thus, CXCR3 is thought to have a dual role in recruiting peripheral blood γδ T to sites of microbial infection as well to secondary lymphoid organs (Brandes et al. 2003; Caccamo et al. 2006a; Jenh et al. 2001).

Expression of CXCR5 (or CXCR3) would permit Vγ9Vδ2 T cells responsive to CXCL13 resulting in relocation from the T cell zone towards the B cell follicle. Within GCs Vγ9Vδ2 T cells would respond to the high concentrations of Tfh derived IL-21 by producing CXCL13, which would lead to further recruitment of $CXCR5^+$ B cells and Tfh cells. In support of the model proposed here, a previous study demonstrated on the mRNA level that CXCL13 is expressed by Vγ9Vδ2 T cells stimulated with HMB-PP in the presence of IL-21 but not of IL-2 or IL-4, and that secretion of CXCL13 protein into the supernatants of PBMC stimulated with HMB-PP and IL-21 depends on the presence of Vγ9Vδ2 T cells (Vermijlen et al. 2007). In order to directly confirm γδ T cells as the source of CXCL13, I established a flow cytometric method to detect intracellular CXCL13. As positive control in these experiments, a proportion of tonsillar $CD4^+$ T cells stained positive for CXCL13 when stimulated with PMA and ionomycin. Data presented in this thesis unambiguously show that activated, $CD25^+$ Vγ9Vδ2 T cells expressed CXCL13 when co-cultured with B cells in the presence of HMB-PP and IL-21. These findings were further validated by immunofluorescence microscopy, where intracellular CXCL13 was clearly detectable in a proportion of peripheral Vγ9Vδ2 T cells co-cultured with autologous peripheral B cells in

the presence of HMB-PP and IL-21. However, attempts to detect intracellular CXCL13 in tonsillar Vγ9Vδ2 T cells failed due to the small frequency of Vγ9Vδ2 T cells obtained from tonsils and methodology used. Despite the low percentage of peripheral $CXCL13^+$ Vγ9Vδ2 T cells under these co-culture conditions, levels of CXCL13 secreted into the supernatant were substantial. Of note, substituting IL-21 with IL-2 almost completely abrogated the CXCL13 production, confirming IL-21 as the main co-stimulatory factor in inducing CXCL13 expression by Vγ9Vδ2 T cells. These findings demonstrated that IL-21 drives expression of CXCL13 in Vγ9Vδ2 T cells, thus suggesting a role for IL-21 stimulated Vγ9Vδ2 T cells in orchestrating immune cell trafficking to the GC.

Secretion of CXCL13 by HMB-PP activated Vγ9Vδ2 T cells would provide as a mechanism of attracting circulating $CXCR5^+$ B cells and Tfh cells towards Vγ9Vδ2 T cells. This would allow Vγ9Vδ2 T cells to provide direct cell-to-cell contact with B cells via presentation of co-stimulatory molecules. Such interactions would be essential for bidirectional communication, mutual co-stimulation and activation of cells (Frauwirth and Thompson 2002). Earlier studies involving the use of a weaker Vγ9Vδ2 T cell activator (IPP) in the absence of cytokine stimulation have shown up-regulation of some co-stimulatory molecules (CD40L, OX40, ICOS and CD70) involved in B cell help (Brandes et al. 2003). Here, by using IL-21 and HMB-PP, this study applied a more physiologically relevant and novel method of investigating Vγ9Vδ2 T cell expression of several Tfh-associated co-stimulatory molecules. Furthermore, in contrast to previous studies, the use of more recent and powerful technology such as 9-colour flow cytometry provided a means of analysing co-expression of several markers, not only on Vγ9Vδ2 T cells but also simultaneously (as a control) on $CD4^+$ T cells. Here, surface expression of co-stimulatory molecules CD40L, OX40, ICOS, CD70, CD28 and CD27 was studied on peripheral blood and tonsillar Vγ9Vδ2 T cells.

