

T cell phenotype and function in Spondyloarthritis

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Abstract

Th17 cells are implicated in a variety of inflammatory disorders including Spondyloarthritis (SpA). Ankylosing spondylitis (AS), the most common SpA, is genetically associated with HLA-B27 and IL-23R polymorphisms, although the link remains unexplained. In addition to classically folded heterotrimers, HLA-B27 can form β_2m -free homodimers (B27₂). B27₂, as well as free heavy chains, are able to interact with KIR3DL2.

I have shown an expansion of KIR3DL2⁺ CD4⁺ T cells in peripheral blood of AS patients and HLA-B27⁺ healthy controls. Although KIR3DL2⁺ CD4⁺ T cells comprise a minority of CD4⁺ T cells in peripheral blood, KIR3DL2⁺ CD4⁺ T cells account for the majority of peripheral blood CD4⁺ T cell IL-23R expression. KIR3DL2⁺ CD4⁺ T cells from synovial fluid are also enriched for IL-23R expression compared to matched peripheral blood samples. Moreover, treatment exposure to rIL-1/23 significantly increases IL-17 production by KIR3DL2⁺ CD4⁺ T cells in AS patients but not RA patients and healthy controls.

KIR3DL2⁺ CD4⁺ T cells from AS patients produce more IL-17 than KIR3DL2⁺ CD4⁺ T cells from HLA-B27⁻ healthy controls. KIR3DL2⁺ CD4⁺ T cells from AS patients and healthy controls are also enriched for dual production of IL-17 and IFN γ , consistent with the theory that AS is a Th17 or a Th17/1 driven disease.

I have shown that naïve CD4⁺ T cells express KIR3DL2 *de novo* after activation. Interaction with B27₂-expressing cells acts to maintain and/or further increase KIR3DL2 expression. Interaction with B27₂-expressing cells also increases expression of Bcl-2. I propose that interaction of KIR3DL2⁺ CD4⁺ T cells with B27₂ preferentially promotes the survival of these pro-inflammatory cytokine-producing KIR3DL2⁺ CD4⁺ T cells in SpA. HD6, a B27₂-specific monoclonal antibody, decreases IL-17 production by PBMCs from AS patients, making it an attractive candidate therapeutic reagent in the treatment of AS and other HLA-B27-associated SpA.

Declaration

I declare that the work presented in this D.Phil thesis entitled “T cell phenotype and function in Spondyloarthritis” is entirely my own work, except for where the contributions of my collaborators have been clearly acknowledged. No part of my thesis has been submitted for any degree or other qualification in this University or elsewhere.

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Hilary Term 2013

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Publications arising from this thesis

Bowness, P., **Ridley, A.**, Shaw, J., Chan, A. T., Wong-Baeza, I., Fleming, M., Cummings, F., McMichael A. and Kollnberger, S. (2011). "Th17 cells expressing KIR3DL2+ and responsive to HLA-B27 homodimers are increased in ankylosing spondylitis." J Immunol **186**(4): 2672-80.

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Other related publications

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Contents

Title	i
Abstract	ii
Declaration	iii
Publications arising from this thesis	iv
Other related publications	iv
Acknowledgments	v
Contents	vii
List of Tables	xiv
List of Figures	xv
List of Abbreviations	xxiii
Chapter 1: Introduction and Aims	1
1.1. Spondyloarthritides (SpA)	1
1.1.1. Clinical Features	1
1.1.2. Ankylosing Spondylitis	2
1.1.3. Diagnosis	3

1.1.4. Treatment	6
1.2. AS Susceptibility	7
1.2.1. Environmental associations with AS	7
1.2.2. Genetic Associations with SpA	8
1.2.2.1. MHC complex genes and SpA	8
1.2.2.2. Non-MHC complex genes and SpA	9
1.3. HLA-B27 and AS disease pathogenesis	13
1.4. T Cell Immunology	19
1.4.1. T cell development, differentiation and death	19
1.4.2. T cell plasticity	24
1.5. Th17 cells	29
1.5.1. Th17 cell discovery and differentiation	29
1.5.2. Cytokine production and phenotype of Th17 cells	32
1.5.3. Th17 cells in SpA	37
1.6. Killer Immunoglobulin-like Receptors (KIRs)	40
1.6.1. KIRs in Health and Disease	40
1.6.2. KIR3DL2	43
1.7 Project Aims	46
 Chapter 2: Materials and Methods	 48
2.1. Patient and Control Recruitment	48
2.2. Laboratory Methods	48
2.2.1 Mononuclear cell separation from peripheral blood and synovial fluid	48
2.2.2. Cryopreservation of peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCS)	49

2.2.3. Thawing of PBMCs and SFMCs	50
2.2.4. Cell lines	50
2.2.5. DNA preparation for HLA typing	52
2.2.6. Cell isolation using magnetic beads	53
2.2.6.1. CD4+ T cell isolation	53
2.2.6.2. Naive CD4+ T cell isolation	53
2.2.7. Conjugation of DX31 or isotype control antibody	54
2.2.8. Fluorescent Activated Cell Sorting (FACS)	55
2.2.8.1. FACS staining	56
2.2.8.2. Intracellular cytokine staining (ICS)	58
2.2.8.3. Carboxyfluorescein succinimidyl ester (CFSE) labelling	59
2.2.8.4. FACS sorting	59
2.2.9. T cell activation and expansion	60
2.2.9.1. Anti-CD2/3/28 stimulation	60
2.2.9.2. SEB stimulation	61
2.2.9.3. Th17 polarization for multiplex bead analysis	61
2.2.10. Antibody-co-culture experiments	61
2.2.11. Enzyme-linked immunosorbent assay (ELISA)	62
2.2.12. Multiplex bead analysis	62
2.2.13. Quantitative PCR (qPCR) and sample preparation	63
2.2.13.1. Dead Cell removal	63
2.2.13.2. RNA extraction	63
2.2.13.3. Complementary DNA (cDNA) synthesis	64
2.2.13.4. qPCR	65
2.3. Analytical methods	66

Chapter 3: CD4+ T cell phenotypic marker expression in inflammatory arthritis	68
3.1. Introduction	68
3.2. Aim	69
3.3. Results	70
3.3.1. Expansion of KIR3DL2+ CD4+ T cells in peripheral blood of AS patients	70
3.3.2. KIR3DL2+ CD4+ T cells are enriched for Th17 markers	72
3.3.3. IL-23R is enriched on KIR3DL2+ CD4+ T cells from synovial fluid	87
3.3.4. KIR3DL2+ CD4+ T cells are enriched for the activation marker CD134	90
3.3.5. KIR3DL2+ CD4+ T cells are enriched for the gut homing marker CCR9	93
3.4. Discussion	96
 Chapter 4: CD4+ T cell cytokine production in inflammatory arthritis	 106
4.1. Introduction	106
4.2. Aim	107
4.3. Results	108
4.3.1. KIR3DL2+ CD4+ T cells are enriched for IL-17 production	108
4.3.2. IL-17 production from KIR3DL2+ CD4+ T cells in SpA patients is further increased by addition of recombinant (r)IL-1/IL-23	114
4.3.3. KIR3DL2+ CD4+ T cells are enriched for IFN γ production by ICS but not multiplex bead analysis	118

4.3.4. KIR3DL2+ CD4+ T cells are enriched for dual production of IL-17 and IFN γ	126
4.3.5. TNF α production by KIR3DL2+ and KIR3DL2- CD4+ T cells is increased by the addition of rIL-1/IL-23	129
4.3.6. KIR3DL2+ CD4+ T cells from AS patients and HLA-B27+ healthy controls are enriched for IL-6 production; for HLA-B27+ healthy controls IL-6 is further increased by the addition of rIL-1/IL-23	132
4.3.7. IL-22 production by KIR3DL2+ CD4+ T cells from RA patients and healthy controls is increased by the addition of rIL-1/IL-23	138
4.3.8. IL-10 production by KIR3DL2+ CD4+ T cells from RA patients, but not other groups, is significantly greater than IL-10 production by KIR3DL2- CD4+ T cells	142
4.3.9. KIR3DL2+ CD4+ T cells are not enriched for IL-5 production, nor is IL-5 production increased by the addition of rIL-1/IL-23	146
4.3.10. GM-CSF production by both KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells from HLA-B27- healthy controls increases with the addition of rIL-1/IL-23	149
4.5. Discussion	153
 Chapter 5: Effects of activation on KIR3DL2 expression by CD4+ T cells	166
5.1. Introduction	166
5.2. Aim	168
5.3. Results	169
5.3.1. KIR3DL2 expression is increased by SEB stimulation of PBMCs	169
5.3.2. IL-17 production is upregulated by SEB stimulation of PBMCs and is greater for AS patients	179

5.3.3. TNF α production is upregulated by SEB stimulation of PBMCs, but does not differ between inflammatory arthritis patients and healthy controls	182
5.3.4. KIR3DL2 expression is upregulated by anti-CD2/3/28 bead stimulation of CD4+ T cells	185
5.3.5. Increased KIR3DL2 expression on CD4+ T cells after SEB stimulation is not inhibited by SEB stimulation in the presence of DX31	188
5.3.6. IL-17 production by PBMCs is inhibited in the presence of an anti-HLA-B27 dimer antibody	193
5.3.7. KIR3DL2 is induced on naive CD4+ T cells following activation	196
5.4. Discussion	201
 Chapter 6: Effects of naïve CD4+ T cell co-culture with HLA-B27-expressing lines on CD4+ T cell development and differentiation	 209
6.1. Introduction	209
6.2. Aim	211
6.3. Results	212
6.3.1. Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells partially inhibits CD45RO acquisition	212
6.3.2. Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells increases KIR3DL2 expression	214
6.3.3. Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells partially inhibits proliferation	221

6.3.4. Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells	
increases RORC and T-bet expression	224
6.3.5. Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells	
increases Bcl-2 expression	227
6.4. Discussion	229
 Chapter 7: Final discussion and future directions	238
 Appendix	250
 References	253

List of Tables

Table 1.1	Assessment in Spondyloarthritis International Society (ASAS) classification criteria for the diagnosis of axial and peripheral spondyloarthritis	5
Table 1.2	Cytokines involved in reinforcement and inhibition of CD4+ T cell subsets	26
Table 2.1	Media used in experiments	49
Table 2.2	Antibodies used in experiments	57
Table 2.3	Isotype control antibodies used in experiments	58
Table 2.4	Master mix components for cDNA synthesis	64
Table 2.5	Master mix components for qPCR	65
Table 2.6	Genes investigated by qPCR	66

List of Figures

Figure 1.1	Hypotheses to explain the role of HLA-B27 in AS	14
Figure 1.2	Simplified diagram of CD4+ T cell lineage differentiation	20
Figure 1.3	Simplified model of CD4+ T cell plasticity	25
Figure 1.4	Shared subunits of IL-12 and IL-23 and the IL-12 receptor and IL-23 receptor	32
Figure 1.5	IL-17 signaling pathways	34
Figure 1.6	Simplified diagram of KIR3DL2 and a prototypic group A KIR haplotype	41
Figure 2.1	.220 and .221 cell lines expressing HLA-B27 stain with HD6	51
Figure 2.2	Testing of DX31 and IgG2a isotype control antibodies following conjugation	55
Figure 2.3	KIR3DL2 expression on CD4+ T cells after FACS sorting	60
Figure 3.1	A higher percentage of CD4+ T cells from AS patients and HLA-B27+ healthy controls express KIR3DL2 compared to HLA-B27- healthy controls	71
Figure 3.2	Enrichment of IL-23R on KIR3DL2+ CD4+ T cells from an AS patient	73
Figure 3.3	KIR3DL2+ CD4+ T cells are enriched for IL-23R expression	74
Figure 3.4	Enrichment of IL-1R on KIR3DL2+ CD4+ T cells from an AS patient	76
Figure 3.5	KIR3DL2+ CD4+ T cells are enriched for IL-1R expression	77
Figure 3.6	Enrichment of CCR6 on KIR3DL2+ CD4+ T cells from an AS	

	patient	79
Figure 3.7	KIR3DL2+ CD4+ T cells are enriched for CCR6 expression	80
Figure 3.8	Enrichment of CD161 on KIR3DL2+ CD4+ T cells from an AS patient	82
Figure 3.9	KIR3DL2+ CD4+ T cells are enriched for CD161 expression	83
Figure 3.10	KIR3DL2+ CD4+ T cells are enriched for CCR6, IL-23R and IL-1R mRNA	85/86
Figure 3.11	Enrichment of CCR6, CD161 and IL-23R on KIR3DL2+ CD4+ T cells from synovial fluid compared to a matched peripheral blood sample from an AS patient	87
Figure 3.12	Enrichment of IL-23R on KIR3DL2+ CD4+ T cells from synovial fluid compared to matched peripheral blood samples from SpA patients	89
Figure 3.13	Enrichment of CD134 on KIR3DL2+ CD4+ T cells from an AS patient	91
Figure 3.14	KIR3DL2+ CD4+ T cells are enriched for CD134 expression	92
Figure 3.15	Enrichment of CCR9 on KIR3DL2+ CD4+ T cells from an AS Patient	94
Figure 3.16	KIR3DL2+ CD4+ T cells are enriched for CCR9 expression	95
Figure 4.1	A higher percentage of KIR3DL2+ CD4+ T cells from an AS patient produce IL-17 compared to KIR3DL2- CD4+ T cells	109
Figure 4.2	Higher percentages of KIR3DL2+ CD4+ T cells produce IL-17 compared to KIR3DL2- CD4+ T cells from AS patients and healthy controls	110
Figure 4.3	KIR3DL2+ CD4+ T cells from patients with inflammatory arthritis produce more IL-17 than KIR3DL2- CD4+ T cells	112

Figure 4.4	Higher IL-17 production by KIR3DL2- CD4+ T cells from AS patients compared to healthy and inflammatory disease controls	113
Figure 4.5	IL-17 production by KIR3DL2+ CD4+ T cells from AS patients is significantly increased by rIL-1/23 treatment	115
Figure 4.6	IL-17 production by KIR3DL2- CD4+ T cells is not significantly altered by rIL-1/23 treatment	116
Figure 4.7	Higher IL-17 production by KIR3DL2+ CD4+ T cells from AS patients in response to rIL-23/1 compared to healthy controls and RA patients	117
Figure 4.8	A higher percentage of KIR3DL2+ CD4+ T cells from an AS patient produce IFN γ compared to KIR3DL2- CD4+ T cells	119
Figure 4.9	Higher percentages of KIR3DL2+ CD4+ T cells produce IFN γ compared to KIR3DL2 CD4+ T cells from AS patients and healthy controls	120
Figure 4.10	KIR3DL2+ CD4+ T cells from AS patients and HLA-B27+ healthy controls do not produce more IFN γ than KIR3DL2- CD4+ T cells	122
Figure 4.11	No significant difference in IFN γ production by KIR3DL2+ CD4+ T cells or KIR3DL2 CD4+ T cells from AS patients compared to healthy controls and inflammatory disease controls	123
Figure 4.12	IFN γ production by KIR3DL2+ CD4+ T cells is significantly increased by rIL-1/23 treatment	124
Figure 4.13	IFN γ production by KIR3DL2- CD4+ T cells is significantly increased by rIL-1/23 treatment	125
Figure 4.14	Higher percentages of KIR3DL2+ CD4+ T cells produce IL-17	

	and IFN γ compared to KIR3DL2- CD4+ T cells from AS patients and healthy controls	127
Figure 4.15	Higher percentages of KIR3DL2+ CD4+ T cells produce IL-17 and IFN γ compared to KIR3DL2- CD4+ T cells from AS patients and healthy controls	128
Figure 4.16	No significant difference in the production of TNF α between KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells	130
Figure 4.17	TNF α production by KIR3DL2+ CD4+ T cells from inflammatory arthritis patients and HLA-B27- healthy controls is significantly increased by rIL-1/23 treatment	131
Figure 4.18	TNF α production by KIR3DL2- CD4+ T cells is significantly increased by rIL-1/23 treatment	132
Figure 4.19	KIR3DL2+ CD4+ T cells from AS patients and HLA-B27+ healthy controls are enriched for IL-6 production	134
Figure 4.20	No significant difference in IL-6 production by KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells from AS patients compared to healthy or inflammatory disease controls	135
Figure 4.21	IL-6 production by KIR3DL2+ CD4+ T cells from healthy controls is significantly increased by rIL-1/23 treatment	136
Figure 4.22	IL-6 production by KIR3DL2- CD4+ T cells is significantly increased by rIL-1/23 treatment	137
Figure 4.23	No significant difference in the production of IL-22 between KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells	139
Figure 4.24	IL-22 production by KIR3DL2+ CD4+ T cells from healthy controls and RA patients is significantly increased by rIL-1/23 treatment	140

Figure 4.25	IL-22 production by KIR3DL2- CD4+ T cells from AS and RA patients and HLA-B27+ healthy controls is significantly increased by rIL-1/23 treatment	141
Figure 4.26	KIR3DL2+ CD4+ T cells from RA patients produce significantly more IL-10 than KIR3DL2- CD4+ T cells	143
Figure 4.27	No significant difference in IL-10 production by KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells from AS patients compared to healthy controls and inflammatory disease controls	144
Figure 4.28	IL-10 production by KIR3DL2+ CD4+ T cells is not significantly increased by rIL-1/23 treatment	145
Figure 4.29	No significant difference in the production of IL-5 between KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells	147
Figure 4.30	IL-5 production by KIR3DL2+ or KIR3DL2- CD4+ T cells is not significantly increased by rIL-1/23 treatment	148
Figure 4.31	No significant difference in the production of GM-CSF between KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells	150
Figure 4.32	GM-CSF production by KIR3DL2+ CD4+ T cells from RA patients and HLA-B27- healthy controls is significantly increased by rIL-1/23 treatment	151
Figure 4.33	GM-CSF production by KIR3DL2- CD4+ T cells from HLA-B27- healthy controls is significantly increased by rIL-1/23 treatment	152
Figure 5.1	Assay optimization for KIR3DL2 expression on CD4+ T cells after activation with SEB	171
Figure 5.2	A higher percentage of CD4+ T cells from an AS patient express KIR3DL2, before and after stimulation with SEB, than CD4+ T cells from a HLA-B27- healthy control	172

Figure 5.3	A higher percentage of CD4+ T cells express KIR3DL2 following stimulation with SEB	174
Figure 5.4	Higher percentage of CD4+ T cells from AS patients express KIR3DL2 following stimulation with SEB, compared to HLA-B27- healthy controls	175
Figure 5.5	Elevated number of CD4+ T cells expression KIR3DL2 following stimulation with SEB	176
Figure 5.6	No significant difference in the numbers of CD4+ T cells expressing KIR3DL2 before or after SEB stimulation	177
Figure 5.7	No significant difference in the expression of other KIRS on CD4+ T cells from AS patients and HLA-B27- healthy controls, before and after stimulation with SEB	178
Figure 5.8	Significantly greater production of IL-17 by PBMCs after stimulation with SEB	180
Figure 5.9	PBMCs from AS patients produce significantly more IL-17 than PBMCs from HLA-B27+ healthy controls following stimulation with SEB	181
Figure 5.10	Significantly higher production of TNF α by PBMCs after stimulation with SEB	183
Figure 5.11	No significant difference in TNF α production from PBMCs from AS or RA patients or inflammatory disease controls	184
Figure 5.12	A higher percentage of CD4+ T cells from a HLA-B27- healthy control express KIR3DL2 after stimulation with anti-CD2/3/28 beads at a bead:cell ratio of 1:1, than after stimulation with anti-CD2/3/28 beads at a bead:cell ratio of 1:10	186
Figure 5.13	A higher percentage of CD4+ T cells express KIR3DL2+ following	

	stimulation with anti-CD2/3/28 beads, but this does not differ between AS patients and HLA-B27- healthy controls	187
Figure 5.14	PBMCs from a healthy control and an AS patient were stimulated for 5 days in the presence of SEB alone or with SEB and a KIR3DL2 blocking antibody (DX31) or a control antibody (IgG2a)	189
Figure 5.15	Increased KIR3DL2 expression on CD4+ T cells is not inhibited by SEB stimulation in the presence of DX31	190
Figure 5.16	Increased KIR3DL2 expression on CD4+ T cells is not inhibited by SEB stimulation in the presence of DX31	191
Figure 5.17	Increased IL-17 production by SEB-stimulated PBMCs is not inhibited by SEB stimulation in the presence of DX31	192
Figure 5.18	Blocking with an anti-B27 dimer antibody (HD6) significantly reduces production of IL-17 by PBMCs after stimulation with SEB	194
Figure 5.19	Blocking with an anti-B27 dimer antibody does not significantly alter production of TNF α by PBMCs after stimulation with SEB	195
Figure 5.20	KIR3DL2 expression is restricted to memory CD4+ T cells	197
Figure 5.21	KIR3DL2 expression is induced by CD4+ T cells by PMA and ionomycin stimulation	199
Figure 5.22	KIR3DL2 expression is induced by CD4+ T cells by PMA and ionomycin stimulation but does not significantly differ between AS patients and HLA-B27- healthy controls	200
Figure 6.1	Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells partially inhibits CD45RO acquisition	213
Figure 6.2	KIR3DL2 expression, induced by naïve CD4+ T cells from a healthy control, is greater after co-culture with 220.B27 compared to .220B8 cells	215

Figure 6.3	KIR3DL2 expression, induced by naïve CD4+ T cells from an AS patient, is greater after co-culture with 220.B27 compared to 220.B8 cells	216
Figure 6.4	KIR3DL2 expression, induced by naïve CD4+ T cells from a cord blood sample, is greater after co-culture with 220.B27 compared to 220.B8 cells	218
Figure 6.5	KIR3DL2 expression, induced by naïve CD4+ T cells from a leukocyte cone, is greater after co-culture with 220.B27 compared to 220.B8 cells	220
Figure 6.6	Co-culture of naïve CD4+ T cells and 220.B27 cells partially blocks naïve CD4+ T cell proliferation	222
Figure 6.7	Co-culture of naïve CD4+ T cells and 221.B27 cells partially blocks naïve CD4+ T cell proliferation	223
Figure 6.8	Co-culture of naïve CD4+ T cells with 220.B27 cells for 8 days increase expression of T-bet, compared to co-culture with 220 or 220.B7 cells	225
Figure 6.9	Co-culture of naïve CD4+ T cells with 220.B27 cells for 8 days increase expression of RORC, compared to co-culture with 220 or 220.B7 cells	226
Figure 6.10	Greater expression of Bcl-2 after 24 hours co-culture of naïve CD4+ T cells with 221.B27 cells compared to 221 cells	228
Figure 7.1	Model to explain KIR3DL2 and HLA-B27 interaction in the pathogenesis of AS.	244

List of Abbreviations

AAU	Acute anterior uveitis
ACAD	Activated autonomous cell death
AHR	Aryl hydrocarbon receptor
AICD	Activation induced cell death
ANOVA	Analysis of variance
ANTXR2	anthrax toxin receptor 2 gene
APCs	Antigen presenting cells
APC	Allophycocyanin
AS	Ankylosing spondylitis
ASAS	Assessment in Spondyloarthritis International Society
BASDI	Bath Ankylosing Spondylitis Disease Activity Index
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
β2m	β ₂ -microglobulin
BID	BH3 interacting-domain death agonist
BIM	B-cell lymphoma 2 interacting mediator of cell death
BiP	ER chaperone immunoglobulin heavy-chain binding protein
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CARD9	Caspase recruitment domain-containing protein 9
CCL	CC chemokine
CCR	CC chemokine receptor

CHOP	C/EBP homologous protein
CXCR	CXC-chemokine receptor
CFSE	Carboxyfluorescein succinimidyl ester
CRP	C-reactive protein
DAS	Disease activity score
DISC	Death-inducing signaling complex
DMARDs	Diseases modifying anti-rheumatic drugs
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERAP	Endoplasmic reticulum aminopeptidase
ESR	Erythrocyte sedimentation rate
ESSG	European Spondyloarthropathy Study Group
EULAR	European League Against Rheumatism
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
GM-CSF	Granulocyte macrophage colony-stimulating factor
GWAS	Genome wide association studies

HC	Healthy control
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICS	Intracellular staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILAR	International League of Associations for Rheumatology
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibition motifs
JAK	Janus kinase
JIA	Juvenile idiopathic arthritis
KIR	Killer immunoglobulin-like receptor
LILR	Leukocyte immunoglobulin-like receptors
LRC	Leukocyte receptor complex
LTBR	Lymphotoxin beta receptor
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NK	Natural killer
NSAIDs	Nonsteroidal anti-inflammatory drugs
nsSNPs	Nonsynonymous single nucleotide polymorphisms
OA	Osteoarthritis

PB	Pacific blue
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR-SSP	Polymerase chain reaction sequence specific primers
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PGE2	Prostaglandin E2
PHA	Phytohaemagglutinin
PMA	Phorbol-12-myristate-13-acetate
PsA	Psoriatic arthritis
PTGER4	Prostaglandin E receptor 4
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
ReA	Reactive arthritis
rIL-1/23	Recombinant IL-1/23
RPM	Revolutions per minute
SCID	Severe combined immunodeficiency
SEB	Staphylococcal enterotoxin B
SFMC	Synovial fluid mononuclear cells
SLE	Systemic lupus erythematosus
SpA	Spondyloarthritis
SS	Sézary syndrome
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TASC	Australo-Anglo-American Spondyloarthritis Consortium
TCR	T cell receptor

TGF	Transforming growth factor
Th	T helper cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
UPR	Unfolded protein response
uSpA	Undifferentiated-arthritis
WIMM	Weatherall Institute of Molecular Medicine
WTCCC	Wellcome Trust Case Control Consortium

Chapter 1: Introduction and Aims

1.1. Spondyloarthritides (SpA)

1.1.1. Clinical Features

The spondyloarthritides encompass a group of chronic, inflammatory disorders, including ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), enteropathic arthritis, and undifferentiated-arthritis (uSpA). There is an estimated prevalence of 1.9% in Caucasian populations (Braun, Bollow et al. 1998). The spondyloarthritides share a number of clinical features including inflammation of axial joints (particularly of the sacroiliac joints), asymmetric oligoarthritis (particularly of the lower limbs) and dactylitis. Enthesitis, or inflammation at the sites of attachment of ligaments or tendon to bone, is considered the primary pathological abnormality. Extra-articular features can involve almost any organ system. The most common extra-articular feature is acute anterior uveitis (AAU), which can occur in 25-30% patients (Singh, Lawrence et al. 2008). The course of the disease is highly variable but a poor prognosis is associated with young age at onset (less than 16 years), hip arthritis, dactylitis, oligoarthritis, erythrocyte sedimentation rate (ESR) >30 mm/h, poor efficacy of nonsteroidal anti-inflammatory (NSAIDs) drugs and limitation of lumbar spine (Amor, Santos et al. 1994).

Interestingly there is a degree of crossover with inflammatory bowel disease (IBD), with a quarter of patients with chronic SpA exhibiting features of Crohn's disease (Leirisalo-Repo, Turunen et al. 1994) and up to 70% of patients displaying microscopic signs of inflammation in the colon and/or ileum (Mielants, Veys et al. 1995). Conversely one study showed features

of SpA were present in 46% of patients with Crohn's disease and 10% of patients with ulcerative colitis (Turkcapar, Toruner et al. 2006).

SpA occurring in children can be particularly challenging to diagnose as symptoms at disease onset often differ from those in adults. Hip and peripheral arthritis, together with enthesitis, are more common presenting features in children, whilst inflammatory back pain is less prominent, reflecting the infrequent involvement of the sacroiliac and other vertebral joints in juvenile disease (reviewed in (Hofer 2006)). The International League of Associations for Rheumatology (ILAR) system for juvenile idiopathic arthritis, classifies most childhood SpA as enthesitis-related arthritis (Petty, Southwood et al. 2004).

1.1.2. Ankylosing Spondylitis

AS involves inflammation of the sacroiliac joints and chronic spinal inflammation (spondylitis). This chronic inflammation can eventually lead to fusion of the vertebrae (ankylosis), giving rise to the characteristic 'bamboo spine', which is accompanied by stiffness, pain and a loss of spinal mobility. Pain usually improves with activity, and returns with inactivity. Onset of symptoms, most commonly buttock and lower back pain, typically begin between the ages of 18 and 30 years. AS occurs more frequently in males, with a male-to-female ratio of 2:1 (Feldtkeller, Khan et al. 2003) and there is evidence to suggest disease can be more severe in men (Jimenez-Balderas and Mintz 1993). Prevalence ranges worldwide with the highest prevalence in Haida Indians, where one study showed that almost 50% of the Haida community were HLA-B27+ and 20% of HLA-B27+ adult Haida males showed evidence of sacroiliitis (Gofton, Chalmers et al. 1975), and the lowest prevalence in people of Afro-Caribbean descent (Baum and Ziff 1971).

Disease course can be highly variable, however evidence suggests that a predictable pattern of AS emerges within the first 10 years of disease (Carette, Graham et al. 1983). In this study the natural course of AS was examined over 23 years in 51 patients, with mean disease duration of 38 years. Of the patients with mild spinal restriction after 10 years, 74% did not progress to severe spinal involvement. In contrast, 81% of the patients who had severe spinal restriction were severely restricted within the first 10 years. The most serious complication encountered in patients with AS is increased mortality primarily due to an increased incidence of cardiovascular disease (Peters, van der Horst-Bruinsma et al. 2004). Spinal fracture, amyloidosis and pulmonary fibrosis are further rare complications (reviewed in (Sieper, Braun et al. 2002)).

Quality of life and socioeconomic impacts are further considerations. A longitudinal cross-sectional survey of 175 patients with AS identified aspects of the disease that adversely affected quality of life (Ward 1999). The majority of patients surveyed were men (68%). The patients had a mean age of 51 years and had AS for a mean duration of 23.7 years. The most prevalent quality of life concerns included stiffness (90%), pain (83%), fatigue (62%), sleep problems (54%), appearance (51%), future outcome (50%), and side effects of drugs (51%). Work status was analysed in a systemic review of the literature (Boonen, de Vet et al. 2001). In patients with AS employment ranged from 34 to 96% after 45 and 5 years disease duration, respectively, and work disability from 3 to 50% after 18 and 45 years disease duration, respectively. Days of sick leave varied from 12 to 46 days per patient per year amongst those in employment.

1.1.3. Diagnosis

Diagnosis of AS can be difficult as patients typically present with non-specific inflammation and pain that can be attributed to a wide range of causes. C-reactive protein (CRP) and

erythrocyte sedimentation rate (ESR), both biomarkers of inflammation, lack sensitivity and specificity for a diagnosis of AS. As a result of difficulty in diagnosing the disease a diagnostic delay of between 5-7 years has been documented (Braun and Pincus 2002) during which time damage to joints can occur, highlighting the need for specific biomarkers associated with disease activity.

A number of groups have set criteria to aid diagnosis and classification of AS. The first criteria set for classification, developed in 1961 in Rome, did not include radiographs of the sacroiliac joints to make a diagnosis, but the Modified New York Criteria (1984) combines both clinical and radiological criteria, whereupon a diagnosis of AS can be made when changes in the sacroiliac joints are accompanied by either inflammatory back pain or restriction of spinal mobility. Further sets of criteria have been subsequently developed, including the European Spondyloarthropathy Study Group (ESSG) criteria (Dougados, van der Linden et al. 1991) and the Amor criteria (Amor, Dougados et al. 1995). Radiographic evidence of sacroiliitis is included in both criteria but is not a prerequisite for diagnosis. More recently the Assessment in Spondyloarthritis International Society (ASAS) classification criteria for axial and peripheral SpA has been established (Rudwaleit, van der Heijde et al. 2011), and can be seen in Table 1.1.

Axial Spondyloarthritis: ≥3 months back pain and age at onset <45 years, with either A or B:	
A: Sacroiliitis on imaging plus ≥1 of	B: HLA-B27 plus ≥2 of
• inflammatory back pain • arthritis • enthesitis • uveitis • dactylitis • psoriasis • Crohn's disease / ulcerative colitis • Good response to NSAIDs • Family history of SpA • HLA-B27 • Elevated CRP	• inflammatory back pain • arthritis • enthesitis • uveitis • dactylitis • psoriasis • Crohn's disease / ulcerative colitis • Good response to NSAIDs • Family history of SpA • Elevated CRP
Peripheral Spondyloarthritis: Arthritis or enthesitis or dactylitis, with either A or B:	
A: Plus ≥1 of	B: Plus ≥2 of the remaining
• psoriasis • inflammatory bowel disease • preceding infection, human • HLA- B27 • Uveitis • sacroiliitis on imaging	• arthritis • enthesitis • dactylitis • inflammatory back pain in the past • family history of SpA

Table 1.1: Assessment in Spondyloarthritis International Society (ASAS) classification criteria for the diagnosis of axial and peripheral spondyloarthritis (adapted from (Rudwaleit, van der Heijde et al. 2011))

Once a diagnosis has been reached various measures of disease activity exist, providing indicators of disease course and response to treatment. The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) is a questionnaire, which allows patients to rate symptoms including discomfort, pain, and fatigue. Magnetic resonance imaging (MRI) is thought to be the best available indirect measure of disease activity (Bollow, Fischer et al. 2000).

1.1.4. Treatment

Recommendations for the management of ankylosing spondylitis have been proposed by a combined ASsessment of Ankylosing Spondylitis (ASAS) and European League Against Rheumatism (EULAR) task force. To summarise management of patients is centred on clinical presentation, with physiotherapy often being highly effective. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used as a first line of drug treatment to reduce the pain and stiffness associated with AS. As a second line of treatment disease modifying anti-rheumatic drugs (DMARDs), such as methotrexate, can be used to slow disease process. More recently biological agents have been used to treat the disease, with anti-TNF therapies showing the most success. These include infliximab, an anti-TNF-monoclonal antibody, adalimumab, a humanized anti-TNF-monoclonal antibody, and etanercept, a soluble TNF-receptor conjugated to human IgFC, which acts as a receptor antagonist. Surgical intervention is a last resort and is usually performed to repair or replace damaged peripheral joints, although spinal surgery is considered useful in some patients.

1.2. AS Susceptibility

1.2.1. Environmental associations with AS

The entheses are the site of primary pathology in AS and mechanical stress in this area could potentially play a role in the development of AS. There is some data to suggest that mechanical stress may result in activation of pathways involved in inflammation and spur formation (Benjamin, Toumi et al. 2007).

A further possibility is an infectious agent playing a causative role in the development of AS. Reactive arthritis, a type of SpA, develops after infection, most commonly of the gastrointestinal or genitourinary tract, with Gram-negative intracellular bacteria including *Salmonella*, *Shigella*, *Campylobacter* and *Chlamydia trachomatis*. Infections are typically resolved before symptoms of arthritis develop making identification of the causative species particularly challenging. Infections with *Klebsiella pneumoniae* have been implicated in the pathogenesis of AS (Ebringer, Cawdell et al. 1978) however a more recent study did not find evidence to support this hypothesis (Stone, Payne et al. 2004).

Studies into HLA-B27 transgenic rats support a role for microbial infection in disease initiation since rats raised in germ-free microenvironments do not develop the joint or gut inflammation seen in rats raised under conventional conditions (Taurog, Richardson et al. 1994). Moreover, reintroduction of commensal gut flora induced arthritis in these rats, lending further weight to the idea that bacterial infection plays a role in the pathogenesis of AS.

1.2.2. Genetic Associations with SpA

It is estimated that 90% of susceptibility to AS can be attributed to genetic factors. This is based largely upon twin studies, which have shown that monozygotic twin pairs have 75% disease concordance compared to 27% for HLA-B27+ dizygotic twin pairs and 12.5% for dizygotic twin pairs overall (Brown, Kennedy et al. 1997). Numerous susceptibility loci have been identified through extensive genome wide association studies (GWAS) (Burton, Clayton et al. 2007; Australo-Anglo-American Spondyloarthritis Consortium (TASC), Reveille JD et al. 2010; Evans, Spencer et al. 2011). These include genes within the major histocompatibility complex (MHC), most notably HLA-B27, and a number of genes outside of the MHC complex.

1.2.2.1. MHC complex genes and SpA

The association of HLA-B27 with AS is the strongest known association of an HLA type with a disease, with as many as 94% of individuals with AS being HLA-B27+ (Brewerton, Hart et al. 1973; Caffrey and James 1973). HLA-B27 is also associated with the development of other types of SpA, although to a less extent than AS. At least 75 alleles of HLA-B27 have been identified, based on nucleotide sequence differences, but at the translated protein level, there are 62 known subtypes (Khan 2010). However not all HLA-B27 subtypes are associated with AS. B*2709, a subtype seen almost exclusively in the Sardinian population, is not associated with disease, but differs only from B*2705 by a single amino acid change (Asp116His). It is thought that peptides that are shared between B*2705 and B*2709 may be bound differently, with the former subtype able to bind several peptides in two distinct conformations that show different T cell reactivities (Hulsmeier, Fiorillo et al. 2004).

Some of the strongest evidence for a role of B27 in the pathogenesis of AS comes from studies into transgenic models of disease. A multisystem inflammatory disease that includes

arthritis and colitis, is seen to develop in transgenic rats with a high copy number of HLA-B27 (Hammer, Maika et al. 1990; Taurog, Maika et al. 1993). Interestingly these rats also exhibit defective co-stimulatory function (Hacquard-Bouder, Falgarone et al. 2004) with defective stimulation of allogeneic and syngeneic T lymphocytes by antigen presenting cells (APC) from HLA-B27 but not HLA-B7 rats. A spontaneous inflammatory disease also develops in HLA-B27 transgenic mice lacking beta-2(β_2) microglobulin, but only after they are transferred from the specific pathogen-free mouse colony, suggesting disease develops when mice are exposed to an appropriate environmental trigger (Khare, Luthra et al. 1995).

In addition to HLA-B27, other HLA molecules have been shown to be associated with AS. Analysis of 9,065 West African patients revealed a significant association between AS and HLA-B14, with this allele being found in 62.5% of AS patients but only 2% of healthy controls (Lopez-Larrea, Mijiyawa et al. 2002). Furthermore, analysis of B14 subtypes showed that B*1403 was the predominant allele in AS patients, and but was absent in healthy controls. HLA-B60 and HLA-B61 have also shown to be strongly associated with AS in Taiwan Chinese patients (Wei, Tsai et al. 2004). HLA class II molecules have also been associated with AS. HLA-typing of 363 white British AS patients showed a significant association between HLA-DR1 and AS (Brown, Kennedy et al. 1998). A weakly significant association between HLA-DR8 and AS was also seen. HLA-DR7 was associated with younger age at onset of disease but no other associations with disease severity or clinical manifestations were observed.

1.2.2.2. Non-MHC complex genes and SpA

The majority of individuals who are HLA-B27+ do not develop disease suggesting that AS is a polygenic disorder with numerous genes conferring risk of disease development. A number of non-MHC genes, implicated by GWAS, have been brought to light. A study by the Wellcome Trust Case Control Consortium (WTCCC) and Australo-Anglo-American Spondyloarthritis

Consortium (TASC) looked at 14,500 nonsynonymous single nucleotide polymorphisms (nsSNPs) in 1000 cases and 1500 controls (Burton, Clayton et al. 2007). The study found 7 nsSNPs in the gene encoding the IL-23 receptor (*IL23R*) were associated with AS. Interestingly *IL23R* polymorphisms have also been shown to be associated with inflammatory bowel disease (IBD). In ulcerative colitis, Crohn's disease and psoriasis a nsSNP (Arg381Gln; *IL23R* variant rs11209026) confers protection against disease (Duerr, Taylor et al. 2006; Capon, Di Meglio et al. 2007; Cargill, Schrodi et al. 2007; Dubinsky, Wang et al. 2007).

Support of the involvement of the IL-23R comes from investigations into other proteins involved in the signalling pathway following IL-23R engagement, both in AS and related diseases. Janus kinases (JAK) are intracellular tyrosine kinases, which transduce cytokine-mediated signals through the JAK-STAT pathway. In the Chinese Han population *JAK2* polymorphisms are associated with susceptibility to AS (Chen, Zhang et al. 2010). Moreover, GWAS have identified STAT3 (signal transducer and activator of transcription 3), a transcription factor through which Th17 cells signal, as being associated with susceptibility to Crohn's disease (Cenit, Alcina et al. 2010).

An additional finding of the WTCCC/TASC study was an association of *ERAP1* (Endoplasmic reticulum aminopeptidase 1) with AS. *ERAP1* is expressed primarily in the endoplasmic reticulum (ER), with two known functions. Firstly it acts as a 'molecular ruler' for trimming peptides to 9 amino acids in length for optimal MHC class I presentation (Saric, Chang et al. 2002; Chang, Momburg et al. 2005). Secondly *ERAP1* may function as a 'shedase', cleaving cell surface cytokine receptors, including IL-6R, IL-1R2 and TNFR, thereby downregulating their signalling (Cui, Hawari et al. 2002; Cui, Rouhani et al. 2003). The polymorphism in *ERAP1* was seen to alter susceptibility to AS but not to Crohn's disease, whilst a further study

showed no association of this SNP with psoriasis (Nair, Duffin et al. 2009). Interestingly the association between ERAP1 and AS is only seen in HLA-B27+ individuals (Evans, Spencer et al. 2011).

In a further study by TASC 2053 AS patients and 5140 controls were genotyped, and the findings were replicated in an independent cohort of 898 AS patients and 1,518 controls (Australo-Anglo-American Spondyloarthritis Consortium (TASC), Reveille JD et al. 2010). In addition to confirming associations with *IL23R* and *ERAP1*, they found associations with SNPs in 2 gene deserts at 2p15 and 21q22, as well as in the genes *ANTXR2* (anthrax toxin receptor 2) and *IL1R2*. A multicenter study identified *IL1A* as an AS susceptibility gene (Sims, Timms et al. 2008), although a subsequent GWAS was not able to confirm this (Burton, Clayton et al. 2007; Australo-Anglo-American Spondyloarthritis Consortium (TASC), Reveille JD et al. 2010). However, a role for genes within the IL-1 region in AS susceptibility were supported by the TASC GWAS, with the identification of the *IL1R2* gene being associated with a risk of developing AS. *IL1R2* encodes IL-1 receptor type 2, which inhibits IL-1 activity by acting as a decoy target for IL-1. The involvement of the *ANTXR2* gene and the associations with SNPs in 2 gene deserts at 2p15 and 21q22, and the association with AS are less clear.

A further GWAS identified *RUNX3* as being associated with AS susceptibility (Evans, Spencer et al. 2011). *RUNX3* encodes a transcription involved in CD8+ T cell differentiation (Park, Adoro et al. 2010). In the same GWAS *IL12B* was shown to be associated with AS. *IL12B* encodes the IL-12p40 protein, a shared component of both IL-12 and IL-23, adding further support to the involvement of the IL-23 axis in AS. This latter finding is of particular interest since ustekinumab, a monoclonal antibody that targets IL-12p40, has been successful used in the treatment of patients with psoriasis (Gottlieb, Menter et al. 2009). Interestingly, in the same GWAS study *PTGER4*, the gene encoding prostaglandin E receptor 4 (PTGER4), was

identified as being associated with AS susceptibility (Evans, Spencer et al. 2011). Again, this gene is involved in the IL-17/23 pathway, with prostaglandin E2 (PGE2) acting through PTGER4 to induce the production of IL-23 and IL-17. SNPs in or near *PTGER4* are also associated with Crohns disease (Libioulle, Louis et al. 2007).

Nonsynonymous SNPs in *CARD9* have also been identified as having a role in susceptibility to AS by GWAS (Australo-Anglo-American Spondyloarthritis Consortium (TASC), Reveille JD et al. 2010; Evans, Spencer et al. 2011). *CARD9* encodes caspase recruitment domain-containing protein 9 (hCARD9), an adaptor molecule that transduces signaling to the canonical NFκB pathway and has an essential role in protection against fungal and bacterial pathogens (reviewed in (Ruland 2008)). Interestingly hCARD9 plays an important role in the IL-17/23 pathway, with signaling via NFκB inducing production of TNF, IL-6 and IL-23, and differentiation of T cells secreting IL-17 and IL-23 (LeibundGut-Landmann, Grosz et al. 2007).

A number of genes associated with TNF signalling have also been shown to be associated with AS (Evans, Spencer et al. 2011) which is of note given the success of anti-TNFα therapies in some patients. In this GWAS the authors observed association with SNPs at chromosome 12p13 between the *LTBR* (lymphotoxin beta receptor) and *TNFRSF1A* (tumor necrosis factor receptor 1 (TNFR1) genes. However it remains to be determined if the genetic polymorphisms at this locus are a result of effects on *LTBR*, or *TNFRSF1A* or both genes. *TBKBP1*, which encodes an adaptor protein that binds to serine/threonine-protein kinase TBK1, and is part of the interaction network in the TNF–NFκB pathway (Bouwmeester, Bauch et al. 2004), was also identified by GWAS to be involved in AS (Evans, Spencer et al. 2011). *TRADD*, a further component of the TNF signalling pathway also showed a potential association with AS (Pointon, Harvey et al. 2010).

In conclusion, extensive GWAS have identified genes in the IL-17/23 axis as being strongly associated with AS. This is consistent with the theory that Th17 cells play a significant role in the pathogenesis of AS. This is a dynamic field with further large-scale GWAS underway, which are vital to confirm the association of genes that have already been identified, as well as bringing to light new candidate genes.

1.3. HLA-B27 and AS disease pathogenesis

Classically MHC class I molecules exist as heterotrimers on the surface of all nucleated cells, formed by an interaction of the $\alpha 3$ heavy chain domain with β_2 -microglobulin. This allows presentation of endogenous antigen to CD8+ T cells. As previously mentioned up to 94% of AS patients carry at least one HLA-B27 allele (Brewerton, Hart et al. 1973; Caffrey and James 1973), however it is currently unknown how HLA-B27 is involved in the disease. Various mechanisms have been proposed to explain how HLA-B27 might be associated with SpA and these can be seen in Figure 1.1.

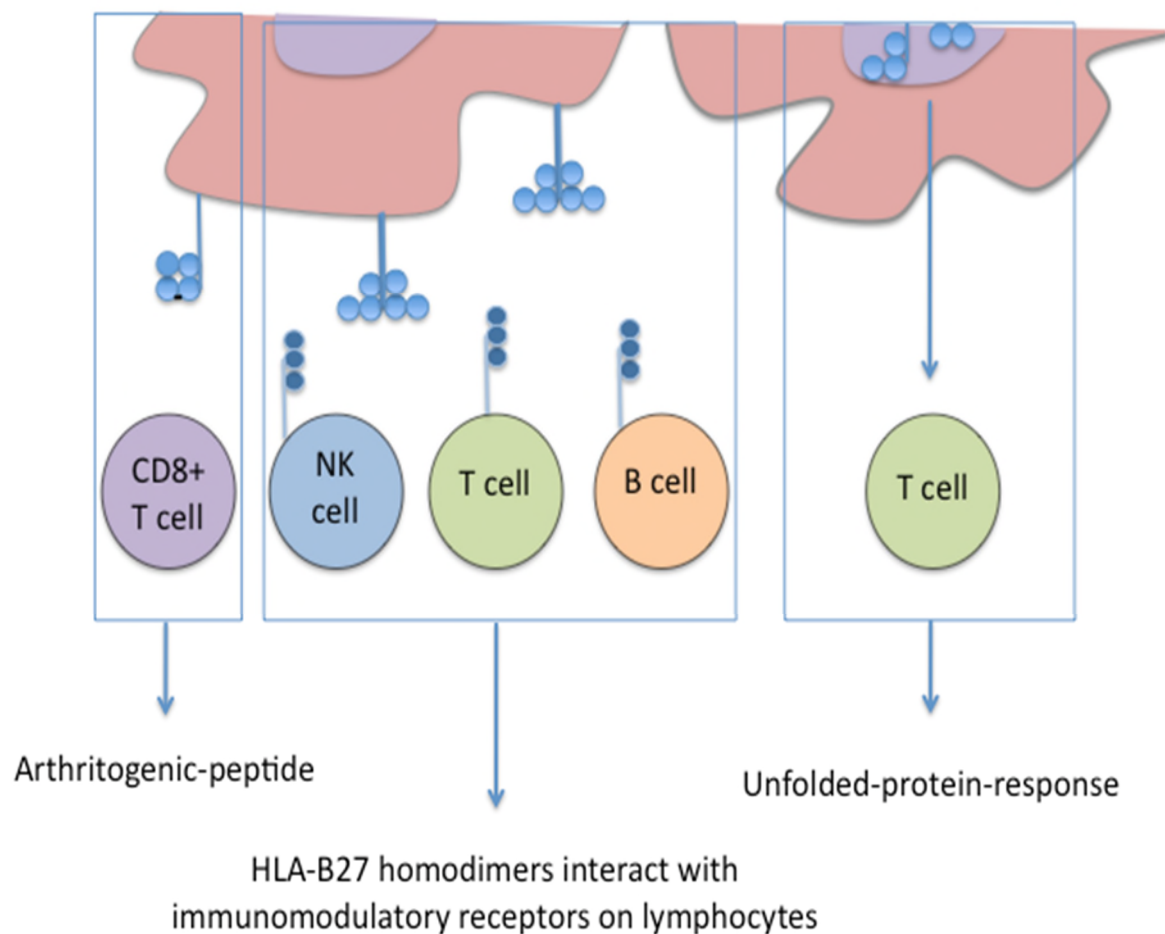


Figure 1.1. Hypotheses to explain the role of HLA-B27 in AS. Three main theories exist to link HLA-B27 and AS. The arthritogenic peptide theory (left) postulates that an as yet unidentified peptide drives deregulation of HLA-B27 restricted CD8+ T cells. The unfolded protein response (UPR) hypothesis (right) postulates that misfolding of HLA-B27 in the ER activated the UPR, which leads to production of pro-inflammatory cytokines and Th17 polarization. The third theory relates to the ability of HLA-B27 to form aberrantly folded forms, including HLA-B27 homodimers and free heavy chains which can interact with immunomodulatory receptors on lymphocytes, which leads to the survival of pro-inflammatory cytokines producing cells (adapted from (Tam, Gu et al. 2010)).

The 'arthritogenic-peptide' hypothesis postulates that HLA-B27 has the ability to bind a specific peptide, or set of peptides, and that presentation of these peptides to HLA-B27 restricted CD8⁺ T cells drives cellular dysregulation and disease pathogenesis. In this scenario an initial immune response against a peptide derived from an infection could deviate to a self-peptide. This is a form of molecular mimicry, first described by Benjam and Parnham, who postulated that HLA-B27-related disease may be the result of the generation of a suitable, "self-mimicking" HLA-B27-binding peptide by certain bacteria, plus an unusual propensity for the HLA-B27 molecule to bind and present such peptides (Benjamin and Parham 1992).

In support of this theory HLA-B27-restricted bacteria-specific CD8⁺ T cells have been identified in the synovial fluid of patients with ReA (Hermann, Yu et al. 1993). HLA-B27-restricted CD8⁺ T cells, able to react with collagen derived peptides, have also been observed in synovial fluid from AS patients (Atagunduz, Appel et al. 2005). *Yersinia*-derived peptides have also been identified in the synovial fluid of patients with ReA (Ugrinovic, Mertz et al. 1997). A further related theory is that HLA-B27 itself, or peptides derived from it, could act as autoantigens for recognition by CD4⁺ T cells via presentation by MHC class II molecules (Boyle, Goodall et al. 2001).

However despite decades of research a candidate peptide remains to be elucidated and there is now substantial evidence to suggest that HLA-B27-restricted CD8⁺ T cells are not required in disease pathogenesis. For example CD8 α -b27/human β_2 -microglobulin transgenic rats still have disease even in the absence of any functional CD8⁺ T cells (Taurog, Dorris et al. 2009). In support of this depletion of CD8 $\alpha\beta$ T cells failed to suppress either arthritis or colitis in HLA-B27 transgenic rats (May, Dorris et al. 2003). Furthermore, anti-CD8 α treatment led to a reduction of two non-T-cells populations, namely NK cells and a CD4⁺CD8⁺CD11b/c⁺

CD161a+CD172a+ monocyte population, both of which have been shown to be expanded in diseased B27 transgenic rats. This suggests these cells may be involved in the disease but that CD8+ T cells are not essential in disease development.

Other hypotheses to explain the link between HLA-B27 and AS refer to the cell biology of HLA-B27. The 'unfolded protein response' (UPR) hypothesis is based on the observation that HLA-B27 molecules fold at a slower rate than other HLA molecules (Mear, Schreiber et al. 1999). In addition to the slower rate of folding, this study showed that the HLA-B27 B pocket caused a portion of the pool of assembling HLA-B27 heavy chains in the ER to misfold. These HLA-B27 heavy chains undergo ER-associated degradation (ERAD) or form disulfide-linked complexes (Dangoria, DeLay et al. 2002). It is proposed that the subsequent accumulation of these misfolded molecules in the ER may initiate the UPR. This pathway acts to restore normal function of the cell by halting protein translation and activating signaling pathways that lead to increased production of molecular chaperones involved in protein folding. Activating transcription factor 6 (ATF6), inositol-requiring transmembrane kinase/endonuclease 1 (IRE1) and pancreatic ER kinase (PERK) are all effector molecules in the UPR. Under favorable physiological conditions these molecules are sequestered in the ER, in an inactive state, by the ER chaperone immunoglobulin heavy-chain binding protein (BiP). ER stress leads to the release of the effector molecules, leading to their oligomerization, autophosphorylation and subsequent activation. Activation of IRE1 then activates the transcription factor XBP1 for the upregulation of UPR-associated genes, whilst activation of PERK activates the transcription factor eIF2 α , again for up-regulation of UPR-associated genes. One such gene is C/EBP homologous protein (CHOP), which in turn suppresses Bcl-2, for induction of apoptosis, which occurs if cellular function cannot be restored.

Support for this hypothesis comes from studies into transgenic animals, where the UPR was activated in macrophages derived from the bone marrow of HLA-B27 transgenic rats with inflammatory disease (Turner, Sowders et al. 2005). Evidence of the UPR in the joints of AS patients was observed in a study showing raised expression of GRP78, a marker of the UPR, in the affected peripheral joints of AS, as compared with osteoarthritis (OA) patients (Dong 2008). Enhanced expression of ER chaperone immunoglobulin heavy-chain binding protein (BiP) in SpA has also been reported (Gu, Rihl et al. 2002), with highest expression observed in the adherent fraction of SpA synovial fluid mononuclear cells (SFMC). Activation of an UPR may lead to upregulation of proinflammatory cytokines, which may polarize T cells. In support of this it was shown that the IL-23 gene is a target of CHOP and enhanced binding of CHOP to the IL-23 gene was seen following ER stress and TLR stimulation (Goodall, Wu et al. 2010). However more recent evidence from a study investigating IL-23 production by LPS stimulated macrophages from AS patients did not see an increase BiP or CHOP expression following stimulation, thus the relationship between the UPR and cytokine production remains unclear (Zeng, Lindstrom et al. 2011).

A further hypothesis to explain the link between HLA-B27 and AS refers to the recognition of aberrantly folded HLA-B27 at the cell surface. In addition to classically folded heterotrimers HLA-B27 can exist as β 2-microglobulin-free homodimers expressed on the cell surface, linked through a disulphide bond of unpaired cysteine residues at position 67 in the α 1 heavy chain domain (Allen, O'Callaghan et al. 1999), or as HLA-B27 free heavy chains (Kollnberger, Bird et al. 2002; Wong-Baeza, Ridley et al. 2013). Although this unpaired cysteine residue is not unique to HLA-B27, being found also in HLA-B14, -B15, -B38, -B39 and -B73, few studies have investigated dimer formation in these other alleles, with one report of HLA-B14 forming homodimers in a transfected B-cell line (Merino, Galocha et al. 2008). HLA-B27 dimer expression has been documented on peripheral blood mononuclear cells (PBMC) from

patients with SpA, as well as in HLA-B27-transfected cell lines and in HLA-B27 transgenic disease models (Tsai, Chen et al. 2002; Kollnberger, Bird et al. 2004; Lan, Tsai et al. 2004; Raine, Brown et al. 2006). HLA-B27 dimers can form via two distinct pathways, either in the ER or in endosomal compartments. However HLA-B27 dimers formed in the ER are thought to be unable to egress to the cell surface in human cells, suggesting the observed surface homodimers are formed through the latter pathway, possibly during endosomal recycling (Bird, Peh et al. 2003).

HLA-B27 dimers and free heavy chains bind immunomodulatory receptors including killer immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs) in humans, as well as paired immunoglobulin receptors (PIRs), a LILR homologue in rats (Allen, Raine et al. 2001; Kollnberger, Bird et al. 2004; Wong-Baeza, Ridley et al. 2013). Indeed HLA-B27 homodimers and multimers are stronger ligands for LILRB2 than classically folded HLA class I heterotrimers (Giles, Shaw et al. 2012). Interestingly the interaction of HLA-B27 dimers with both KIR3DL1 and KIR3DL2 is independent of the sequence of bound peptide (Kollnberger, Chan et al. 2007). Immunomodulatory receptors are expressed on a wide variety of cells including NK cells, NKT cells, T cells, monocytes, macrophages and dendritic cells (Lanier 1998; Colonna, Nakajima et al. 1999; Brown, Trowsdale et al. 2004). It is proposed that the interaction of HLA-B27 dimers with immunomodulatory receptors may explain the association of HLA-B27 with AS.

1.4. T Cell Immunology

1.4.1. T cell development, differentiation and death

T cells develop in the thymus, where they encounter self-antigen and go through a process of positive and negative selection which allows cells with functioning T cell receptors (TCR), that are not reactive to self, to move out into the periphery where they circulate until they encounter antigen displayed by APCs (reviewed in (Starr, Jameson et al. 2003)). Depending on the microenvironment in which they experience antigen they can then develop into distinct lineages, as shown in Figure 1.2. During naïve T cell differentiation transcription factors act to commit a T cell to a specific lineage. T-bet, Gata3, ROR γ t and FoxP3 are the master regulators of Th1, Th2, Th17 and T regulatory (Treg) cell differentiation respectively.

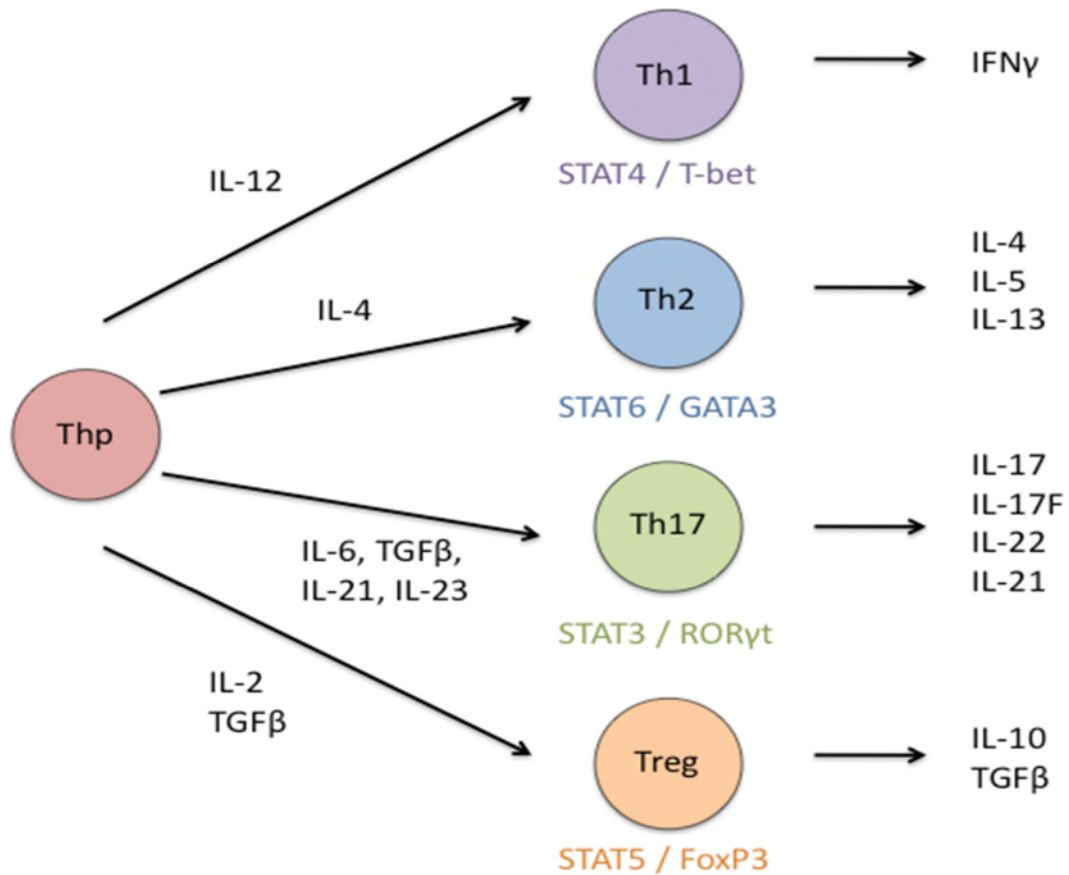


Figure 1.2. Simplified diagram of CD4⁺ T cell lineage differentiation. CD4⁺ T cells differentiate into distinct lineages from naïve T cell precursors (Thp) under the influence of different cytokines. The activation of distinct signaling cascades and transcription factors results in the induction of additional cytokines and chemokines and cytokine and chemokine receptors. These cytokines and transcription factors may favor one cell lineage, while inhibiting another (not indicated but discussed in section 1.4.2). Adapted from (Jetten 2009).

It was originally thought that T cells belonged to one of two lineages, becoming either T helper 1 (Th1) cells, which are important in cellular immunity, or Th2 cells, which are

important in humoral immunity and are involved in allergic disease. More recently additional T cell subsets have come to light, including Th17 and T regulatory (Treg) cells. Th17 cells are important in protection against infection with various bacteria and fungal species, and have been implicated in autoimmune and inflammatory diseases. T regulatory cells are proposed to regulate the actions of other T cells, by production of anti-inflammatory cytokines, specifically IL-10 and TGF β , and through cell-cell contact mediated inhibition.

T cells are further subdivided based on the expression of CD4 or CD8, with the majority of T cells expressing one of these surface glycoproteins. CD4+ T cells interact with exogenous antigen presented on MHC class II molecules and CD8+ T cells interact with endogenous antigen presented on MHC class I molecules. CD4+ T cells are known as T helper cells. They provide help to B cells for antibody production, as well as producing a variety of cytokines for activation and recruitment of cells pivotal to the immune response. CD8+ T cells are known as cytotoxic T cells, and upon stimulation they release perforin and granzyme from cytoplasmic vesicles, for lysis of target cells. Certain antigenic challenge favors the expansion of different subsets. For example *Mycobacterium tuberculosis* favors clonal expansion of CD4+ T cells (Silva, Bonato et al. 1999), whilst Epstein-Barr virus (EBV) favors clonal expansion of CD8+ T cells (Maini, Gudgeon et al. 2000).

After activation naïve T cells undergo clonal expansion and differentiation. This requires priming of naïve T cells with antigen and co-stimulatory signals, resulting in expansion and differentiation to effector cells, within 7-9 days (reviewed in (Lees and Farber 2010)). Once activated T cells home to sites of inflammation and this is largely mediated by chemokines. Th1 cells preferentially express the CXC-chemokine receptor (CXCR)-3 and CC-chemokine receptor (CCR)-5 and Th2 cells preferentially express CCR4 and CCR8 (Bonecchi, Bianchi et al. 1998; Nagata, Tanaka et al. 1999).

Following resolution of infection the pool of effector T cells contracts in order to restore homeostasis (reviewed in (Russell 1995)). Activation induced cell death (AICD) occurs following TCR restimulation of expanded T cells in the absence of co-stimulatory signals (Krammer 2000), after ligation of cell death receptors including CD95 (Dhein, Walczak et al. 1995) and TNFR1 (Sytwu, Liblau et al. 1996), as well as through mechanisms independent of death receptors, including granzyme B in the AICD of Th2 cells (Devadas, Das et al. 2006). Signaling through cell-death receptors results in the formation of a death-inducing signaling complex (DISC) (Kischkel, Hellbardt et al. 1995), bringing pro-caspase-8 and pro-caspase-10 into close proximity for their autoproteolytic activation and release into the cytosol (Chang, Xing et al. 2003). Activated caspase-8 and -10 then activate further caspases including down-stream effector caspase-3, -6 and -7 for cleavage of cellular proteins (Mashima, Naito et al. 1997). As well as directly activating down stream effector caspases, caspase-8 and -10 can cleave the Bcl-family member BID, which in its truncated form causes aggregation of pro-apoptotic molecules BAX and BAK, and release of cytochrome c from the mitochondria (Korsmeyer, Wei et al. 2000). Cytochrome c release leads to the formation of the apoptosome and activation of pro-caspase-9, for subsequent activation of down-stream effector caspases (Li, Nijhawan et al. 1997).

A further mechanism of cell death is activated autonomous cell death (ACAD). ACAD occurs in the absence of appropriate survival signals. Bcl-2-interacting mediator of cell death (BIM) is essential for ACAD (Hildeman, Zhu et al. 2002). BIM binds Bcl-2 or Bcl-X_L on mitochondrial membranes, which then bind BAX or BAK, abrogating Bcl-2- or Bcl-X_L-mediated inhibition of cell-death by causing the release of cytochrome c from the mitochondria (Sandalova, Wei et al. 2004; Willis, Fletcher et al. 2007).

Although the vast majority of the T cell effector pool is removed by apoptosis a number of antigen-specific T cells remain as a memory pool, thus providing a patrolling pool of cells that are able to recognize and respond to various antigenic challenges much more rapidly than naïve T cells. Two main theories have been proposed to explain which T cells survive to make up the pool of circulating memory T cells. One theory suggests that cellular and molecular regulators control the lineage fate and functional capacities of memory T cells early after priming. The alternative theory proposes that populations of memory T cells are inherently plastic and subject to alterations in function and/or survival at many stages during their long-term maintenance (reviewed in (Lees and Farber 2010)). Once generated, CD4+ memory T cells can persist for a several years and are thought to be more stable than CD8+ memory T cells (Hammarlund, Lewis et al. 2003).

Two memory T-cell subsets have been delineated based on expression of CD62 and CCR7 (Sallusto, Lenig et al. 1999). The CD62L^{lo} CCR7^{lo} memory subtype is defined as the T effector-memory (T_{EM}) cell population, while the CD62L^{hi} CCR7^{hi} memory subtype is defined as the T central-memory (T_{CM}) cell population. The majority of studies have focused on memory CD8+ T cells, where markers including IL-7R, CD27, CD122, Bcl-2 and CXCR3 have been used to further define memory T cells and differentiate them from the effector T cells population (reviewed in (Kaech and Wherry 2007)). Initial findings identified the T_{EM} subset as the subset responsible for producing effector cytokines, with the T_{CM} subset predominantly producing IL-2 (Sallusto, Lenig et al. 1999), however subsequent studies suggested both populations are able to exhibit effector function (Baron, Bouneaud et al. 2003). Whether the T_{EM} and T_{CM} subsets represent stable or dynamic populations is not yet clear. There is evidence that homeostatic proliferation of T_{CM} results in both maintenance of T_{CM} subsets and conversion of some progeny to T_{EM} phenotypes, mostly based on results with CD8+ T cells (Marzo, Yagita et al. 2007). T_{EM} have been shown to convert to T_{CM} cells during persistence or

following activation of CD8⁺ and CD4⁺ T cells (Schwendemann, Choi et al. 2005), suggesting the specific subsets are not fixed.

1.4.2. T cell plasticity

The Th1-Th2 paradigm is based on the concept that Th1 and Th2 cells differentiate along distinct pathways, with specific transcription factors and cytokines playing key roles in this differentiation into separate lineages. Thus it stipulates that Th1 and Th2 cells represent terminal products of their respective developmental programs. However T cell lineage commitment now appears to be more complex than originally proposed with the discovery of new T cell subsets, particularly since both Th17 cells and Tregs require TGF β for differentiation. Th1 cells are now thought to be relatively flexible in terms of lineage commitment, as compared to Th2 cells, which are rapidly stabilized. This rapid stabilization of Th2 cells is proposed, in part, to be the result of early extinction of IL-12 signaling (Szabo Immunity 1995). In addition repeated stimulation in chronic disease may lead to the irreversible commitment of CD4⁺ T cells (reviewed in (O'Garra 1998)).

A hierarchy of T cells subsets and their stability or subsequent transition to a different subset was described in a review by Murphy and Stockinger (Murphy and Stockinger 2010). They propose a model whereby naïve T cells are able to develop into other subsets dependant on the microenvironment, as illustrated in Figure 1.3, with cells moving from an unstable to a more stable phenotype. In this model naïve T cells move through a hierarchy, first becoming Tregs cells, then moving to Th17 then Th1 cells, in permissive microenvironments.

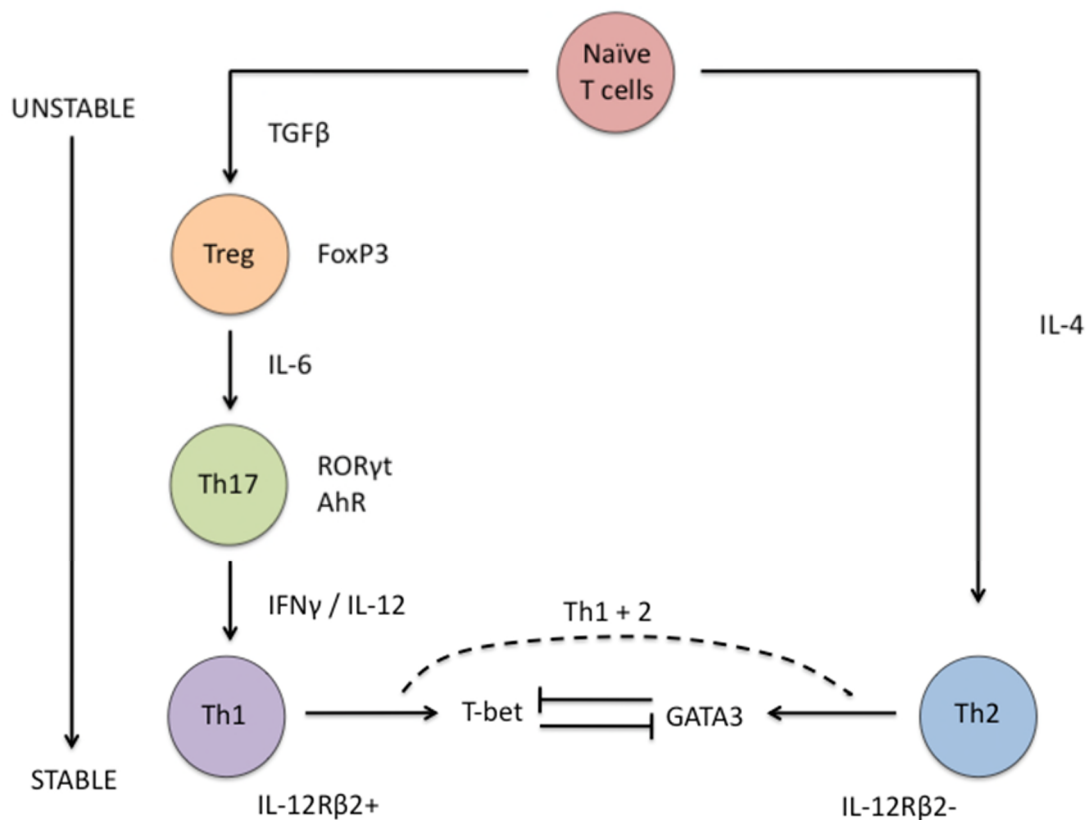


Figure 1.3. Simplified model of CD4⁺ T cell plasticity. In a model proposed by K. Murphy and B. Stockinger. Naïve CD4⁺ T cells transition into various other subsets, from a more unstable to a more stable state. This is mediated by cytokines and involves interactions between transcription factors, as indicated, which, in some cases, inhibit the differentiation of other subsets. Adapted from (Murphy and Stockinger 2010).

Numerous factors effecting T cell stability are important determinants of T cell subset plasticity. One of the most widely studied of these is the conditions favoring specific subsets of T cells, encompassing both cytokines and co-stimulation, provided by cells of the adaptive and innate system. Table 1.2 lists some of the key cytokines involved in reinforcement and destabilization of different T cell subsets. Cytokines that polarize T cells to a specific lineage

can suppress the development of different types of T helper subsets. For example IL-12 favors Th1 differentiation, whilst destabilizing Th2 and Th17 differentiation.

Subset	Reinforcement		Inhibition	
	Molecule	Activity	Molecule	Activity
Th1	IL-2 IL-18	IFN γ ↑		
Th2	TSLP IL-2 Helminth products	IL-12p40↓	TGF- β IL-12	Th9 Th1+2
Th17	AhR ligands IL-23 IL-1 TNF IL-21	IL-22 IL-17 & IL-22↑	IL-12 Retinoic acid	Th1
Treg	Retinoic acid Vitamin D		IL-6 IL-2	Th17 Th1

Table 1.2: Cytokines involved in reinforcement and inhibition of CD4+ T cell subsets
(adapted from (Murphy and Stockinger 2010))

Interaction of transcription factors also plays a role in stability and plasticity of T cell subsets. For example GATA-3, the master transcription factor in Th2 cells, acts in a self-reinforcing positive feedback loop, promoting the stability of Th2 cells (Ouyang, Lohning et al. 2000). However GATA-3 and T-bet act against each other and one many neutralize the transcriptional activities of the other, promoting divergence of one subset to another (Hwang, Szabo et al. 2005).

Epigenetic modifications are another important determinant of plasticity and stability, and studies of epigenetic markers have suggested that Th17 cells have greater plasticity with regards to developmental programming than Th1 and Th2 cells. Epigenetic studies have shown that the promoters of both *IL17* and *IL17F* genes in Th17 cells, but not Th1 or Th2 cells, have histone modifications associated with activated, transcribing loci (Akimzhanov, Yang et al. 2007). In another study activating and repressive trimethylations of histone 3 were investigated in the proximal promoters of various T cell lineage transcription factor and cytokines genes (Wei, Wei et al. 2009). Here the authors found that promoters of *Tbx21* and *Gata3*, key transcription factors in Th1 and Th2 development respectively, were incompletely silenced in the context of the ROR γ t-dominated program that is characteristic of Th17 development. Conversely the *Rorc* promoter was marked only by repressive methylation in Th1 or Th2 cells.

There is much evidence to support the theory of repolarisation of “committed” Th17 precursors. A study using a reporter mouse system showed that Th17 cells stimulated with IL-12 undergo a STAT4 and T-bet-dependent transition to a Th1-like profile, with suppression IL-17 and upregulation of IFN γ (Lee, Turner et al. 2009). The same study showed that developing Th17 cells expressed both the IL-23R and IL-12R β 2, resulting in their being responsive to IL-23 and IL-12. Additional evidence suggests that a combination of IFN γ and IL-12 signalling is required for conversion of Th17 cells into Th1/Th17 cells (Lexberg, Taubner et al. 2010). Here the authors showed that IFN γ was required for upregulation of IL-12R β 2, whilst IL-12 was required for Th1 polarization. It has been shown that IL-12 can have a repolarizing effect on Th17 cells, causing a transition from a Th17 to a Th1 phenotype in Th17 precursor cells (Annunziato, Cosmi et al. 2007). The same can be

seen with a repolarizing effect on IL-4 on Th17 cells, shifting them towards a Th2 phenotype (Lexberg, Taubner et al. 2008).

A further study has suggested that Th17 cells have distinct plasticity in different inflammatory settings (Hirota, Duarte et al. 2011). In this study the authors used a reporter mouse strain designed to map the fate of cells that had, at some point, switched on an IL-17 program. In EAE, a chronic inflammatory condition, there was a switch from IL-17A to IFN γ production, whereas in acute cutaneous infection with *Candida albicans* production of IL-17A was also shut down but there was no deviation to IFN γ production. The authors termed these cells that had deviated from IL-17 production 'ex-Th17 cells' and showed that they required IL-23 for this conversion and maintained expression of IL-1R1 even after production of IL-17A was shut off.

A number of studies have investigated cytokine production by T cells taken from sites of inflammation in various disease settings, showing the ability of these cells to co-produce cytokines associated with different T cell subsets, lending further weight to the theory that T cells are relatively plastic in terms of effector function. Annunziato *et al* showed the presence of Th17 cells producing either IL-17 alone or IL-17 and IFN γ in combination in the gut of patients with Crohn's disease (Annunziato, Cosmi et al. 2007). A study in mice, using an adoptive transfer approach, showed that although Th17 cells were able to promote pancreatic inflammation, they only induced diabetes in lymphopenic hosts after conversion to Th1 cells (Martin-Orozco, Chung et al. 2009).

Tregs are also thought to be relatively plastic in terms of development, with reports of IL-17 and IFN γ expression from natural and induced Tregs. FoxP3, the major transcription factor associated with Treg development, is dominant and antagonizes ROR γ t function unless IL-6

is present (Zhou, Lopes et al. 2008), such that in a pro-inflammatory microenvironment the balance between the Tregs and Th17 cells may be shifted in favor of the latter. IL-6 stimulates IL-17 expression from Tregs, whilst down-regulating Foxp3 expression (Xu, Kitani et al. 2007). Upon activation purified CD25⁺ T cells produced high amounts of soluble TGF β and when cultured with CD4⁺CD25⁻Foxp3⁻ T cells, in the presence of IL-6, the former cells induced the latter to differentiate into Th17 cells. Moreover, upon activation CD4⁺CD25⁺Foxp3⁺ T cells were able to differentiate into Th17 cells. This effect was abrogated in STAT3-deficient mice, consistent with the requirement for STAT3-mediated suppression of Foxp3 in developing Th17 cells (Yang, Nurieva et al. 2008).

1.5. Th17 cells

1.5.1. Th17 cell discovery and differentiation

The presence of Th17 cells first came to light after studies into experimental allergic encephalomyelitis (EAE). Initial investigations had pointed to EAE as a Th1 mediated disease, however this was disputed when further investigation showed that despite abrogation of IFN γ expression, a classical Th1 cytokine, disease still developed in these mice (Krakowski and Owens 1996). This was followed by the discovery of p19, a novel cytokine chain, able to form heterodimers with the p40 chain of IL-12 (Oppmann, Lesley et al. 2000), and this biologically active cytokine was named IL-23. IL-12 is formed by a heterodimer of p35 and p40, and when IL-23p19 knockout mice were compared to IL-12p35 knockout mice it was shown that IL-23 was involved in the initiation of EAE, and not IL-12 as previously thought (Cua, Sherlock et al. 2003).

Th17 cells are thought to be protective against extracellular pathogens and fungal infections. Evidence to support this comes from investigations into patients with Hyper IgE syndrome. Here a dominant negative mutation in STAT3 leads to impaired Th17 differentiation and a subsequent deficiency of these cells, leaving patients with increased susceptibility to infections with *Staphylococcus aureus* and *Candida albicans* (Ma, Chew et al. 2008).

So called because of the production of IL-17, Th17 cells develop from naïve CD4⁺ CD161⁺ T cells following exposure to IL-1 β and IL-6. IL-1 β acts in synergy with IL-6 for expression of ROR γ t, the transcription factor responsible for driving Th17 differentiation (Wilson, Boniface et al. 2007). Following initial differentiation IL-21, induced by IL-6, acts as a positive feedback amplifier for Th17 differentiation (Nurieva, Yang et al. 2007). IL-23 is required for the terminal differentiation of IL-17 producing effector T helper cells *in vivo* (McGeachy, Chen et al. 2009).

The aryl hydrocarbon receptor (AhR) is also important for the differentiation of Th17 cells. It is specifically induced in naïve T cells under Th17-polarizing conditions and its ligation results in the production of IL-22 (Quintana, Basso et al. 2008; Veldhoen, Hirota et al. 2008). The AhR has been shown to interact with STAT1 and STAT5, and after stimulation with TGF β and IL-6 STAT1 was seen to remain activate in AhR-deficient naïve T cells (Kimura, Naka et al. 2008), leading the authors to conclude that the AhR was involved in Th17 cell differentiation through regulating STAT1 activation. A further study showed that in addition to acting as a transcription factor, AhR functions as a ligand-dependent E3 ubiquitin ligase, thus it is possible that AhR marks activated STAT1 for degradation in Th17 cells (Ohtake, Baba et al. 2007).

A requirement for TGF β in the development of Th17 cell was initially unclear. Studies in mice demonstrated a requirement for TGF β (Veldhoen, Hocking et al. 2006), but in humans the first studies suggested it was not required (Acosta-Rodriguez, Napolitani et al. 2007; Wilson, Boniface et al. 2007). However subsequent studies found contradictory results, suggesting TGF β is in fact essential for Th17 differentiation (Volpe, Servant et al. 2008; Yang, Anderson et al. 2008). It was proposed that previous studies had underestimated the role of TGF β since the CD4⁺ T cells in these experiments were cultured in human or bovine serum, which usually contain TGF β . TGF β is produced by many cells of the immune system, as well as stromal cells. Interestingly there is also a requirement for TGF β in the development of induced Treg cells, but where TGF β is present with the proinflammatory cytokine IL-6, T cells are skewed towards Th17 differentiation. Dendritic cells, which have been activated by microbial products, provide a source of IL-6 (Zhou, Ivanov et al. 2007).

IL-23 is produced by many cells of the immune system, including dendritic cells and macrophages (Oppmann, Lesley et al. 2000; Korn, Bettelli et al. 2009). Its receptor, IL-23R, is also a heterodimer of IL-12R β 2 & IL-23R, and is expressed on memory T cells but not on naïve T cells (Wilson, Boniface et al. 2007). Figure 1.4 shows the cytokines IL-12 and IL-23, highlighting the common p40 chains, and the shared β 1 subunit of the IL-12 and IL-23 receptors.

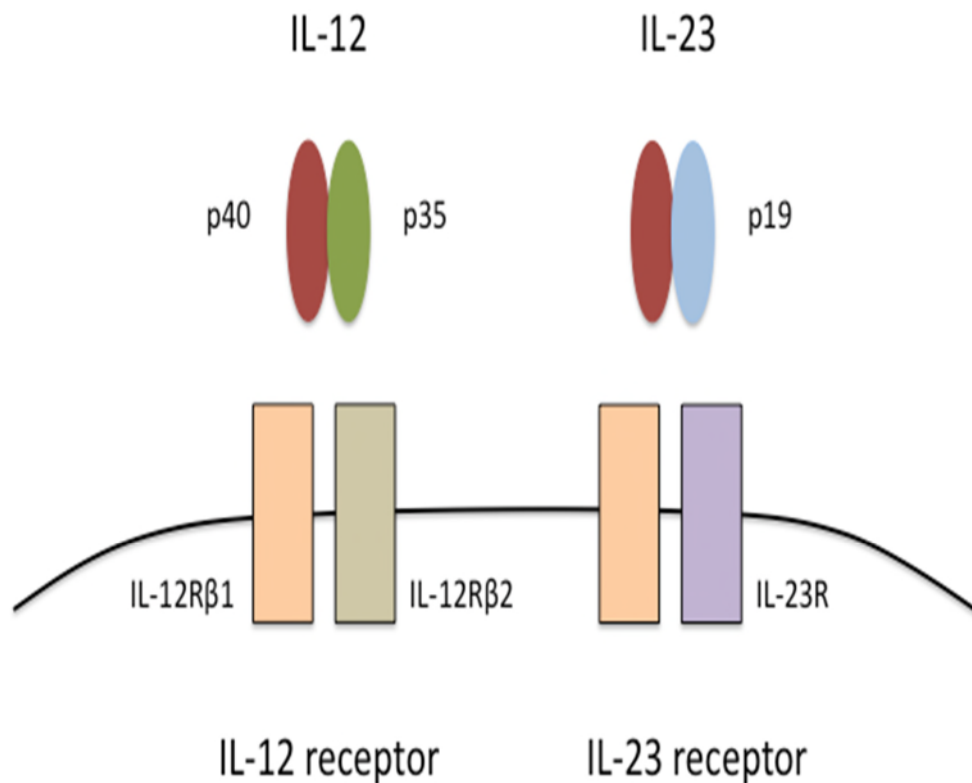


Figure 1.4. Shared subunits of IL-12 and IL-23 and the IL-12 receptor and IL-23 receptor. IL-12 and IL-23 are heterodimers, which share a common p40 subunit. Their downstream effects reflect the cells that express their respective receptors, which also share a common chain (IL-12Rβ1). Adapted from (Bell, Reynolds et al. 2011).

1.5.2. Cytokine production and phenotype of Th17 cells

In addition to Th17 cells a number of other cells produce IL-17. This includes $\gamma\delta$ T cells (Lockhart, Green et al. 2006), a subset of NKT cells (Lee, Kang et al. 2008), mast cells (Noordenbos, Yeremenko et al. 2011) and neutrophils (Ferretti, Bonneau et al. 2003). IL-17 has effects on a wide range of target cells, including epithelial and endothelial cells,

fibroblasts, keratinocytes, osteoblasts, monocytes and macrophages (reviewed in (Onishi and Gaffen 2010)). IL-17 acts on target cells leading to the release of various cytokines, including TNF α and IL-1 β (Jovanovic, Di Battista et al. 1998). It can also induce chemokine expression, including CXCL-1, -8 and -10, and matrix metalloproteinase expression (Laan, Cui et al. 1999; Chabaud, Garnerio et al. 2000).

Six subtypes of IL-17 are recognized, classified as IL-17A to IL-17F. IL-17A, henceforth termed IL-17, was the first family member to be discovered and much work has focused on this cytokine and its association with various autoimmune diseases. It should be noted that IL-17E is produced by Th2 cells and not by Th17 cells (Fort, Cheung et al. 2001). The most recently discovered family member, IL-17F, has the highest homology to IL-17A, as well as being localized to the same region on chromosome 6 (Hymowitz, Filvaroff et al. 2001). In addition to forming homodimers, IL-17 and IL-17F are able to form heterodimeric complexes (Chang and Dong 2007; Wright, Guo et al. 2007), as seen in Figure 1.5. To what extent IL-17F plays a role in both health and disease compared to IL-17 remains to be elucidated. One study revealed that like IL-17, IL-17F is able to induce CXCL1, IL-6, CCL2, CCL7, and MMP13, although levels of gene expression were more moderate, compared to IL-17, after treatment with IL-17F (Yang, Chang et al. 2008). The same study showed that mice deficient in IL-17F, but not IL-17, had defective airway neutrophilia in response to allergen challenge, but that IL-17, and not IL-17F, was required for the initiation of EAE.

It is clear that numerous cells are capable of producing IL-17 but less clear is the relative contribution of each of these cell types. In addition the relative levels of IL-17, as compared to IL-17F and IL-17A/F heterodimers is still unknown and work needs to be carried out to determine this as it is relevant when targeting cytokines in both monitoring and treating diseases.

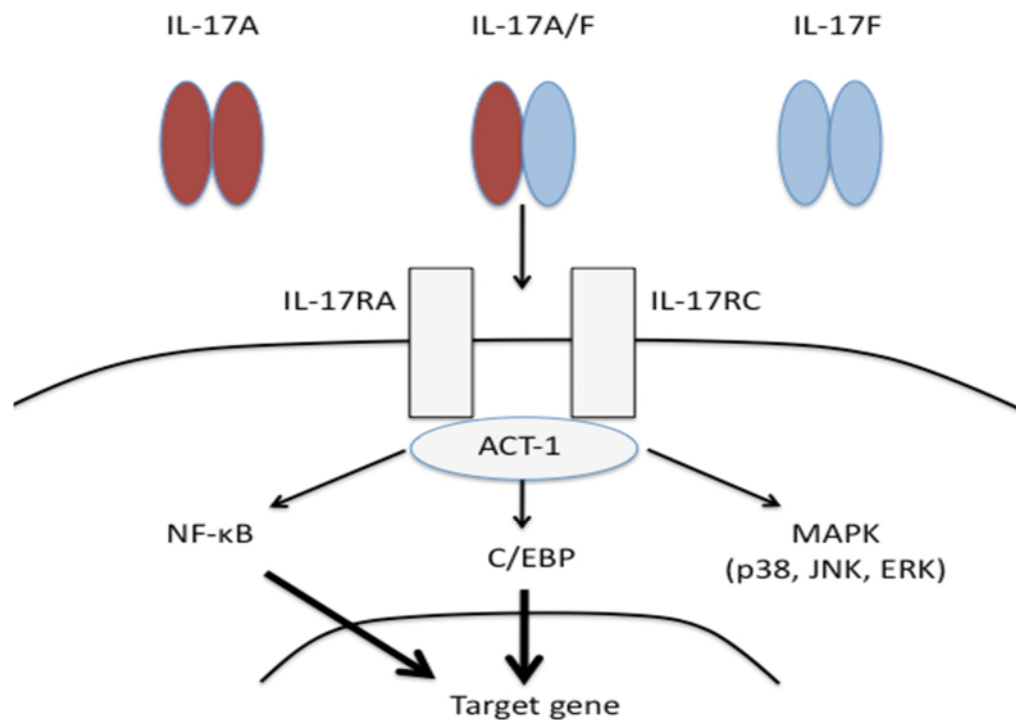


Figure 1.5. IL-17 signaling pathways. IL-17A and IL-17F are homodimer but can also form heterodimer, able to bind to the IL-17R (a heterodimer of IL-17RA and IL-17RC). Upon binding Act1 associates with IL-17RA and/or IL-17RC through its SEFIR domains. This then allows the complex to associate with TRAF6, leading to the activation of NF-κB, MAPK-AP-1, and C/EBP. Act1-independent ERK activation also contributes to IL-17R signaling via an unknown molecule. Adapted from (Iwakura, Ishigame et al. 2011).

There are five receptors for IL-17, termed IL-17RA to IL-17RE, each with one transmembrane domain and a large intracellular C terminus (reviewed in (Korn, Bettelli et al. 2009). IL-17RA is the cognate receptor for IL-17 and IL-17RC is the cognate receptor for IL-17F.

Furthermore, IL-17RA can form heterodimers with IL-17RC, thereby allowing binding of IL-17A and IL-17F. IL-17RA is expressed ubiquitously, although highest expression is seen on hematopoietic cells (Yao, Fanslow et al. 1995). In contrast IL-17RC is expressed at high levels in glandular tissues but is not detectable on hematopoietic tissues (Kuestner, Taft et al. 2007).

IL-22, an IL-10 family member, is another cytokine secreted by Th17 cells, whereupon it acts predominantly on epithelial cells (Liang, Tan et al. 2006). Overexpression of IL-22 has been observed in various chronic inflammatory conditions, including IBD, RA and psoriasis (Andoh, Zhang et al. 2005; Ikeuchi, Kuroiwa et al. 2005; Wolk, Witte et al. 2006). In the latter case the elevation in IL-22 correlated with disease activity and was reduced by anti-psoriatic therapy. As well as pro-inflammatory effects anti-inflammatory roles of IL-22 have been described, particularly in host defence against extracellular bacteria, which is thought to be via the actions of β -defensins and/or S100 proteins (Liang, Tan et al. 2006; Wolk, Witte et al. 2006). Moreover IL-22 induces proliferation and induces anti-apoptotic pathways in responsive cells suggesting a role in tissue regeneration (Radaeva, Sun et al. 2004). Given the dual nature of IL-22, it is thought its physiological action likely depends on the inflammatory context, for example the cytokine microenvironment, tissue localization and the amount of IL-22 produced (reviewed in (Zenewicz and Flavell 2011)).

IL-26, a further member of the IL-10 family, has been shown to be secreted by Th17 cells in the inflamed colon of individuals with Crohn's disease (Dambacher, Beigel et al. 2009). In this study the authors demonstrated that stimulation of intestinal epithelial cell lines with IL-26 increased mRNA expression of proinflammatory cytokines TNF α and IL-8, but decreased cell proliferation.

A number of putative surface markers have been used to identify Th17 cells. Once such marker is the chemokine receptor CCR6 (Acosta-Rodriguez, Rivino et al. 2007). CCR6 is thought to be involved in the movement of Th17 cells through the choroid plexus, into the CNS, where they are reported to be required for the initiation of EAE (Reboldi, Coisne et al. 2009) and for localization to the gut (Wang, Kang et al. 2009). Th17 cells also express CCL20 (Wilson, Boniface et al. 2007). Interestingly CCL20 is a ligand for CCR6 (Hoover, Boulegue et al. 2002; Acosta-Rodriguez, Rivino et al. 2007). Since IL-23R is expressed on Th17 cells but not on naïve CD4⁺ T cells it is also considered useful for the identification of Th17 cells (Wilson, Boniface et al. 2007). RANKL is another marker associated with Th17 cells (Acosta-Rodriguez, Rivino et al. 2007), as is the chemokine receptor CCR2, but only in the absence of CCR5 (Sato, Aranami et al. 2007). A further marker of Th17 cells was described by Cosmi *et al*, who showed that CD161 was highly up-regulated by Th17 clones (Cosmi, De Palma et al. 2008). The authors also demonstrated that these IL-17-producing cells originated from CD161⁺ naïve CD4⁺ T cells in cord blood in response to IL-1 β and IL-23. Further studies have shown the presence of CD161⁺CD4⁺ T cells in both the intestine (O'Keefe, Doherty et al. 2004) and peripheral blood (Takahashi, Dejbakhsh-Jones et al. 2006) of healthy adults, as well as accumulations of these cells in Crohn's disease lesions (Kleinschek, Boniface et al. 2009) and in the joints of patients with rheumatoid arthritis (Warrington, Takemura et al. 2001).

In my view no single surface marker of Th17 cells has thus far been identified, however using a combination of cell surface markers, including CCR6, CD161 and IL-23R, together with intracellular cytokine staining (ICS) for the detection of IL-17, is perhaps the best way of defining this subset of cells.

There is evidence of raised levels of IL-17 in a wide variety of disease. Th17 cells that infiltrate inflamed tissue have been observed in patients with psoriasis, Crohn's disease, rheumatoid arthritis and asthma (Pene, Chevalier et al. 2008). Although the majority of studies have focused on IL-17, there is some evidence to suggest that IL-17F may also have a role in various autoimmune and inflammatory diseases. IL-17F has been shown to be elevated in serum from psoriasis patients (Wilson, Boniface et al. 2007). Here the authors looked at punch biopsies' from psoriasis patients and healthy controls and showed significantly higher expression of IL-17 and IL-17F, as well as IL-22, IL-26 and IFN γ , in lesional skin compared to nonlesional skin and to health controls (Sato, Suematsu et al. 2006). In Crohn's disease IL-17F was found to be raised in sera and colonic biopsies (Seiderer, Elben et al. 2008).

1.5.3. Th17 cells in SpA

A number of studies have investigated levels of IL-17 and characterised IL-17-producing T cells in patients with SpA. In one such study IL-17 and bone morphogenic protein (BMP)-7 were studied in serum from 28 patients with AS and compared to healthy controls (Wendling, Cedoz et al. 2007). IL-17 was significantly elevated in AS patients as compared to controls, and there was a weak correlation of serum IL-17 and TRAP, a marker of bone resorption. As such the authors hypothesised that IL-17 may play a role in the mechanism that leads to a loss in bone mineral density seen in AS patients. More recently the same group investigated p40, the shared subunit of IL-12 and IL-23, in SpA patients and compared serum and synovial levels to those in osteoarthritis patients and healthy controls (Wendling, Cedoz et al. 2009). They found no difference in the levels of p40 IL-12/23 in serum between SpA patients and controls, however comparison of synovial fluid levels of p40 IL-12/23 between SpA patients and osteoarthritis patients revealed higher levels in SpA patients. Levels in synovial fluid were also raised compared to paired serum samples.

In another study Wang *et al* looked at IL-23 and IL-17 in serum and supernatants from cultured PBMC and noted that patients with active AS had raised levels of both cytokines, as compared to healthy controls (Wang, Lin et al. 2009). At a molecular level greater expression of IL-23p19 mRNA was detected in PBMC from AS patients. Furthermore, production of IL-17 was enhanced in the supernatant of cultured PBMC by the addition of recombinant IL-23. A further report was able to correlate the raised levels of IL-17 observed in the serum of patients with AS to those individuals defined as rapid progressors, as compared to patients with no progression despite considerable duration of disease (Maksymowych, Morency et al. 2010).

A more recent study has also observed significantly elevated serum levels of both IL-17 and IL-23 in AS patients as compared with healthy controls, although no associations of serum IL-17 and IL-23 levels with clinical parameters could be made (Mei, Pan et al. 2011).

A further investigation demonstrated raised levels of IL-17 in synovial fluid samples from non-psoriatic arthritis patients (non-PsA SpA), as compared to psoriatic arthritis (PsA) patients and RA patients (Melis, Vandooren et al. 2009). Similar levels of IL-23 were observed in the synovial fluid samples from all disease groups. In the RA group there was elevated IL-23 in serum, which correlated with disease activity. Moreover, CCL20, a chemokine involved in recruitment of Th17 cells, was elevated in the joints of RA patients as compared with SpA patients, and compared to paired serum samples.

Singh *et al* investigated synovial fluid samples from patients with reactive arthritis (ReA), undifferentiated spondyloarthritis (uSpA), RA and osteoarthritis (OA), looking specifically at levels of IL-17, IL-6, p40 IL-12/23, IFN γ , IL-10 and TNF β (Singh, Aggarwal et al.

2007). In synovial fluid samples from ReA/uSpA patients there was significantly more IL-17, IL-6, TGF β , and IFN γ compared to RA patients. Furthermore, in patients with ReA/uSpA IL-17, IL-6, IL-10, and IFN γ synovial fluid levels were significantly higher than paired serum samples.

A further study investigated Th17 cells in peripheral blood samples from healthy controls and patients with PsA, AS, RA and vitiligo (Jandus 2008). Raised numbers of circulating Th17 cells were detected only in the peripheral blood of patients with seronegative spondylarthritides and AS. These Th17 cells were polyfunctional, with up to 87% producing IL-17 and TNF α , and up to 20% of these cells simultaneously producing IL-2. The cells showed advanced differentiation, as defined by a lack of CD45RA, CCR7 and CD27 expression. More recently Shen *et al* investigated IL-17 production by PBMC from patients with AS, RA and healthy controls following *ex vivo* stimulation with phorbol myristate acetate (PMA) and ionomycin (Shen, Goodall et al. 2009). They discovered an increased frequency of IL-17+ CD4+ and IL-22+ CD4+ T cells in RA and AS patients, as compared to controls, with these cells being found in excess of IL-10+ CD4+ T cells. This was supported by an increase in the concentration of IL-17 from cultured supernatants, which was only significant when comparing AS patients and healthy controls. Phenotypically the IL-17 producing cells were CD45RO+ CD4+ T cells, with the majority expressing CCR4 and CCR6, and 50% expressing the IL-23 receptor.

In conclusion there is considerable evidence of raised levels of IL-17 in the serum, synovial fluid and supernatants of cultured PBMC from SpA patients. This is further supported by the description of increased numbers of IL-17-producing CD4+ T cells in the peripheral blood of SpA patients. In addition, given the substantial evidence provided by GWAS for a link

between genes in the IL-17/23 axis and a strong association with AS, it makes Th17 cells a crucial subset of cells to investigate when trying to understand disease pathogenesis.