Consistent with previous reports (Brandes et al. 2003), the co-stimulatory molecules CD40L, OX40, ICOS and CD70 were found to be absent on resting peripheral blood Vγ9Vδ2 T cells. However, in line with previous human studies a large proportion of resting

peripheral Vγ9Vδ2 T cells were found to express CD28 (67%) and CD27 (92%) (Lafont et al. 2000; Ribot et al. 2011; Takamizawa et al. 1995; Testi and Lanier 1989). In comparison, a minor population of freshly isolated tonsillar Vγ9Vδ2 T cells readily expressed ICOS (6%) and OX40 (3%), but not CD40L, CD70 or CD28. The expression of ICOS and OX40 on freshly isolated tonsillar Vγ9Vδ2 T cells could be explained due to the findings that a minority of freshly isolated tonsillar Vγ9Vδ2 T cells readily expressed CD25 (3%) and CD69 (11%), thus indicating the presence of pre-activated Vγ9Vδ2 T cells in inflamed infected tonsils. Furthermore the majority of tonsillar Vγ9Vδ2 T cells became rapidly activated (within 24 hours) in response to *in vitro* HMB-PP stimulation alone. This indicated that *in vitro* activation of tonsillar Vγ9Vδ2 T cells was far more robust in comparison to peripheral blood Vγ9Vδ2 T cells. Here, I also studied co-stimulatory molecule expression on *in vitro* activated peripheral blood and tonsillar Vγ9Vδ2 T cells.

The early co-stimulatory molecules CD40L, is required during pre-Tfh cell and B cell interactions. Ligation of CD40 on B cells by CD40L on Tfh cells induces GC B cell activation, survival, proliferation and in the presence of specific cytokines isotype switching (Daoussis et al. 2004; King 2009; Yong et al. 2009). Here, the early co-stimulatory molecules CD40L was not found on HMB-PP or IL-21 co-stimulated peripheral or tonsillar Vγ9Vδ2 T cells, but was significantly up-regulated (24%) upon 3 hours stimulation with PMA+ionomycin. CD40L is rapidly up-regulated at the very early stages (4-6 hours) of T cell stimulation (Daoussis et al. 2004). Thus the time-points used here (1-2 days post HMB-PP/IL-21 stimulation) may have been too late for detection of surface CD40L.

OX40:OX40L and ICOS:ICOS-L interactions between Tfh and B cells follow CD40L:CD40 interactions (Croft et al. 2009; Deenick et al. 2011; Salek-Ardakani and Croft 2006). OX40 and ICOS are mainly found on activated T cells and are up-regulated following TCR engagement and CD28 signalling (Croft et al. 2009; Salek-Ardakani and Croft 2006). However, ICOS can be found at low levels on the surface of resting naïve $CD4^+$ T cells. Co-expression of high levels of CXCR5 and ICOS is a defining feature of Tfh cells (Rasheed et al. 2006), and the ability of Tfh cells (Akiba et al. 2005) as well as

Vγ9Vδ2 T cells (Caccamo et al. 2006a) to provide follicular B cell help is in part mediated through ICOS. Strikingly, IL-21 showed a strong co-stimulatory effect on ICOS, with expression levels reaching 50% of all Vγ9Vδ2 T cells over a period of 6 days, at a time point of considerable proliferative expansion (Vermijlen et al. 2007). In parallel with ICOS, activated Vγ9Vδ2 T cells also transiently expressed the co-stimulatory molecule OX40, although the effect of IL-21 on OX40 levels was less pronounced. However, the effects of IL-21 co-stimulation maintained expression of OX40 for a further 24 hours.

When comparing the responsiveness of peripheral and tonsillar Vγ9Vδ2 T cells, tonsillar cells showed an increased sensitivity to IL-21 with regard to ICOS expression, while OX40 was preferentially induced on peripheral Vγ9Vδ2 T cells. These time-course experiments helped elucidate the unique role of IL-21 in regulating ICOS expression on HMB-PP activated Vγ9Vδ2 T cells, which has never been shown before. Tfh cells secrete IL-21 in an autocrine manner in response to ICOS stimulation which is thought to drive ICOS expression through a positive feedback mechanism (Bauquet et al. 2009). IL-21 may also have similar effects in driving ICOS expression on Vγ9Vδ2 T cells.

Other co-stimulatory molecules such as CD70 are also involved in stimulating B cell activation and promoting plasma cell differentiation (Borst et al. 2005; Elgueta et al. 2010; Jacquot 2000; Nolte et al. 2009). Here, CD70 was also found to be up-regulated on both peripheral and tonsillar Vγ9Vδ2 T cells when exposed to a combination of HMB-PP and IL-21. Taken together, our data demonstrate a role for IL-21 in the acquisition by Vγ9Vδ2 T cells of markers associated with B cell help. In particular, activation of peripheral and tonsillar Vγ9Vδ2 T cells in the presence of IL-21 leads to the expansion of $ICOS^+$ Vγ9Vδ2 T cells with a Tfh cell-like phenotype.

Cytokines also have an influence on the outcome of humoral immune responses (Crotty 2011). γδ T cells have been well established as producers of pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10) (Caccamo et al. 2006a; Hayday 2000; Vermijlen et al. 2007). Secretion of such cytokines by Vγ9Vδ2 T cells within

secondary lymphoid tissues may modulate the outcome of humoral immune responses. For example, IL-4 is known to assists in T:B interactions, promotes B cell proliferation and plasma cell differentiation (King et al. 2008; Yong et al. 2009). IL-4 also has an inhibitory effect on IgA production but drives production of IgE, and IFN-γ has been shown to inhibit switching of certain isotypes such as IgE (Konforte et al. 2009; Pistoia and Cocco 2009). Previous studies have shown activated human Vγ9Vδ2 T cells to provide help to B cells via secretion of IL-4 and IL-10 (Caccamo et al. 2006a). Here, peripheral $CXCL13^+$ Vγ9Vδ2 T cells were found to be IL-4 and IFN-γ producing cells. IL-21 enhanced the expression of both IL-4 and IFN-γ in HMB-PP activated peripheral $CXCL13^+$ Vγ9Vδ2 T cells. Similarly, a small percentage of tonsillar Vγ9Vδ2 T cells upon PMA+ionomycin stimulation expressed IL-4, whilst a higher percentage expressed TNF-α, and IFN-γ. In tonsils, the percentage of IFN-$\gamma^+$ Vγ9Vδ2 T cells was significantly higher in comparison to IFN-$\gamma^+$ CD4 T cells. Levels of cytokines secreted by Tfh cells within GCs are thought to be dependent on environmental conditions (Crotty 2011). Thus, inflammatory conditions such as tonsillitis may favour secretion of IFN-γ by Vγ9Vδ2 T cells but not by $CD4^+$ T cells. Secretion of IFN-γ by Vγ9Vδ2 T cells may be a way of modulating function of Tfh cells and B cell antibody responses.

Vγ9Vδ2 T cells may also contribute to the outcome of antibody responses by presenting antigen to Tfh cells. Several studies have already reported activated human Vγ9Vδ2 T cells to express important markers required for antigen-presentation and to act as professional APCs (Brandes et al. 2009; Brandes et al. 2005; Himoudi et al. 2012; Landmeier et al. 2009; Meuter et al. 2010; Moser and Eberl 2011; Wu et al. 2009). Here, consistent with previous studies, CCR7, HLA-DR and CD86 was absent or found on a very low percentage of freshly isolated peripheral Vγ9Vδ2 T cells. However, CD40 was readily expressed on freshly isolated peripheral Vγ9Vδ2 T cells, which was further up-regulated along with CCR7, HLA-DR and CD86 upon HMB-PP stimulation. In comparison to peripheral blood, an almost two-fold higher percentage of Vγ9Vδ2 T cells from tonsils readily expressed CCR7, CD40 and HLA-DR. Culturing of mononuclear tonsillar cells with HMB-PP resulted in up-regulation of CD86 and further up-regulation of CD40 and HLA-DR on tonsillar Vγ9Vδ2 T cells. The response to HMB-PP stimulation was unique to Vγ9Vδ2 T

cells, as CD40, HLA-DR and CD86 found readily expressed by tonsillar $CD4^+$ T cells were not further up-regulated in response to HMB-PP.