1.6. Killer Immunoglobulin-like Receptors (KIRs)

1.6.1. KIRs in Health and Disease

KIRs are immunoglobulin (Ig) superfamily members, expressed on the surface of natural killer (NK) cells, and a subset of $\gamma\delta$ T cells, and memory and effector $\alpha\beta$ T cells (Mingari, Schiavetti et al. 1996; Battistini, Borsellino et al. 1997; van Bergen, Thompson et al. 2004). KIRs are characterized on the basis of structural features, including the number of extracellular Ig-like domains, of which they have two (2D) or three (3D), and the length of the cytoplasmic tail, which is either long (L) or short (S), as seen in Figure 1.6. Those with long cytoplasmic tails are referred to as inhibitory receptors owing to their immunoreceptor tyrosine-based inhibition motifs (ITIMs), which recruit SH2-domain containing protein tyrosine phosphatases. KIRs with short cytoplasmic tails contain a charged amino acid, most commonly a lysine or arginine residue, in the transmembrane domain. This permits interaction with the adaptor protein DAP12, which itself contains two immunoreceptor tyrosine-based activation motifs (ITAMs) (Rajagopalan and Long 2005). These KIRs are described as activating receptors since signalling through them results in tyrosine phosphorylation of cellular proteins and upregulation of early-activation antigens (Lanier, Corliss et al. 1998). The ligands for KIRs are HLA molecules, some of which are listed in Figure 1.6. KIRs are predominantly recognized by HLA-class I molecules but can also be recognised by non-classical HLA molecules, such as HLA-G, which is a ligand for KIR2DL4.

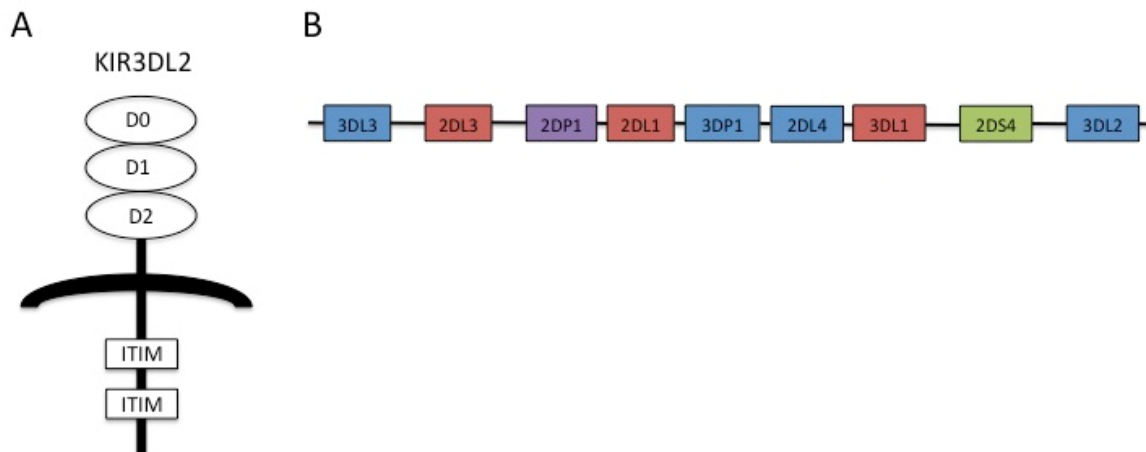


Figure 1.6. Simplified diagram of KIR3DL2 and a prototypic group A KIR haplotype. (A) KIR3DL2 protein structure, showing three extracellular domains and two intracellular immunoreceptor tyrosine-based inhibition motifs (ITIM). (B) A prototypical group A KIR haplotype, where blue boxes indicate framework genes, purple boxes pseudogenes, red boxes indicate inhibitory KIR and green boxes represent activating KIR genes. The KIR gene family consists of 15 gene loci encoded within a 150 Kb stretch of the Leukocyte Receptor Complex (LRC) on chromosome 19. KIR genes vary in length from 4 to 16 Kb and are organised into two broad haplotypes (A and B), which both include four framework genes (see text for further detail) (Adapted from <http://www.ebi.ac.uk/ipd/kir/introduction.html>).

After the HLA system, the KIR family is thought to be the most highly polymorphic system within the human genome. KIRs are encoded by a family of genes located on the leukocyte receptor complex (LRC) on chromosome 19q13.4 (Wende, Colonna et al. 1999), with a total of 15 genes, and two pseudogenes (denoted 'P') thus far described. Four of these (KIR2DL4, KIR3DL2, KIR3DL3 and KIR3DP1), termed framework genes, are present in all individuals (Middleton and Gonzelez 2010). Two KIR haplotypes exist in humans, termed A and B. The

A haplotype has fewer genes than the B haplotype but exhibits significant allelic variation (Shilling, Guethlein et al. 2002). In contrast the B haplotype has more genes but with relatively little allelic polymorphism (Yawata, Yawata et al. 2002). The frequency of these haplotypes is relatively even in Caucasian populations (Uhrberg, Valiante et al. 1997), although there are reports of variability in other populations, with the A haplotype being more prevalent in the Japanese population (Yawata, Yawata et al. 2002) and the B haplotype being more prevalent in the Afro-Caribbean population (Cook, Moss et al. 2003).

Allelic polymorphism generates further diversity, with some KIR genes having over 50 different alleles (Traherne, Martin et al. 2010). Point mutations and homologous recombination are frequently localized to loops of the Ig-like domains, suggesting selection of polymorphisms that contribute to interaction with ligands (Snyder, Brooks et al. 1999; Fan, Long et al. 2001; Gardiner, Guethlein et al. 2001).

In contrast to NK cells, which express KIRs throughout development, T cells are proposed to express KIRs only after rearrangement of the *Tcr* genes (Vely, Peyrat et al. 2001). Interestingly KIRs have been shown to be differentially expressed amongst TCR-clonal lineages, both in terms of receptor type and density (Snyder, Muegge et al. 2002).

An increase in KIR expression is seen with age and *de novo* KIR expression has been described on CD28^{null} CD8⁺ T cells, a phenotype associated with pre-senescent and senescent T cells (Tarazona, DelaRosa et al. 2000; Vallejo 2005). Elevation of KIR⁺ T cells has also been shown in chronic inflammatory conditions, including RA, where CD28^{null} CD4⁺ T cells preferentially express KIR2DS2 (Yen, Moore et al. 2001), which costimulates TCR-mediated IFN γ production and proliferation (Namekawa, Snyder et al. 2000; Warrington, Takemura et al. 2001). Moreover, an association has been seen between the expression of specific KIRs

and HLA-class-I molecules and the development of autoimmune disease and control of certain infections. One such study documented an increased susceptibility to PsA in individuals with activating KIR2DS1 and/or KIR2DS2 genes, only when HLA ligands for their homologous inhibitory receptors, KIR2DL1 and KIR2DL2/3, were missing (Martin, Nelson et al. 2002). The same authors reported that the activating KIR KIR3DS1, in combination with HLA-B alleles, is associated with delayed progression to acquired immunodeficiency syndrome (AIDS) in individuals infected with human immunodeficiency virus type 1 (Martin, Gao et al. 2002).

1.6.2. KIR3DL2

KIR3DL2, also termed CD158K, is one of four framework genes. It has a long cytoplasmic tail containing two ITIMs and is known to bind to HLA-B27 dimers and free heavy chains but not conventionally folded HLA-B27 (Kollnberger, Bird et al. 2002). KIR3DL2 also binds to HLA-A3 and HLA-A11 (Dohring, Scheidegger et al. 1996; Hansasuta, Dong et al. 2004), with binding dependent on the sequence of complexed viral peptide (Hansasuta, Dong et al. 2004). KIR3DL2 is expressed as a 140kD disulphide-linked dimer (Pende, Biassoni et al. 1996). The KIR3DL2 gene incorporates nine exons, encoding for a protein with D0, D1, and D2 Ig-like domains, stem, transmembrane and cytoplasmic tail region (Colonna and Samaridis 1995).

NK and CD4⁺ T cells expressing KIR3DL2 are expanded in the peripheral blood of patients with SpA, as compared to healthy controls (Chan, Kollnberger et al. 2005), with an average of 11.2% CD4⁺ T cells and 43.5% NK cells expressing this particular KIR in peripheral blood. When looking specifically at patients with HLA-B27-associated SpA this number increased to 12.4% on CD4⁺ T cells and 47.5% on NK cells. Overall levels of KIR expression on NK cells in synovial fluid was lower than in peripheral blood and this was attributed to the predominance of the CD56^{bright} population in synovial fluid, whilst KIRs are predominantly

expressed on the CD56^{dim} population. Immunophenotyping of these KIR3DL2+ CD4+ T cells demonstrated increased expression of CD45RO and low or absent expression of CD28 and CCR7, suggesting an effector memory phenotype. KIR3DL2+ NK cells co-expressed CD38, indicating they had recently been activated, as well as perforin, which is important for cytotoxicity. KIR3DL2+ NK cells also expressed higher levels of β 7 integrin, a marker of cells that have recirculated from the gut, compared to KIR3DL2- NK cells. In functional studies KIR3DL2+ NK cells were protected from apoptosis by culture with LBL.721.220B27 cells, an EBV immortalized B cell line known to express elevated levels of B27. These cells also exhibited greater cytotoxicity against K562 targets than healthy controls. A further study showed that NK and T cell function was altered upon binding of KIR3DL2 to HLA-B27 dimers (Kollnberger, Chan et al. 2007). Culture of KIR3DL2+ NK cells with LBL.721.220B27 cells resulted in secretion of IFN γ . This effect was not observed with control LBL.721.220 cells expressing either HLA-B7 or HLA-B8, or in LBL.721.220 cells transfected to overexpress human tapasin, which reduces HLA-B27 dimer formation by stabilizing the peptide-loading complex for the formation of HLA-B27 heterotrimers.

Genetic techniques have been employed to investigate a possible association between specific KIR genes and AS (Harvey, Pointon et al. 2009). The authors genotyped 200 patients with AS and a further 405 healthy controls for 14 different KIR genes and found that the frequency of both KIR3DL1 and KIR3DS1 were similar between patients and controls. Sequence-specific oligonucleotide probes were used to subtype 368 patients with AS and 366 controls, for a total of 12 KIR3DL2 alleles. The study was unable to determine a significant difference in the frequency of any alleles between the patient and control groups, although it did not exclude the possibility that a rare allelic variant of KIR3DL2 is associated with disease. At present no information on the function of these different allelic variants has been identified.

KIR3DL2⁺ CD4⁺ T cell expansions are also seen in Sézary syndrome, a cutaneous T-cell lymphoma, with malignant CD4⁺ T cells (SS cells) in skin, lymph nodes, and blood (Bagot, Moretta et al. 2001). Both circulating and infiltrating T cell lymphoma cells expressing this particular KIR are observed in patients with Sézary syndrome (Musette, Michel et al. 2003; Poszepczynska-Guigne, Schiavon et al. 2004). Of note, these SS CD4⁺ T cells demonstrate constitutive phosphorylation of STAT3 (van der Fits, Out-Luiting et al. 2012) and produce IL-17 (Ciree, Michel et al. 2004).

1.7 Project Aims

Despite extensive research into the pathogenesis of AS and the known association with HLA-B27, the mechanisms underlying this association remain unclear. There are three main theories to explain the link between AS and HLA-B27, as described in section 1.3. The work described herein relates to the ability of aberrantly folded forms of HLA-B27 to bind immunomodulatory receptors, with particular focus on the interaction of HLA-B27 with KIR3DL2, and its functional effects.

The specific objectives of my research are detailed at the start of each chapter. The overall aims of the work are

1. To investigate surface markers on KIR3DL2+ CD4+ T cells to more fully determine the phenotype of KIR3DL2+ CD4+ T cell in the peripheral blood of patients with SpA as compared to healthy and inflammatory disease controls.
2. To investigate cytokine production by KIR3DL2+ CD4+ T cells in order to more fully determine the function of KIR3DL2+ CD4+ T cell in the peripheral blood of patients with SpA as compared to healthy and inflammatory disease controls.
3. To investigate whether activation induces and/or increases KIR3DL2 expression on CD4+ T cells.
4. To investigate the effect that co-culturing naïve CD4+ T cells with HLA-B27 expressing cell lines has on CD4+ T cell development and differentiation.

Characterization of KIR3DL2+ CD4+ T cells, in terms of expression of cell surface markers and production of cytokines, will provide additional insight into whether these cells play a role disease, and how they might be doing so. Exploring the mechanisms that initiate and

maintain KIR3DL2 expression is valuable to explain the observed increase in KIR3DL2+ CD4+ T cells in SpA patients. Moreover, determining what the interaction of aberrantly folded HLA-B27 with KIR3DL2 is doing in disease is essential to improve understanding of AS pathogenesis and to target therapies in HLA-B27 related disorders.

Chapter 2: Materials and Methods

2.1. Patient and Control Recruitment

AS and RA patients were recruited from clinics in Oxford and Reading, with ethical permission to participate in the study entitled 'Immune function in inflammatory arthritis' (REC reference number 06/Q1606/139). All patients were HLA-B27+ and none had received biological treatment. Healthy controls were recruited from the same area. Leukocyte cones were obtained from the National Blood Service.

2.2. Laboratory Methods

2.2.1 Mononuclear cell separation from peripheral blood and synovial fluid

Venous blood or synovial fluid was collected into tubes containing sodium heparin ($\geq 1\text{U/ml}$; BD Vacutainer) as an anticoagulant or into 50ml polypropylene tubes (Falcon) prepared with 100 μl (1000U/ml) of sodium heparin (Wockhardt). Samples were diluted 1:1 in R0 (see Table 2.1 for a description of all cell culture media used in the project) and a maximum of 35mls was layered over 15mls lymphoprep (Axis Shield) in a 50ml falcon tube (BD Falcon). Tubes were centrifuged at 2000 rpm for 20 minutes without the brake. The cellular interface was collected with a Pasteur pipette and resuspended in R10. Cells were washed twice in R10 by centrifugation at 1800 rpm for 10 minutes, then at 1500rpm for 10 minutes.

To count cells they were resuspended in R10 and diluted 1:1 in 0.4% Trypan blue solution (Sigma), added to a FastRead disposable cell counting system (Immune Systems, BVS100)

and counted under a microscope (Nikon). Alternatively the automated cell counting machine (ABX Pentra 60) was used.

Name	Composition
R0	RPMI-1640 (Sigma) + 2mM l-glutamine (Sigma), 50 µg/ml penicillin (PAA) + 50 µg/ml streptomycin (PAA)
R10	R0 + 10% sterile filtered heat inactivated FCS (Sigma)
R10-G418	R10 + 0.5mg/ml Geneticin® (Gibeco)
D10	Dulbecco's Modified Eagle Medium (DMEM; Sigma) + 2mM l-glutamine, 50 µg/ml penicillin and 50 µg/ml streptomycin + 10% sterile filtered heat inactivated FCS
D10 rIL-2	D10 + 50 units/ml IL-2

Table 2.1: Media used in experiments

2.2.2. Cryopreservation of peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC)

For storage PBMC and SFMC were pelleted by centrifugation at 1800 rpm for 10 minutes, the supernatant removed and cells resuspended at $7-10 \times 10^6$ cells/ml in freezing medium (10% dimethyl sulphoxide, Sigma, in FCS). Cells were frozen in 1ml aliquots in labeled cryovials (Alpha laboratories) and transferred to storage at -80°C in a freezing container (Nalgene). For long-term storage cells were transferred to liquid nitrogen.

2.2.3. Thawing of PBMC and SFMC

PBMC or SFMC were thawed by placing cryovials in a water bath at 37°C for rapid thawing, before transferring to a 15ml falcon. Cells were washed twice in R10, by centrifugation at 1500rpm for 10 minutes, before use.

2.2.4. Cell lines

LCL.721.220 (.220) and LCL.721.221 (.221) cells, either transfected or lentivirally-transduced to express HLA-B27 or control HLA alleles, were made by Simon Kollnberger and Jackie Shaw. .220 cells are a human B-lymphoblastoid cell line (B-LCL) derived by mutagenesis, where HLA-A and HLA-B are deleted and low levels of a non-functional mutant tapasin are expressed (Shimizu and DeMars 1989). HLA-Cw1 remains intact on one chromosome, however the surface expression of HLA-Cw4 is reduced to low levels since the cells lack expression of functional tapasin. .221 cells are a human B-LCL derived by mutagenesis in which HLA-A, HLA-B, and HLA-C are deleted, but functional tapasin is retained (Shimizu and DeMars 1989). Transfection of HLA-class I genes into .221 cells restores surface expression to levels comparable to other B-LCLs, indicating intact and functional antigen presentation machinery (Shimizu and DeMars, 1989). In contrast, surface expression of class I molecules in .220 cells is only seen for some alleles, including HLA-B27, suggesting HLA-B27 transport to the cell surface is less dependant on tapasin (Peh et al., 1998). Experiments using surface iodination and antibodies staining β_2m -free heavy chains shows that HLA-B27 dimers and multimers are expressed at the surface of stably transected with HLA-B27 (Bird, Peh et al. 2003). Figure 2.1. shows representative FACS staining of .220 and .221 cells expressing HLA-B27 or HLA-B7, where indicated. FACS staining was carried out with HD6, an antibody that recognized HLA-B27 dimers, and W6/32, an antibody that reacts with correctly folded HLA-class I molecules (Barnstable, Bodmer et al. 1978). Transected cell lines express similar levels of W6/32 but expression of HD6 is higher on HLA-

B27 transfected cell lines.

All cell lines were maintained in R10, with the exception of transfected .220 and .221 cells, which were maintained in R10-G418, and all cell lines were split twice weekly. Cells were cultured in sterile tissue culture flasks at 37°C with 5% CO₂. Before use in experiments cell lines were irradiated with 3300 rads using the irradiator in the Nuffield Department of Surgery in the John Radcliffe Hospital.

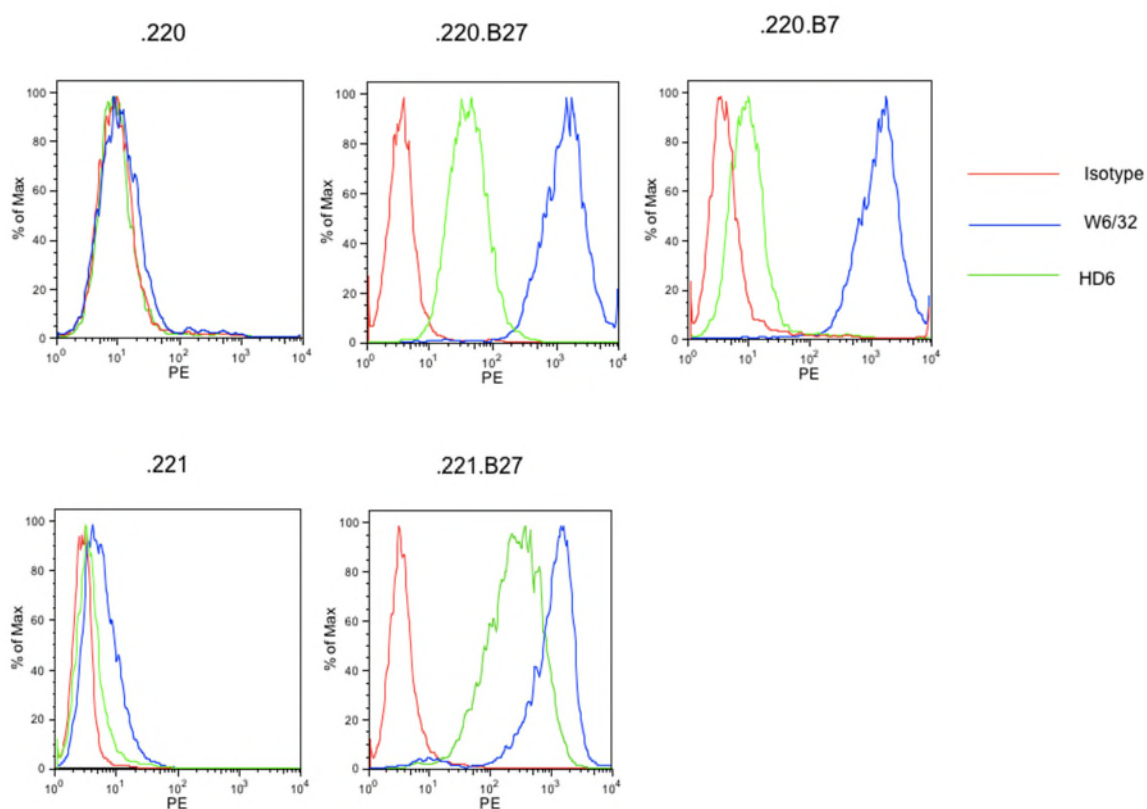


Figure 2.1. .220 and .221 cell lines expressing HLA-B27 stain with HD6. Representative FACS staining showing staining with HD6, an antibody that recognized HLA-B27 dimers, and ME-1, an antibody that reacts with correctly folded HLA-class I molecules, on .220 cells (top left) or .220 cells transfected with HLA-B27 (top middle) or HLA-B7 (top right), or on .221 cells (bottom left) or .221 cells transfected with HLA-B27 (bottom right).

Jurkat cells expressing KIR3DL2, for testing of conjugated DX31 antibody, were made by Jacqueline Shaw and Simon Kollnberger.

2.2.5. DNA preparation for HLA typing

DNA extraction was performed using a PureLink™ Genomic DNA Mini Kit (Invitrogen). 5×10^6 PBMC were pelleted by centrifugation for 5 minutes at 1500rpm and resuspended in 200µl sterile PBS. This was added to 20µl proteinase K in a 1.5ml eppendorf, with 20µl RNase A. After vortexing and incubation at room temperature for 2 minutes 200µl genomic lysis/binding buffer was added and samples were vortexed and heated to 55°C for 10 minutes. 200µl 96-100% ethanol was added and the lysate was added to a spin column in a collection tube and centrifuged at 10,000g for 1 minute. This was followed by washing with 500µl wash buffer one, a 1 minute centrifugation at 10,000g, and a further wash with 500µl wash buffer two, with a 3 minute centrifugation at 10,000g. The spin column was transferred to a 1.5ml microtube, 100µl elution buffer was added and incubated for 1 minute at room temperature. A final centrifugation at 10,000g was performed and the DNA in the microtube was quantified by the ND100 NanoDrop Spectrophotometer (NanoDro) at 260nm.

Tissue typing was carried out on DNA samples by Tim Rostron from the HLA Typing Laboratory at the Weatherall Institute of Molecular Medicine (WIMM). HLA typing was carried out by polymerase chain reaction-sequence specific primers (PCR-SSP) analysis. All known specificities of HLA-A, HLA-B, HLA-C, DRB1, DRB3, DRB4, DRB5 and DQB1 were detected using SSP in an allele or group specific manner.

2.2.6. Cell isolation using magnetic beads

2.2.6.1. CD4+ T cell isolation

Isolation was performed using the CD4+ T cell isolation kit II (Miltenyi Biotec) by a process of negative selection. This method uses a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ , and glycophorin A, leaving untouched CD4+ T cells. 1×10^7 cells/ml were suspended in 40 μ l MACS buffer. 10 μ l biotin-antibody cocktail was added per 1×10^7 cells/ml, mixed and incubated on ice for 10 minutes. Following incubation 30 μ l MACS buffer and 20 μ l anti-biotin microbeads were added per 1×10^7 cells/ml, mixed and incubated for a further 15 minutes on ice. Cells were washed by adding 10 times the labelling volume of MACS buffer and centrifuging at 1800 rpm for 10 minutes. After washing cells were resuspended in MACS buffer, with up to 1×10^8 cells/ml being resuspended in 500 μ l MACS buffer.

LS or MS columns were used for magnetic separation, depending on cell numbers, with MS columns being used for isolation with up to 1×10^7 labelled cells and LS columns being used for isolation with up to 1×10^8 labelled cells (Miltenyi Biotec). To prepare the columns 500 μ l MACS buffer was applied to a MS column or 3ml MACS buffer was applied to a LS column. The cell suspension was applied to a pre-separation filter (Miltenyi Biotec) to remove cell clumps and passed through the column. The column was washed 3 times in 500 μ l MACS buffer. The eluted fraction, containing the CD4+ T cells, was washed twice with MACS buffer. Using this method a purity of >95% was achieved.

2.2.6.2. Naive CD4+ T cell isolation

Naïve CD45RO- CD4+ T cells were obtained from PBMC using the Naive CD4+ T Cell Isolation Kit II (Miltenyi Biotec) by a process of negative selection. This method uses a cocktail of biotin-conjugated antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCR γ/δ , HLA-DR and glycophorin A, leaving untouched naïve CD4+ T cells. 1×10^7 cells/ml were suspended in 40 μ l MACS buffer. 10 μ l biotin-antibody cocktail was added per 1×10^7 cells/ml, mixed and incubated on ice. After 10 minutes cells were washed by adding 2mls of MACS buffer/ 1×10^7 cells and centrifuging at 1800 rpm for 10 minutes. After removing the supernatant 30 μ l MACS buffer and 20 μ l anti-biotin microbeads were added per 1×10^7 cells/ml, mixed, and incubated for a further 15 minutes on ice. Cells were washed by adding 10 times the labelling volume of MACS buffer and centrifuging at 1800 rpm for 10 minutes. After washing cells were resuspended in buffer, with up to 1×10^8 cells/ml in 500 μ l buffer.

To increase purity labelled cells were first added to a LS column (Miltenyi Biotec) and then to a MS column (Miltenyi Biotec). Cells were passed through a pre-separation filter to remove cell clumps and then passed through the column. The column was washed 3 times in 500 μ l MACS buffer. The eluted fraction, containing the CD45RO-CD4+ T cells, was washed twice with MACS buffer. Using this method a purity of >90% was achieved.

2.2.7. Conjugation of DX31 or isotype control antibody

DX31, an IgG2a KIR3DL2-specific monoclonal antibody, was manufactured in the WIMM by members of the Laboratory Management team in the Human Immunology Unit (HIU). DX31 or an isotype control antibody were conjugated to Alexa Fluor 647 using an Alexa Fluor® 647 monoclonal antibody labeling kit (Invitrogen). A 1M solution of sodium bicarbonate was prepared by adding 1ml of deionized water to one vial of sodium bicarbonate. 10 μ l of this solution was added to the DX31 or IgG2a antibody and 100 μ l of this solution was

transferred to a vial of reactive dye, mixed and incubated at room temperature for 1 hour, then incubated at 4°C overnight. A spin column was placed on a 5ml polystyrene tube and 1.5ml of purification resin added to the column. The tube was centrifuged for 3 minutes at 1100g and the flow through was discarded. 100µl of the reactive dye-protein-sodium bicarbonate mix was added to the spin column and centrifuged for 5 minutes at 1100g. The absorbance at A280 and A650 was measured using the Ultraspec 2000 (Pharmacia Biotech) to determine the concentration. Conjugated antibodies were tested by FACS staining Jurkat cells expressing KIR3DL2 or KIR3DL1, as shown in Figure 2.2. Labeled antibody was stored at 4°C, in the dark until use.

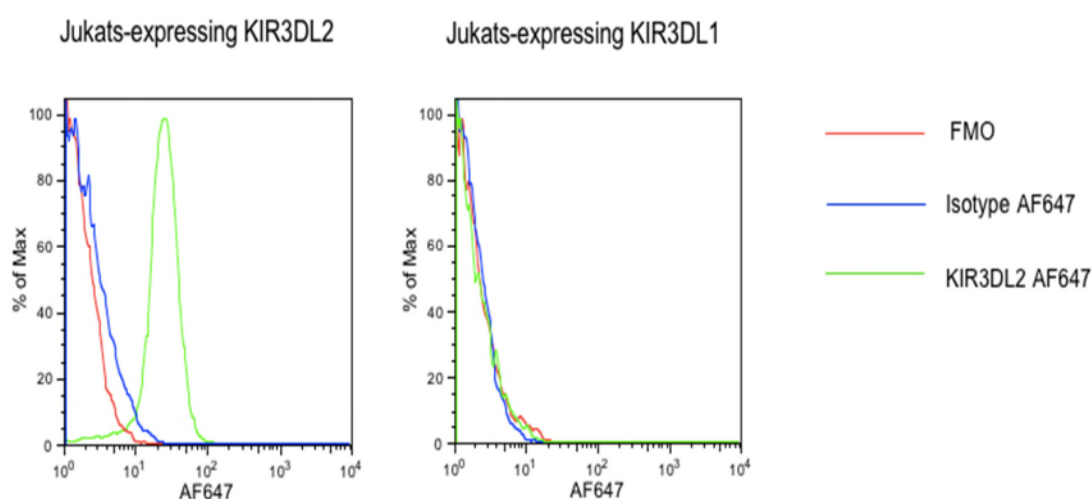


Figure 2.2. Testing of DX31 and IgG2a isotype control antibodies following conjugation. Representative FACS plots showing binding of DX31, an anti-KIR3DL2 monoclonal antibody, to Jurkat cells expressing KIR3DL2 (left) but not to Jurkat cells expressing KIR3DL1 (right). Isotype control antibody or fluorescence minus one (FMO; no AF674 conjugated antibody) shows no staining of either Jurkat cells.

2.2.8. Fluorescent Activated Cell Sorting (FACS)

FACS acquisition was performed on a CyAn ADP (Beckman Coulter) using Summit software version 3.4 and analyzed using FlowJo software version 8.8.6.

2.2.8.1. FACS staining

Table 2.2 shows all the antibodies used during the project, which includes a live/dead exclusion marker (ViViD violet; Invitrogen) in all samples, as well as appropriate isotype control antibodies, as detailed in Table 2.3.

Antibody	Fluorochrome	Catalogue Number	Supplier	Clone	Isotype
Anti-CD3	APC	300412	BioLegend	UCHT1	Mouse IgG1κ
Anti-CD3	PE	300408	BioLegend	UCHT1	Mouse IgG1κ
Anti-CD3	PerCP	345766	BD Biosciences	SK7	Mouse IgG1κ
Anti-CD3	AF647	300322	BioLegend	HIT3a	Mouse IgG2aκ
Anti-CD4	PB	300521	BioLegend	RPA-T4	Mouse IgG1κ
Anti-CD4	FITC	300506	BioLegend	RPA-T4	Mouse IgG1κ
Anti-CD4	PE-Cy7	25-0049	eBioscience	RPA-T4	Mouse IgG1κ
Anti-CD45RO	FITC	555492	BD Pharmingen	UCHL1	Mouse IgG2aκ
Anti-CD69	FITC	555530	BD Pharmingen	FN50	Mouse IgG1κ
Anti-CD134	PE	555838	BD Pharmingen	ACT35	Mouse IgG1κ
Anti-CD158a	PE	556063	BD Biosciences	HP-3E4	Mouse IgM
Anti-CD158b	PE	559785	BD Biosciences	CH-L	Mouse IgG2b
Anti-CD158b	PE	IM3292	Beckman Coulter	Z27.3.7	Not specified
Anti-CD161	PE	IM3450	Beckman Coulter	191B8	Mouse IgG2a
Anti-196 (CCR6)	PE	559562	BD Pharmingen	11A9	Mouse IgG1κ
Anti-CD199 (CCR9)	FITC	FAB179F	R&D Systems	112509	Mouse IgG2a
Anti-KIR3DL2	N/A	N/A	In-house	DX31	Mouse IgG2a
Anti-IFN γ	FITC	340449	BD FastImmune	25723.11	Mouse IgG2bκ
Anti-IL-1R	FITC	FAB269F	R&D Systems	Not specified	Goat IgG1
Anti-IL-17A	PE	12-7178	eBioscience	eBio64CAP17	Mouse IgG1κ
Anti-IL-23R	FITC	FAB14001F	R&D Systems	218213	Mouse IgG2bκ
Anti-TCR V β 3	FITC	IM2372	Beckman Coulter	CH92	Mouse IgM
Anti-TCR V β 12	FITC	IM1587	Beckman Coulter	VER2.32.1	Mouse IgG2a
Anti-TCR V β 13.2	PE	IM3603	Beckman Coulter	H132	Mouse IgG1κ
Anti-TCR V β 14	PE	IM2047	Beckman Coulter	CAS.1.1.3	Mouse IgG1κ
Anti-TCR V β 17	PE	IM2048	Beckman Coulter	E17.5F3.15.15	Mouse IgG1κ
Anti-TCR V β 20	FITC	IM1562	Beckman Coulter	ELL1.4	Mouse IgG2a
LIVE/DEAD fixable stain	Violet	L34955	Invitrogen	N/A	N/A

Table 2.2: Antibodies used in experiments

Antibody	Fluorochrome	Catalogue Number	Supplier	Clone
IgG1 isotype control	FITC	553443	BD Biosciences	A85-1
IgG2a isotype control	FITC	IC003F	R & D Systems	20102
IgG2b isotype control	FITC	MAB004	R & D Systems	20116
IgG1 isotype control	PE	550083	BD Biosciences	A85-1
IgG2a isotype control	PE	IM3046	Beckman Coulter	Not specified
IgG2b isotype control	PE	559785	BD Biosciences	CH-L

Table 2.3: Isotype control antibodies used in experiments

A total of 2×10^5 cells/well were stained in FACS wash (PBS + 1% FCS) in a total volume of 50 μ l per well. All staining was carried out for 20 minutes on ice and in the dark. After staining cells were washed twice in 200 μ l FACS wash and resuspended in 200 μ l FACS fix (FACS wash + 2% paraformaldehyde; Sigma), transferred to 5ml polypropylene tube and kept at 4°C until acquisition.

For some experiments Flow-Count Flurospheres (Beckman Coulter) were added to determine the absolute cell number. In these experiments 100 μ l FACS fix was added to each well after FACS staining and 100 μ l Flow-Count Flurospheres, diluted at 1:10 in FACS wash, were added to each well, before being transferred to 5ml polypropylene tube for acquisition.

2.2.8.2. Intracellular cytokine staining (ICS)

5×10^5 cells/well were stimulated with phorbol myristate acetate (125ng/ml; Sigma-Aldrich) and ionomycin (1 μ g/ml; Sigma-Aldrich), in the presence of brefeldin A (10 μ g/ml; Sigma-

Aldrich) and incubated at 37°C in 5% CO₂. After 6 hours plates were centrifuged at 1500 rpm for 5 minutes to pellet cells and the supernatant removed. Staining was carried out in FACS wash in a total volume of 50µl per well. Cells were stained on ice and in the dark for 20 minutes. Following FACS staining plates were centrifuged at 1500 rpm for 5 minutes and washed twice in 200µl FACS wash. Cells were permeabilised by resuspending in 130µl Cytofix/Cytoperm™ fixation and permeabilization solution (BD Bioscience) for 30 minutes on ice. Cells were washed twice in 200µl Perm/Wash™ buffer (BD Bioscience). Cells were then stained for intracellular cytokines of interest in a total volume of 50µl per well of Perm/Wash™ buffer for 30 minutes, and then washed twice in 200µl Perm/Wash™ buffer. Cells were resuspended in 200µl FACS fix, transferred to 5ml polypropylene tubes and kept at 4°C until acquisition.

2.2.8.3. Carboxyfluorescein succinimidyl ester (CFSE) labelling

CFSE labelling of PBMC was carried out using the CellTrace™ CFSE cell proliferation kit (Invitrogen) with a final labelling concentration of 10µM CFSE. PBMC were suspended in prewarmed buffer (PBS/0.1% BSA/2mM EDTA) at a final concentration of 1x10⁶ cells/ml. A 5mM CellTrace™ CFSE stock solution was prepared dissolving the contents of a vial in 18µl DMSO immediately prior to use. 2µl of this stock was then added per milliliter of cells and incubated at 37°C for 10 minutes. The reaction was quenched by addition of 5 volumes of ice-cold R10. Cells were pelleted by centrifugation at 1800 rpm for 10 minutes and washed 3 times in R10.

2.2.8.4. FACS sorting

Staining for FACS sorting was carried out in the dark and on ice, in a laminar flow hood. Compensation controls were set up for each fluorochrome used in the sort, including an

unstained sample and single stains. Wash steps were performed using R10 with the exception of the last wash, which was performed using PBS+1% FCS. After FACS staining cells were resuspended in 1 ml PBS+1% FCS. Cells were filtered into a 50ml Falcon using a 70µm nylon cell strainer (BD Falcon) and transferred to 5ml polystyrene FACS tubes for sorting (BD Falcon). Sorting was carried out using an automated cell sorter (MoFlo, Dako) by Craig Waugh and Kevin Clark from the flow cytometry facility in the WIMM. Using this method a purity of >96% was achieved, as seen in Figure 2.3

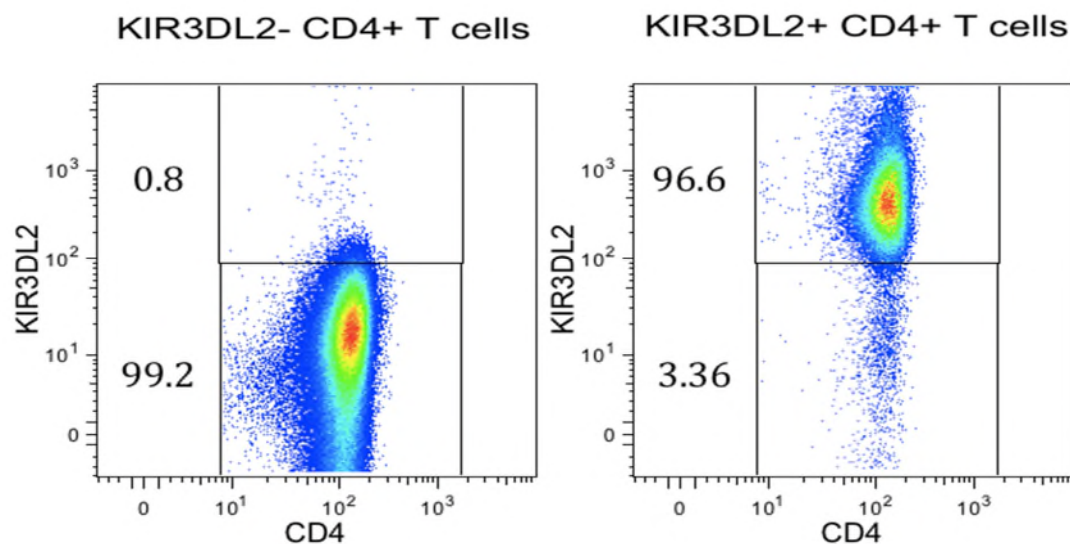


Figure 2.3. KIR3DL2 expression on CD4+ T cells after FACS sorting. PBMCs were stained for CD3, CD4 and KIR3DL2 to obtain KIR3DL2- CD4+ T cells (left) and KIR3DL2+ CD4+ T cells (right).

2.2.9. T cell activation and expansion

2.2.9.1. Anti-CD2/3/28 stimulation

A T cell activation/expansion kit (Miltenyi Biotech) was used for T cell stimulation. MACSiBead particles were prepared according to the manufactures instructions. Briefly

100µl CD2-biotinylated antibody, 100µl CD3-biotinylated antibody and 100µl CD28-biotinylated antibody were added to 500µl of anti-biotin MACSiBead particles, MACS buffer was added to make the volume up to 1ml and the mix was gently rotated for 2 hours at 2-8°C. MACSiBead particles were used at a ratio of one MACSiBead particle to two cells unless otherwise stated.

2.2.9.2. Staphylococcal enterotoxin B (SEB) stimulation

SEB (Sigma) was used at a final concentration of 100ng/ml. For wells without SEB equal volumes of culture medium was added.

2.2.9.3. Th17 polarization for multiplex bead analysis

For experiments using Th17 polarizing conditions, 10µl recombinant (r) IL-1β (20ng/ml; AMS Biotechnology) and 10µl rIL-23 (20ng/ml; R and D systems) were added to 10mls D10 rIL-2. 100µl was added per well or 100µl D10 rIL-2 was added where rIL-23/1 was not used. Cells were incubated at 37°C in 5% CO₂. After 1 day 100µl supernatant was collected, with medium being replaced as appropriate, and plates were incubate at 37°C in 5% CO₂ for a further 4 days, before supernatant was collected. Supernatant was stored at -20°C before analysis by multiplex bead analysis.

2.2.10. Antibody-co-culture experiments

DX31 or control IgG2a antibody, or HD6, an antibody that recognizes HLA-B27 dimers and HLA-B27 free heavy chains, or control IgG1 antibody, were added to appropriate wells, at a final concentration of 50µg/ml, when the experiment was set up (D0) and were topped up on day 3.

2.2.11. Enzyme-linked immunosorbent assay (ELISA)

Human ELISA Ready-SET-Go! (eBioscience) kits were used in all experiments. Washing steps were performed in PBS + 0.05% Tween-20 (Sigma-Aldrich) using the Wellwash plate washer (Thermo Scientific). 96 well flat bottom plates (Nunc) plates were coated with 100µl/well capture antibody and incubated overnight at 4°C. Plates were washed 5 times with 300µl/well wash buffer and blocked for an hour with assay diluent. Standards were prepared with the top standard at a concentration of 500pg/ml. The top standard was double diluted to give a 7-point curve, ranging from 500pg/ml to 7.81pg/ml. After 5 washes 50µl/well of samples, standards or matrix media was added and plates were incubated overnight at 4°C. Samples were either added neat or diluted in assay diluent, as determined by titration experiments. After 5 washes 100µl/well of detection antibody was added and incubated for an hour at room temperature. After a further 5 washes 100µl/well of avidin-HRP was added and plates were incubated for 30 minutes at room temperature. After a further 7 washes 100µl/well substrate solution was added and incubated in the dark at room temperature for 15 minutes. 50µl/well 2M sulphuric acid (Fisher Scientific) was added to stop the assay. Plates were read at 450nm on the 6800 plate reader (Bio-Rad), using the microplate manager 5.2.1 software. Files were imported into Microsoft Excel for analysis.

2.2.12. Multiplex bead analysis

Multiplex-cytokine detection kits were obtained from BioRad. A variety of human cytokines were investigated, including IL-5, IL-6, IL-10, IL-17, TNFα, IFNγ and GM-CSF. Detailed instructions for carrying out the assay can be found in the manufactures handbook. Briefly wells were pre-wet and 50µl thoroughly mixed beads were added to each well and washed twice. Standards were prepared by 4 fold dilutions of a premixed standard. 60µl standards, sample or blanks of matrix medium were added to appropriate wells and incubate on a plate shaker in the dark for 2 hours. The plate was washed 3 times and incubated for a further 30

mins with 50µl detection antibody. After washing, plates were incubated for 10 mins with 50µl streptavidin-HRP. Following a final wash step plates were kept in the fridge until they were read on the Bio-Plex™ 200 System (BioRad) using Bio-Plex Manager software version 5.0. Files were imported into Microsoft Excel for analysis.

2.2.13. Quantitative PCR (qPCR) and sample preparation

2.2.13.1. Dead Cell removal

A dead cell removal kit (Miltneyi Biotech) was used to remove dead cells before RNA extraction was performed. Cells were incubated at room temperature for 15 minutes with 100µl dead cell removal microbeads, washed once in binding buffer and passed over an MS column. The flow-through, containing the live cells, was collected and washed once in binding buffer.

2.2.13.2. RNA extraction

The Absolutely RNA microprep Kit (Agilent Technologies) was used for RNA extraction. 0.7µl of β2-mercaptoethanol was added to 100µl lysis buffer for each sample of up to 5×10^5 cells. Cells were centrifuged at 1500rpm for 15 minutes, the supernatant removed and cells resuspended in lysis buffer plus β2-mercaptoethanol, and mixed. 100µl of 70% ethanol was added and mixed thoroughly. This mixture was transferred to an RNA-binding spin cup seated in a 2ml collection tube and spun at 1300rpm for 1 minute in a microcentrifuge. The filtrate was discarded, 600µl of 1x low salt buffer was added to the spin cup and then tube was spun at 1300rpm for 1 minute. The filtrate was removed and the tube was spun for a further 2 minutes at 1300rpm to dry the fiber matrix. A DNase solution was prepared by adding 5µl RNase-free DNase I with 25µl DNase digestion buffer for each sample. This DNase solution was added directly to the fibre matrix in the spin cup and incubated at 37°C

for 15 minutes. 500µl 1x high salt buffer was added to the spin cup and the tube was spun for 1 minute at 1300rpm. The filtrate was discarded, 600µl 1x low salt buffer added and the sample was spun at 1300rpm for 1 minute. A second low salt wash was performed by adding 300µl 1x low salt buffer and spinning at 1300rpm for 2 minute. The spin cup was transferred to a fresh 1.5ml collection tube and 10µl elution buffer was added to the fiber matrix. The sample was incubated at room temperature for 2 minutes before being spun at 1300rpm for 2 minutes. Samples were either used directly for cDNA synthesis or stored at -20°C for a month or at -80°C for long-term storage.

2.2.13.3. Complementary DNA (cDNA) synthesis

The SuperScript® VILO™ cDNA synthesis kit (Invitrogen) was used to synthesis cDNA from mRNA samples. Reaction mixes were made by adding the components detailed in Table 2.4. For multiple reactions a master mix was prepared, without RNA, by scaling up the amounts stated below accordingly and adding RNA where appropriate.

Reaction Component	Volume
5x VILO™ reaction mix	4µl
10x SuperScript® enzyme mix	2µl
RNA	8µl
Nuclease-free water	6µl

Table 2.4: Master mix components for cDNA synthesis

The components were combined in a 0.5µl PCR tube, on ice. Following mixing tubes were incubated at 25°C for 10 minutes and then for 42°C for 1 hour, in a Tetrad 2 thermal cycler

(Bio-Rad). The reaction was terminated by raising the temperature to 85°C for 5 minutes. cDNA was used for qPCR, as described below, or stored at -20°C until further use.

2.2.13.4. qPCR

The TaqMan® Gene Expression Assay (Applied Biosystems) was used. A master mix of all reagents except cDNA was prepared, using the reagents in Table 2.5. A table of the genes investigated, with ID numbers, can be seen in Table 2.6.

Reaction Component	Volume
TaqMan® Gene Expression Assay mix (x20) containing • Forward PCR primer (18µM) • Reverse PCR primer (18µM) • TaqMan probe (5µM)	1µl
cDNA template	5µl
TaqMan® Fast Universal PCR master mix (x2), no AmpErase® UNG	10µl
RNase-free water	4µl

Table 2.5: Master mix components for qPCR

Gene	Applied Biosystems ID	Entrez Gene ID
Bcl2	Hs00153350_m1	596
CCR6	Hs01890706_s1	1235
CD3d	Hs00174158_m1	915
IL-1R1	Hs00991002_m1	3554
IL-23R	Hs00332759_m1	149233
KIR3DL2	Hs00601497_gH	3812
RORC	Hs01076122_m1	6097
Tbx21 (T-bet)	Hs00203436_m1	30009

Table 2.6: Genes investigated by qPCR

cDNA stock was diluted in RNase-free water to prepare a 200ng/μl solution and added to wells of an Optical 96-well fast plate (Applied Biosystems). 15μl master mix was added, plates were sealed with an optical adhesive cover (Applied Biosystems) and centrifuged at 1500rpm for 4 minutes. The amplification reactions were performed in a 7500 Fast Real-Time PCR system (Applied Biosystems) and consisted of one cycle of 95°C for 20 seconds, then 40 cycles of 95°C for 3 seconds followed by 60°C for 30 seconds. The $\Delta\Delta CT$ method was used to determine the relative expression of the genes, with CD3d as the endogenous control.

2.3. Analytical methods

Prism version 5 was used for statistical analysis. Help with statistical analysis was provided by Dr Lunn from the Department of Statistics. For data on CD4+ T cell phenotype, in chapter

3, box-and-whisker plots were used. These show the smallest observation (sample minimum), lower quartile, median, upper quartile and largest observation (sample maximum).

When comparing two groups with normal distribution of data a two-tailed student t test was used for statistical analysis. When comparing two groups with data that was not normally distributed a Wilcoxon signed-rank test was used for statistical analysis. When comparing more than two groups, where the data was normally distributed, a one-way analysis of variance (ANOVA) with Bonferroni's correction was used for statistical analysis. Where the data was not normally distributed a Kruskal-Wallis test with Dunn's multiple column analysis was used for statistical analysis. Significance was defined as $p \leq 0.05$.

Chapter 3: CD4+ T cell phenotypic marker expression in inflammatory arthritis

3.1. Introduction

KIRs are predominantly expressed on NK cells and on subsets of T cells (reviewed in (Vilches and Parham 2002)). KIR+ CD8+ T cells are proposed to have a memory phenotype characterized by a lack of CCR7, CD27 and CD28 (Anfossi, Pascal et al. 2001). Less work has focused on the phenotype of KIR+ CD4+ T cells. One study found that, in contrast to KIR- CD4+ T cells, the majority of KIR+ CD4+ T cells lacked expression of CD27, CD28, CCR4, or CCR7 (van Bergen, Thompson et al. 2004). Moreover, in this study, these KIR+ CD4+ T cells produced mainly IFN γ , with little IL-4, IL-10, or IL-17 production upon TCR triggering. The KIR+ CD4+ T cell population also contained cells that responded to recall antigens in an HLA-class II restricted fashion, thus leading the authors to classify them as effector memory Th1 cells. However it should be noted that the authors investigated KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, KIR2DS2, KIR2DS4, and KIR3DL1 but not KIR3DL2.

Following the observation that there are expansions of both CD4+ T cells and NK cells expressing KIR3DL2 in patients with SpA (Chan, Kollnberger et al. 2005), I wanted to further investigate the phenotype of KIR3DL2+ CD4+ T cells from inflammatory arthritis patients and healthy controls.

3.2. Aim

The overall aim of this chapter was to investigate surface markers on KIR3DL2+ CD4+ T cells to more fully determine the phenotype of KIR3DL2+ CD4+ T cell in the peripheral blood of patients with spondyloarthritis, as compared to healthy and inflammatory disease controls. To achieve this I had three objectives.

- To determine whether KIR3DL2+ CD4+ T cells from AS patients are enriched for phenotypic markers associated with Th17 cells as compared to KIR3DL2+ CD4+ T cells from healthy controls and RA patients.
- To determine whether KIR3DL2+ CD4+ T cells from AS patients are enriched for a phenotypic marker associated with activation as compared to KIR3DL2+ CD4+ T cells from healthy controls and RA patients.
- To determine whether KIR3DL2+ CD4+ T cells from AS patients are enriched for a phenotypic marker associated with homing to the gut as compared to KIR3DL2+ CD4+ T cells from healthy controls and RA patients.

3.3. Results

3.3.1. Expansion of KIR3DL2+ CD4+ T cells in peripheral blood of AS patients

Expansions of NK cells and CD4+ T cells expressing KIR3DL2 in the peripheral blood of patients with SpA have previously been reported (Chan, Kollnberger et al. 2005). Figure 3.1.A shows FACS staining of PBMC from 15 AS patients, 15 HLA-B27- healthy controls, 8 HLA-B27+ healthy controls and 9 RA patients (used for investigation of phenotypic markers in section 3.3.2, 3.3.4 and 3.3.5) confirming that a significantly higher percentage of CD4+ T cells from AS patients express KIR3DL2 compared to HLA-B27- healthy controls and patients with RA. Interestingly there was no significant difference between KIR3DL2 expression on CD4+ T cells from AS patients and HLA-B27+ healthy controls. Moreover, a significantly higher percentage of CD4+ T cells from HLA-B27+ healthy controls express KIR3DL2 compare to HLA-B27- healthy controls.

FACS staining for a panel of other KIRs, specifically KIR3DL1, KIR2DL1 and KIR2DL2, revealed no significant difference in the percentage of CD4+ T cells expressing these KIRs between disease and control groups (Figure 3.1.B).

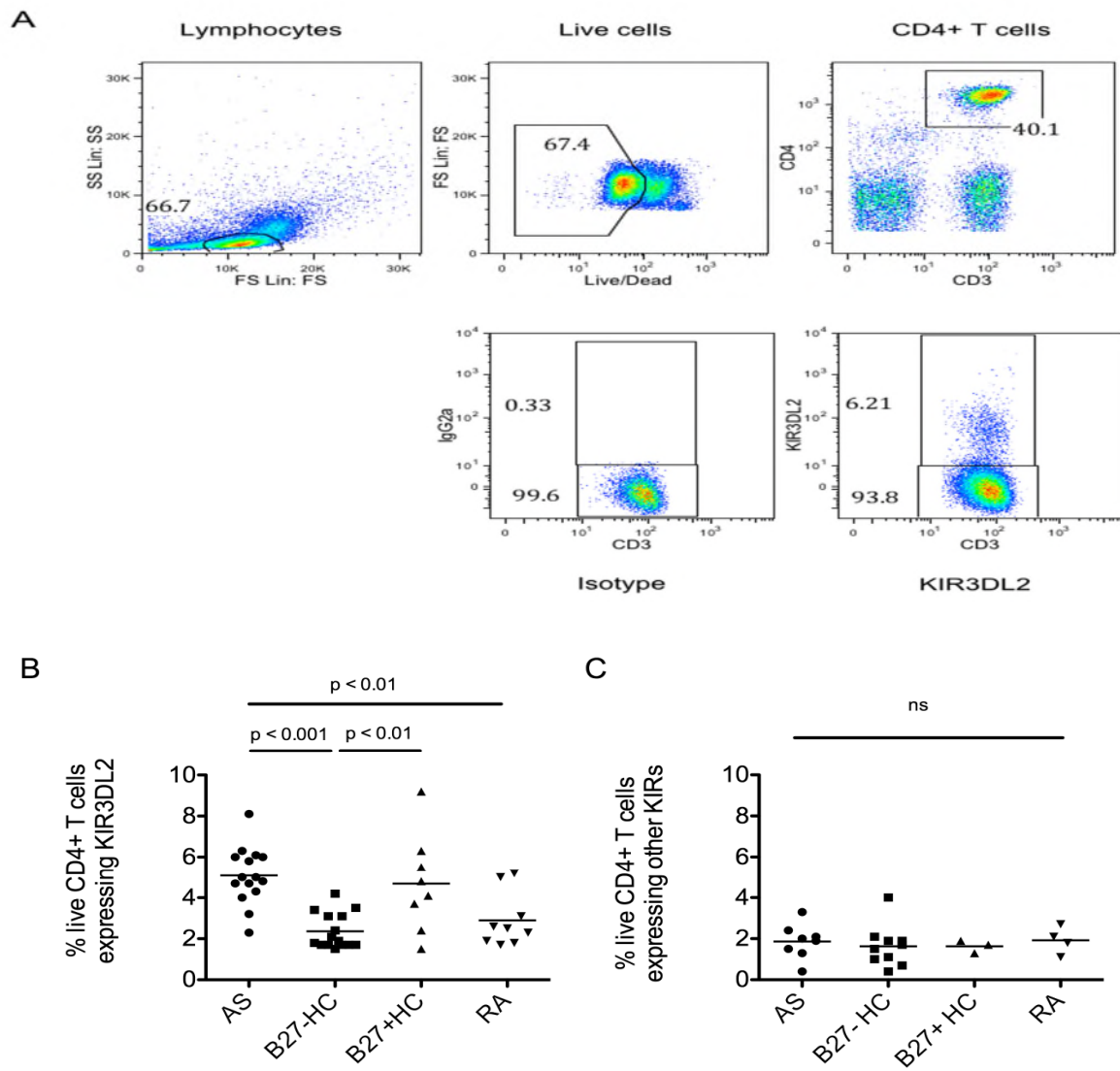


Figure 3.1: A higher percentage of CD4+ T cells from AS patients and HLA-B27+ healthy controls express KIR3DL2 compared to HLA-B27- healthy controls. (A) Representative FACS staining from one AS patient showing gating strategy (top panel) and KIR3DL2 expression on CD4+ T cells (bottom right panel) compared to isotype staining (bottom left panel). Note all subsequent experiments were gated using the same gating strategy. (B) FACS staining for KIR3DL2 on CD4+ T cells from AS patients (n=15), HLA-B27- and (n=15) and HLA-B27+ (n=8) healthy controls and RA patients (n=9). A one-way ANOVA was used for statistical analysis. (B) FACS staining for a panel of other KIRs (KIR3DL1, KIR2DL1 and KIR2DL2) on CD4+ T cells from AS patients (n=8), HLA-B27- (n=10) and HLA-B27+ (n=3) healthy controls and RA patients (n=4). A one-way ANOVA was used for statistical analysis.

3.3.2. KIR3DL2+ CD4+ T cells are enriched for Th17 markers

To further characterise KIR3DL2+ CD4+ T cells, surface markers associated with a Th17 phenotype were investigated by flow cytometry, specifically IL-23R, IL-1R, CCR6 and CD161. Figure 3.2 shows representative FACS staining of the IL-23R on PBMC from an AS patient, showing an enrichment of IL-23R on KIR3DL2+ CD4+ T cells, compared to KIR3DL2- CD4+ T cells (21.3% v 0.6%). Gating was performed as shown in Figure 3.1.A.

Figure 3.3.A shows the data from the all groups investigated, which included AS patients, HLA-B27- and HLA-B27+ healthy controls, and RA patients. A significantly higher percentage of KIR3DL2+ CD4+ T cells express the IL-23R compared to KIR3DL2- CD4+ T cells, in all groups investigated. When taking into account the percentage of KIR3DL2+ expression on CD4+ T cells, which is significantly higher in AS patients compared to HLA-B27- healthy controls and RA patients (as shown Figure 3.1), we see that a significantly higher percentage of IL-23R+ CD4+ T cells express KIR3DL2 in AS patients compared to RA patients and HLA-B27- healthy controls (Figure 3.3.B). In AS patients over half of all IL-23R+ CD4+ T cells co-express KIR3DL2. In addition, a significantly higher percentage of IL-23R+ CD4+ T cells from HLA-B27+ healthy controls express KIR3DL2, compared to RA patients.

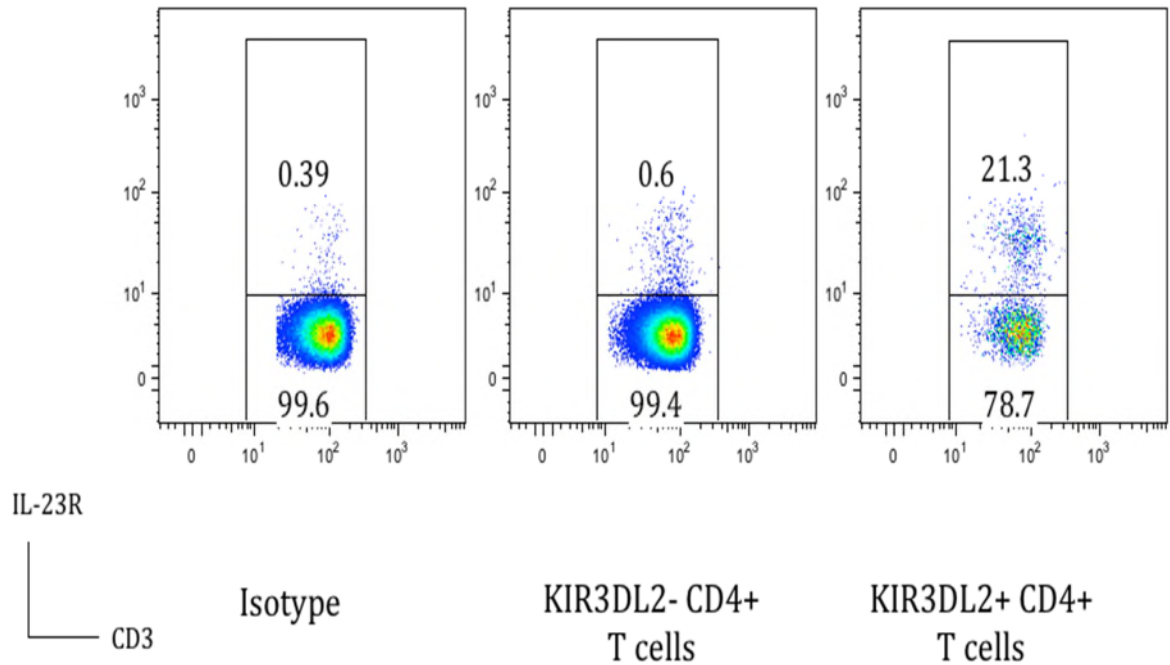
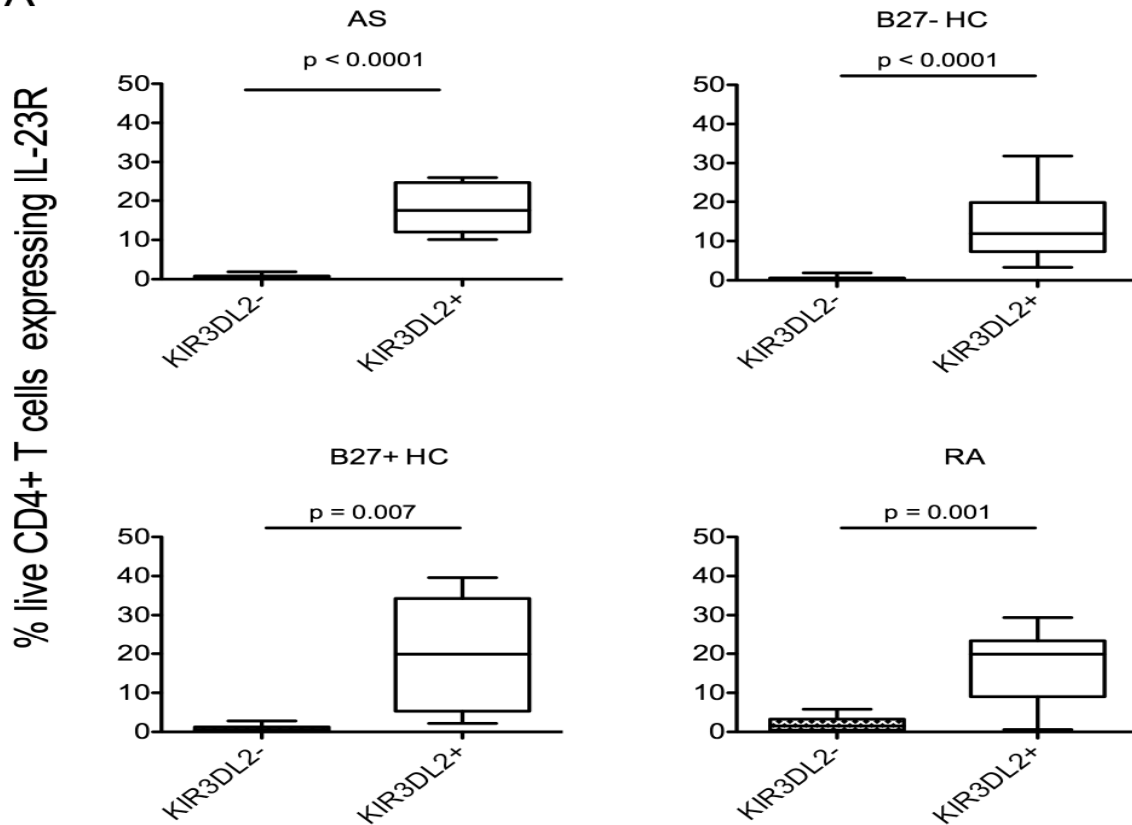


Figure 3.2: Enrichment of IL-23R on KIR3DL2+ CD4+ T cells from an AS patient. Representative FACS staining from one AS patients showing IL-23R expression on KIR3DL2- CD4+ T cells (center) and KIR3DL2+ CD4+ T cells (right) compared to isotype control (left).

A



B

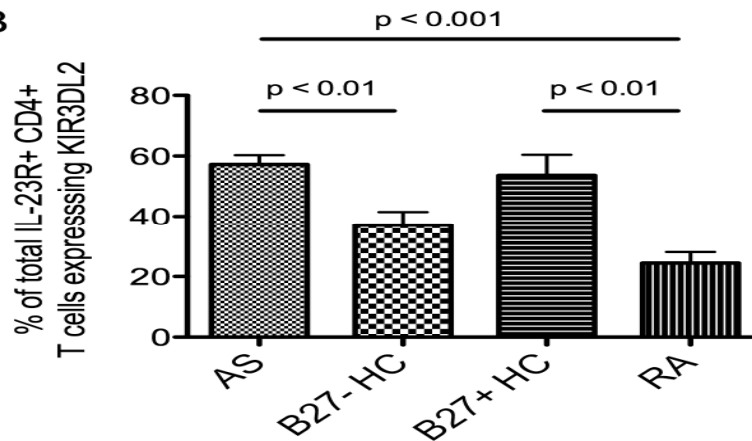


Figure 3.3: KIR3DL2+ CD4+ T cells are enriched for IL-23R expression. (A) Percentage of KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells expressing IL-23R in peripheral blood from AS patients (n=15), HLA-B27- (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=9). A two-tailed student t-test was used for statistical analysis. (B) Percentage of IL-23R+ CD4+ T cells expressing KIR3DL2 in peripheral blood from AS patients (n=15), HLA-B27- healthy controls (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=9). Bars represent mean values ($\pm$ SD). A one-way ANOVA was used for statistical analysis.

Figure 3.4 shows representative FACS staining of the IL-1R on PBMC from an AS patient, showing an enrichment of IL-1R on KIR3DL2+ CD4+ T cells, compared to KIR3DL2- CD4+ T cells (21.6% v 6.62%) from the same patient.

Figure 3.5.A shows that a significantly greater percentage of KIR3DL2+ CD4+ T cells express the IL-1R compared to KIR3DL2- CD4+ T cells, in all groups investigated. Furthermore, a significantly higher percentage of IL-1R+ CD4+ T cells from AS patients and HLA-B27+ healthy controls express KIR3DL2 compared to HLA-B27- healthy controls (Figure 3.5.B).

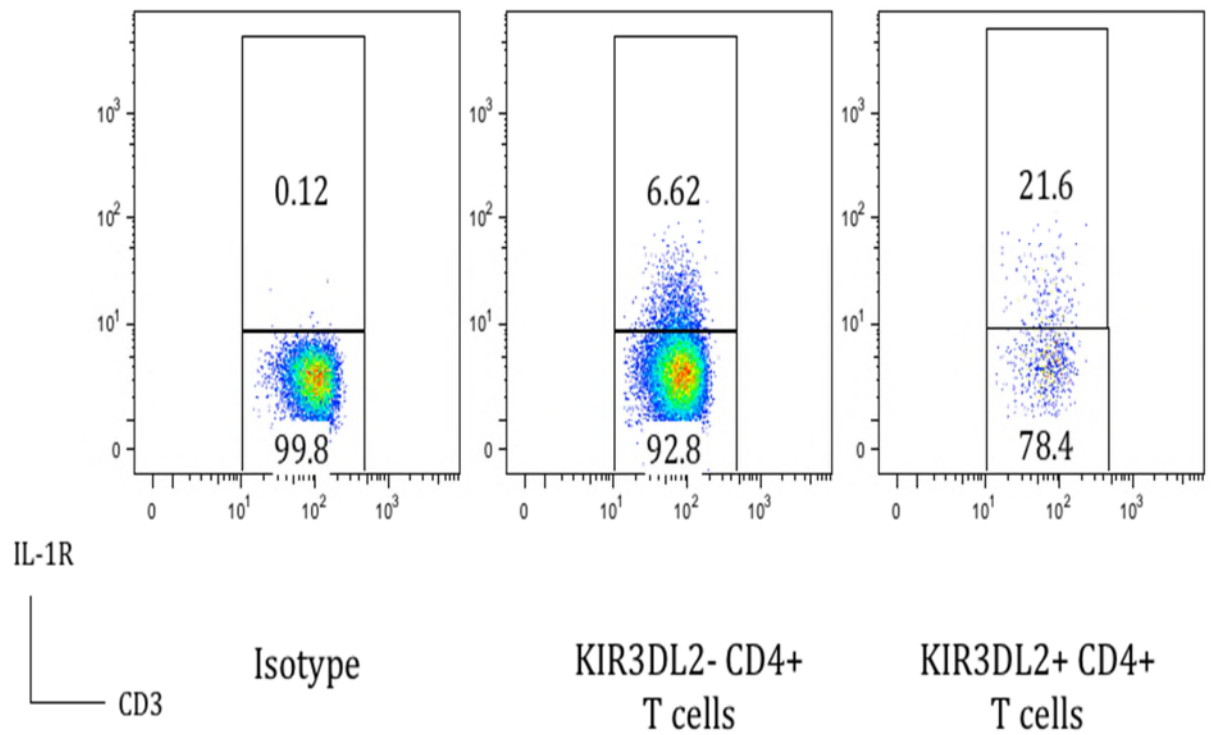


Figure 3.4: Enrichment of IL-1R on KIR3DL2+ CD4+ T cells from an AS patient. Representative FACS staining from one AS patients showing IL-1R expression on KIR3DL2- CD4+ T cells (center) and KIR3DL2+ CD4+ T cells (right) compared to isotype control (left).

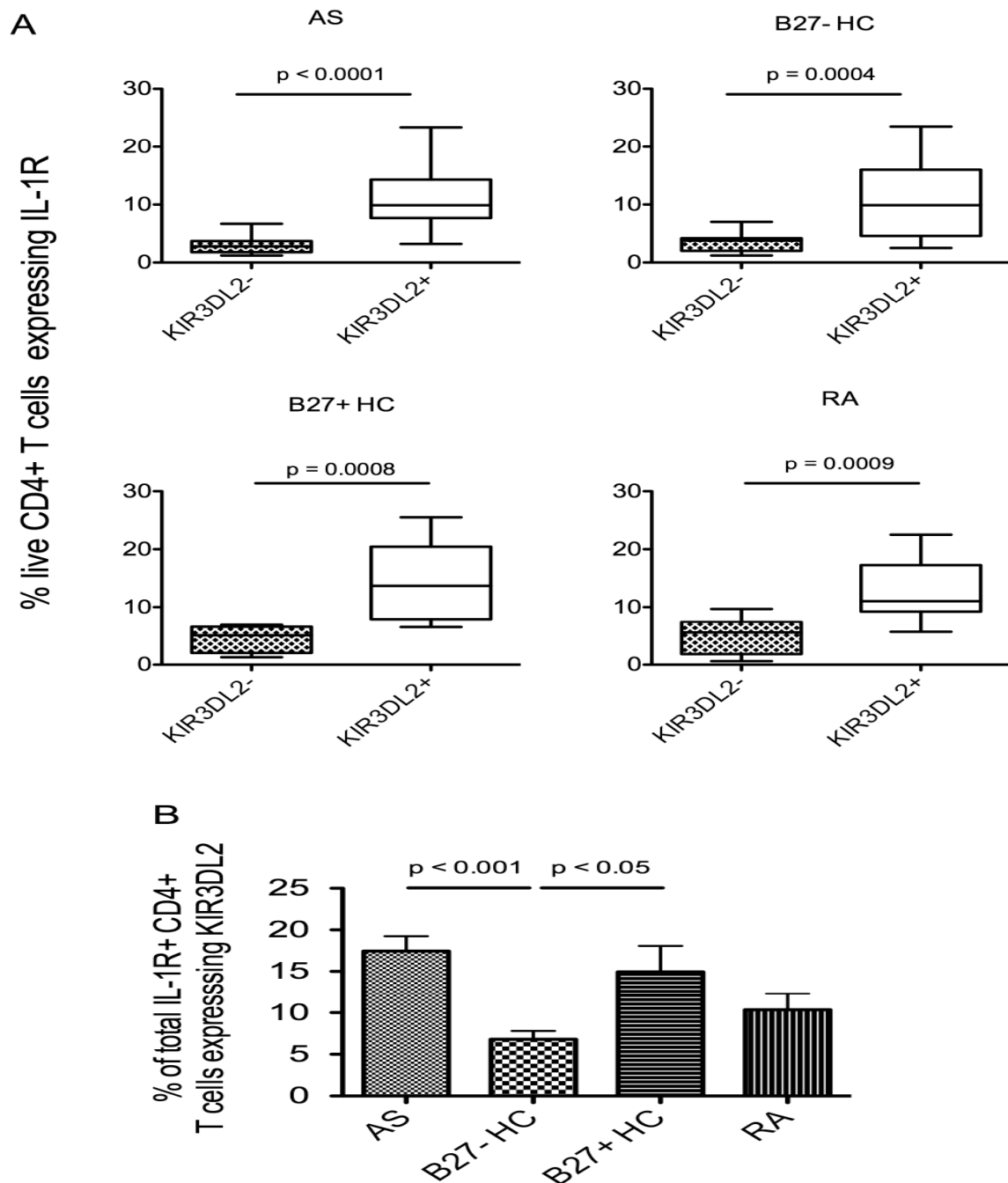


Figure 3.5: KIR3DL2+ CD4+ T cells are enriched for IL-1R expression. (A) Percentage of KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells expressing IL-1R in peripheral blood from AS patients (n=15), HLA-B27- (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=8). A two-tailed student t-test was used for statistical analysis. (B) Percentages of IL-1R+ CD4+ T cells expressing KIR3DL2 in peripheral blood from AS patients (n=15), HLA-B27- (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=8). Bars represent mean values ($\pm$ SD). A one-way ANOVA was used for statistical analysis.

Figure 3.6 shows representative FACS staining of CCR6 on PBMC from an AS patient, showing an enrichment of CCR6 on KIR3DL2⁺ CD4⁺ T cells, compared to KIR3DL2⁻ CD4⁺ T cells (28.4% v 15%).

Figure 3.7.A shows that a significantly higher percentage of KIR3DL2⁺ CD4⁺ T cells express CCR6 compared to KIR3DL2⁻ CD4⁺ T cells, in all groups investigated. Moreover, a significantly higher percentage of CCR6⁺ CD4⁺ T cells from inflammatory arthritis patients and HLA-B27⁺ healthy controls express KIR3DL2 compared to HLA-B27⁻ healthy controls (Figure 3.7.B).

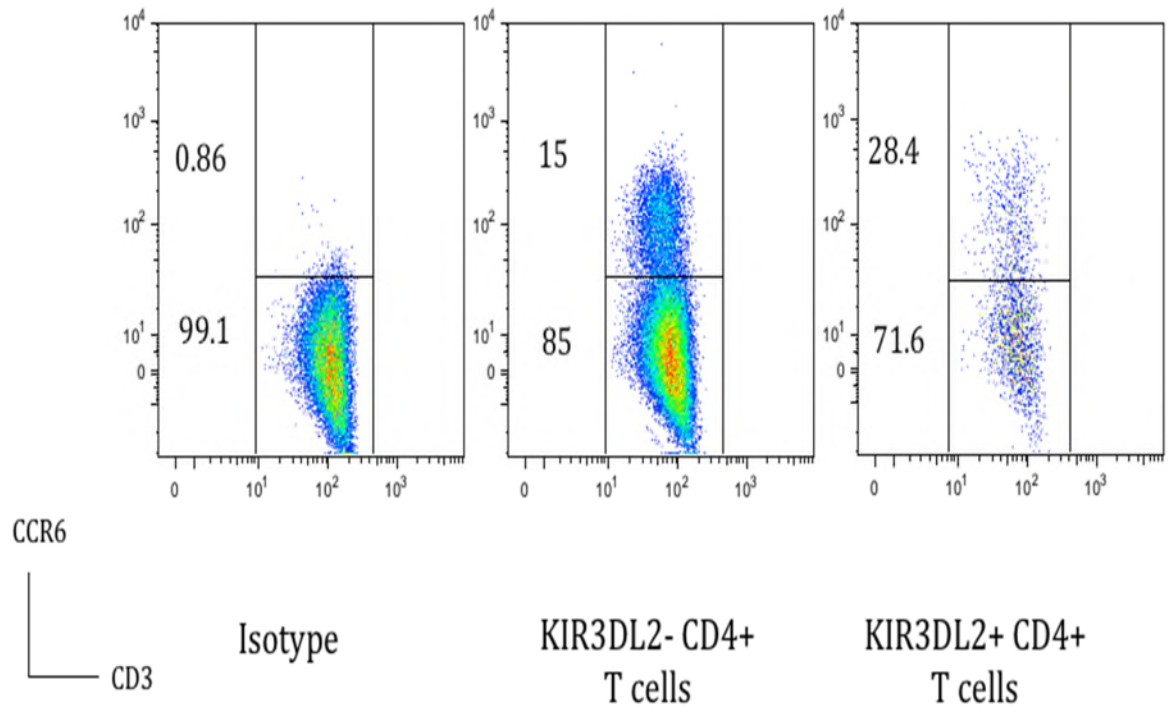


Figure 3.6: Enrichment of CCR6 on KIR3DL2+ CD4+ T cells from an AS patient. Representative FACS staining from one AS patients showing CCR6 expression on KIR3DL2- CD4+ T cells (center) and KIR3DL2+ CD4+ T cells (right) compared to isotype control (left).

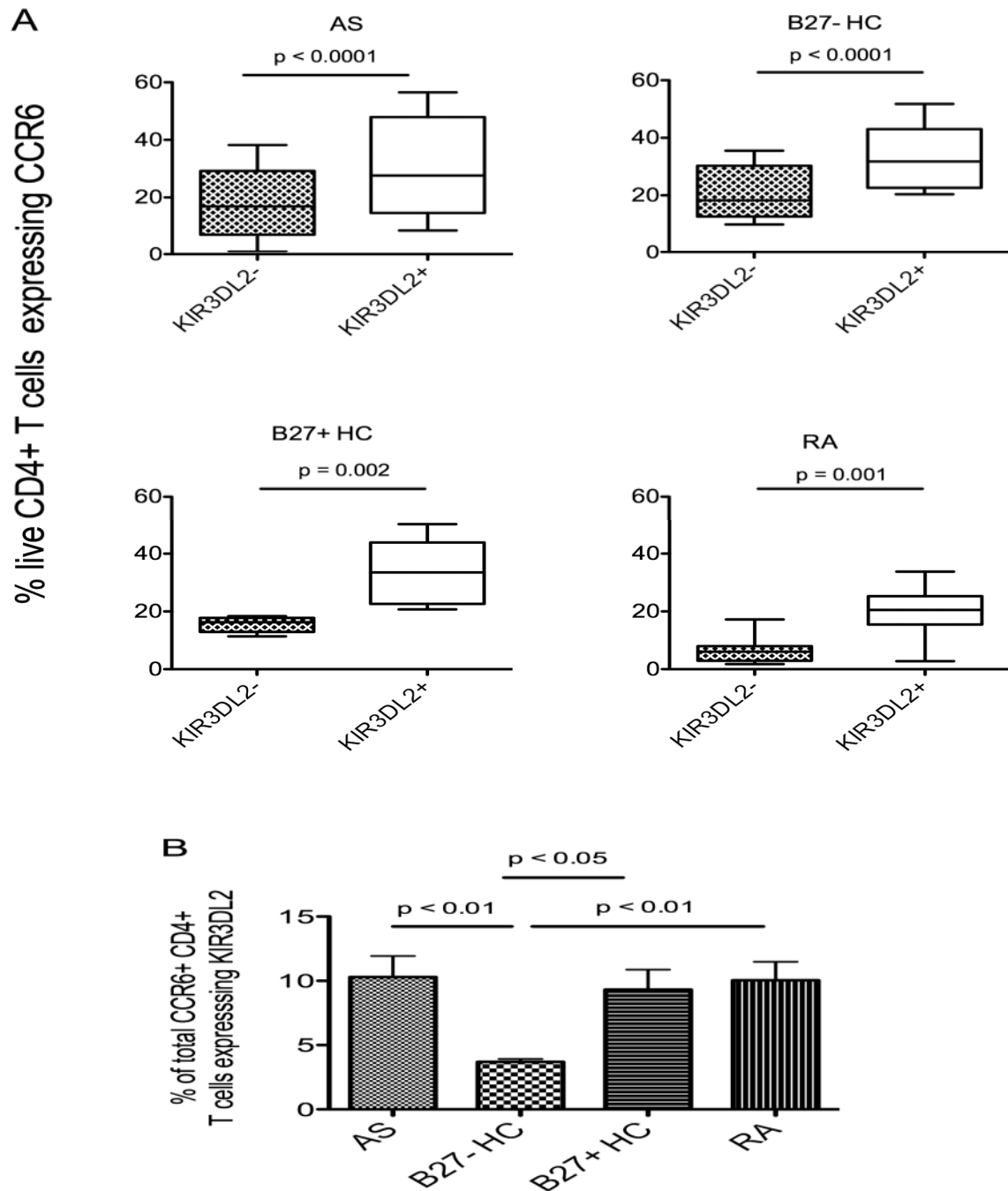


Figure 3.7: KIR3DL2+ CD4+ T cells are enriched for CCR6 expression. (A) Percentage of KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells expressing CCR6 in peripheral blood from AS patients (n=15), HLA-B27- (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=9). A two-tailed student t-test was used for statistical analysis. (B) Percentage of CCR6+ CD4+ T cells expressing KIR3DL2 in peripheral blood from AS patients (n=15), HLA-B27- (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=9). Bars represent mean values (±SD). A one-way ANOVA was used for statistical analysis.

Figure 3.8 shows representative FACS staining of CD161 on PBMC from an AS patient, showing an enrichment of CD161 on KIR3DL2+ CD4+ T cells, compared to KIR3DL2- CD4+ T cells (49.7% v 24.9%).

Figure 3.9.A shows all the data from the 4 groups investigated showing a significantly higher percentage of KIR3DL2+ CD4+ T cells express CD161 compared to KIR3DL2- CD4+ T cells. However, there is no significant difference in the percentage of CD161+ CD4+ T cells expressing KIR3DL2 in AS patients compared to HLA-B27- or HLA-B27+ healthy controls or RA patients (Figure 3.9.B).

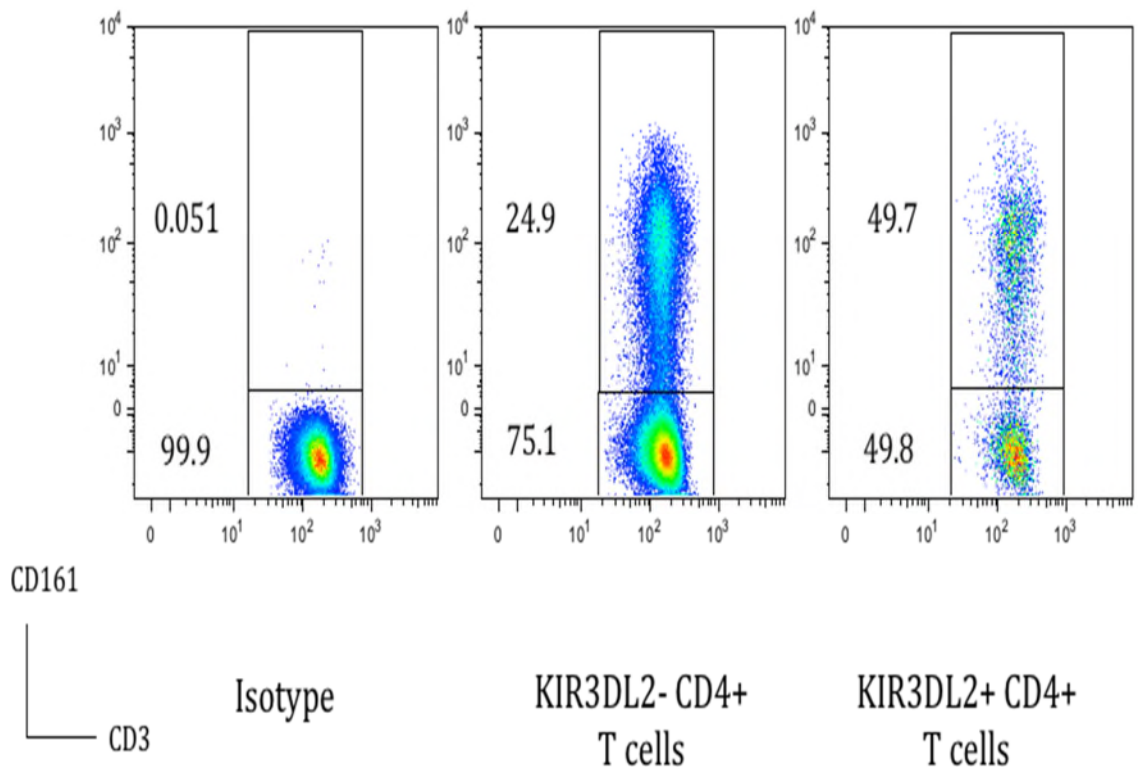
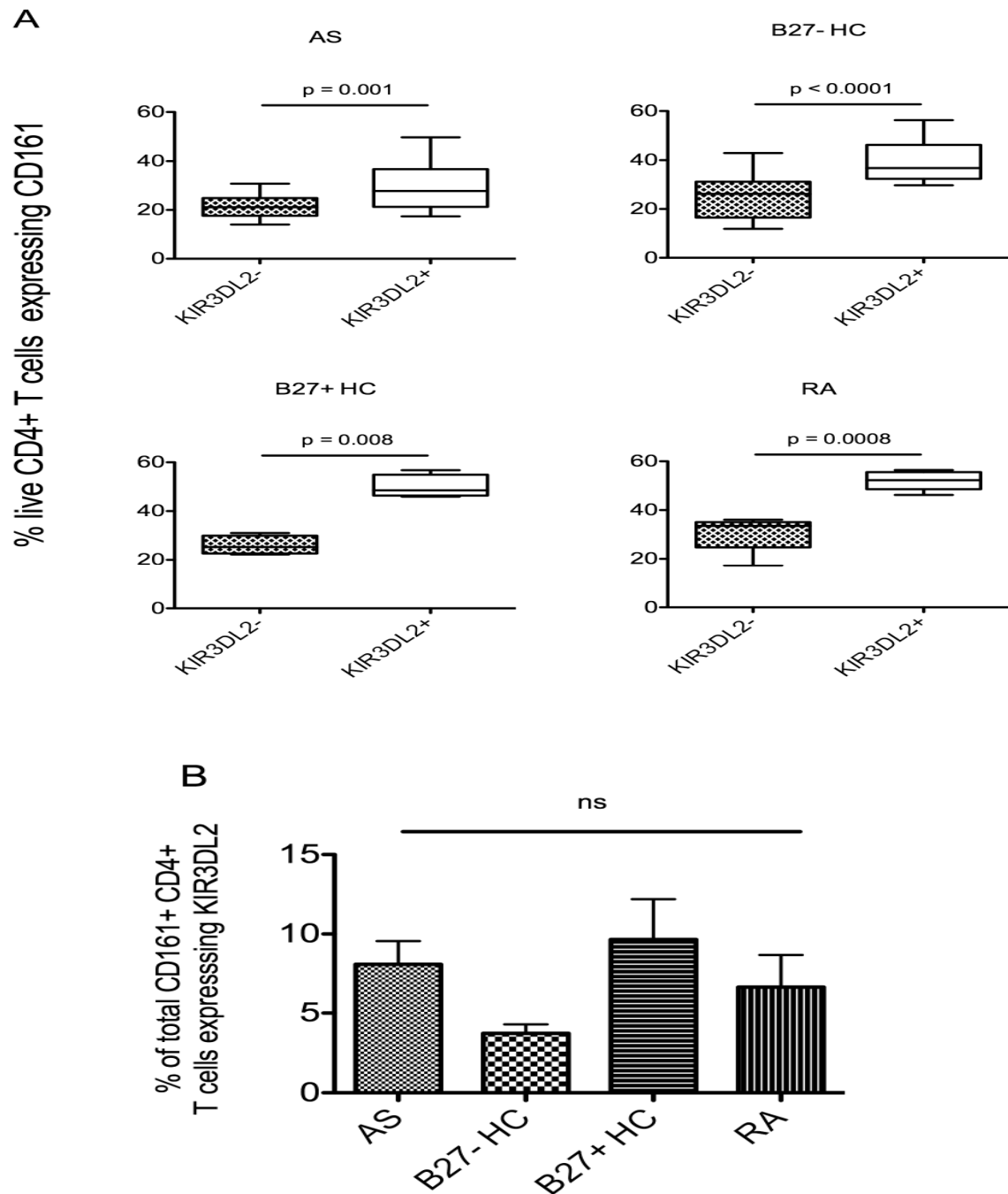


Figure 3.8: Enrichment of CD161 on KIR3DL2+ CD4+ T cells from an AS patient. Representative FACS staining from one AS patients showing CD161 expression on KIR3DL2- CD4+ T cells (center) and KIR3DL2+ CD4+ T cells (right) compared to isotype control (left).



In addition to FACS staining qPCR was used to quantify expression of mRNA of genes associated with Th17 cells. KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells were isolated by FACS sorting from one HLA-B27⁻ healthy control and one AS patient (Figure 3.10.A), as described in section 2.2.8.4. This was repeated on a further two AS patients (Figure 3.10.B and 3.10.C). RNA was collected, converted to cDNA, and qPCR was used to compare the expression of IL-23R, IL-1R and CCR6 between KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells, as described in section 2.2.13.

Figure 3.10 confirms that FACS-sorted KIR3DL2⁺ CD4⁺ T cells express KIR3DL2, whilst KIR3DL2 was not detectable in KIR3DL2⁻ CD4⁺ T cells following FACS sorting. CCR6, IL-23R and IL-1R expression is either exclusively, or higher, in KIR3DL2⁺ CD4⁺ T cells from the healthy control or AS patients.

A

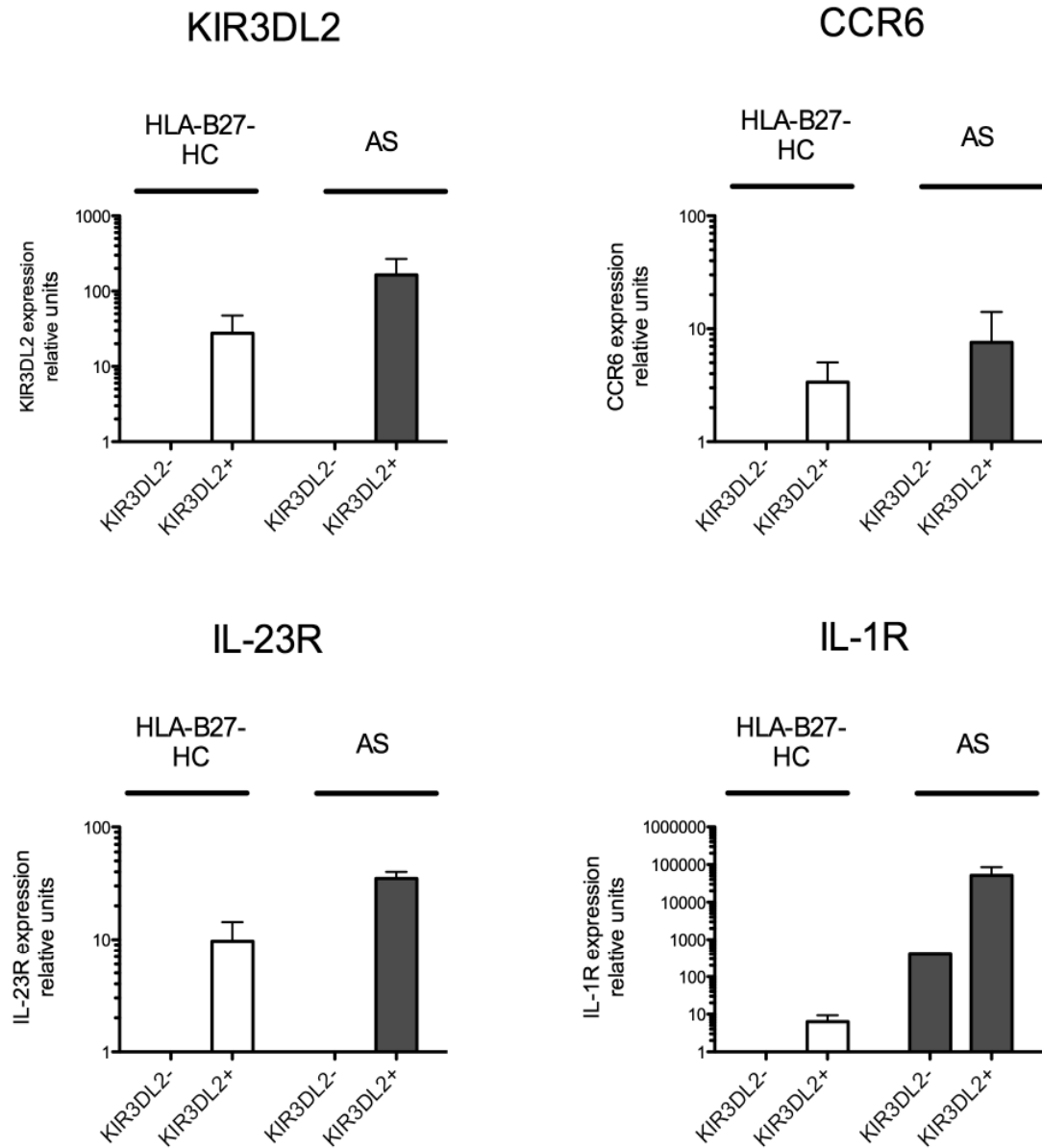


Figure 3.10.A: KIR3DL2+ CD4+ T cells are enriched for CCR6, IL-23R and IL-1R mRNA. qPCR analysis of mRNA from FACS sorted KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells from peripheral blood from a HLA-B27- healthy control (clear bars) and an AS patient (dark bars) confirming KIR3DL2+ expression in FACS sorted cells and showing expression of CCR6, IL-23R and IL-1R, normalized to CD3. Bars represent mean values ($\pm$ SD). Data was analyzed using the $\Delta\Delta$ CT method.

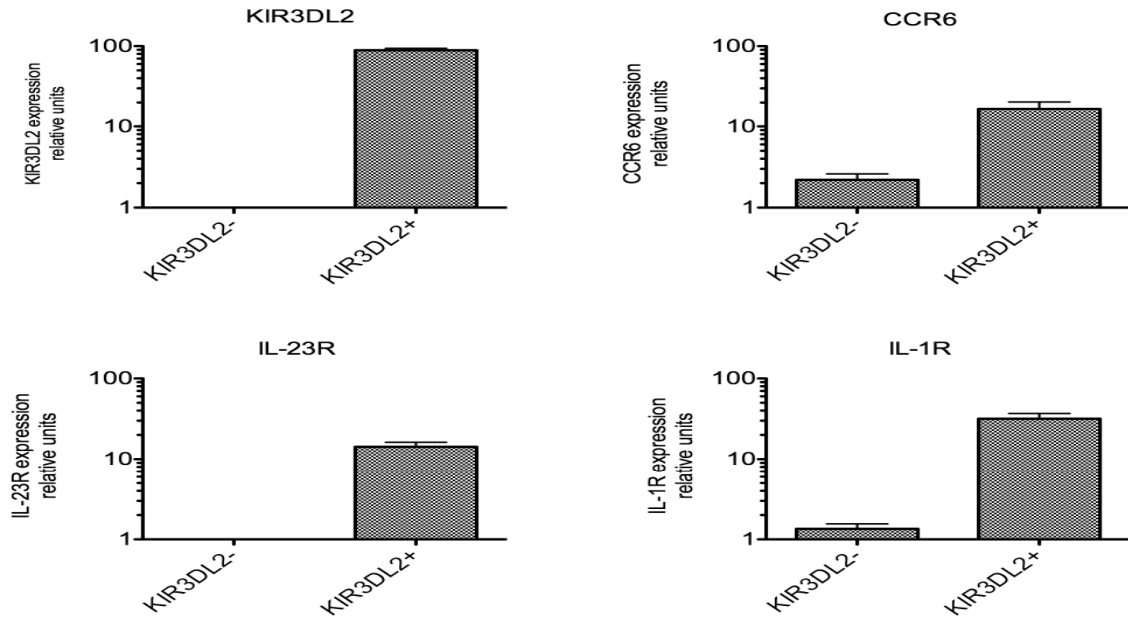
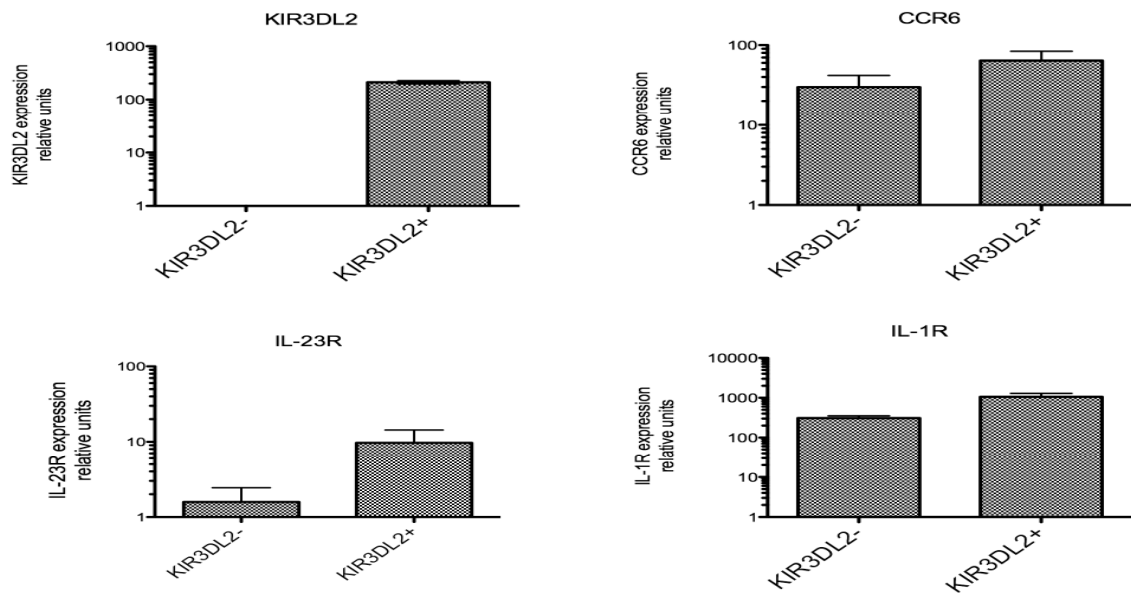
B**C**

Figure 3.10.B & C: KIR3DL2⁺ CD4⁺ T cells are enriched for CCR6, IL-23R and IL-1R mRNA. qPCR analysis of mRNA from FACS sorted KIR3DL2⁺ CD4⁺ T cells and KIR3DL2⁻ CD4⁺ T cells from peripheral blood from two AS patients confirming KIR3DL2⁺ expression in FACS sorted cells and showing expression of CCR6, IL-23R and IL-1R, normalized to CD3. Bars represent mean values ($\pm$ SD). Data was analyzed using the $\Delta\Delta$ CT method.

3.3.3. IL-23R is enriched on KIR3DL2+ CD4+ T cells from synovial fluid

Expression of Th17 markers, including IL-23R, IL-1R, CCR6 and CD161, on CD4+ T cells from matched peripheral blood and synovial fluid samples were investigated by FACS. Figure 3.11 shows representative FACS staining of KIR3DL2+ CD4+ T cells from matched peripheral blood and synovial fluid sample from one AS patient. In this example there is enrichment of CCR6, CD161 and IL-23R on synovial fluid KIR3DL2+ CD4+ T cells compared to peripheral blood KIR3DL2+ CD4+ T cells. In contrast there is a higher percentage of peripheral blood KIR3DL2+ CD4+ T cells expressing IL-1R compared to KIR3DL2+ CD4+ T cells from synovial fluid (48.1 v 38.3%).