Collectively, these data indicate that IL-21 drives Vγ9Vδ2 T cells to assume a Tfh-like phenotype, thus evoking the crucial effect of IL-21 in the generation of $CD4^+$ Tfh cells (Vogelzang et al. 2008). Furthermore, in the presence of HMB-PP, Vγ9Vδ2 T cells underwent potent crosstalk with B cells as evidenced by up-regulation of CD25, CD69, CD40 and CD86 on co-cultured B cells. This effect was comparable to the effect of treatment of B cells with the mitogen SAC, indicating that HMB-PP-activated Vγ9Vδ2 T cells provided a strong stimulus for B cells. The phenotypic characterisation of Vγ9Vδ2 T cells was further supported by the functional demonstration that co-stimulation with IL-21 of co-cultures of peripheral Vγ9Vδ2 T cells and B cells enhanced the production of IgM, IgG and IgA in the presence of HMB-PP. Of note, the stimulatory effect of peripheral γδ T cells on autologous B cells depended on the γδ T cell:B cell ratio, confirming the exquisite ability of HMB-PP-activated γδ T cells to provide B cell help, especially when co-stimulated in the presence of IL-21. A similar co-stimulatory effect of IL-21 was seen on IgM levels produced by co-cultures of tonsillar Vγ9Vδ2 T cells and B cells. We are aware that the interpretation of these co-culture assays is hampered by a possible direct effect of IL-21 on B cells (Bryant et al. 2007; Linterman et al. 2010). Although IL-21 on its own had no significant effect on antibody production by peripheral B cells, partial activation of B cells by IL-21 may have played a role in these experiments. In co-cultures of tonsillar cells, there was a clear effect of IL-21 alone, suggesting that under those conditions HMB-PP and IL-21 acted synergistically. In order to rule out any direct effect on B cells, we pre-stimulated purified Vγ9Vδ2 T cells with HMB-PP and IL-21 before adding them to autologous B cells. However, no conclusive data were obtained, most likely because any soluble factors of importance for supporting antibody production released during this pre-stimulation period would have been washed away before setting up Vγ9Vδ2 T cell-B cell co-cultures.

Taken together, our present findings complement and extend previous observations on the potential of γδ T cells to provide B cell help, and ascribe an important role to IL-21 in contributing to this potential. The interaction between HMB-PP-responsive Vγ9Vδ2 T cells with IL-21 producing Tfh cells and with B cells in secondary lymphoid tissues is likely to impact on the generation of high affinity, class-switched antibodies in microbial infections. Furthermore, the nature of γδ T cells to recognise non-peptide antigens on a diverse array of pathogens in concert with the recognition of a diverse array of peptide antigens by αβ ($CD4^+$ and $CD8^+$) T cells and lipid antigens by NKT cells, may allow for a more diverse repertoire of antibodies to be generated. In conclusion, based on the major findings presented in this PhD thesis **Figure 6.1** illustrates the model proposed here.