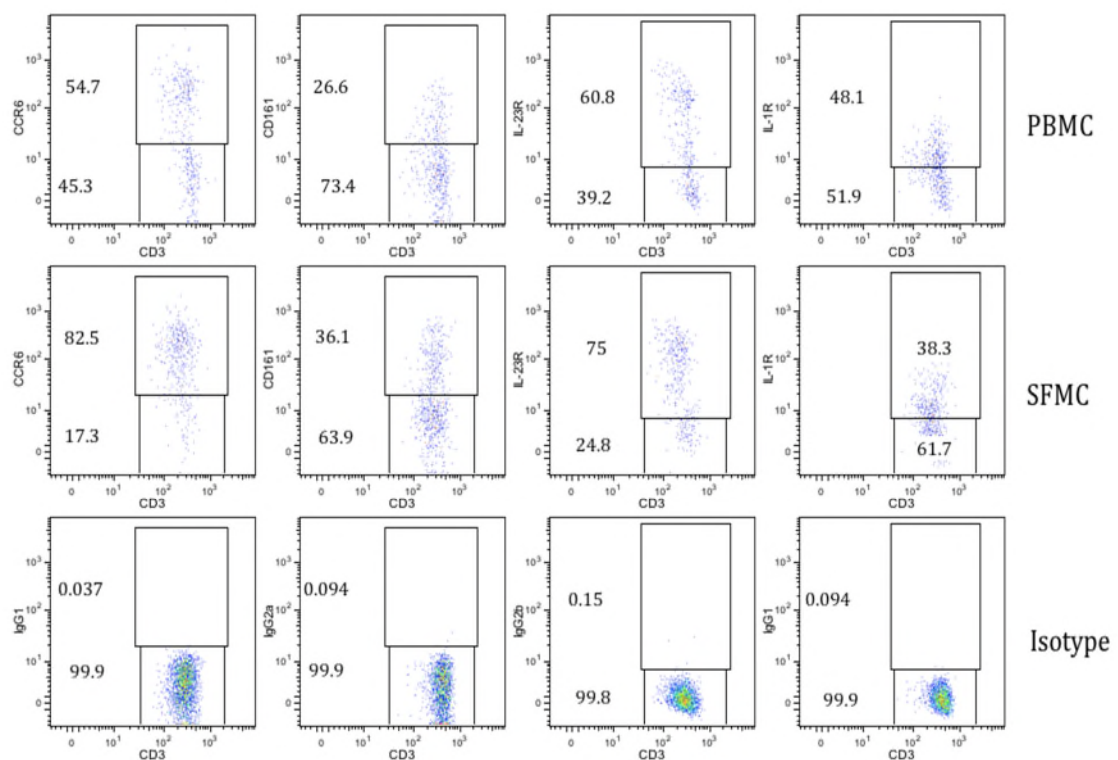


Figure 3.11: Enrichment of CCR6, CD161 and IL-23R on KIR3DL2+ CD4+ T cells from synovial fluid compared to a matched peripheral blood sample from an AS patient. Representative FACS staining from one AS patients showing CCR6, CD161, IL-23R and IL-1R expression on KIR3DL2+ CD4+ T cells from synovial fluid (bottom panel) compared to KIR3DL2+ CD4+ T cells from a matched peripheral blood sample (top panel) from the same AS patient.

When looking at all the matched peripheral blood and synovial fluid samples from SpA patients, as seen in Figure 3.12.A, there is no significant difference between the percentage of CD4+ T cells expressing KIR3DL2 in peripheral blood and synovial fluid. It is likely that the small number of matched synovial fluid and peripheral blood samples available for investigation are too low to detect a statistically significant difference, and as such it would be of interest to extend this study in the future to compare more matched synovial fluid and peripheral blood samples. When looking at expression of Th17-associated markers we see that there is significantly higher expression of IL-23R on KIR3DL2+ CD4+ T cells from synovial fluid compared to KIR3DL2+ CD4+ T cells from peripheral blood samples from the same patients (Figure 3.12.D).

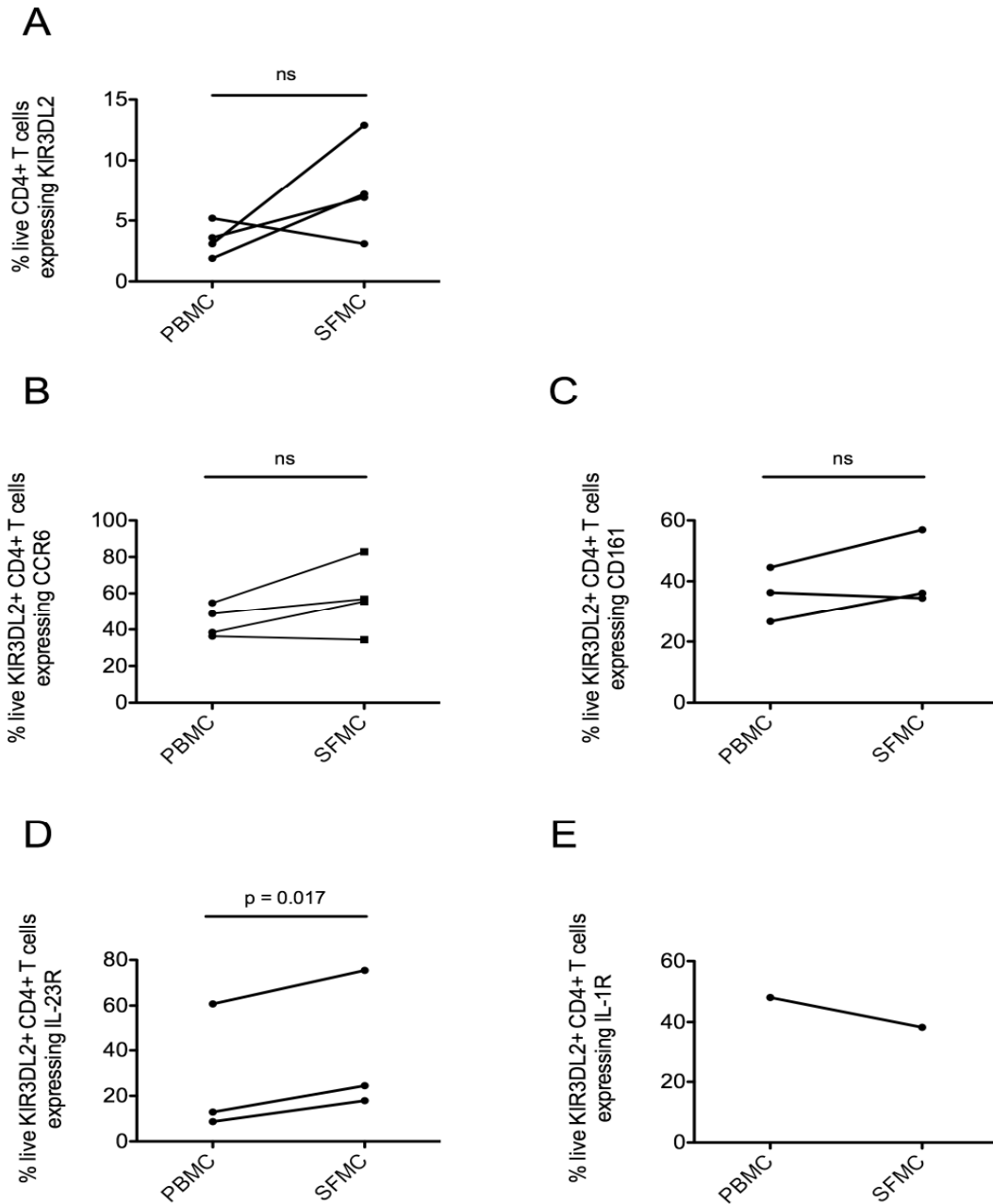


Figure 3.12: Enrichment of IL-23R on KIR3DL2+ CD4+ T cells from synovial fluid compared to matched peripheral blood samples from SpA patients. (A) Percentage of KIR3DL2+ CD4+ T cells in peripheral blood expressing compared to synovial fluid (n=4). Percentage of KIR3DL2+ CD4+ T cells from peripheral blood expressing (B) CCR6 (n=4), (C) CD161 (n=3), (D) IL-23R (n=3) and (E) IL-1R (n=1), compared to matched synovial fluid samples. A two-tailed student t-test was used for statistical analysis.

3.3.4. KIR3DL2+ CD4+ T cells are enriched for the activation marker CD134

To further investigate the phenotype of the KIR3DL2+ CD4+ T cells the activation marker CD134 was investigated by FACS. CD134, a TNFR-superfamily member, is not constitutively expressed on resting naïve T cells but is upregulated upon activation (Baum, Gayle et al. 1994).

Figure 3.13 shows representative FACS staining of CD134 on PBMC from an AS patients, showing an enrichment of CD134 on KIR3DL2+ CD4+ T cells, compared to KIR3DL2- CD4+ T cells from the same patient (25.6% v 0.76%). A significantly greater percentage of KIR3DL2+ CD4+ T cells express CD134 compared to KIR3DL2- CD4+ T cells, in all groups investigated (Figure 3.14.A). Moreover, a significantly higher percentage of CD134+ CD4+ T cells from AS patients express KIR3DL2 compared to HLA-B27- healthy controls and RA patients (Figure 3.14.B).

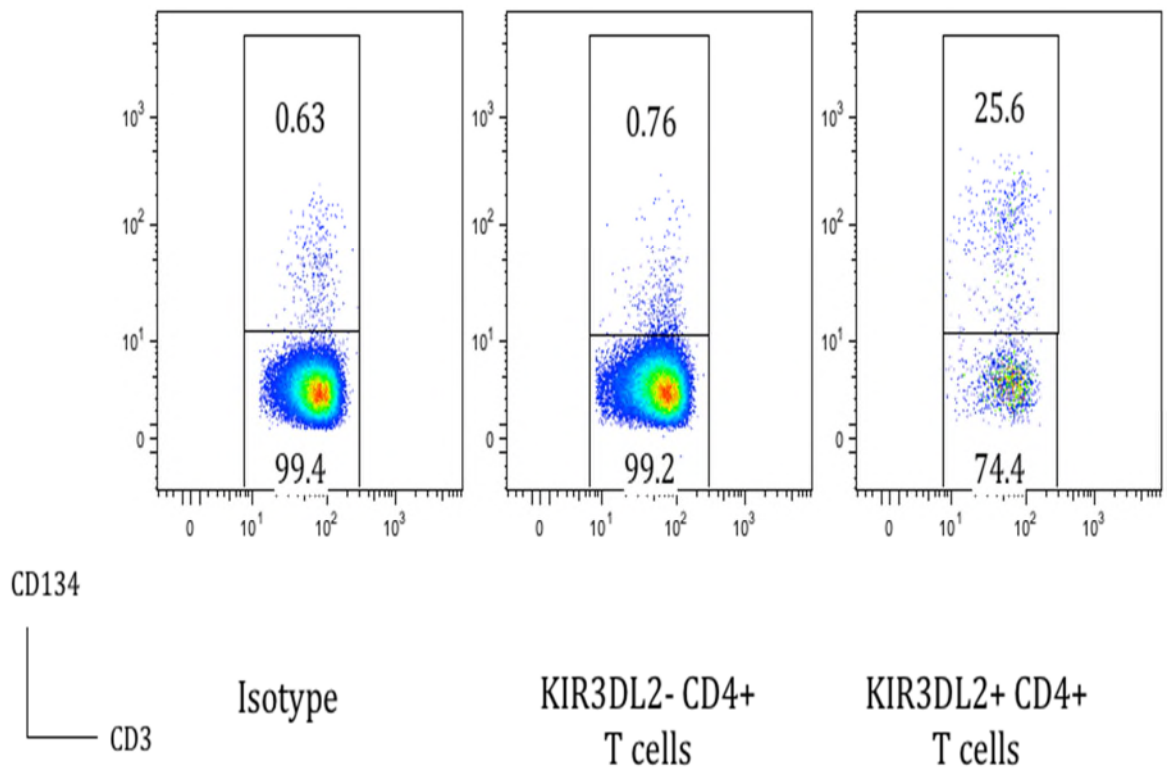


Figure 3.13: Enrichment of CD134 on KIR3DL2+ CD4+ T cells from an AS patient. Representative FACS staining from one AS patients showing CD134 expression on KIR3DL2- CD4+ T cells (center) and KIR3DL2+ CD4+ T cells (right) compared to isotype control (left).

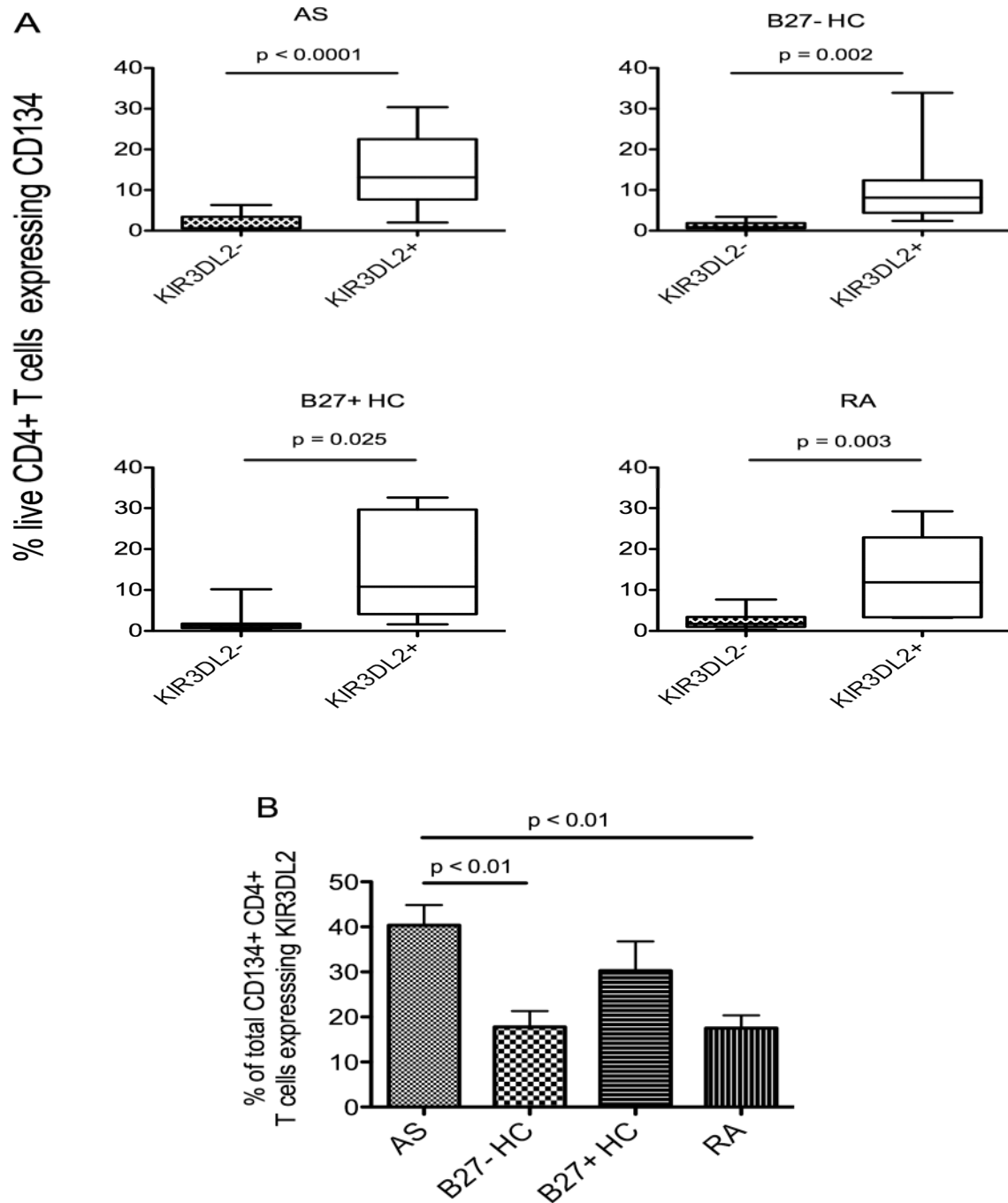


Figure 3.14: KIR3DL2+ CD4+ T cells are enriched for CD134 expression. (A) Percentage of KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells expressing CD134 in peripheral blood from AS patients (n=15), HLA-B27- (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=9). A two-tailed student t-test was used for statistical analysis. (B) Percentage of CD134+ CD4+ T cells expressing KIR3DL2 in peripheral blood from AS patients (n=15), HLA-B27- (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=9). Bars represent mean values ($\pm$ SD). A one-way ANOVA was used for statistical analysis.

3.3.5. KIR3DL2+ CD4+ T cells are enriched for the gut homing marker CCR9

To more fully characterise KIR3DL2+ CD4+ T cells, CCR9, a chemokine receptor associated with homing to the gut, was investigated by FACS.

Representative FACS staining in Figure 3.15 shows an enrichment of CCR9 on KIR3DL2+ CD4+ T cells from a AS patient, compared to KIR3DL2- CD4+ T cells (25.8% v 0.73%). Figure 3.16.A shows the data from all the groups investigated. A significantly higher percentage of KIR3DL2+ CD4+ T cells express CCR9 compared to KIR3DL2- CD4+ T cells, in all groups investigated. Furthermore, a significantly higher percentage of CCR9+ CD4+ T cells from AS patients and HLA-B27+ healthy controls express KIR3DL2 compared to HLA-B27- healthy controls (Figure 3.16.B). The percentage of CCR9+ CD4+ T cells expressing KIR3DL2 from AS patients is also significantly greater than in RA patients.

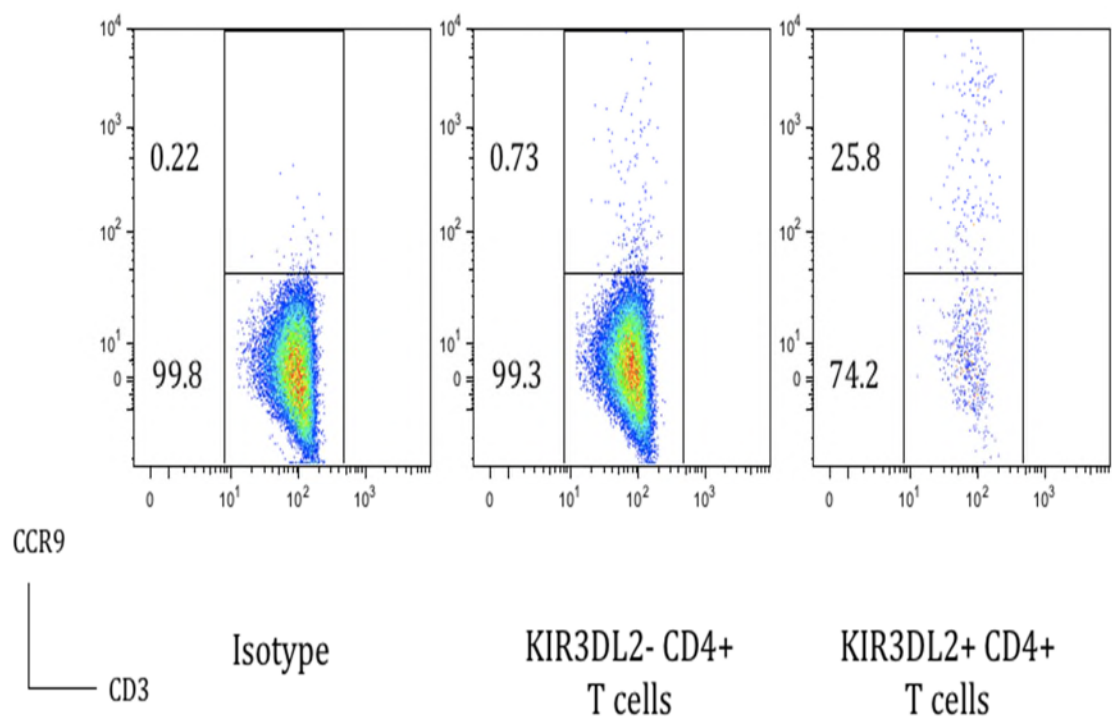


Figure 3.15: Enrichment of CCR9 on KIR3DL2⁺ CD4⁺ T cells from an AS patient. Representative FACS staining from one AS patients showing CCR9 expression on KIR3DL2- CD4⁺ T cells (center) and KIR3DL2⁺ CD4⁺ T cells (right) compared to isotype control (left).

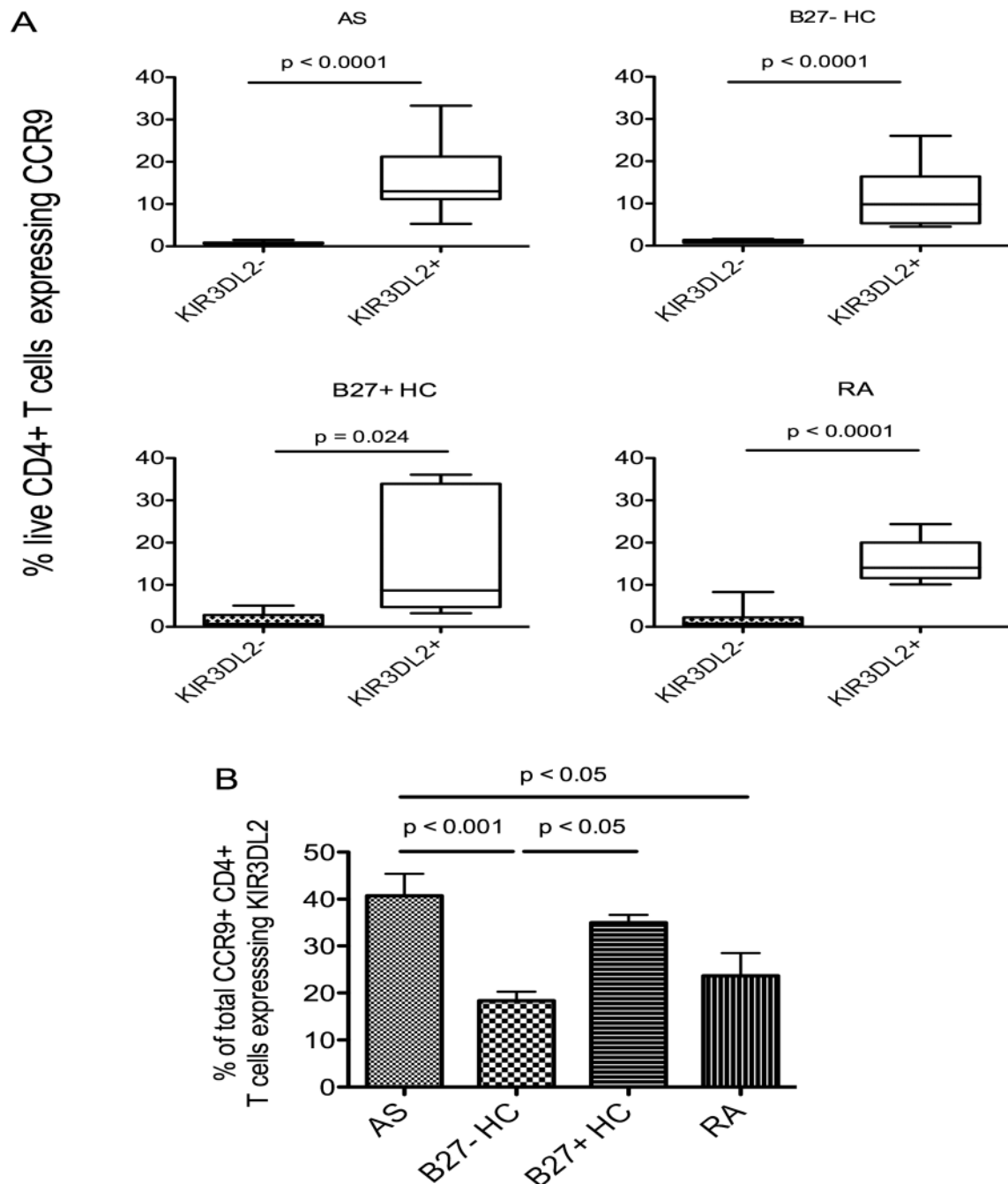


Figure 3.16: KIR3DL2+ CD4+ T cells are enriched for CCR9 expression. (A) Percentage of KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells expressing CCR9 in peripheral blood from AS patients (n=15), HLA-B27- (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=9). A two-tailed student t-test was used for statistical analysis. (B) Percentage of CCR9+ CD4+ T cells expressing KIR3DL2 in peripheral blood from AS patients (n=15), HLA-B27- (n=15) and HLA-B27+ healthy controls (n=8) and RA patients (n=9). Bars represent mean values ($\pm$ SD). A one-way ANOVA was used for statistical analysis.

3.4. Discussion

The primary aim of this chapter was to investigate the phenotype of KIR3DL2+ CD4+ T cells. This was achieved by FACS staining PBMC, from patients with AS and comparing this to PBMC from HLA-B27- and HLA-B27+ healthy controls and patients with RA. Pivotal to this work is the observation that KIR3DL2+ CD4+ T cells are enriched in the peripheral blood from patients with AS, compared to HLA-B27- healthy controls (Chan, Kollnberger et al. 2005). My FACS analysis confirms this finding. Moreover, I have shown that a significantly higher percentage of CD4+ T cells from HLA-B27+ healthy controls express KIR3DL2 compared to HLA-B27- healthy controls, with similar a similar percentage of CD4+ T cells expressing KIR3DL2 in the peripheral blood of AS patients and HLA-B27+ healthy controls. Interestingly this increase in KIR expression seems to be unique to KIR3DL2 since FACS staining with a panel of other KIRs showed no significant difference between the expression of these KIRs on CD4+ T cells from peripheral blood from inflammatory arthritis patients or healthy controls. The primary aim was subdivided into three objectives, which will be discussed in detail below.

1) To determine whether KIR3DL2+ CD4+ T cells from AS patients are enriched for phenotypic markers associated with Th17 cells as compared to KIR3DL2+ CD4+ T cells from healthy controls and RA patients.

A number of cell surface markers have been used to define Th17 cells, although a definitive marker remains to be elucidated. I have shown for the first time that KIR3DL2+ CD4+ T cells in inflammatory arthritis patients and healthy controls patients have raised expression of IL-23R, IL-1R, CCR6 and CD161 when compared to KIR3DL2- CD4+ T cells from the same

individuals and this is of particular interest since these are all markers associated with Th17 cells.

Although KIR3DL2+ CD4+ T cells comprise a minority of CD4+ T cells in peripheral blood, a mean of 57% of the total number of IL-23R+ CD4+ T cells from AS patients express KIR3DL2, compared to a significantly lower proportion in HLA-B27- healthy controls and RA patients (37% and 25%, respectively). A similar proportion of IL-23R+ CD4+ T cells from HLA-B27+ healthy controls to AS patients express KIR3DL2 (54%). IL-23R is not expressed on naïve CD4+ T cells, but is expressed on memory CD4+ T cells and is required for the terminal differentiation and expansion of Th17 cells *in vivo* (Wilson, Boniface et al. 2007; McGeachy, Chen et al. 2009).

A strong genetic association between AS and IL-23R polymorphisms have been reported by GWAS (Burton, Clayton et al. 2007; Australo-Anglo-American Spondyloarthritis Consortium (TASC), Reveille JD et al. 2010). Similar associations have been found between IL-23R polymorphisms and other diseases including ulcerative colitis, Crohn's disease and psoriasis (Duerr, Taylor et al. 2006; Capon, Di Meglio et al. 2007). Studies are now underway to investigate the functional consequences of these SNPs. One such study looked at the rs11209026 variant of the IL-23R gene, a variant in the initial portion of the IL-23R cytoplasmic region, known to be associated with protection against AS, IBD and psoriasis (Pidashveva, Trifari et al. 2011). The authors used primary T cells from healthy donors to show that the IL23R R381Q variant was associated with a reduction in STAT3 phosphorylation following stimulation with IL-23. They also observed decreased numbers of IL-23 responsive T cells providing evidence that the IL23R R381Q variant is a loss-of-function variant. A further study looking at a variant of the IL-23R gene associated with inflammatory bowel disease reported the rs10889677 variant in the 3'-untranslated region

of the IL-23R gene displayed enhanced levels of both mRNA and protein production of IL-23R (Zwiers, Kraal et al. 2012). This could be attributed to a loss of binding capacity for the microRNAs Let-7e and Let-7f by the variant allele, demonstrating a role for microRNA-mediated dysregulation of IL-23R signaling.

IL-23 is produced by cells of the innate immune system including macrophages and dendritic cells in the gut (Oppmann, Lesley et al. 2000; Becker, Wirtz et al. 2003). In a study by Zeng *et al* *in vitro*-differentiated macrophages from AS patients produced higher levels of IL-23 in response to LPS, compared to healthy controls (Zeng, Lindstrom et al. 2011). A further study looked at dendritic cells, revealing that dendritic cells derived from AS patients had increased production of IL-23 compared to RA patients (Prevosto, Goodall et al. 2009). Interestingly human fibroblasts support the expansion of IL-17-producing T cells via up-regulation of IL-23 production by dendritic cells (Schirmer, Klein et al. 2010).

Given the role of IL-23 in the differentiation and expansion of Th17 cells, targeting IL-23 signaling is an attractive therapeutic option. Ustekinumab is a human monoclonal antibody that binds to the p40, the shared subunit of IL-12 and IL-23. Results from two phase III trials, PHOENIX 1 and PHOENIX 2, showed efficacy of ustekinumab in patients with moderate to severe psoriasis (Leonardi, Kimball et al. 2008; Papp, Langley et al. 2008). Moreover, subcutaneous injection every 12 weeks maintained efficacy for at least a year in most patients. Ustekinumab is now licensed for the treatment of moderate to severe psoriasis. A phase II trial with Ustekinumab for the treatment of AS is underway, with results expected in mid-2013.

My FACS analysis showed raised IL-1R expression on KIR3DL2+ CD4+ T cells compared to KIR3DL2- CD4+ T cells in inflammatory arthritis and healthy controls. A higher proportion of

IL-1R⁺ CD4⁺ T cells from AS patients and HLA-B27⁺ healthy controls express KIR3DL2, compared to that seen in HLA-B27⁻ healthy controls. IL-1 is important in the development of Th17 cells (Acosta-Rodriguez, Napolitani et al. 2007; Wilson, Boniface et al. 2007; Lee, Kang et al. 2011). Moreover, the expression of the IL-1R is critical for the induction of EAE (Chung, Chang et al. 2009). In addition to its expression on Th17 cells the IL-1R is also proposed to be expressed on ex-Th17 cells (Hirota, Duarte et al. 2011). Using a reporter mouse strain designed to map the fate of cells that have activated IL-17A, it was shown that cells that had begun a Th17 programme but had subsequently switched off production of IL-17 could be identified by continued expression of the IL-1R.

A further marker of Th17 cells was described by Cosmi *et al*, who showed that CD161 was highly up-regulated in Th17 clones (Cosmi, De Palma et al. 2008). They demonstrated that these IL-17-producing cells originated from CD161⁺ naive CD4⁺ T cells from cord blood in response to IL-1 β and IL-23. Further studies have showed the presence of these CD161⁺CD4⁺ T cells in both the intestine (O'Keeffe, Doherty et al. 2004) and peripheral blood (Takahashi, Dejbakhsh-Jones et al. 2006) of healthy adults, as well as accumulations of these cells in Crohn's disease lesions (Kleinschek, Boniface et al. 2009) and in the joints of patients with RA (Warrington, Takemura et al. 2001). More recently the same authors looked at CD161⁺ CD4⁺ T cells in synovial fluid from the affected joints of children with oligoarticular-onset juvenile idiopathic arthritis (JIA) and found an accumulation of CD4⁺CD161⁺ T cells producing both IL-17 and IFN γ or IFN γ alone as compared to CD161⁺ CD4⁺ T cells from matched peripheral blood samples (Cosmi, Cimaz et al. 2011). They hypothesized that this population originated from a shift of IL-17 producing cells to a population capable of producing IL-17 and IFN γ . Interestingly spectratyping and clonotype analyses showed a similar skewing of the T cell receptor V β repertoire in both CD161⁺ Th17 cells and CD161⁺ Th1 cells derived from the SF of the same JIA patient, suggesting a common

cellular origin. This work supports an earlier study of T cell subsets in the joints of JIA patients (Nistala, Adams et al. 2010). With conditions that mimicked disease, more specifically low TGF β and high IL-12, the authors showed a skewing from IL-17 production to dual production of IL-17 and IFN γ by CD4 $^{+}$ T cells in the joints of JIA patients, with clonality studies again suggesting a common cellular origin. Interesting a high frequency of the Th1 cells, in addition to Th17 and Th1/17 cells, in the joints of the patients co-expressed CD161, and further analysis revealed a shared clonal origin of Th1 and Th17 cells expressing CD161. Both studies lend support to the hypothesis that Th17 cells are relatively unstable and that conversion to a population able to produce IFN γ alone, or in combination with IL-17, may define the pathogenic population in joint disease.

I have shown that KIR3DL2 $^{+}$ CD4 $^{+}$ T cells from inflammatory arthritis patients and healthy controls are enriched for expression of CD161 as compared to KIR3DL2 $^{-}$ CD4 $^{+}$ T cells from the same individuals. However the percentage of CD161 $^{+}$ CD4 $^{+}$ T cells expressing KIR3DL2 is not significantly different between inflammatory arthritis patients and healthy controls. It would be interesting to look at cytokine production by CD161 $^{+}$ KIR3DL2 $^{+}$ CD4 $^{+}$ T cells in the synovial fluid of AS patients to see the distribution of T cell subsets expressing KIR3DL2. Interestingly some initial data suggests restricted V β usage, with more oligoclonal TCR expression by KIR3DL2 $^{+}$ CD4 $^{+}$ T cells from the peripheral blood of AS patients (Kollnberger, unpublished data).

Th17 cells have been shown to express CCR6 (Acosta-Rodriguez, Rivino et al. 2007). The fact that Th17 cells express CCR6 is interesting in the context of IBD and AS given that CCR6 on these cells is proposed to regulate Th17 cell migration into the gut. More specifically CCR6 is important for localization to Peyer's patches and to other related tissue sites of the intestine where its ligand, CCL20, is expressed (Wang, Kang et al. 2009). Moreover, preferential

recruitment of CCR6⁺ Th17 cells to inflamed joints in RA has been observed (Hirota, Yoshitomi et al. 2007).

In addition to showing that KIR3DL2⁺ CD4⁺ T cells from inflammatory arthritis patients and healthy controls are enriched for CCR6, I have shown that a significantly higher proportion of CCR6⁺ CD4⁺ T cells from AS and RA patients and HLA-B27⁺ healthy controls express KIR3DL2 compared to HLA-B27⁻ healthy controls. This suggests that KIR3DL2⁺ CD4⁺ T cells may have homed to the gut, or become activated in the gut, and subsequently be recruited to the joints in inflammatory arthritis patients.

2) To determine whether KIR3DL2⁺ CD4⁺ T cells from AS patients are enriched for a phenotypic marker associated with activation as compared to KIR3DL2⁺ CD4⁺ T cells from healthy controls and RA patients.

In addition to the expression of phenotypic markers associated with Th17 cells a further objective was to investigate expression of a marker associated with activation on KIR3DL2⁺ CD4⁺ T cells. CD134 (OX40), a TNF-receptor family molecule, is expressed on the surface of CD4⁺ T cells following activation (Mallett, Fossum et al. 1990; Baum, Gayle et al. 1994), with peak expression between 48-72 hours (Gramaglia, Weinberg et al. 1998). Its ligand, CD252 (OX40L), is expressed on activated vascular endothelial cells, dendritic cells, B cells and NK cells (Imura, Hori et al. 1996; Ohshima, Tanaka et al. 1997; Flynn, Toellner et al. 1998; Zingoni, Sornasse et al. 2004). My FACS staining showed an enrichment of CD134 on KIR3DL2⁺ CD4⁺ T cells compared to KIR3DL2⁻ CD4⁺ T cells from inflammatory arthritis patients and healthy controls. Overall 40% of CD134⁺CD4⁺ T cells from AS patients express KIR3DL2, which is significantly higher than the percentage of CD134⁺CD4⁺ T cells in HLA-B27⁻ healthy controls and RA patients expressing KIR3DL2.

Ligation of CD134 is proposed to augment clonal expansion of CD134 expressing T cells as a result of increased survival of these cells, through upregulation of anti-apoptotic proteins including survivin (Song, So et al. 2005) and Bcl-2 and Bcl-xL (Rogers, Song et al. 2001). This is interesting if signalling through CD134 is potentially increasing the expression of anti-apoptotic molecules by KIR3DL2+ CD4+ T cells, conceivably leading to survival of these cells.

Expression of CD134 has been described on Th17 cells from mice in an ovalbumin-induced uveitis model of disease (Zhang, Zhong et al. 2010). Signalling through CD134 enhanced the effector function of CD134 expressing Th17 cells, with CD134-activating antibody significantly augmenting the production of Th17 cytokines. Enrichment of CD134 on IL-17 producing T cells has been described in patients with SLE (Dolff, Quandt et al. 2010). Inhibition of CD134-mediated signalling has been shown to reduce inflammation in other models including animal models of MS (Weinberg, Bourdette et al. 1996), diabetes (Pakala, Bansal-Pakala et al. 2004) and colitis (Higgins, McDonald et al. 1999).

CD134 has been shown to induce CCL20 expression by T cells (Zhong, Zhang et al. 2010). Using DO11.10 mice whose transgenic T cell receptor specifically recognizes ovalbumin, the authors demonstrated that ovalbumin induced CD134 expression on CD4+ T cells, and that stimulation of CD134 by CD134 activating antibody up-regulated CCL20 production. Intranasal administration of cell lysates derived from cells following CD134 stimulation resulted in more severe leukocyte infiltration in the lungs of DO11.10 mice, which was substantially attenuated by CCL20 blocking antibody, suggesting a role for CD134 in chemotaxis, as well as its classical role in co-stimulation and cell survival.

3) To determine whether KIR3DL2+ CD4+ T cells from AS patients are enriched for a phenotypic marker associated with homing to the gut as compared to KIR3DL2+ CD4+ T cells from healthy controls and RA patients.

I show here that KIR3DL2+ CD4+ T cells from inflammatory arthritis patients and healthy controls are enriched for expression of CCR9 as compared to KIR3DL2- CD4+ T cells from the same individuals. Overall 41% of CCR9+CD4+ T cells from AS patients express KIR3DL2, significantly higher than in HLA-B27- healthy controls and RA patients. Moreover, the percentage of CCR9+CD4+ T cells from HLA-B27+ healthy controls expressing KIR3DL2 is significantly higher than in HLA-B27- healthy controls.

CCR9 is a chemokine receptor expressed on most small intestinal lamina propria and intraepithelial lymphocytes, as well as on thymocytes (Zabel, Agace et al. 1999). It signals through thymus-expressed chemokine (TECK or CCL25), which is constitutively expressed in the thymus and small intestine but not the colon (Papadakis, Prehn et al. 2000). More specifically TECK acts as a chemoattractant for PBMC that co-express the integrin $\alpha 4\beta 7$ and CCR9. CCR9 is also expressed by a small subset of peripheral blood lymphocytes in healthy individuals. A study into the phenotype of these CCR9+ CD4+ T cells showed they co-expressed both CD40L and CD134, suggesting an activated phenotype (Papadakis, Landers et al. 2003). A higher fraction of CCR9+CD4+ T cells have been detected in the peripheral blood of patients with small intestinal Crohn's disease and coeliac disease, compared with patients with isolated colonic Crohn's disease and healthy controls (Papadakis, Prehn et al. 2001).

In support of my finding, expression of $\beta 7$ integrin, a surface marker associated with lymphocyte recruitment to intestinal tissue, has previously been reported on KIR3DL2+ NK cells, as compared to KIR3DL2- NK cells (Chan, Kollnberger et al. 2005). Increased $\beta 7$

integrin expression has also been observed in SpA synovium compared with RA synovium (Elewaut, De Keyser et al. 1998).

Given the increase in CCR9 expression on KIR3DL2+ CD4+ T cells, and the increase in β 7 integrin expression on KIR3DL2+ NK cells previously described, it is interesting to hypothesize that these cells may have recirculated from the gut. Of note an enrichment of KIR3DL2+ CD4+ T cells have also been seen in gut samples from Crohn's disease and ulcerative colitis patients (Wong, unpublished data). Since peripheral blood KIR3DL2+ CD4+ T cells have an activated phenotype, with increased expression of CD134, a further hypothesis is that these cells are being activated in the gut, before moving out into circulation. Given that the primary site of pathology in AS is the enthesis it is possible that circulating KIR3DL2+ CD4+ T cells may then home to the joints of AS patients. FACS staining revealed that in 3 of 4 matched peripheral blood and synovial fluid samples from AS patients there was elevated KIR3DL2+ CD4+ T cells in the synovial fluid samples, with an increase from 3.1% to 12.9% in one patient. However more samples need to be investigated to see if the difference in percentages of KIR3DL2+ CD4+ T cells between peripheral blood and synovial fluid is significant. It would be of interest to isolate cells from tissue samples from affected joint in AS patients to look at expression of surface markers and cytokine production for comparison to peripheral blood samples. Moreover, in order to gain more of an insight into the distribution of KIR3DL2+ CD4+ T cells at sites of inflammation it would be interesting to use imaging techniques to look at tissue sections from the inflamed joints from AS patients.

In summary I have confirmed that KIR3DL2+ CD4+ T cells are expanded in the peripheral blood of AS patients. I have shown that KIR3DL2+ CD4+ T cells are enriched for expression of IL-23R, IL-1R, CD161 and CCR6, all surface markers associated with Th17 cells, compared to

KIR3DL2⁻ CD4⁺ T cells. FACS staining revealed that the majority of IL-23R⁺ CD4⁺ T cells from AS patients express KIR3DL2, and this is significantly higher than in HLA-B27⁻ healthy controls. Interestingly synovial fluid samples from SpA patients are further enriched for IL-23R as compared to matched peripheral blood samples. Moreover, significantly higher proportions of IL-1R⁺ CD4⁺ T cells and CCR6⁺ CD4⁺ T cells from AS patients express KIR3DL2, as compared to HLA-B27⁻ healthy controls. In addition my findings that KIR3DL2⁺ CD4⁺ T cells are enriched for CD134 and CCR9 might suggest that these cells are activated and may have homed from the gut.

Chapter 4: CD4⁺ T cell cytokine production in inflammatory arthritis

4.1. Introduction

IL-17 is produced by a wide variety of cells, as discussed in section 1.5.2, including CD4⁺ T cells, $\gamma\delta$ T cells, a subset of NKT cells, mast cells and neutrophils. Elevated levels of IL-17 in both serum and synovial fluid from patients with SpA have been described (Wendling, Cedoz et al. 2007; Melis, Vandooren et al. 2009). In addition, CD4⁺ T cells producing IL-17 have been described in SpA (Jandus 2008; Shen, Goodall et al. 2009). Increasingly there is interest in the ability of T cell at sites of inflammation to be able to produce multiple cytokines. This evidence for plasticity has been observed in a number of different diseases both in animal studies (Martin-Orozco, Chung et al. 2009; Hirota, Duarte et al. 2011) and in sites of inflammation in human diseases, including Crohn's disease and JIA (Annunziato, Cosmi et al. 2007; Nistala, Adams et al. 2010).

Having shown that KIR3DL2⁺ CD4⁺ T cells are enriched for phenotypic markers associated with Th17 cells I sought to look at IL-17 production by KIR3DL2⁺ CD4⁺ T cells after different stimulus. To this end I investigated IL-17 production directly *ex vivo* after PMA and ionomycin stimulation. In addition to IL-17 I look at production of IFN γ . I also investigated cytokine production in cultured supernatants from KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells after 5 days stimulation with anti-CD2/3/28 beads and IL-2 (50U/ml) and investigated the affect of the addition of rIL-23 (10pg/ml) and rIL-1 (10pg/ml).

4.2. Aim

The overall aim of this chapter was to investigate cytokine production by KIR3DL2+ CD4+ T cells in order to more fully determine the function of KIR3DL2+ CD4+ T cell in the peripheral blood of patients with spondyloarthritis as compared to healthy and inflammatory disease controls. To achieve this I had 3 objectives.

- To determine whether KIR3DL2+ CD4+ T cells are enriched for cytokine production as compared to KIR3DL2- CD4+ T cells.
- To determine whether KIR3DL2+ CD4+ T cells from AS patients are enriched for cytokine production as compared to KIR3DL2+ CD4+ T cells from healthy controls and RA patients.
- To determine whether the addition of Th17 polarizing cytokines affects cytokine production by KIR3DL2+ CD4+ T cells.

4.3. Results

4.3.1. KIR3DL2+ CD4+ T cells are enriched for IL-17 production

I performed intracellular cytokine staining (ICS) for IL-17 on PBMC from 16 AS patients and 15 HLA-B27- healthy controls. Figure 4.1 shows representative FACS staining from one AS patient. Following 6 hours stimulation with PMA and ionomycin a higher percentage of KIR3DL2+ CD4+ T cells produce IL-17, compared to KIR3DL2- CD4+ T cells (6% v 2.14%).

Figure 4.2.A shows the results from all AS patients and HLA-B27- healthy controls investigated. A significantly higher percentage of KIR3DL2+ CD4+ T cells from AS patients and healthy controls produce IL-17 compared to KIR3DL2- CD4+ T cells from the same individuals. Furthermore, a significantly higher percentage of KIR3DL2+ CD4+ T cells from AS patients produce IL-17, as compared to KIR3DL2+ CD4+ T cells from HLA-B27- healthy controls, as shown in Figure 4.2.B. There was no difference in IL-17 production when comparing KIR3DL2- CD4+ T cells from AS patients and HLA-B27- healthy controls.

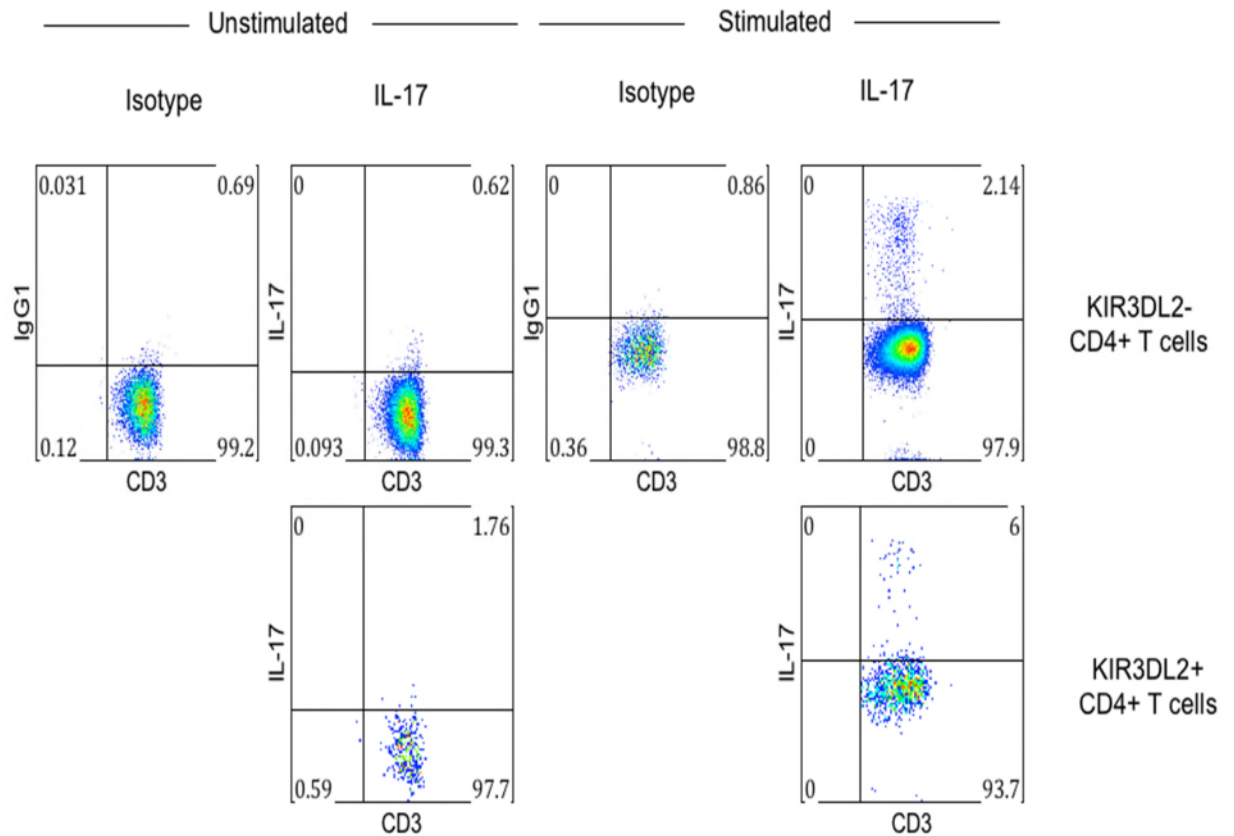


Figure 4.1: A higher percentage of KIR3DL2+ CD4+ T cells from an AS patient produce IL-17 compared to KIR3DL2- CD4+ T cells. Representative FACS staining shows a higher percentage of KIR3DL2+ CD4+ T cells produce IL-17, as compared to KIR3DL2- CD4+ T cells from the same patient, both before and after stimulation with PMA and ionomycin. ICS was performed as described in chapter 2, section 2.2.8.2.

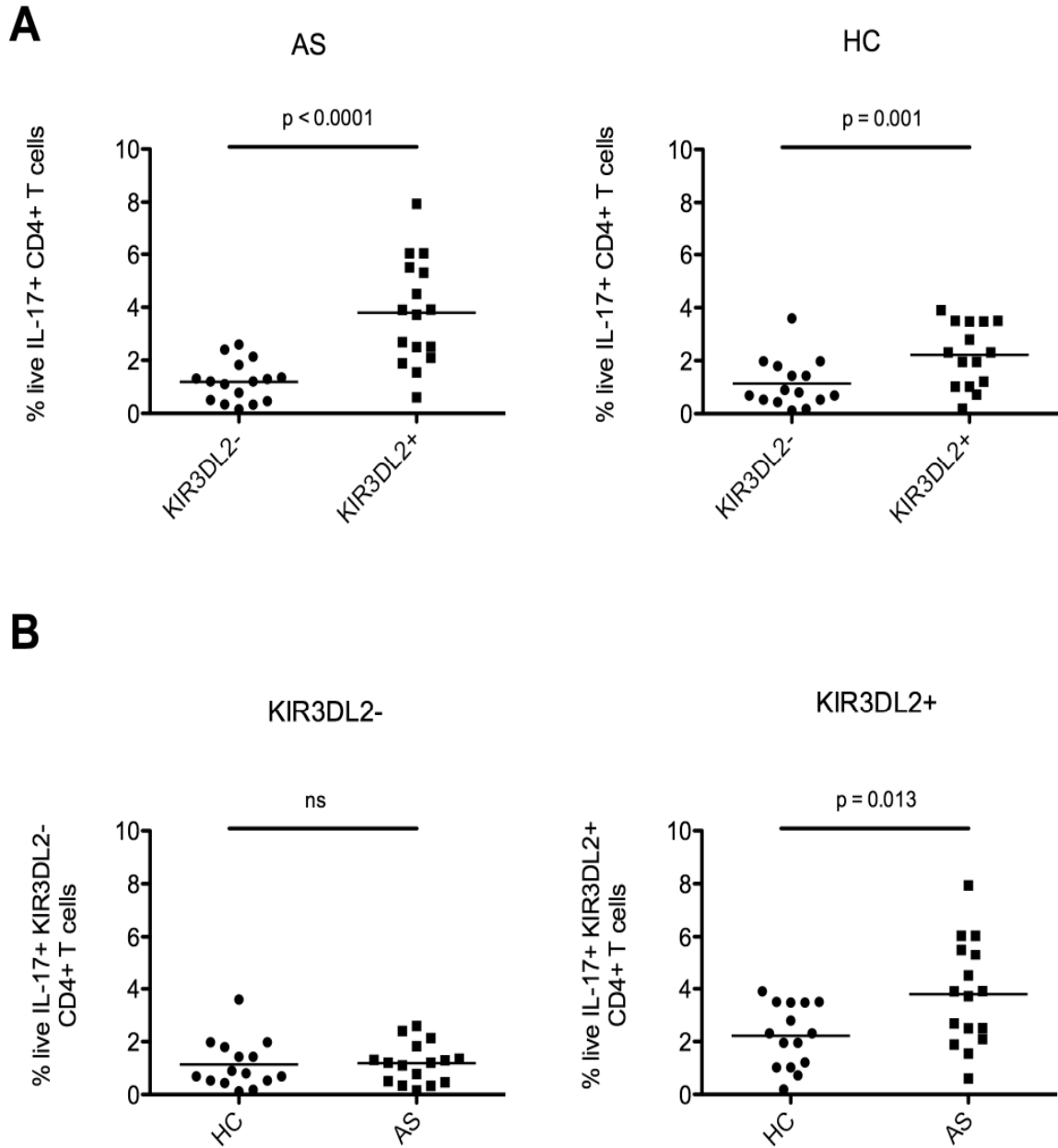


Figure 4.2: Higher percentages of KIR3DL2+ CD4+ T cells produce IL-17 compared to KIR3DL2- CD4+ T cells from AS patients and HLA-B27- healthy controls. (A) Percentages of KIR3DL2+ and KIR3DL2- CD4+ T cells producing IL-17 from peripheral blood of AS patients (n=16) and HLA-B27- healthy controls (n=15) after 6 hours stimulation with PMA and ionomycin. A two-way paired student t test was used for statistical analysis. (B) Comparison of IL-17 production by KIR3DL2+ or KIR3DL2- CD4+ T cells from AS patients and healthy controls, after 6 hours stimulation with PMA and ionomycin. An unpaired t test was used for statistical analysis. Note data in Figure 4.2.B is drawn from Figure 4.2.A.

Having shown that KIR3DL2+ CD4+ T cells from AS patients are enriched for IL-17 directly *ex vivo* after stimulation with PMA and ionomycin I wanted to look at production of IL-17 in supernatants after culture of KIR3DL2+ and KIR3DL2- CD4+ T cells after 5 days stimulation with anti-CD2/3/28 beads. Raw data displayed as a heat map can be seen in appendix 1. Figure 4.3 shows that KIR3DL2+ CD4+ T cells from AS patients and RA patients produce more IL-17, compared to KIR3DL2- CD4+ T cells. KIR3DL2+ CD4+ T cells from AS patients produce an average of 4,422pg/ml/50,000 cells, compared to 2,657pg/ml/50,000 cells by KIR3DL2- CD4+ T cells.

When comparing IL-17 production across disease and healthy control groups, as shown in Figure 4.4.A, we see that there is a trend, although not significant, for greater production of IL-17 by KIR3DL2+ CD4+ T cells from AS patients compared to HLA-B27- and HLA-B27+ healthy controls and RA patients.

Figure 4.4.B shows that KIR3DL2- CD4+ T cells from AS patients produce significantly more IL-17 than KIR3DL2- CD4+ T cells from HLA-B27+ healthy controls and RA patients, with an overall production of IL-17 by KIR3DL2+ CD4+ T cells from AS patients of 2,658pg/ml/50,000 cells.

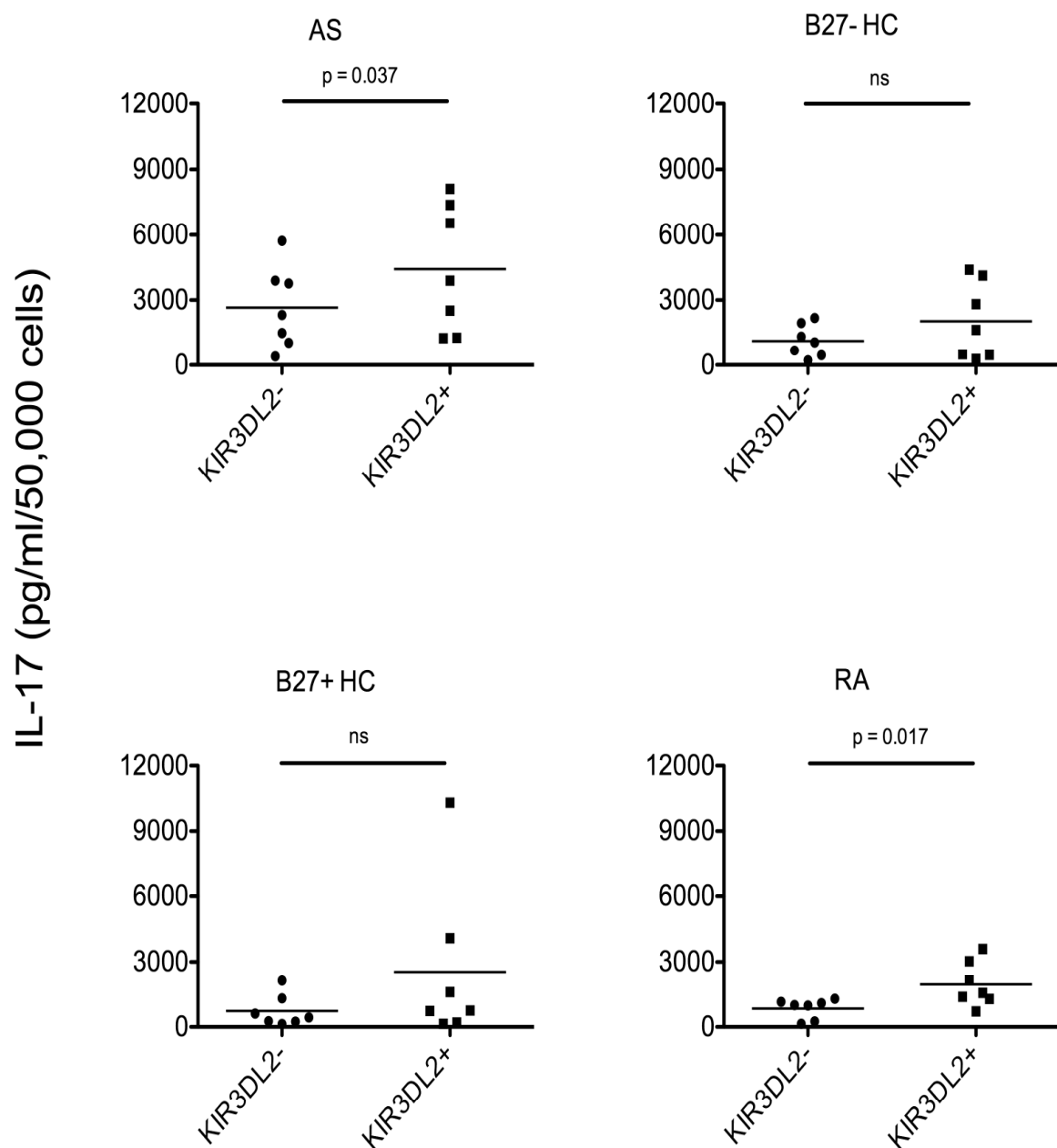
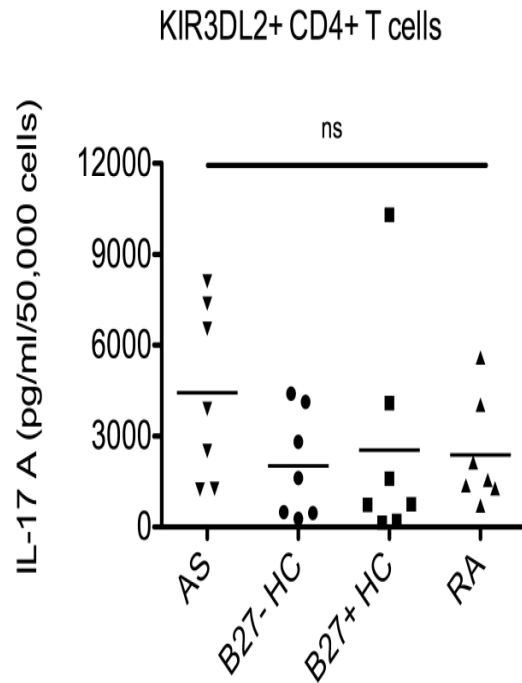


Figure 4.3: KIR3DL2+ CD4+ T cells from patients with inflammatory arthritis produce more IL-17 than KIR3DL2- CD4+ T cells. FACS-sorted KIR3DL2+ and KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants, as described in chapter 2, section 2.2.9.1. and 2.2.12. A two-tailed student t test was used for statistical analysis.

A



B

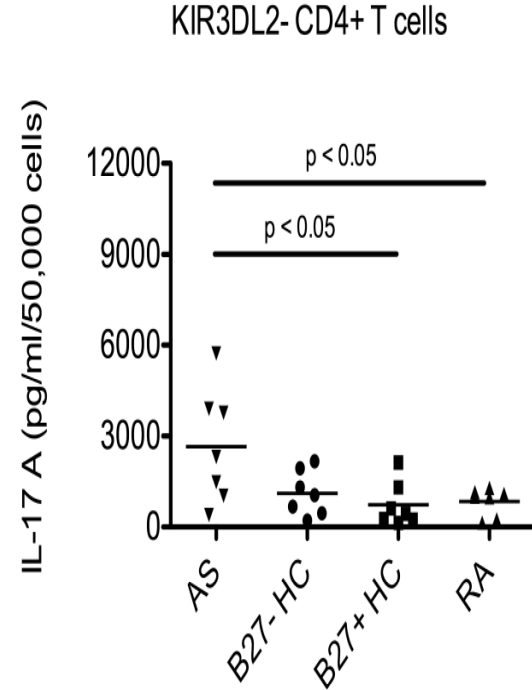


Figure 4.4: Higher IL-17 production by KIR3DL2- CD4+ T cells from AS patients compared to healthy and inflammatory disease controls. (A) FACS-sorted KIR3DL2+ and (B) KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. A Kruskal-Wallis test was used for statistical analysis in (A) and a one-way ANOVA was used for statistical analysis in (B). Note data is drawn from Figure 4.1.

4.3.2. IL-17 production from KIR3DL2+ CD4+ T cells in SpA patients is further increased by addition of recombinant (r)IL-1/IL-23

Having shown that KIR3DL2+ CD4+ T cells are enriched for Th17 markers I asked whether the addition of rIL-1/23 altered the production of IL-17 by these cells. KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells were obtained by FACS sorting as previously described. Figure 4.5 shows that the addition of rIL-1/23 to KIR3DL2+ CD4+ T cells from AS patients, but not the other groups investigated, significantly increases the production of IL-17. The addition of rIL-1/23 to KIR3DL2- CD4+ T cells did not significantly alter the production of IL-17 by these KIR3DL2- CD4+ T cells in any of the groups investigated, as shown in Figure 4.6.

When comparing IL-17 production from KIR3DL2+ CD4+ T cells after treatment with rIL-1/23, across disease and healthy control groups, as shown in Figure 4.7.A, we see that there is no difference in IL-17 production by KIR3DL2+ CD4+ T cells from any of the groups investigated. However three of the seven AS patients produce in excess of 40,000 pg/ml/50,000 cells, which is greater than double the production by KIR3DL2+ CD4+ T cells from the other four AS patients. This suggests there might be two groups in terms of IL-17 production, although analysis of further numbers of AS patients would be required to confirm this. Figure 4.7.B shows that KIR3DL2- CD4+ T cells from AS patients produce significantly more IL-17 than KIR3DL2- CD4+ T cells from RA patients and HLA-B27+ healthy controls after treatment with rIL-1/23.

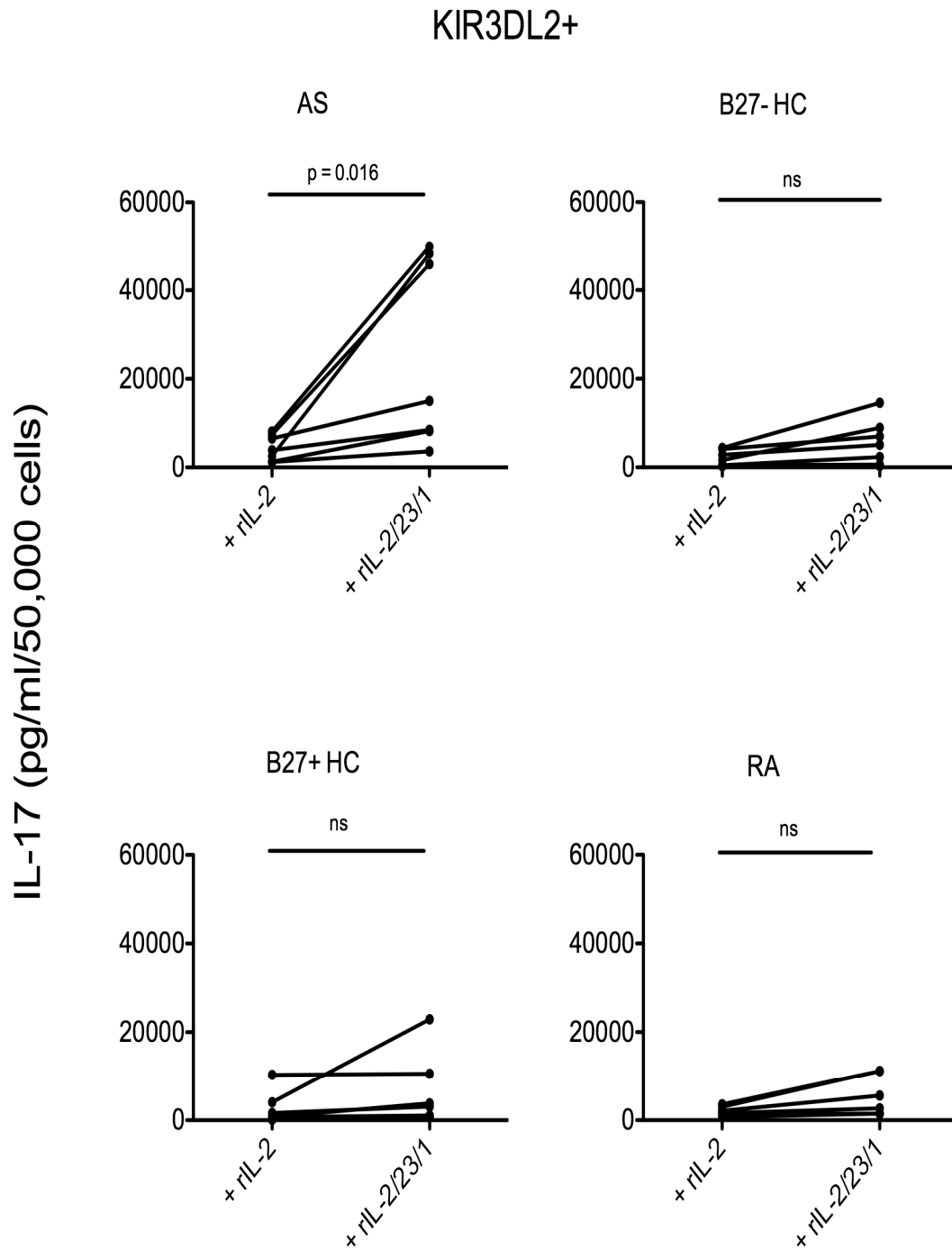


Figure 4.5: IL-17 production by KIR3DL2+ CD4+ T cells from AS patients is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2+ CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads and rIL-2, but without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A Wilcoxon signed-rank test was used for statistical analysis.

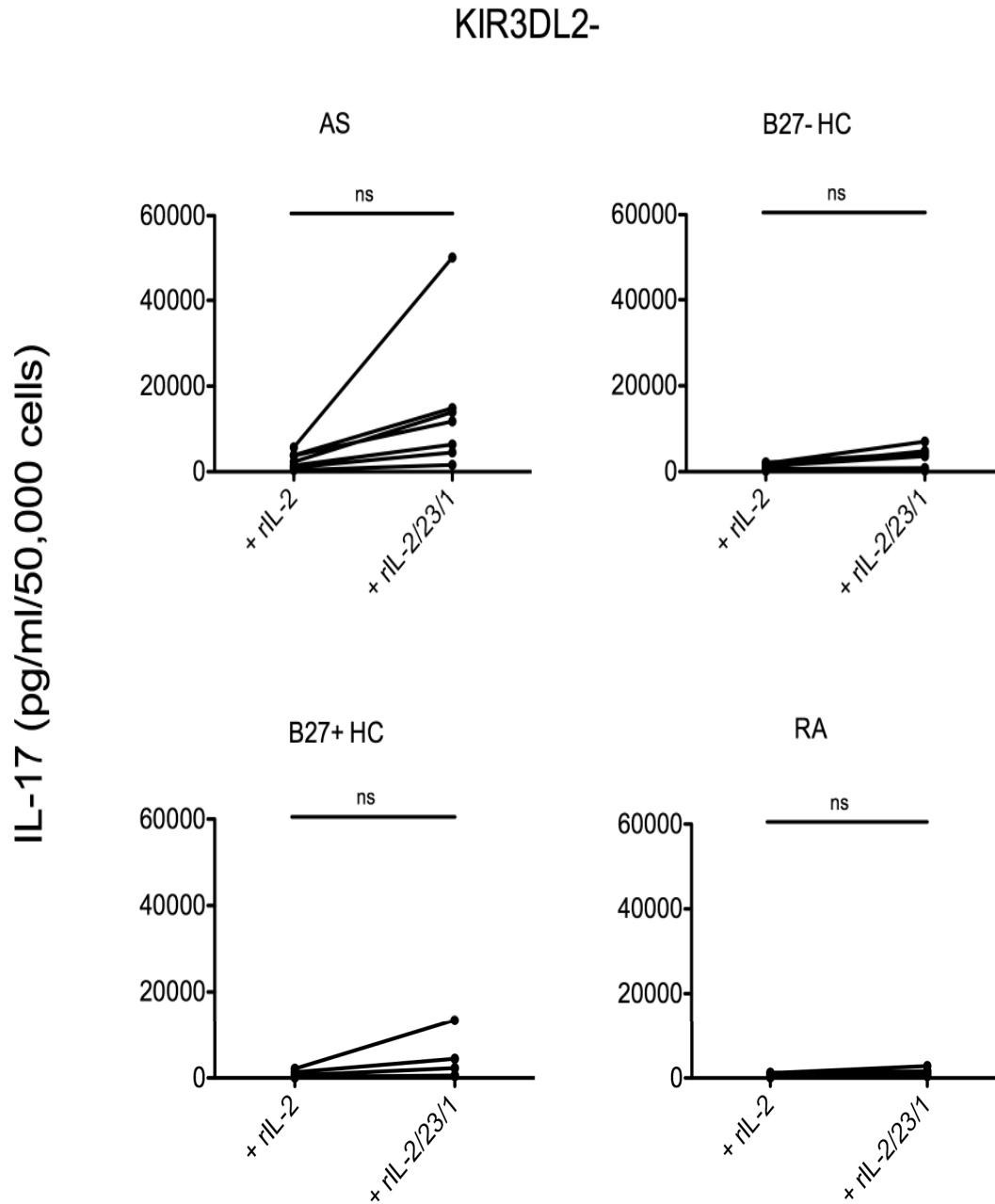


Figure 4.6: IL-17 production by KIR3DL2- CD4+ T cells is not significantly altered by rIL-1/23 treatment. FACS-sorted KIR3DL2+ CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

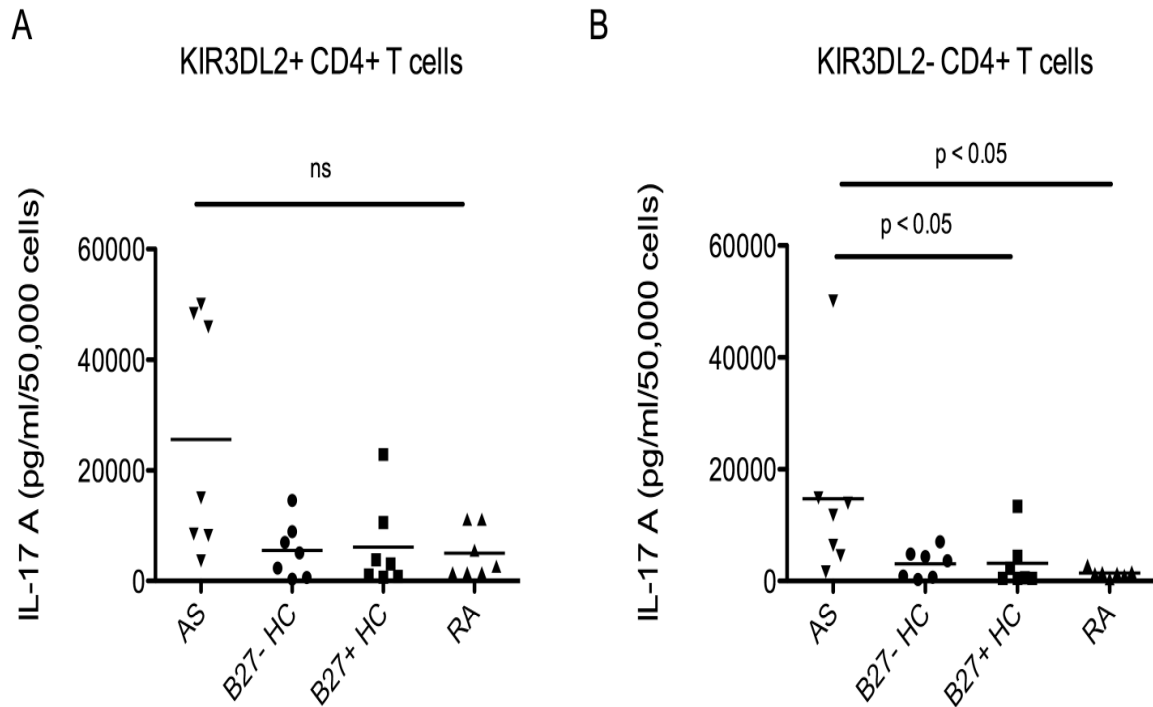


Figure 4.7: Higher IL-17 production by KIR3DL2- CD4+ T cells from AS patients in response to rIL-23/1 compared to healthy controls and RA patients. (A) FACS-sorted KIR3DL2+ and (B) KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days, in the presence of rIL-1/23, prior to multiplex bead analysis of supernatants. A Kruskal-Wallis test was used for statistical analysis in (A) and a one-way ANOVA was used for statistical analysis in (B). Note data is drawn from Figure 4.5. and 4.6.

4.3.3. KIR3DL2+ CD4+ T cells are enriched for IFN γ production by ICS but not multiplex bead analysis

In addition to ICS for IL-17 I also performed ICS for IFN γ on PBMC from 8 AS patients and 8 HLA-B27 healthy controls. Figure 4.8 shows representative FACS staining from one AS patient. Without stimulation there is no IFN γ production. After stimulation a higher percentage of KIR3DL2+ CD4+ T cells produce IFN γ , compared to KIR3DL2- CD4+ T cells (13.7% v 7.14%).

Figure 4.9.A shows the data from all the AS patients and HLA-B27 healthy controls investigated, demonstrating that KIR3DL2+ CD4+ T cells from both AS patients and HLA-B27- healthy controls produce more IFN γ than KIR3DL2- CD4+ T cells from the same individuals. When comparing IFN γ between AS patients and HLA-B27- healthy controls, as seen in Figure 4.9.B, we see that there is no difference in IFN γ production by KIR3DL2+ or KIR3DL2- CD4+ T cells from AS patients or HLA-B27- healthy controls.

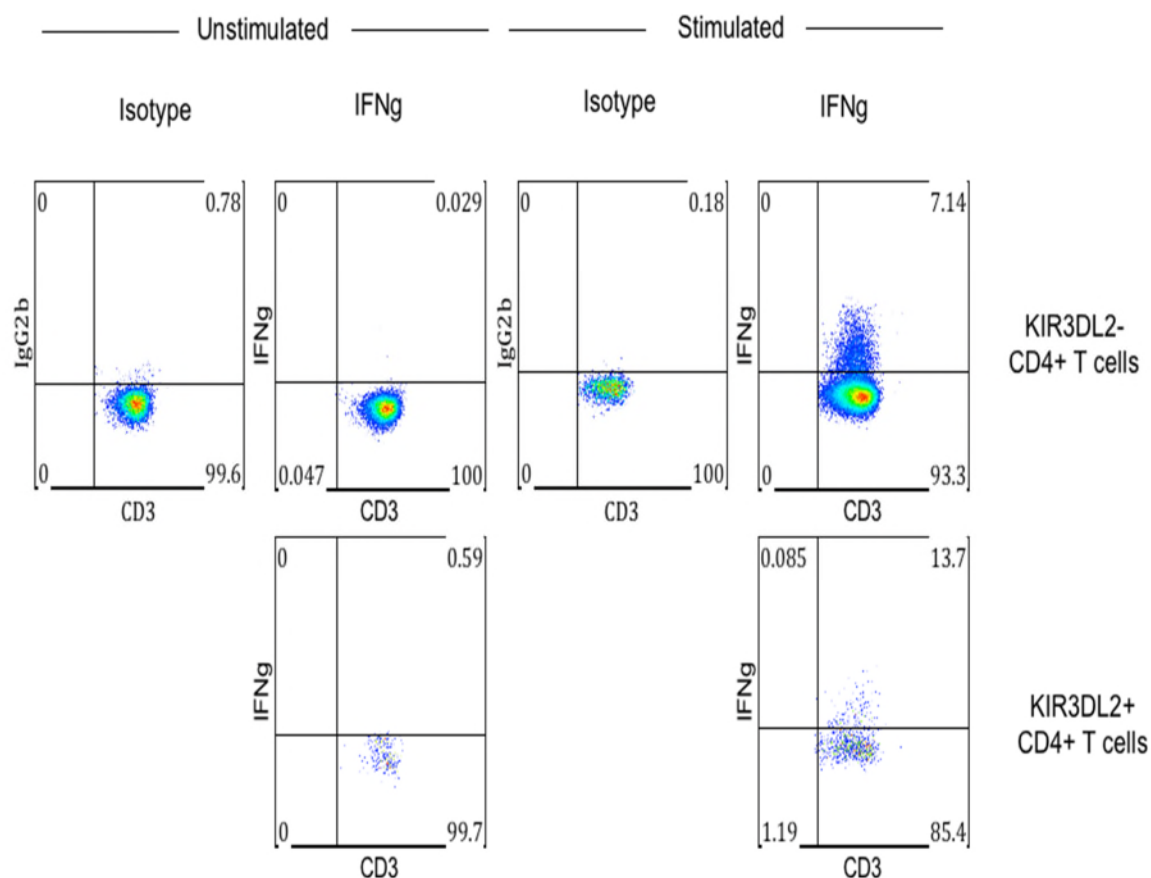
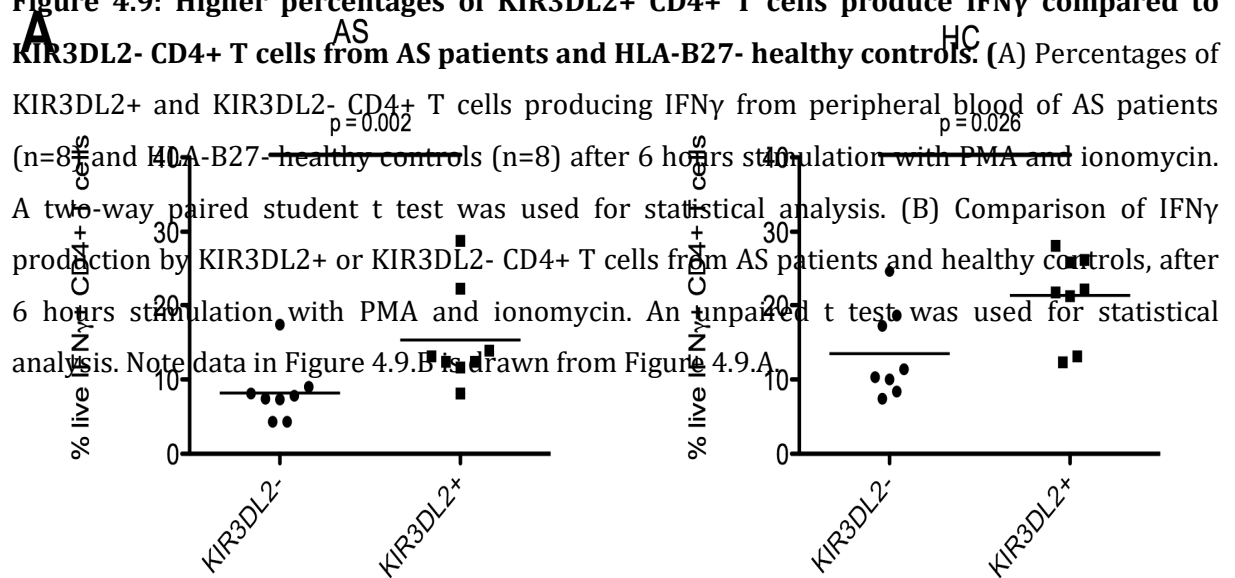


Figure 4.8: A higher percentage of KIR3DL2+ CD4+ T cells from an AS patient produce IFN γ compared to KIR3DL2- CD4+ T cells. Representative FACS staining shows a higher percentage of KIR3DL2+ CD4+ T cells produce IFN γ , as compared to KIR3DL2- CD4+ T cells from the same patient, both before and after stimulation with PMA and ionomycin.

Figure 4.9: Higher percentages of KIR3DL2+ CD4+ T cells produce IFN γ compared to KIR3DL2- CD4+ T cells from AS patients and HLA-B27- healthy controls. (A) Percentages of KIR3DL2+ and KIR3DL2- CD4+ T cells producing IFN γ from peripheral blood of AS patients (n=8) and HLA-B27- healthy controls (n=8) after 6 hours stimulation with PMA and ionomycin. A two-way paired student t test was used for statistical analysis. (B) Comparison of IFN γ production by KIR3DL2+ or KIR3DL2- CD4+ T cells from AS patients and healthy controls, after 6 hours stimulation with PMA and ionomycin. An unpaired t test was used for statistical analysis. Note data in Figure 4.9.B is drawn from Figure 4.9.A.



IFN γ production by anti-CD2/3/28 stimulated KIR3DL2+ and KIR3DL2- CD4+ T cells was investigated by multiplex bead analysis. Figure 4.10 shows that KIR3DL2+ CD4+ T cells from HLA-B27- healthy controls and RA patients produce more IFN γ than KIR3DL2- CD4+ T cells.

When comparing IFN γ production from KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells across all groups investigated, in Figure 4.11.A and 4.11.B respectively, there is no significant difference between the production of IFN γ between the groups.

The addition of rIL-23/IL-1 significantly increases production of IFN γ from KIR3DL2+ CD4+ T cells from AS and RA patients and HLA-B27- healthy controls, as seen in Figure 4.12. The addition of rIL-1/23 to KIR3DL2- CD4+ T cells significantly increases the production of IFN γ from these cells in all of the groups investigated, as seen in Figure 4.13.

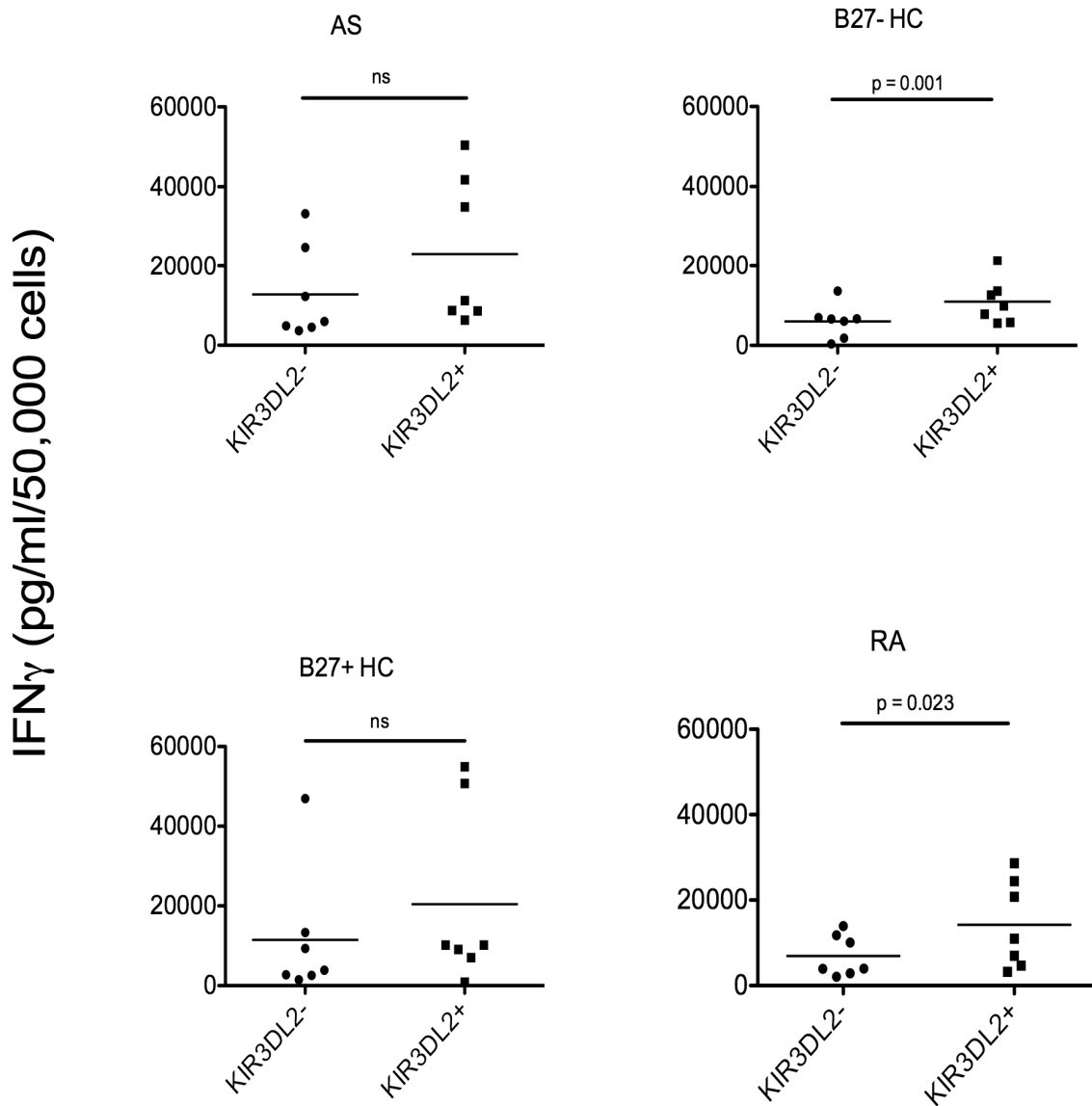
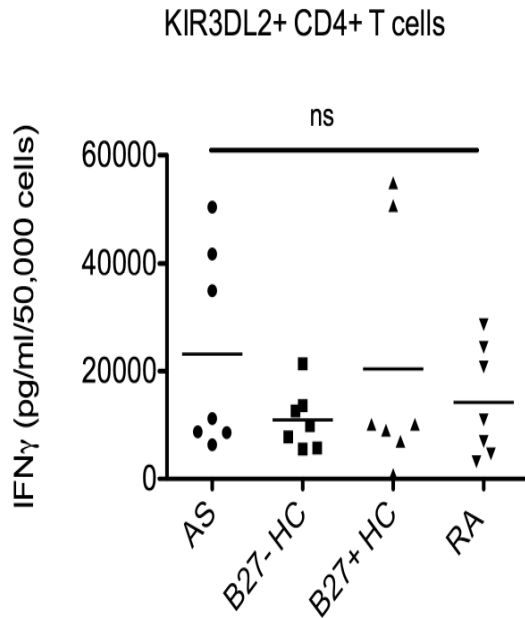


Figure 4.10: KIR3DL2⁺ CD4⁺ T cells from AS patients and HLA-B27⁺ healthy controls do not produce more IFN γ than KIR3DL2⁻ CD4⁺ T cells. FACS-sorted KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells from AS (n=7) and RA (n=7) patients and HLA-B27⁻ (n=7) and HLA-B27⁺ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

A



B

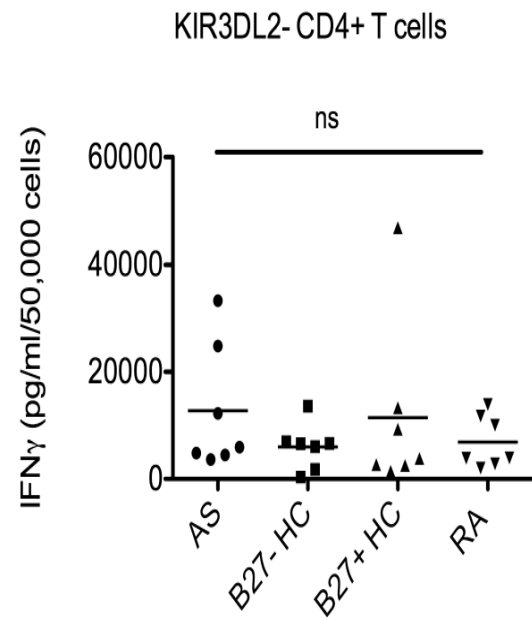


Figure 4.11: No significant difference in IFN γ production by KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells from AS patients compared to healthy controls and inflammatory disease controls. (A) FACS-sorted KIR3DL2+ and (B) KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. A Kruskal-Wallis test was used for statistical analysis. Note data is drawn from Figure 4.10.

KIR3DL2+

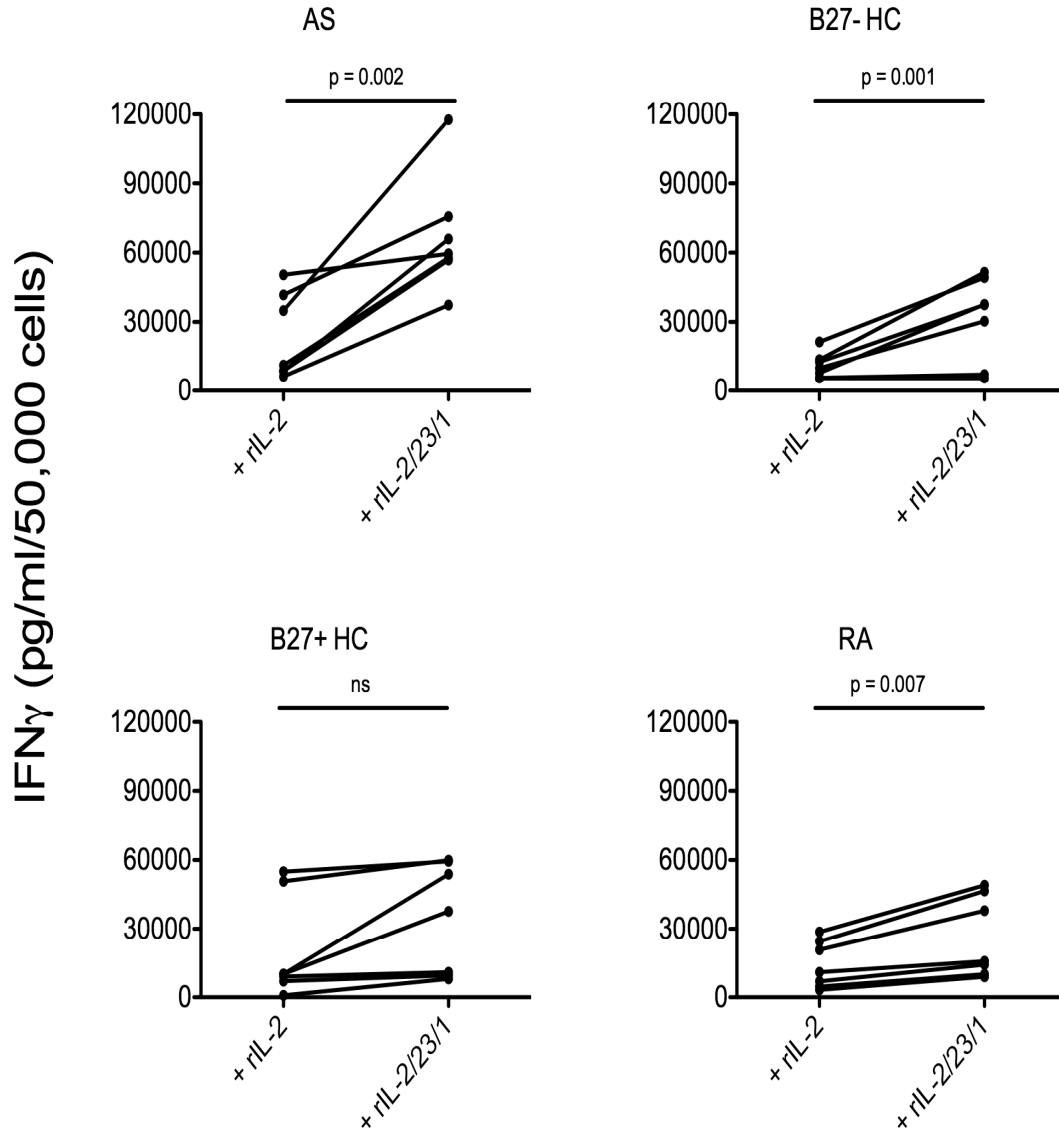


Figure 4.12: IFN γ production by KIR3DL2+ CD4+ T cells is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2+ CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

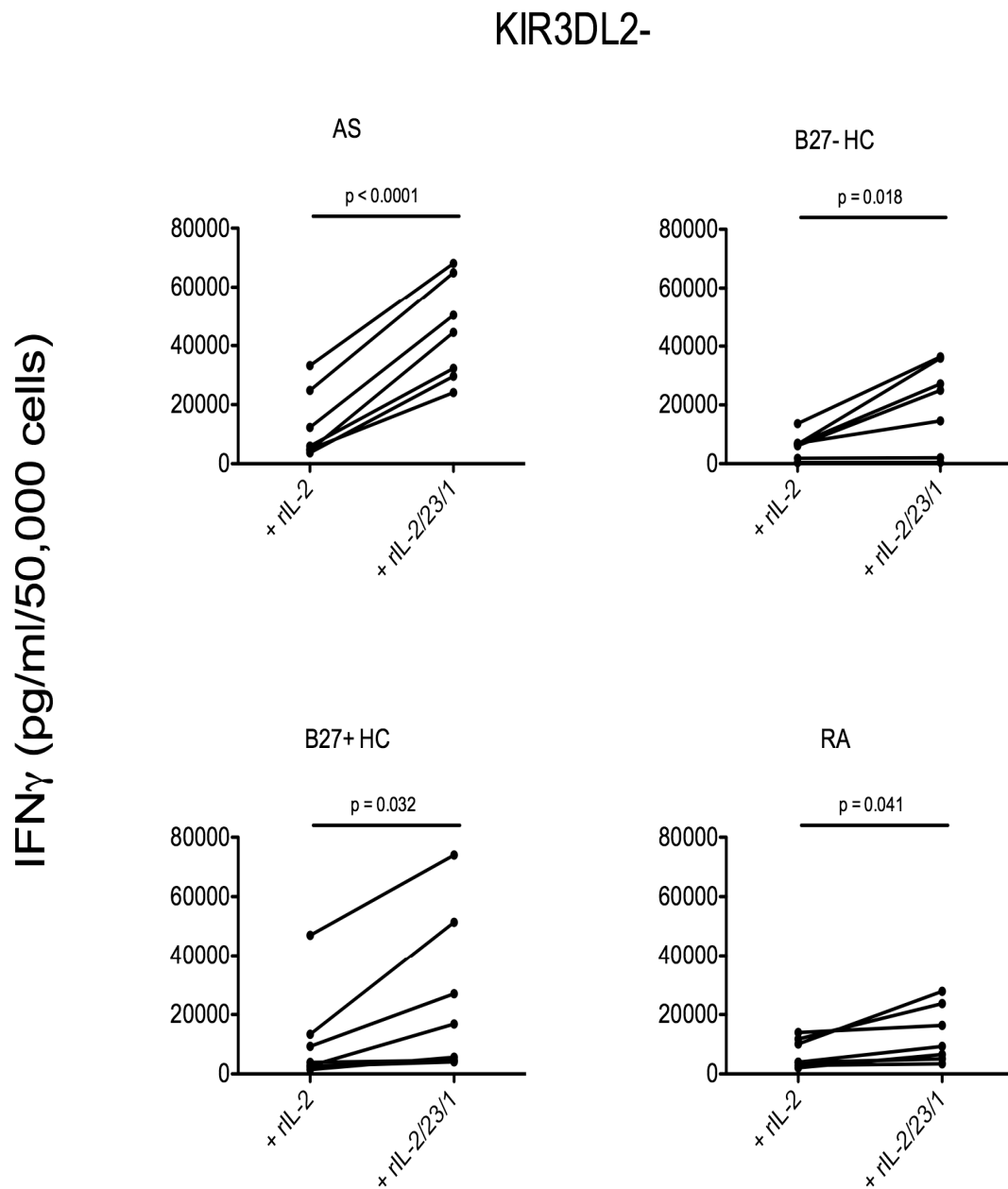


Figure 4.13: IFN γ production by KIR3DL2- CD4+ T cells is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

4.3.4. KIR3DL2+ CD4+ T cells are enriched for dual production of IL-17 and IFN γ

ICS was used to look at simultaneous production of IL-17 and IFN γ by KIR3DL2+ CD4+ T cells from AS patients and HLA-B27- healthy controls. In these experiments ICS has the advantage over multiplex bead analysis in that it is able to measure dual cytokine production on a per cell basis. Figure 4.14 shows representative FACS staining from one AS patient. Without stimulation there is no IL-17 or IFN γ production. After stimulation a higher percentage of KIR3DL2+ CD4+ T cells produce both IL-17 and IFN γ , compared to KIR3DL2- CD4+ T cells (1.73% v 0.26%).

In Figure 4.15.A we see that KIR3DL2+ CD4+ T cells from both AS patients and HLA-B27- healthy controls produce more IL-17 and IFN γ than KIR3DL2- CD4+ T cells from the same individuals. However when comparing IL-17 and IFN γ production between AS patients and HLA-B27- healthy controls, both from KIR3DL2- and KIR3DL2+ CD4+ T cells, as seen in Figure 4.15.B, there is no significant difference in the dual production of IL-17 and IFN γ .