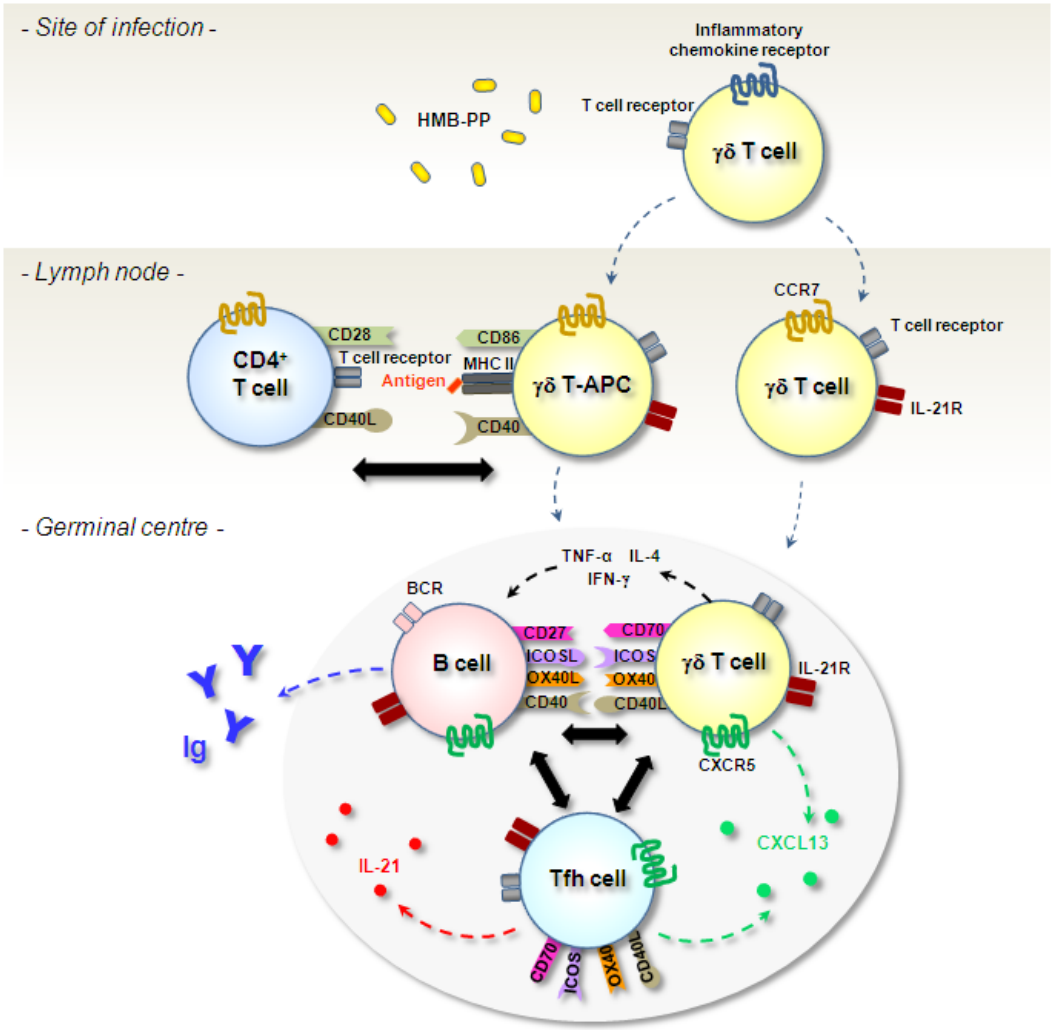


**Figure 6.1. Proposed interaction between γδ T cells, B cells and Tfh cells in secondary lymphoid tissue.**
Vγ9Vδ2 T cells recognise HMB-PP at sites of inflammation resulting in cell activation, up-regulation of the IL-21R and acquisition of a lymph node homing phenotype. Vγ9Vδ2 T cells migrate into secondary lymphoid organs such as the lymph node via expression of CCR7 and into GCs via expression of CXCR5 (or CXCR3). Within GCs Vγ9Vδ2 T cells respond to Tfh derived IL-21. The co-stimulatory effect of IL-21 via IL-21R on HMB-PP-activated Vγ9Vδ2 T cells induces secretion of CXCL13, secretion of pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-4), up-regulation of CXCR5 and co-stimulatory molecules (ICOS, OX40, CD40L and CD70). This allows Vγ9Vδ2 T cells to interact with B cells, influence the outcome of humoral immune responses and support antibody production. Secretion of CXCL13 by Vγ9Vδ2 T cells results in further recruitment of $CXCR5^+$ B cells and Tfh cells. This 'ménage à trois' of Vγ9Vδ2 T cells, Tfh cells and B cells in the GCs of secondary lymphoid tissues is likely to impact on the generation of high affinity, class-switched antibodies in microbial infections. Additionally γδ T cells may also influence the outcome of humoral immune responses by differentiating into γδ T-APCs, which interact with $CD4^+$ T cells (and $CD8^+$ T cells) via CD86:CD28 and CD40:CD40L co-stimulatory molecules, and present processed antigen.

## 6.1 Future directions

Although our present findings complement and extend previous observations, more research is required before any definitive conclusions can be drawn on the exact role and contribution of human Vγ9Vδ2 T cells in humoral immune responses.

*In vivo* studies remain a challenge and limited, as Vγ9Vδ2 T cells and the responsiveness to HMB-PP are only found in higher primates including humans but are absent in all other vertebrates including rodents (Eberl and Moser 2009). For example, one of the biggest challenges of human studies is obtaining suitable fresh tissue material such as lymph nodes, which would be more ideal for study of humoral immune responses. Instead, here and used by many other researchers, tonsils provided the most easily obtained and most accessible form of human lymphoid tissue for study. This was mainly due to routine tonsillectomies being carried out on a daily basis. However, a tonsillectomy is usually carried out as a result of on-going chronic infection and is not performed during an episode of acute tonsillitis but after a response to infection is over. Activated Vγ9Vδ2 T cells have been documented to be elevated during acute infections (Davey et al. 2011; Eberl and Moser 2009; Moser and Eberl 2011). Moreover, the majority of cases of tonsillitis are due to an infection caused by the Gram positive bacteria *Streptococcus pyogenes* (also know as "strep throat") (Murray and Chennupati 2012; Sidell and Shapiro 2012), which do not produce HMB-PP, therefore in such cases does not result in stimulation/activation of γδ T cells (Eberl and Moser 2009). For future studies it would be more appropriate to use a source of secondary lymphoid organ/tissue that represents acute infection caused by i.e. Gram negative HMB-PP producing bacteria (Eberl and Moser 2009).