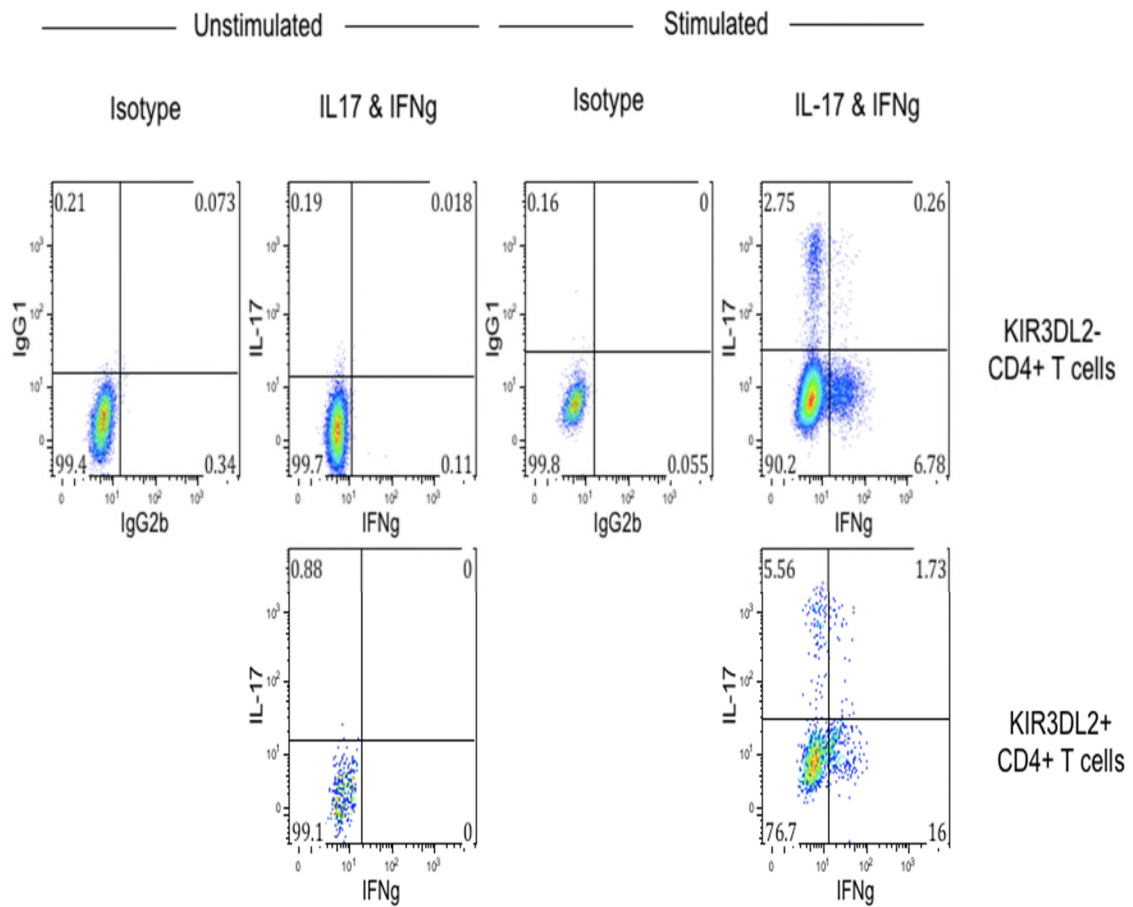


Figure 4.14: A higher percentage of KIR3DL2+ CD4+ T cells from an AS patient produce IL-17 and IFN γ compared to KIR3DL2- CD4+ T cells. Representative FACS staining shows a higher percentage of KIR3DL2+ CD4+ T cells producing both IL-17 and IFN γ , as compared to KIR3DL2- CD4+ T cells from the same patient, after stimulation with PMA and ionomycin.

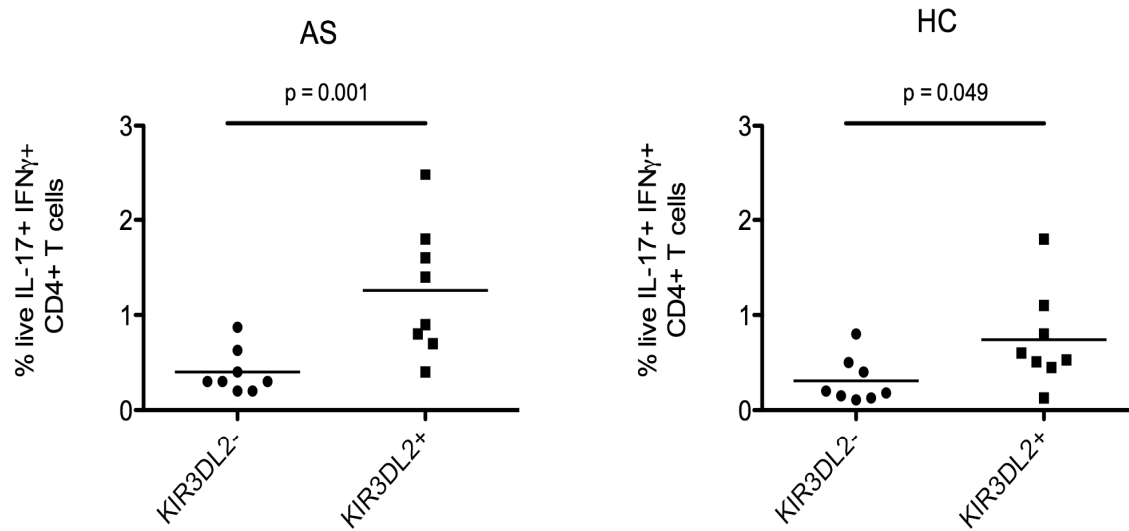
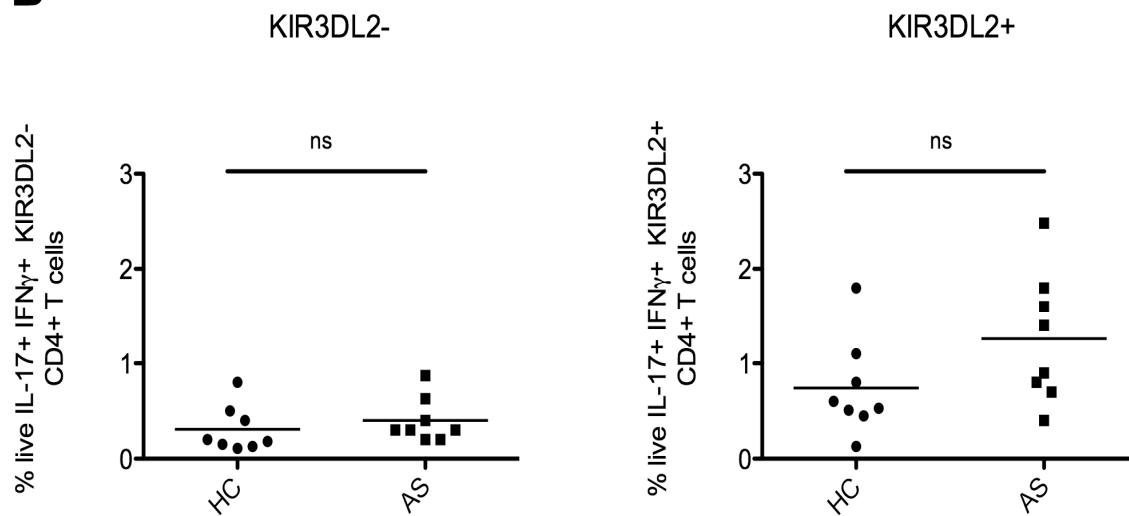
A**B**

Figure 4.15: Higher percentages of KIR3DL2⁺ CD4⁺ T cells produce IL-17 and IFN γ compared to KIR3DL2⁻ CD4⁺ T cells from AS patients and healthy controls. (A) Percentages of KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells producing IL-17 and IFN γ from peripheral blood of AS patients (n=8) and healthy controls (n=8) after 6 hours stimulation with PMA and ionomycin. A two-way paired student t test was used for statistical analysis. (B) Comparison of IL-17 and IFN γ production by KIR3DL2⁺ or KIR3DL2⁻ CD4⁺ T cells from AS patients and healthy controls, after 6 hours stimulation with PMA and ionomycin. An unpaired t test was used for statistical analysis. Note data in Figure 4.15.B is drawn from Figure 4.15.A.

4.3.5. TNF α production by KIR3DL2+ and KIR3DL2- CD4+ T cells is increased by the addition of rIL-1/IL-23

Production of TNF α was also investigated since this cytokine plays a key role in the pathogenesis of AS. Figure 4.16.A shows that there was no significant difference in the production of TNF α by KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells in any of the groups investigated. Moreover, there was no significant difference in the production of TNF α from KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells across all groups investigated, as shown in Figure 4.16.B and 4.16.C respectively.

The addition of rIL-1/23 significantly increases production of TNF α from KIR3DL2+ CD4+ T cells from AS, as seen in Figure 4.17. An increase in TNF α production following the addition of rIL-1/23 to KIR3DL2+ CD4+ T cells from HLA-B27- healthy controls and RA patients was also observed, although the levels of TNF α produced were lower than levels produced by KIR3DL2+ CD4+ T cells from AS patients after the addition of rIL-1/23. There was also a significant increase in the production of TNF α from KIR3DL2- CD4+ T cells after treatment with rIL-1/23 in all groups investigated, as shown in Figure 4.18.

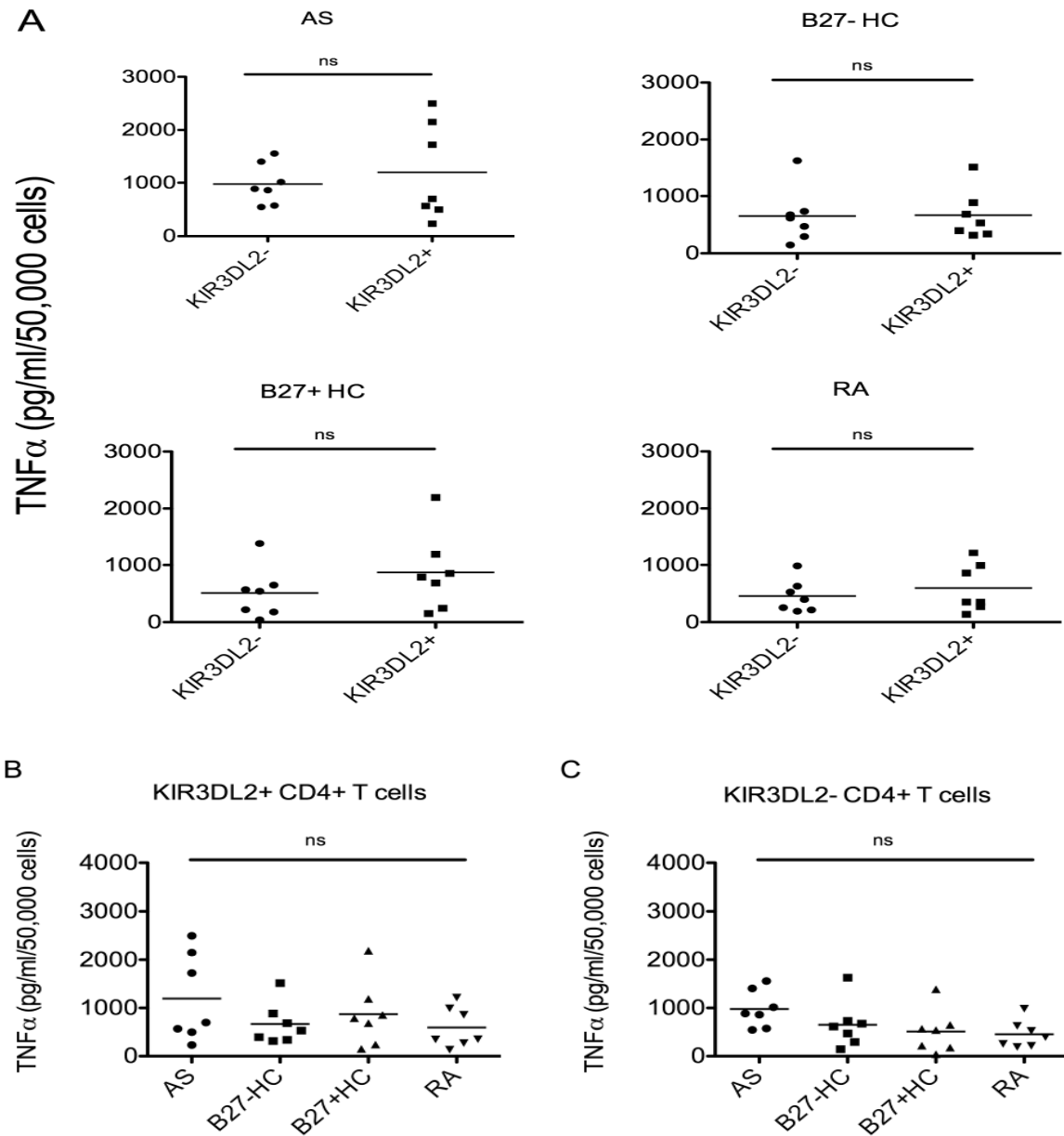
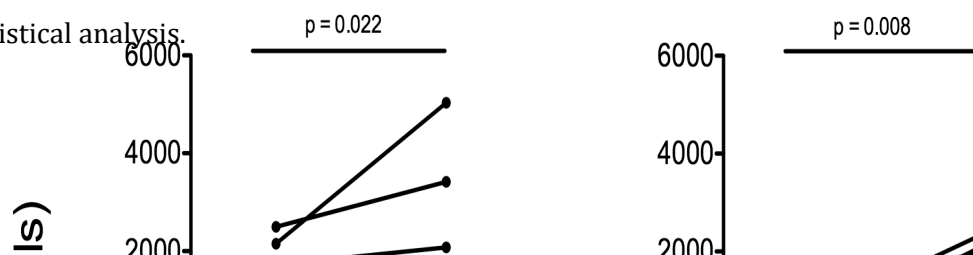


Figure 4.16: No significant difference in the production of TNF α between KIR3DL2⁺ CD4⁺ T cells and KIR3DL2⁻ CD4⁺ T cells. FACS-sorted KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells from AS (n=7) and RA (n=7) patients and HLA-B27⁻ (n=7) and HLA-B27⁺ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. (A) Comparison of TNF α production by KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells. A two-tailed student t test was used for statistical analysis. (B) Comparison of TNF α production from KIR3DL2⁺ CD4⁺ T cells across disease and control groups and (C) TNF α production from KIR3DL2⁻ CD4⁺ T cells across disease and control groups. A Kruskal-Wallis test was used for statistical analysis in (B) and a one-way ANOVA was used for statistical analysis in (C).

Figure 4.17: TNF α production by KIR3DL2+ CD4+ T cells from inflammatory arthritis patients and HLA-B27- healthy controls is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2+ CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.



KIR3DL2-

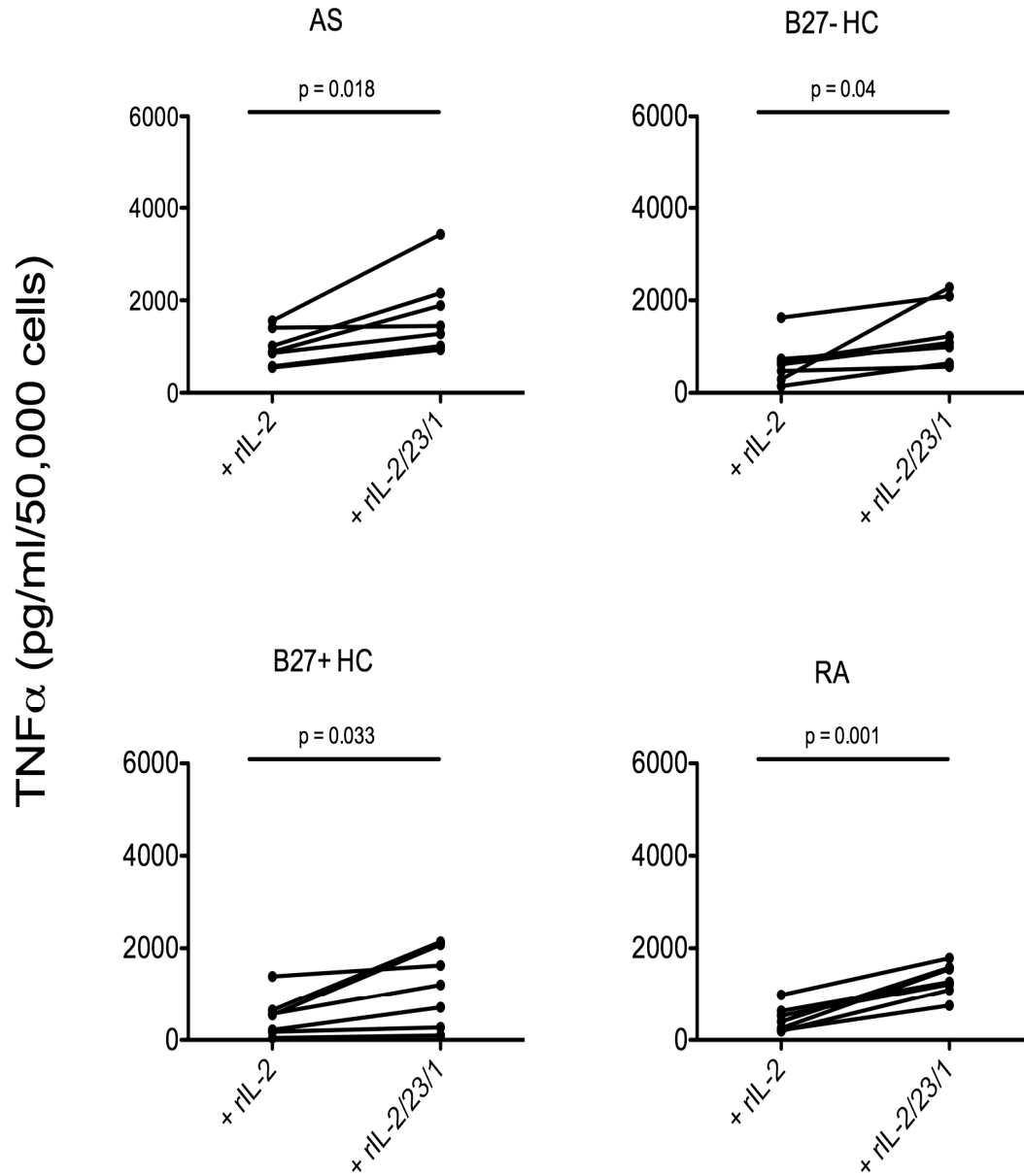


Figure 4.18: TNFα production by KIR3DL2- CD4+ T cells is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

4.3.6. KIR3DL2+ CD4+ T cells from AS patients and HLA-B27+ healthy controls are enriched for IL-6 production; for HLA-B27+ healthy controls IL-6 is further increased by the addition of rIL-1/IL-23

IL-6 is a pro-inflammatory cytokine produced by T cells and macrophages. Figure 4.19 shows that there was greater production of IL-6 after 5 days stimulation with anti-CD2/3/28 by KIR3DL2+ CD4+ T cells from AS patients and HLA-B27+ healthy controls compared to KIR3DL2- CD4+ T cells from the same individuals. When comparing IL-6 production from KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells across disease and healthy control groups, as shown in Figure 4.20.A and 4.20.B respectively, there is no significant difference in the production of IL-6.

The addition of rIL-1/23 caused a significant increase in IL-6 production by KIR3DL2+ CD4+ T cells from HLA-B27- and HLA B27+ healthy controls, as shown in Figure 4.21, with an increase in average IL-6 production from 301 to 1,010pg/ml/50,000 cells in HLA-B27- healthy controls and from 507 to 948pg/ml/50,000 cells in HLA-B27+ healthy controls, after addition of rIL-1/23 to KIR3DL2+ CD4+ T cells. KIR3DL2- CD4+ T cells from all groups investigated produce significantly more IL-6 after addition of rIL-1/23, as shown in Figure 4.22.

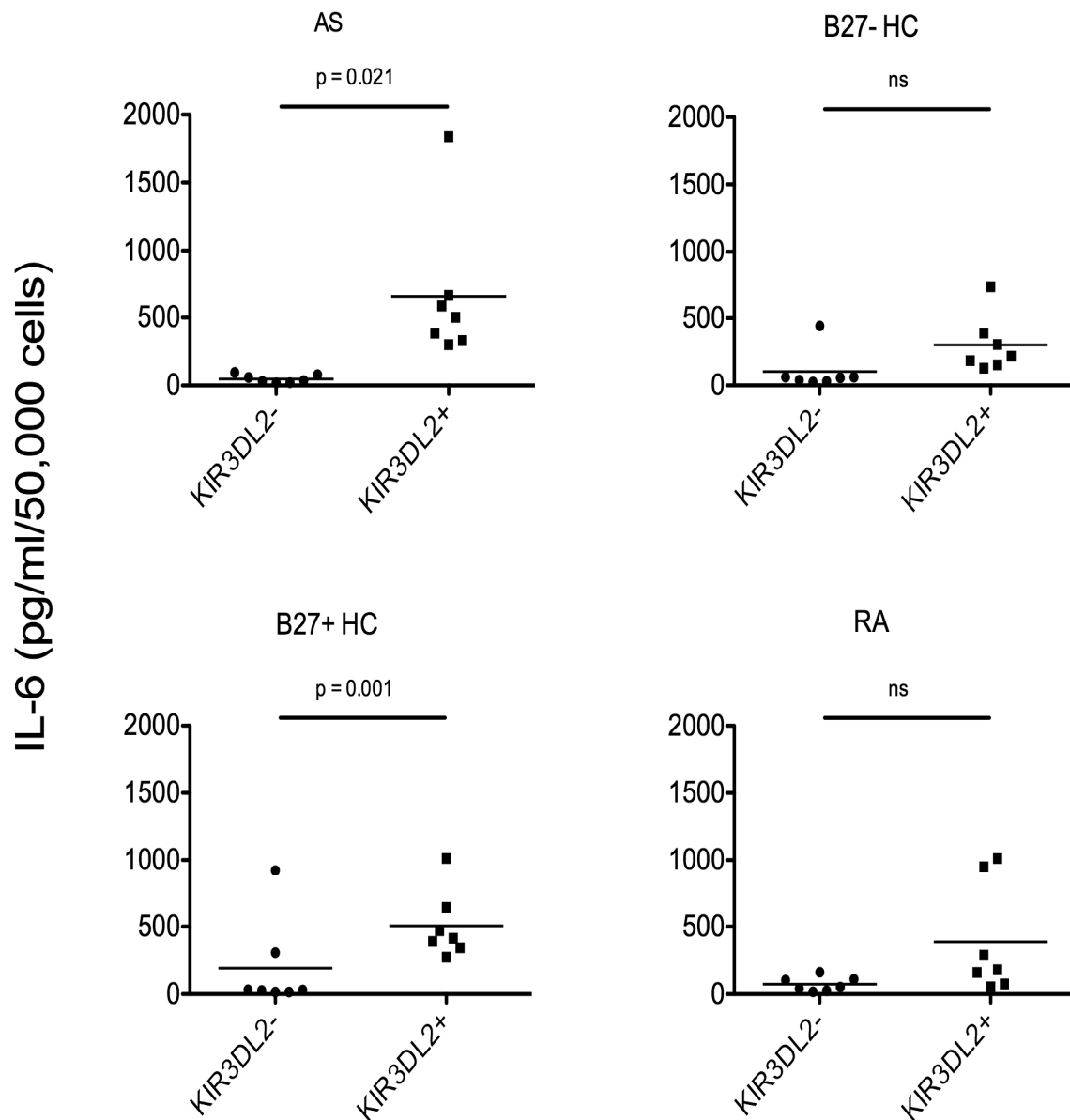


Figure 4.19: KIR3DL2⁺ CD4⁺ T cells from AS patients and HLA-B27⁺ healthy controls are enriched for IL-6 production. FACS-sorted KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells from AS (n=7) and RA (n=7) patients and HLA-B27⁻ (n=7) and HLA-B27⁺ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

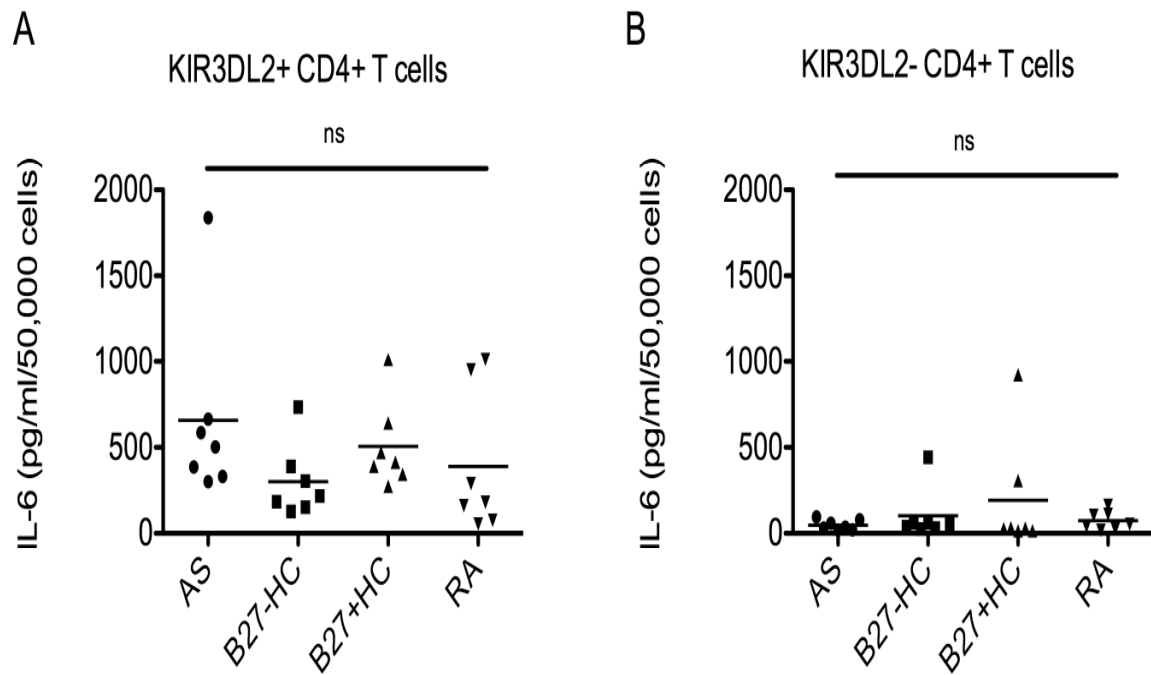


Figure 4.20: No significant difference in IL-6 production by KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells from AS patients compared to healthy or inflammatory disease controls. (A) FACS-sorted KIR3DL2+ and (B) KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. A one-way ANOVA test was used for statistical analysis. Note data is drawn from Figure 4.18.

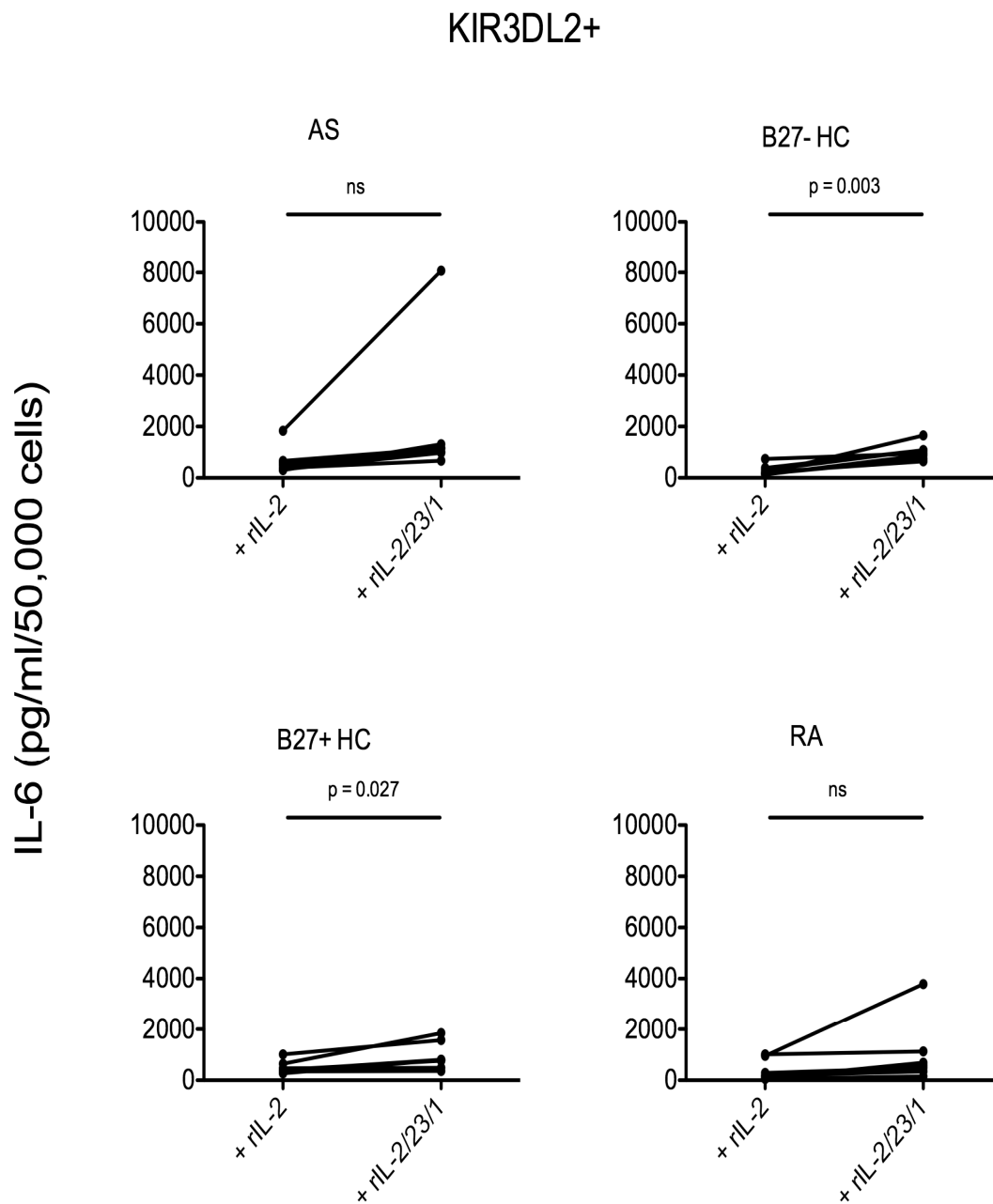


Figure 4.21: IL-6 production by KIR3DL2+ CD4+ T cells from healthy controls is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2+ CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

KIR3DL2-

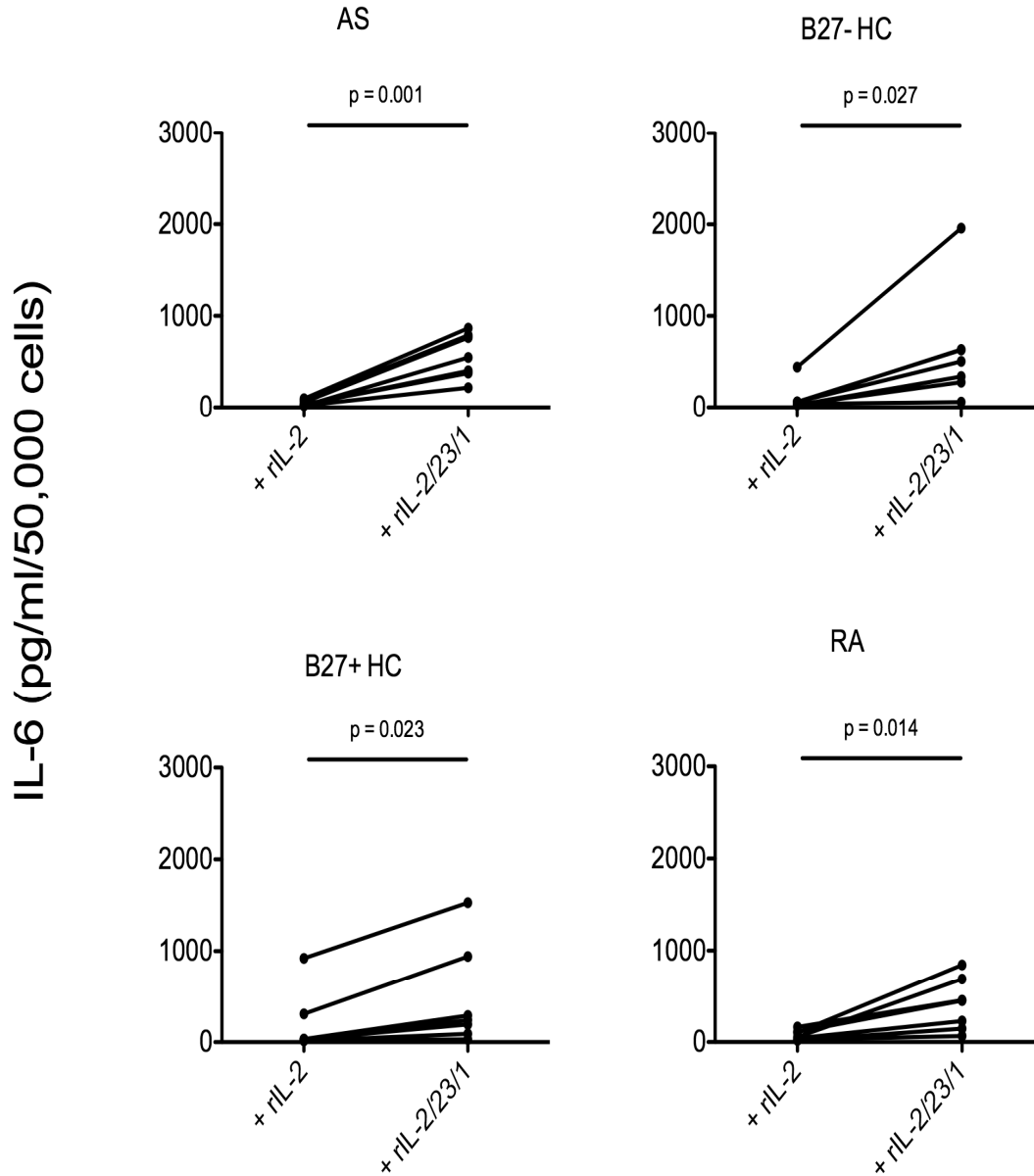


Figure 4.22: IL-6 production by KIR3DL2- CD4+ T cells is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

4.3.7. IL-22 production by KIR3DL2+ CD4+ T cells from RA patients and healthy controls is increased by the addition of rIL-1/IL-23

IL-22 is a further cytokine produced by Th17 cells, and as such I wanted to investigate IL-22 production by KIR3DL2+ CD4+ T cells. IL-22 production was measured by ELISA since multiplex bead analysis of this cytokine was not possible at the time of supernatants analysis. There was no significant difference in the production of IL-22 by KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells in any of the groups investigated, as shown in Figure 4.23.A. Moreover, there is no significant difference in the production of IL-22 from KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells across all groups investigated, as shown in Figure 4.23.B and 4.23.C respectively.

The addition of rIL-1/IL-23 resulted in significantly increased production of IL-22 from KIR3DL2+ CD4+ T cells from RA patients and HLA-B27- and HLA-B27+ healthy controls, as seen in Figure 4.24. Although not significant the average (mean) production of IL-22 by KIR3DL2+ CD4+ T cells from AS patients increased from 636 to 1,412pg/ml/50,000 cells after the addition of rIL-1/23, which is greater than the other groups investigated. The addition of rIL-1/23 significantly increased IL-22 production by KIR3DL2- CD4+ T cells from AS and RA patients and HLA-B27- healthy controls as shown in Figure 4.25.

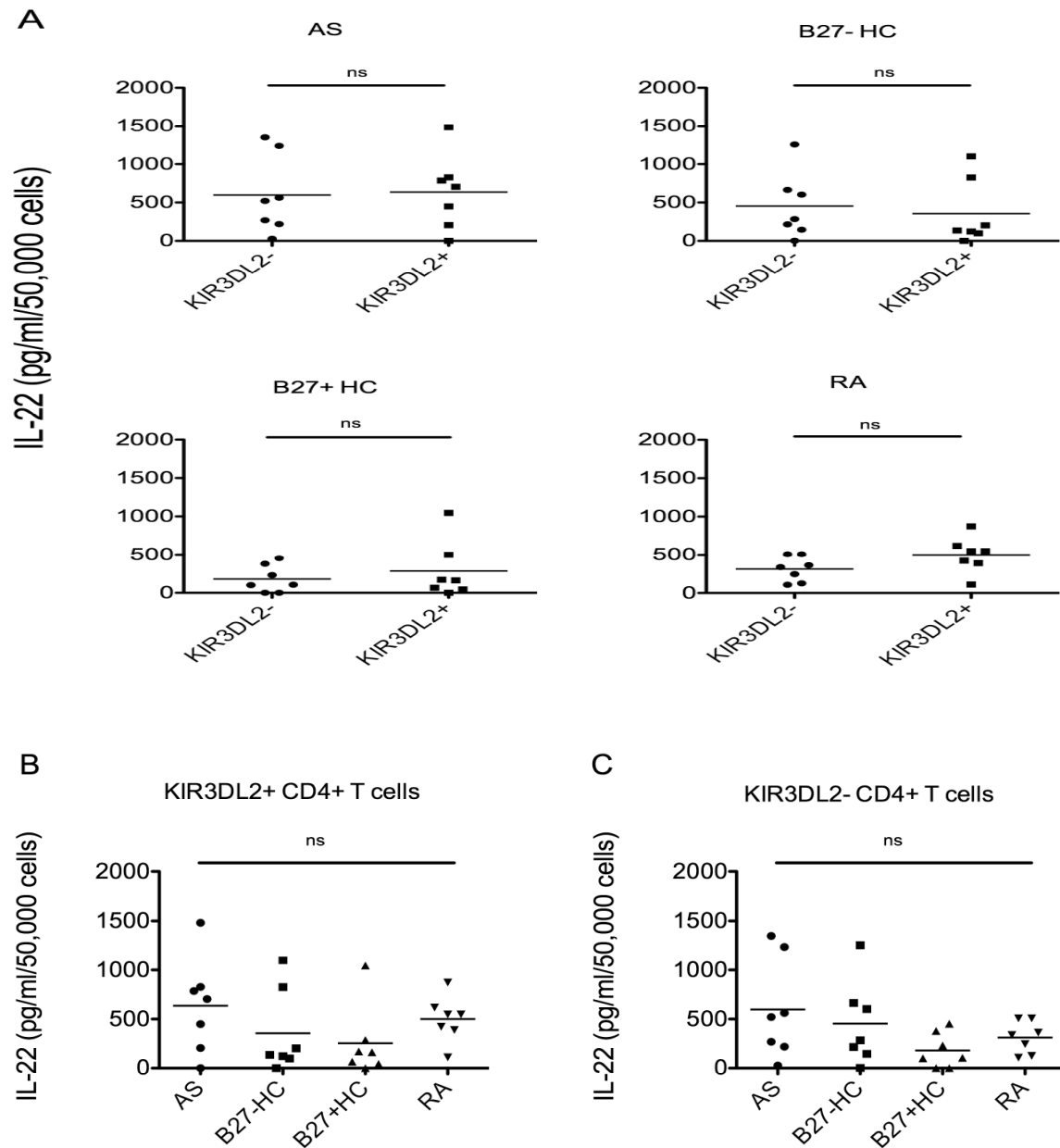


Figure 4.23: No significant difference in the production of IL-22 between KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells. FACS-sorted KIR3DL2+ and KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. (A) Comparison of IL-22 production by KIR3DL2+ and KIR3DL2- CD4+ T cells. A two-tailed student t test was used for statistical analysis. (B) Comparison of IL-22 production from KIR3DL2+ CD4+ T cells across disease and control groups and (C) IL-22 production from KIR3DL2- CD4+ T cells across disease and control groups. A one-way ANOVA was used for statistical analysis.

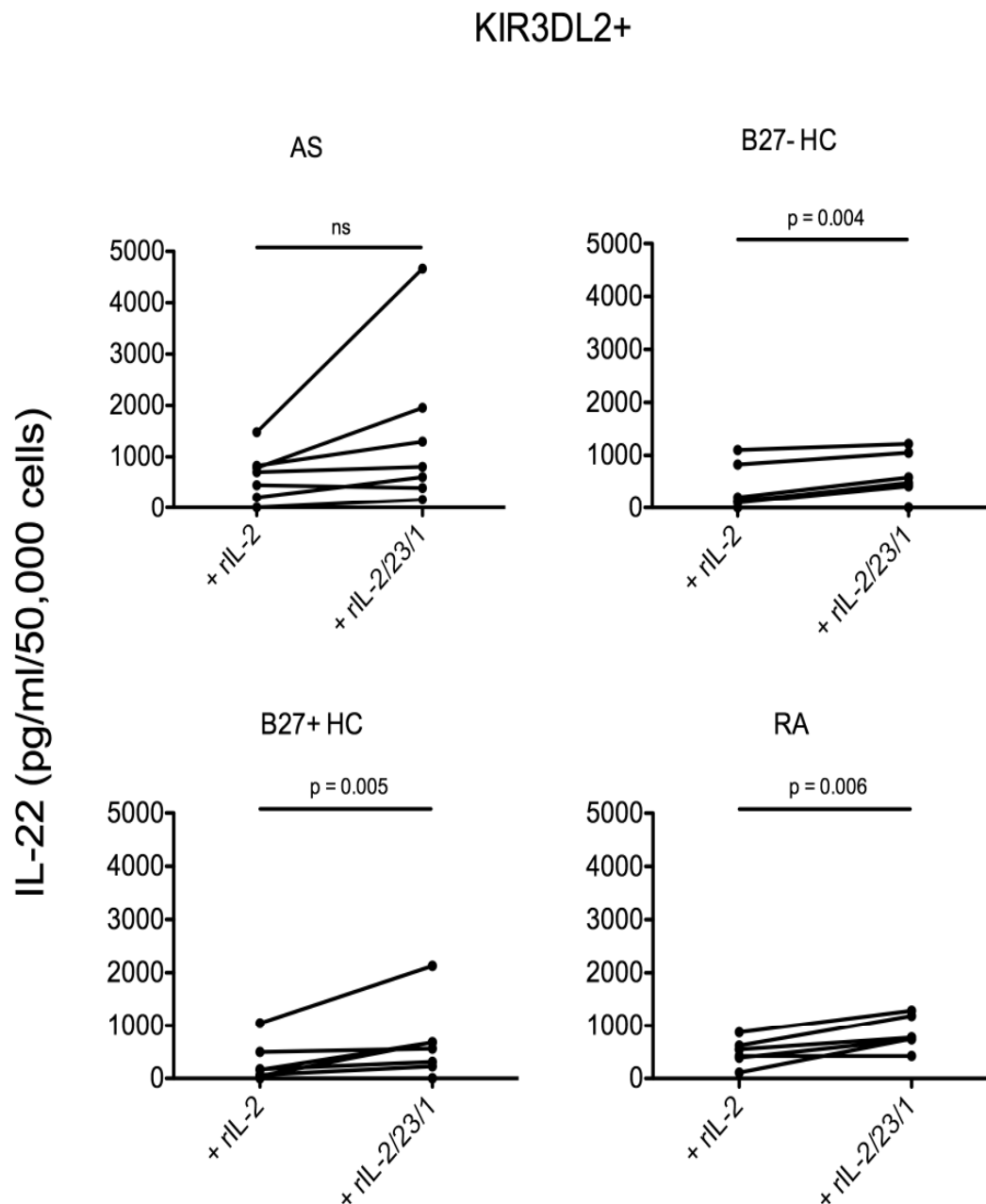


Figure 4.24: IL-22 production by KIR3DL2+ CD4+ T cells from healthy controls and RA patients is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2+ CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to ELISA of supernatants. A two-tailed student t test was used for statistical analysis.

KIR3DL2-

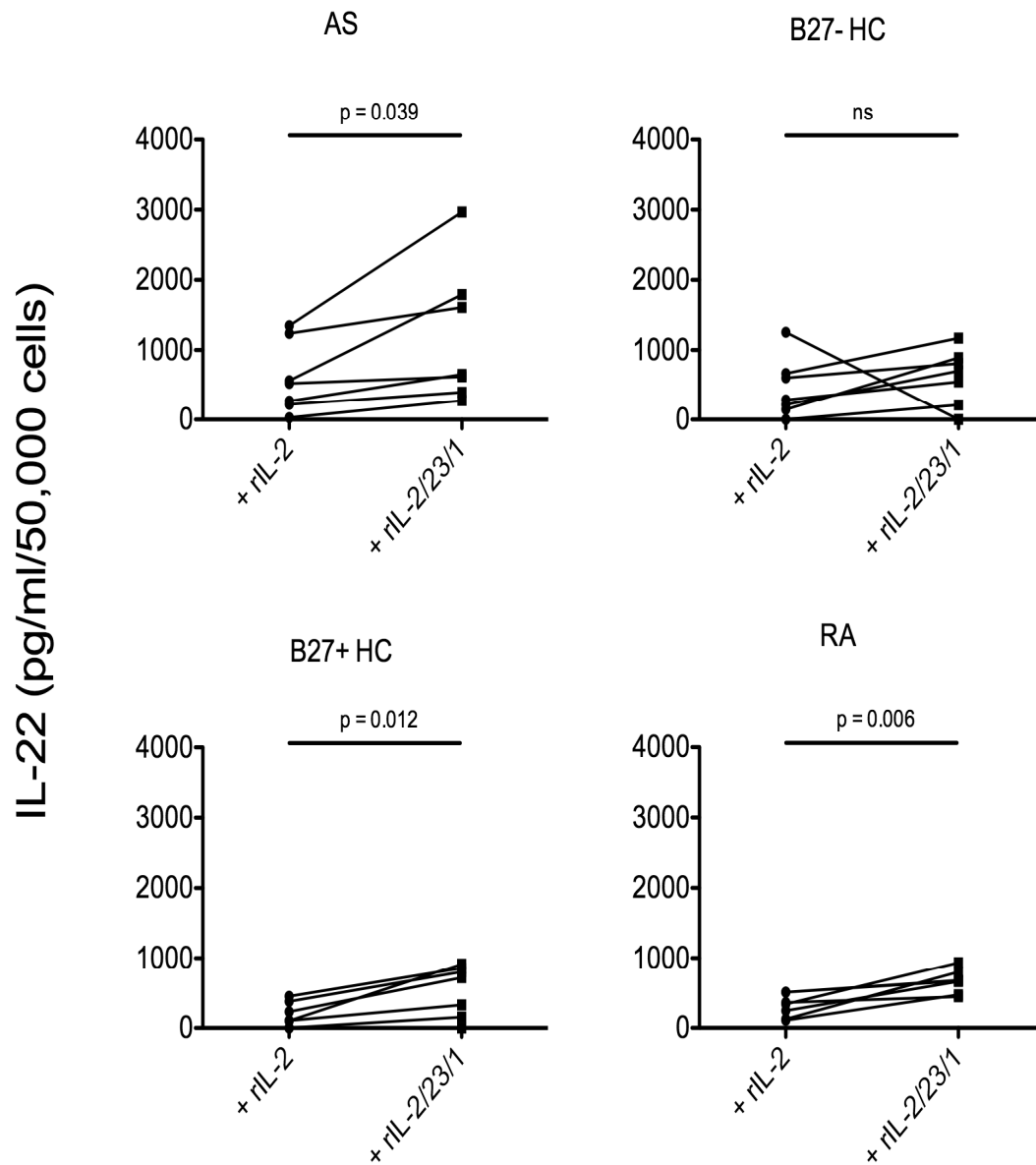


Figure 4.25: IL-22 production by KIR3DL2- CD4+ T cells from AS and RA patients and HLA-B27+ healthy controls is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to ELISA of supernatants. A two-tailed student t test was used for statistical analysis.

4.3.8. IL-10 production by KIR3DL2+ CD4+ T cells from RA patients, but not other groups, is significantly greater than IL-10 production by KIR3DL2- CD4+ T cells

IL-10 is cytokine associated with regulation of the immune response following its production by regulatory T cells. It has also been reported to be produced by pathogen-induced human Th17 cells (Zielinski, Mele et al. 2012). I asked whether KIR3DL2+ CD4+ T cells produce this cytokine. Figure 4.26 shows that KIR3DL2+ CD4+ T cells from RA patients produce significantly more IL-10 than KIR3DL2- CD4+ T cells. There was no significant difference in the production of IL-10 from KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells across all groups investigated, as shown in Figure 4.27.A and 4.27.B respectively.

The addition of rIL-1/IL-23 did not significantly increase production of IL-10 from KIR3DL2+ CD4+ T cells from any of the groups investigated, as seen in Figure 4.28.A. rIL-1/23 did significantly increase the amount of IL-10 production by KIR3DL2- CD4+ T cells from RA patients but not AS patients or healthy controls, as seen in Figure 4.28.B.