Future *ex vivo* studies would involve investigation of Vγ9Vδ2 T cell frequencies, phenotypes and B cell helper functional capabilities from inflamed infection-involved lymph nodes in comparison to non-inflamed. This may not only support current data but also help provide a better insight into the role of Vγ9Vδ2 T cells as APCs and in humoral

immune response. Immunohistochemical and immunofluorescence analysis of infection-involved lymph node tissue sections could be used to demonstrate *in vivo* co-localisation of γδ T cells, B cells, and Tfh cells within GCs, along with staining for co-stimulatory markers, CXCL13 and CXCR5. Direct immunofluorescence staining of markers would be essential. Thus an anti-human CXCL13 antibody that is directly conjugated to a fluorochrome would be essential.

Ideas for future *in vitro* co-culture experimental work would include blocking of IL-21R in revealing the importance of exogenous and Tfh derived IL-21 on Vγ9Vδ2 T cells in delivering co-stimulation for enhancing B cell helper capability. This would also involve investigating crosstalk between γδ T cells and Tfh cells in regulating cytokine production and B cell responses (i.e. antibody production and isotype switching), which would be carried out under several different culture conditions.

Finally, the aspect of antigen specificity deserves attention. Earlier studies in mice demonstrated that GC reactions that solely depend on γδ T cells are somewhat inefficient and result in relatively high titres of class-switched, self-reactive antibodies (Wen and Hayday 1997; Wen et al. 1994). γδ T cells may thus provide general B cell help and enhance humoral immune responses, while MHC restricted αβ T cells ensure antigen specificity by regulating affinity maturation and clonal selection. This view is supported by the fact that HMB-PP is present in the majority of bacterial pathogens, in malaria parasites and in *Toxoplasma*, and hence activation of human Vγ9Vδ2 T cells is likely to occur in a broad range of infections (Eberl and Moser 2009). A 'bystander' role for Vγ9Vδ2 T cells in driving humoral responses may result in different antibody levels, isotypes or affinities upon infection with HMB-PP producing or HMB-PP deficient micro-organisms. While such a role can only be postulated in humans and at best supported by indirect epidemiological evidence, infection and/or vaccination experiments in non-human primates might address the contribution of Vγ9Vδ2 T cells to the generation of high-affinity, class-switched antibodies. Such studies could exploit the availability of convenient pathogen models such as specially engineered HMB-PP deficient strains of *Listeria monocytogenes* (Begley et al.

2004) or HMB-PP overproducing strains of *Mycobacterium tuberculosis* (Brown et al. 2010).

Recent findings also point toward a more antigen-restricted role of Vγ9Vδ2 T cells in providing B cell help, given their potential to take up exogenous antigens and present them to $CD4^+$ and $CD8^+$ T cells (Brandes et al. 2005; Moser and Eberl 2011). Antigen-presenting γδ T cells may thus be able to interact directly with Tfh cells. In mice, epidermal γδ T cells leave the skin upon activation and reach the draining lymph node where they may help the production of antibodies specific for non-self antigen applied onto, or artificially expressed within, the skin (Shimura et al. 2010; Strid et al. 2009). It remains to be investigated whether HMB-PP or related compounds with activity on Vγ9Vδ2 T cells, such as the aminobisphosphonate zoledronate, might be useful in boosting humoral responses. A recent vaccine trial in cynomolgus monkeys did address a possible adjuvant effect of the HMBPP analogue Picostim on the immune response against mycobacterial antigens yet did not measure levels of anti-mycobacterial antibodies (Cendron et al. 2007). Similarly, the effect of aminobisphosphonate treatment on anti-tumour antibody responses in cancer patients has so far been overlooked.

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# Appendix:

## Peer reviewed publication

## arising from the study