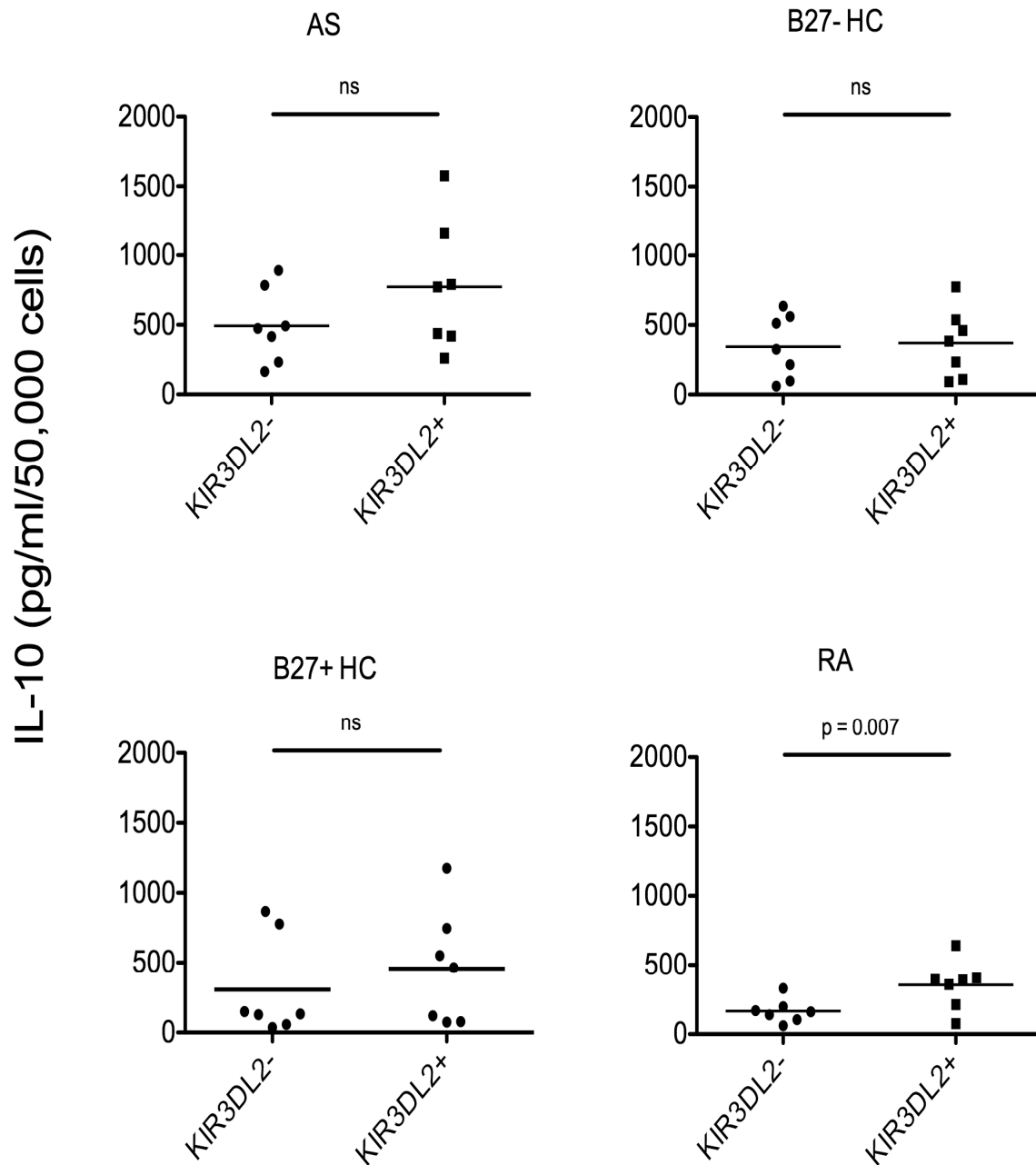


Figure 4.26: KIR3DL2+ CD4+ T cells from RA patients produce significantly more IL-10 than KIR3DL2- CD4+ T cells. FACS-sorted KIR3DL2+ and KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

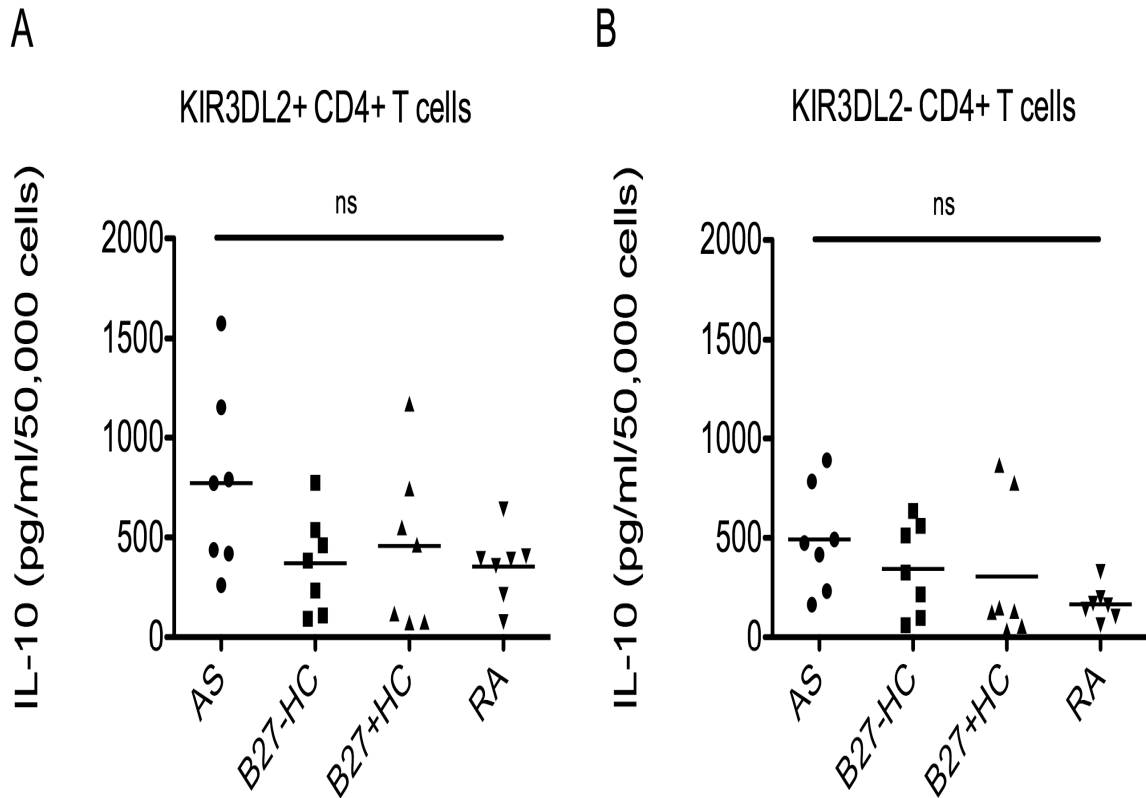


Figure 4.27: No significant difference in IL-10 production by KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells from AS patients compared to healthy controls and inflammatory disease controls. (A) FACS-sorted KIR3DL2+ and (B) KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. A one-way ANOVA test was used for statistical analysis. Note data is drawn from Figure 4.25.

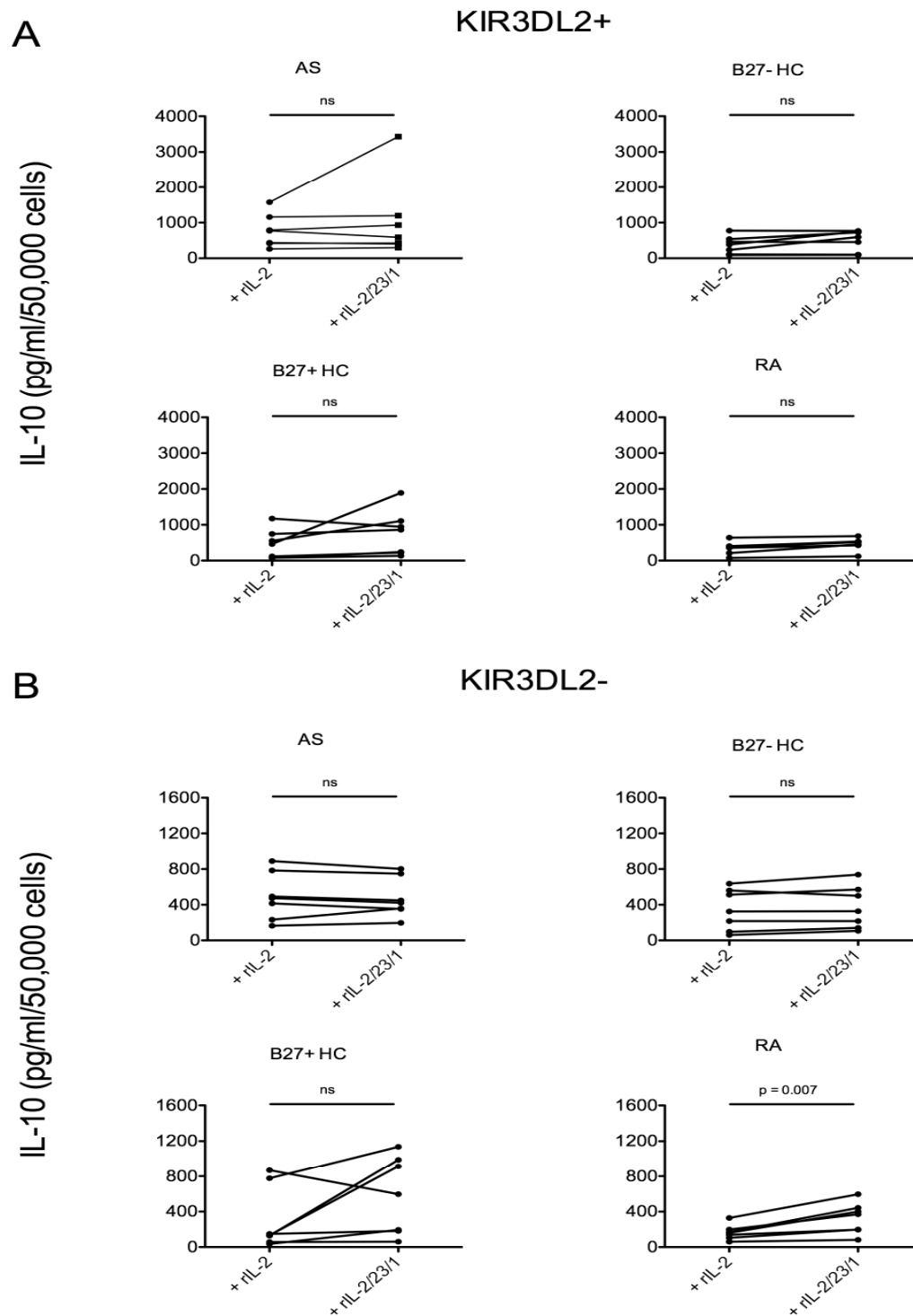


Figure 4.28: IL-10 production by KIR3DL2+ CD4+ T cells is not significantly increased by rIL-1/23 treatment. (A) FACS-sorted KIR3DL2+ and (B) KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

4.3.9. KIR3DL2+ CD4+ T cells are not enriched for IL-5 production, nor is IL-5 production increased by the addition of rIL-1/IL-23

IL-5 is a cytokine produced by Th2 cells and associated with allergy and immune responses to parasites. Figure 4.29.A shows that there was no significant difference in the production of IL-5 by KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells in any of the groups investigated. In fact in HLA-B27- healthy controls and RA patients there is a trend for greater IL-5 production by KIR3DL2- CD4+ T cells as compared to KIR3DL2+ CD4+ T cells. Moreover, there was no significant difference in the production of IL-5 from KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells across all groups investigated, as shown in Figure 4.29.B and 4.29.C respectively.

When looking at the effect of rIL-1/23, in Figure 4.30.A, the results show wide variation, with rIL-1/23 increasing IL-5 production in some cases but decreasing IL-5 production in other cases. Overall there was no significant difference in the production of IL-5 after the addition of rIL-1/23 to KIR3DL2+ CD4+ T cells in any of the groups investigated. Furthermore, rIL-1/23 did not significantly alter the amount of IL-5 production by KIR3DL2- CD4+ T cells, as shown in Figure 4.30.B.

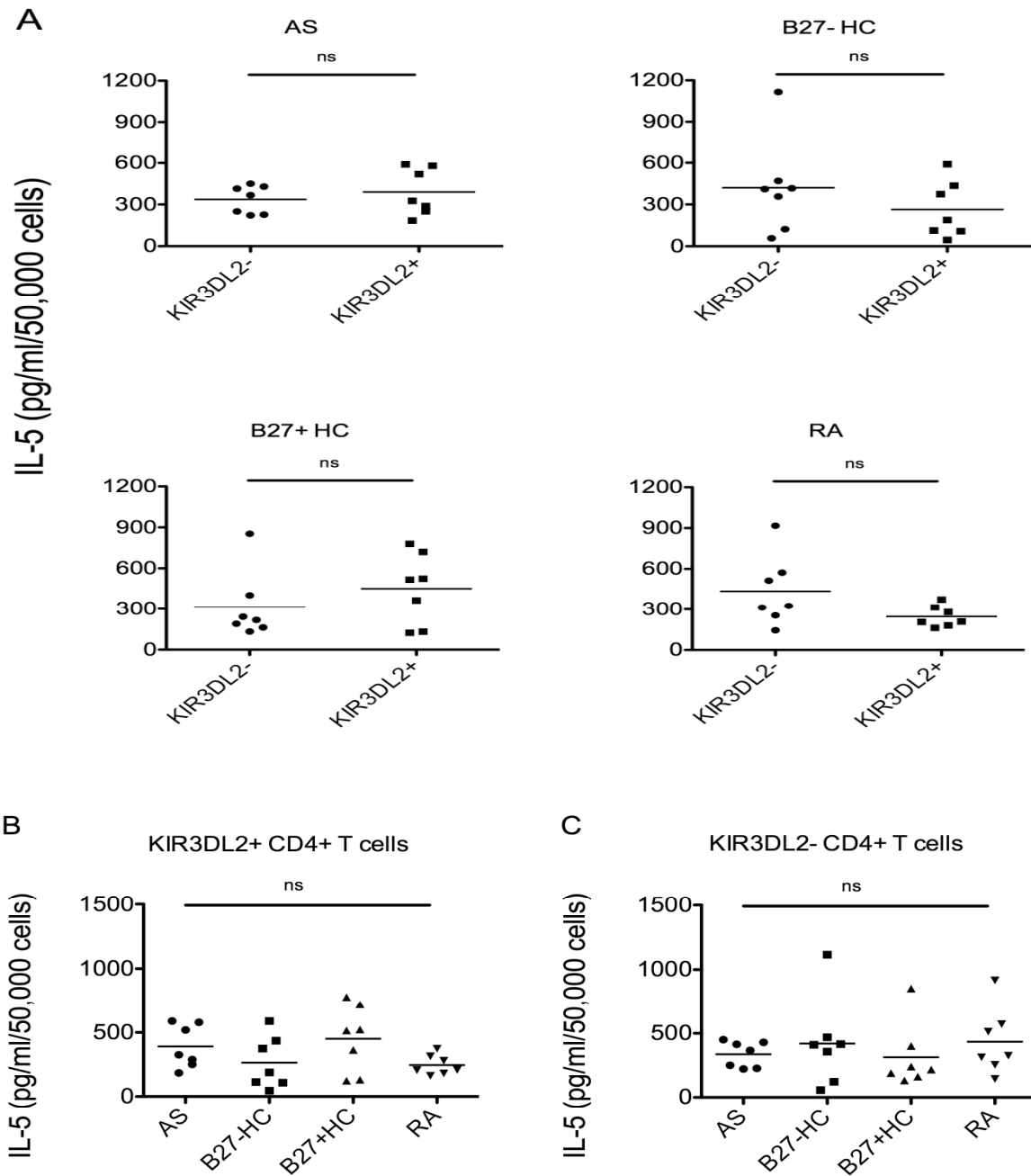
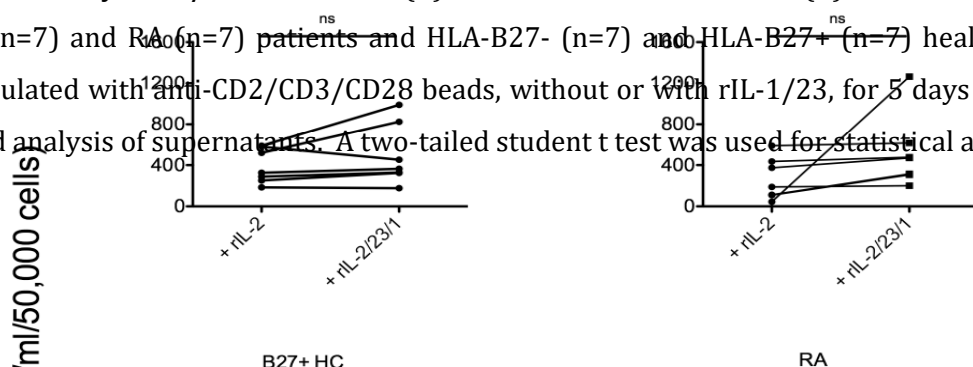


Figure 4.29: No significant difference in the production of IL-5 between KIR3DL2⁺ CD4⁺ T cells and KIR3DL2⁻ CD4⁺ T cells. FACS-sorted KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells from AS (n=7) and RA (n=7) patients and HLA-B27⁻ (n=7) and HLA-B27⁺ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. (A) Comparison of IL-5 production by KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells. A two-tailed student t test was used for statistical analysis. (B) Comparison of IL-5 production from KIR3DL2⁺ CD4⁺ T cells across disease and control groups and (C) IL-5 production from KIR3DL2⁻ CD4⁺ T cells across disease and control groups. A one-way ANOVA was used for statistical analysis.

Figure 4.30: IL-5 production by KIR3DL2+ and KIR3DL2- CD4+ T cells is not significantly increased by rIL-1/23 treatment. (A) FACS-sorted KIR3DL2+ and (B) KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.



4.3.10. GM-CSF production by both KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells from HLA-B27- healthy controls increases with the addition of rIL-1/IL-23

GM-CSF is a cytokine produced by many cells of the immune system, including T cells, macrophages and mast cells. It acts to increase production and maturation of granulocytes and monocytes. There was no significant difference in the production of GM-CSF by KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells in any of the groups investigated, as shown in Figure 4.31.A. Likewise, there was no significant difference in the production of GM-CSF from KIR3DL2+ CD4+ T cells or KIR3DL2- CD4+ T cells across all groups investigated, as shown in Figure 4.31.B and 4.31.C respectively.

The addition of rIL-1/23 significantly increases the production of GM-CSF by KIR3DL2+ CD4+ T cells from RA patients and HLA-B27- healthy controls, as shown in Figure 4.32. The addition of rIL-1/23 also significantly increases the amount of GM-CSF production by KIR3DL2- CD4+ T cells from HLA-B27- healthy controls, as shown in Figure 4.33.

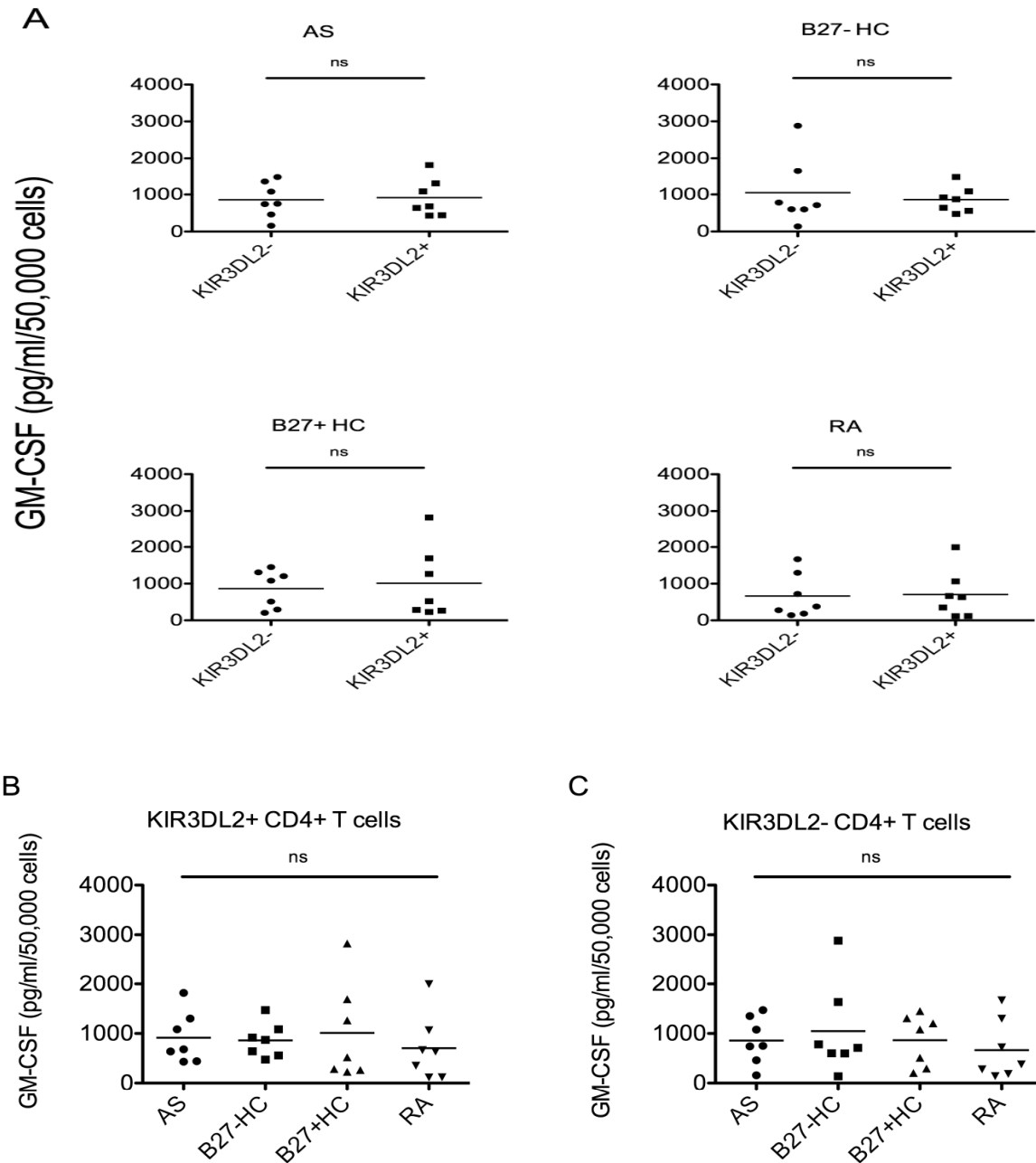


Figure 4.31: No significant difference in the production of GM-CSF between KIR3DL2+ CD4+ T cells and KIR3DL2- CD4+ T cells. FACS-sorted KIR3DL2+ and KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, for 5 days prior to multiplex bead analysis of supernatants. (A) Comparison of GM-CSF production by KIR3DL2+ and KIR3DL2- CD4+ T cells. A two-tailed student t test was used for statistical analysis. (B) Comparison of GM-CSF production from KIR3DL2+ CD4+ T cells across disease and control groups and (C) GM-CSF production from KIR3DL2- CD4+ T cells across disease and control groups. A one-way ANOVA was used for statistical analysis.

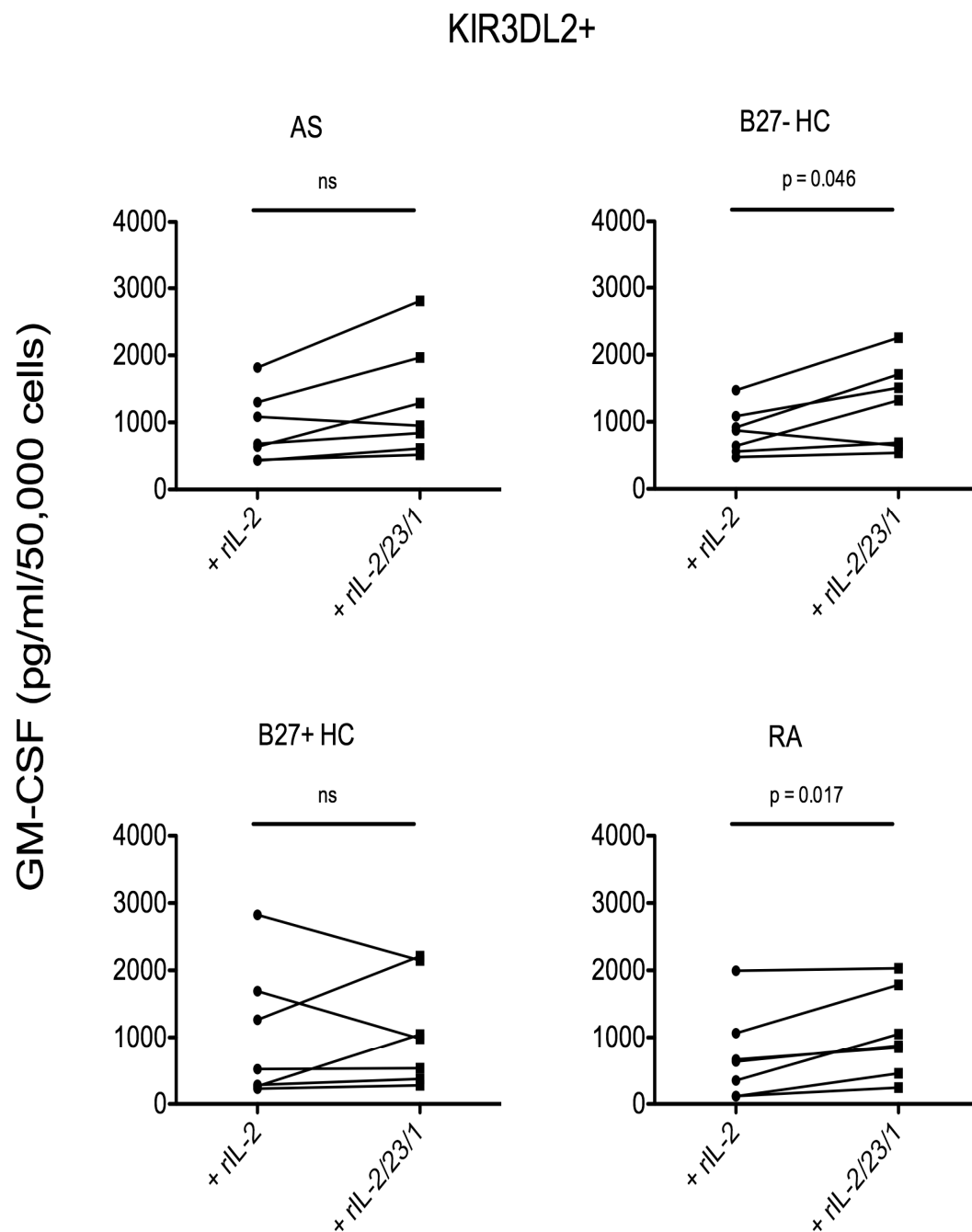


Figure 4.32: GM-CSF production by KIR3DL2+ CD4+ T cells from RA patients and HLA-B27- healthy controls is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2+ CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

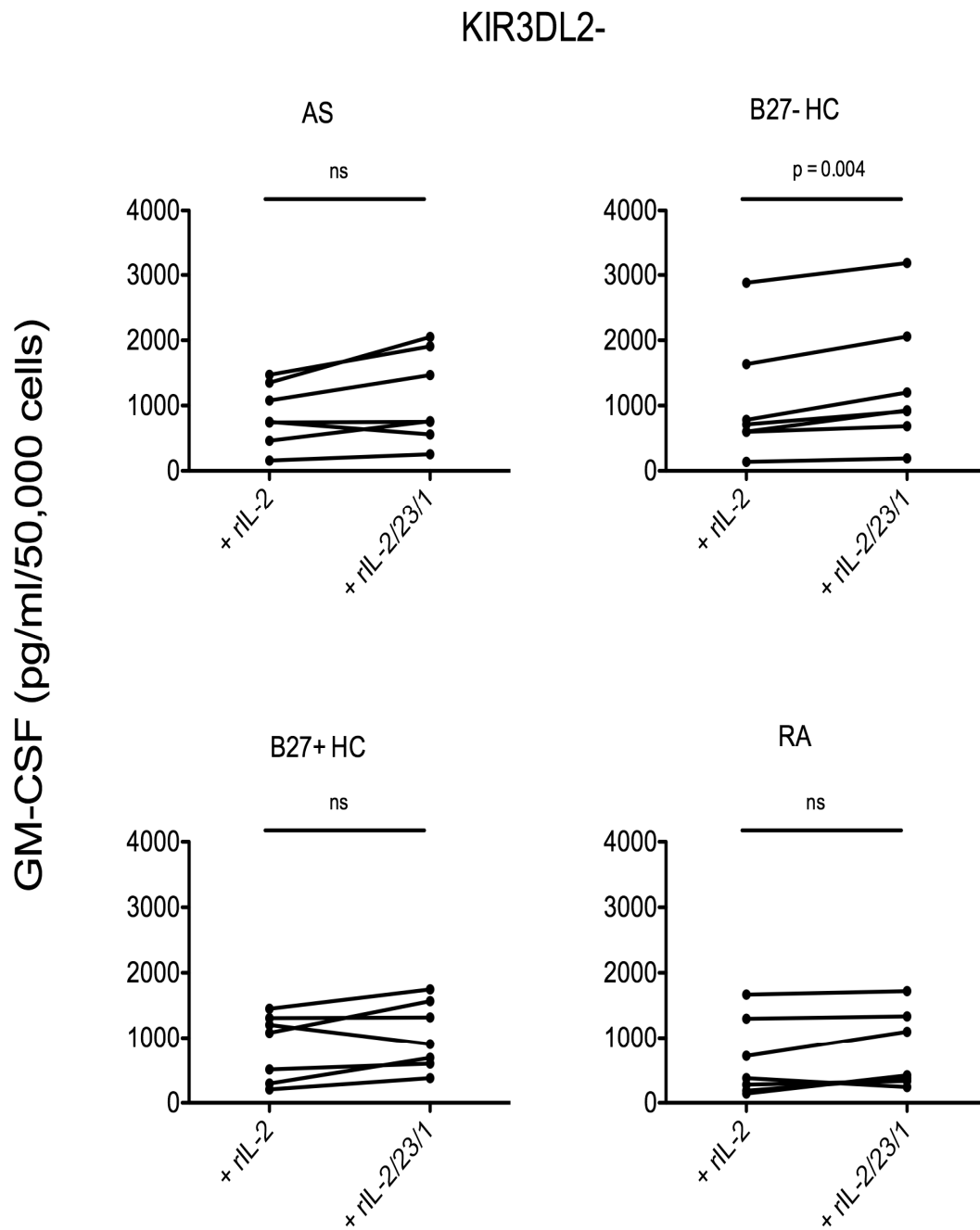


Figure 4.33: GM-CSF production by KIR3DL2- CD4+ T cells from HLA-B27- healthy controls is significantly increased by rIL-1/23 treatment. FACS-sorted KIR3DL2- CD4+ T cells from AS (n=7) and RA (n=7) patients and HLA-B27- (n=7) and HLA-B27+ (n=7) healthy controls were stimulated with anti-CD2/CD3/CD28 beads, without or with rIL-1/23, for 5 days prior to multiplex bead analysis of supernatants. A two-tailed student t test was used for statistical analysis.

4.4. Discussion

The primary aim of this chapter was to investigate cytokine production by KIR3DL2⁺ CD4⁺ T cells. This was addressed by investigating cytokine production by these cells by two different methods. Firstly, I used ICS to determine IL-17 and IFN γ production by KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells from PBMC, after direct *ex vivo* stimulation with PMA and ionomycin. Secondly, I performed multiplex bead analysis to determine cytokine production after 5 days stimulation of FACS sorted KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells with anti-CD2/3/28 beads. The primary aim was subdivided into three objectives, which will be discussed in detail below.

1) To determine whether KIR3DL2⁺ CD4⁺ T cells are enriched for cytokine production as compared to KIR3DL2⁻ CD4⁺ T cells.

I have shown for the first time, by ICS, elevated percentages of IL-17 producing KIR3DL2⁺ CD4⁺ T cells in the peripheral blood of patients with AS compared to HLA-B27⁻ healthy controls. There are numerous reports of raised IL-17 in the serum (Wendling, Cedoz et al. 2007) and synovial fluid (Melis, Vandooren et al. 2009) of inflammatory arthritis patients, as compared to healthy controls, as well as an increase in Th17 cells in the peripheral blood of these patients (Jandus 2008; Shen, Goodall et al. 2009). However the percentages of IL-17 producing CD4⁺ T cells in the peripheral blood of AS patients was lower in the latter 2 studies (mean = 0.65% and 0.94%, respectively) than that seen in my study (mean = 3.8%). Although it is difficult to draw direct comparisons due differences in experimental set up this finding is of interest since these other studies have looked at total CD4⁺ T cells however here I show that there is significantly higher production of IL-17 by CD4⁺ T cells that express KIR3DL2.

ICS also revealed that KIR3DL2⁺ CD4⁺ T cells, from both AS patients and HLA-B27⁻ healthy controls, are enriched for IFN γ production. A study into patients with PsA and AS found that IFN γ -secreting CD4⁺ T cells were significantly raised in the peripheral blood of these patients compared to healthy controls (Jandus 2008). I have also shown, by ICS, that KIR3DL2⁺ CD4⁺ T cells, from both AS patients and HLA-B27⁻ healthy controls, are enriched for dual production of IL-17 and IFN γ , which is of particular interest given the growing evidence for the presence of T cells with the ability to produce both IL-17 and IFN γ at sites of inflammation in various diseases (Annunziato, Cosmi et al. 2007; Martin-Orozco, Chung et al. 2009; Hirota, Duarte et al. 2011). Of note sorted CD161⁺ Th17 cells cultured in the presence of IL-12 to promote plasticity toward Th17/1 cells were shown to maintain CD161 expression, whilst CD161⁻ CD4⁺ T cells cultured under the same conditions failed to up-regulate CD161 (Nistala, Adams et al. 2010). This suggests that CD161 may identify cells within the Th1 population that have a Th17 ancestry. Unfortunately due to technical limitations I was unable to simultaneously FACS stain for phenotypic makers associated with Th17 cells in addition to intracellular cytokines, but this would add value in terms of further characterising KIR3DL2⁺ CD4⁺ T cells.

Using multiplex bead analysis I have shown for the first time that KIR3DL2⁺ CD4⁺ T cells from the peripheral blood of inflammatory arthritis patients produce significantly higher amounts of IL-17 compared to KIR3DL2⁻ CD4⁺ T cells from the same patients. In contrast there was no significant difference between that amount of IL-17 produced by KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells from the peripheral blood of HLA-B27⁻ or HLA-B27⁺ healthy controls.

One important caveat of this work, investigated subsequently to these experiments and detailed in chapter 5, is that activation increases KIR3DL2 expression on CD4⁺ T cells, therefore following activation of KIR3DL2⁻ CD4⁺ T cells it is probable that some of these cells gain expression of KIR3DL2. Thus, cytokine production measured by KIR3DL2⁻ CD4⁺ T cells may

actually represent cytokine production by a mixed population of KIR3DL2⁻ and KIR3DL2⁺ CD4⁺ T cells. This is also of interest when interpreting the results of experiments using the treatment of rIL-23/1 to see the effect of cytokine production, since I have shown in chapter 3 that IL-23R expression is restricted to KIR3DL2⁺ CD4⁺ T cells and IL-1R is enriched on KIR3DL2⁺ CD4⁺ T cells as compared to KIR3DL2⁻ CD4⁺ T cells. However if activation induces KIR3DL2 expression on previously KIR3DL2⁻ CD4⁺ T cells these cells may also gain expression of IL-1R and IL-23R, therefore making them more responsive to rIL-23/1.

I have shown that KIR3DL2⁺ CD4⁺ T cells from RA patients and HLA-B27⁻ healthy controls produce significantly more IFN γ than KIR3DL2⁻ CD4⁺ T cells from the same individuals. Although not significant a similar trend for greater IFN γ production from KIR3DL2⁺ CD4⁺ T cells compared to KIR3DL2⁻ CD4⁺ T cells was also observed in AS patients and HLA-B27⁺ healthy controls. However, this may have not reached significance due to the numbers of individuals investigated. More specifically, IFN γ production by KIR3DL2⁻ CD4⁺ T cells in two AS patients and one HLA-B27⁺ healthy controls was particularly high and it would be of interest to look at more samples to determine if these are outliers or represent elevated IFN γ production by KIR3DL2⁻ CD4⁺ T cells from AS patients and HLA-B27⁺ healthy controls.

A further cytokine investigated was IL-6, which is of particular interest, since it is proposed to be a biomarker of AS disease activity with levels of IL-6 correlating with ESR and CRP (Gratacos, Collado et al. 1994; Bal, Unlu et al. 2007) and to poor prognosis in SpA (Londono, Romero-Sanchez et al. 2012). IL-6 is known to be involved in Th17 differentiation and Th17 cells themselves have been shown to produce IL-6 (Langrish, Chen et al. 2005; Nurieva, Yang et al. 2007). I have shown that KIR3DL2⁺ CD4⁺ T cells from AS patients and HLA-B27⁺ healthy controls produce significantly more IL-6 than KIR3DL2⁻ CD4⁺ T cells from the same individuals.

Significantly more IL-10 was produced by KIR3DL2⁺ CD4⁺ T cells than KIR3DL2⁻ CD4⁺ T cells from RA patients. RA serum and synovial fluid has been shown to contain significantly elevated IL-10 levels compared with levels in healthy controls, but serum levels of IL-10 did not correlate with measures of clinical activity (Cush, Splawski et al. 1995). In the same study patients with SpA had significantly increased serum levels of IL-10 compared to healthy controls, and this is supported by a more recent study which demonstrated elevated serum levels of IL-10 in AS patients as compared to healthy controls (Lv, Wang et al. 2011). I have shown that production of IL-10 by KIR3DL2⁻ and KIR3DL2⁺ CD4⁺ T cells from AS patients is not significantly different. This may represent a true result, although a further possibility is that work is not significantly powered to show differences in the levels of IL-10 production. IL-10 is a cytokine classically associated with Treg cells. An expansion of both peripheral and gut resident Treg cells in patients with AS and Crohn's disease as compared to healthy controls has been reported (Ciccio, Accardo-Palumbo et al. 2010). Here the authors showed a five-fold increase in the proportion of Treg cells observed in the gut of patients with AS, as compared to healthy controls, with up to 80% of these cells producing IL-10. I have only looked at peripheral blood samples in this project but this previous study would support the investigation of cytokine production from KIR3DL2⁺ CD4⁺ T cells from the gut in patients with AS.

The levels of other cytokines I investigated by multiplex bead analysis were not significantly different between KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells. This included TNF α , a proinflammatory cytokine produced primarily by macrophages, but also by a number of other cells including CD4⁺ T cells, eosinophils and mast cells (Costa, Matossian et al. 1993; Bischoff, Lorentz et al. 1999; Jandus 2008). KIR3DL2⁺ and KIR3DL2⁻ CD4⁺ T cells from AS patients produced the highest levels of TNF α , and this is perhaps not surprising since raised levels of TNF α are well documented in serum (Gratacos, Collado et al. 1994) and plasma (Imrich, Rovinsky et al. 2004) from AS patients, and anti-TNF α therapies are proving to be a successful

treatment in many AS patients. In addition to its detection in peripheral samples, TNF α expression has also been demonstrated in synovial tissue samples for patients with juvenile RA and SpA (Grom, Murray et al. 1996). Expression of TNF α receptor was also observed; with the vast majority of synovial tissues cells expressing the p55 form of the receptor. In a small study of sacroiliac biopsies from AS patients those with early disease had more CD3⁺ T cells, as well as more TNF- α ⁺ cells and IL-6⁺ cells, compared to patients with advanced AS, as detected by immunohistochemistry (Francois, Neure et al. 2006). Thus, it would be of interest to investigate cytokine production by KIR3DL2⁺ CD4⁺ T cells in synovial fluid and tissue samples from joints of AS patients.

IL-22 is a further cytokine produced by Th17 cells (Liang, Tan et al. 2006). The same study showed that IL-22 and IL-17 or IL-17F synergistically induced the expression of anti-microbial peptides β -defensin 2 and S100A9. Th22 cells have also been described as IL-22 producing T cells that co-express CCR6 and the skin-homing receptors CCR4 and CCR10, but with low or undetectable expression of T-bet or ROR γ t (Duhon, Geiger et al. 2009; Trifari, Kaplan et al. 2009). Elevated levels of IL-22 have been reported in AS and RA patients compared with OA patients and healthy controls (Zhang, Li et al. 2012). The same study found a positive correlation between IL-22 and Th17 cells in AS patients. Elevated IL-22 has also been described in psoriasis, where elevated IL-22 correlated with disease activity and was reduced by phototherapy (Wolk, Witte et al. 2006). I found that the production of IL-22 by KIR3DL2⁺ CD4⁺ T cells and KIR3DL2⁻ CD4⁺ T cells from inflammatory arthritis and healthy controls did not significantly differ. However, there was a trend for greater production of IL-22 by inflammatory arthritis patients, as compared to healthy controls, although with the number of individuals investigated in this study this did not reach statistical significance.

In addition to cytokines associated with Th17 cells and Th1 cells I looked at IL-5 production, a cytokine associated with Th2 cells. KIR3DL2+ CD4+ T cells were not enriched for IL-5 production in any of the disease or control groups I investigated.

The final cytokine I investigated was GM-CSF, which is produced by a variety of cells including endothelial cells, innate cells and T cells (Golde, Finley et al. 1972; Cline and Golde 1974; Quesenberry and Gimbrone 1980). Innate GM-CSF has been shown to be critical for generation of pathological Th17 cells in a mouse model of autoimmune myocarditis, by stimulating IL-6 and IL-23 production by dendritic cells (Sonderregger, Iezzi et al. 2008). The production of GM-CSF was not significantly different between KIR3DL2+ and KIR3DL2- CD4+ T cells in any of the groups investigated, however rIL-1/23 did increase GM-CSF production by KIR3DL2+ CD4+ T cells from HLA-B27- healthy controls and RA patients, as discussed below.

2) To determine whether KIR3DL2+ CD4+ T cells from AS patients are enriched for cytokine production as compared to KIR3DL2+ CD4+ T cells from healthy controls and RA patients.

Using ICS I have shown that KIR3DL2+ CD4+ T cells from AS patients are enriched for IL-17 production as compared to KIR3DL2+ CD4+ T cells from HLA-B27+ healthy controls. In contrast IFN γ production by KIR3DL2+ CD4+ T cells is not significantly different between AS patients and HLA-B27- healthy controls. In addition, the percentage of KIR3DL2+ CD4+ T cells producing both IL-17 and IFN γ was not significantly different between AS patients and HLA-B27- healthy controls. In a further study looking at co-expression of cytokines by CD4+ T cells from patients with AS, PsA or RA, as well as healthy controls, the authors could not detect significant proportions of cells secreting both IL-17 and IFN γ , in any of the groups investigated (Jandus 2008). Since this study, and my work, has focused on cytokine production by peripheral blood CD4+ T cells it would be of interest to look at CD4+ T cells from sites of inflammation, such as

those infiltrating into synovial fluid, to see if there are differences in dual production of IL-17 and IFN γ production by CD4 $^{+}$ T cells from AS patients and healthy controls.

Multiplex bead analysis of supernatants after 5 days stimulation with anti-CD2/3/28 beads shows there is trend, although not significant, for higher IL-17 production by KIR3DL2 $^{+}$ CD4 $^{+}$ T cells from AS patients, compared to RA patients and healthy controls. Of note IL-17 production by AS patients was variable, with particularly high production by KIR3DL2 $^{+}$ CD4 $^{+}$ T cells from three AS patients, thus given the small numbers of patients investigated it is likely that the study is not sufficiently powered to detect significant differences. However, the data suggests it would be of interest to investigate more inflammatory arthritis patients and healthy controls. Significantly higher production of IL-17 by KIR3DL2 $^{+}$ CD4 $^{+}$ T cells from AS patients compared to RA patients and HLA-B27 $^{+}$ healthy controls was observed.

3) To determine whether the addition of Th17 polarizing cytokines affects cytokine production by KIR3DL2 $^{+}$ CD4 $^{+}$ T cells.

The final objective of this chapter was to investigate whether the addition of Th17 polarizing cytokines effects cytokine production by KIR3DL2 $^{+}$ CD4 $^{+}$ T cells. The Th17 polarizing cytokines I used in this study were IL-23 and IL-1. IL-23 production can be induced by a number of factors. Signalling through various pathogen associated molecular patterns and danger associated molecular patterns, can lead to upregulation of IL-23 (Re and Strominger 2001). Toll-like receptor (TLR) agonists have also been shown to increase levels of IL-23, whilst decreasing IL-12 production, from monocyte-derived dendritic cells (Goodall, Wu et al. 2010). Moreover, B27 heavy chains which misfold in the ER have been shown to induce ER stress (Mear, Schreiber et al. 1999) and the IL-23 gene was found to be a target of the ER stress-induced transcription factor C/EBP homologous protein (CHOP) (Goodall, Wu et al. 2010). This work is

supported by a study using transgenic rats, where IL-23 was synergistically up-regulated by LPS in macrophages undergoing a UPR, induced by pharmacologic agents, or by HLA-B27 misfolding (DeLay, Turner et al. 2009). In addition prostaglandin E₂ (PEG₂) has been shown to induce IL-23 production by dendritic cells, following in release by fibroblasts (Schirmer, Klein et al. 2010). Several studies have shown elevated IL-23 in both serum and synovial fluid from AS patients (Wang, Lin et al. 2009; Wendling, Cedoz et al. 2009; Mei, Pan et al. 2011).

IL-1 β is a pro-inflammatory cytokine released rapidly by cells of the innate immune system following activation. Studies into IL-1 β levels in AS have been contradictory with one study showing increased IL-1 β levels in serum from AS patients compared to healthy controls (Vazquez-Del Mercado, Garcia-Gonzalez et al. 2002) but further studies showing no difference in IL-1 β levels between AS patients and controls with noninflammatory back pain (Gratacos, Collado et al. 1994) or healthy controls (Sonel, Tutkak et al. 2002). Several groups have reported that a gene, or combination of genes, within the IL-1 gene cluster, significantly influence susceptibility to AS. This includes the *IL1RN* gene, which encodes IL-1RA, an IL-1 receptor antagonist (Timms, Crane et al. 2004) and *IL1R2*, which encodes a protein that inhibits IL-1 by acting as a decoy receptor, preventing binding of IL-1 α and IL-1 β to the IL-1R (Australo-Anglo-American Spondyloarthritis Consortium (TASC), Reveille JD et al. 2010). This is of interest since it is possible that these polymorphisms may alter signaling through the IL-1R in AS, which has implications for Th17 development.

Following treatment with rIL-1/23 KIR3DL2⁺ CD4⁺ T cells from AS patients produced significantly higher amounts of IL-17 than KIR3DL2⁺ CD4⁺ T cells, from the same patients, without rIL-1/23. The same treatment did not significantly increase IL-17 production by KIR3DL2⁺ CD4⁺ T cells from RA patients or healthy controls. Staining for IL-23R and IL-1R on KIR3DL2⁺ CD4⁺ cells from these patients would have been valuable to see whether the patients

that produced particularly high levels of IL-17 after treatment with rIL-1/23 expressed higher levels of these receptors. However FACS staining on KIR3DL2+ CD4+ T cells from AS patients, as seen in chapter 3, shows that KIR3DL2+ CD4+ T cells from AS patients are enriched for expression of both IL-23R and IL-1R as compared to KIR3DL2- CD4+ T cells.

Given that KIR3DL2+ CD4+ T cells from AS patients produce more IL-17 after treatment with rIL-1/23, it suggests that, in addition to having raised numbers of KIR3DL2+ CD4+ T cells in peripheral blood, the nature in which KIR3DL2+ CD4+ T cells from AS patients responds to Th17 polarizing cytokines may be important. Moreover since three of the seven AS patients produced over double the levels of IL-17 produced by KIR3DL2+ CD4+ T cells from the remaining AS patients investigated it is possible that in this data set we are seeing responses to rIL-23/1 that are representative of two different groups. A study investigating the IL23R R381Q gene variant found that this particular variant protected against immune-mediated diseases by impairing IL-23-induced Th17 effector response, with Th17 cells from A allele carriers having significantly reduced IL-23-induced IL-17A production and STAT3 phosphorylation as compared to G allele carriers (Di Meglio, Di Cesare et al. 2011), therefore it is possible that difference on the genotypes of the patients may reflect differences in their response to rIL-1/23.

Of the seven AS patients I investigated by multiplex bead analysis information on the genotype of the IL-23R was available for three of these patients. There are two regions of association within the IL-23R gene and in the primary association region the three patients share the same allele in the major coding variant. However in the secondary region of association two of the patients share the same allele in the major gene variant and KIR3DL2+ CD4+ T cells from these patients produced the highest levels of IL-17 after 5 days treatment with rIL-1/23. Conversely the other patient had a different genotype to the other two patients and KIR3DL2+ CD4+ T cells from this patient produced much less IL-17 after the same treatment. Although the numbers are

very low this data suggests that it would be worthwhile investigating greater numbers of AS patients to see if there is a correlation between the genotype of the IL-23R and the function of KIR3DL2+ CD4+ T cells, in terms of IL-17 production.

Treatment with rIL-1/23 significantly increased production of IFN γ by KIR3DL2+ CD4+ T cells from AS and RA patients and HLA-B27- healthy controls. rIL-1/23 are cytokines commonly used to polarize naïve CD4+ T cells down a Th17 differentiation pathway. IFN γ is the classical cytokine produced by Th1 cells, with IFN γ neutralization commonly being employed in the differentiation of naïve T cells to a Th17 phenotype. However, it should be noted that experiments in this chapter focus on cytokine production by KIR3DL2+ CD4+ T cells, the majority of which are memory CD4+ T cells rather than naïve CD4+ T cells. Therefore, the addition of Th17 polarizing cytokines is likely to be much more complex than simply skewing T cells to either a Th1 or a Th17 phenotype. Moreover, production of multiple cytokines is of interest since there are numerous reports in the literature of T cell subsets producing different cytokines in addition to the classical cytokine, or cytokines, associated with that specific subset. In particular there is much literature on the dual production of IL-17 and IFN γ , suggestive of an intermediate phenotype between Th17 and Th1 cells subsets, lending weight to the hypothesis that T cells subsets are relatively plastic in terms of development and differentiation (Aarvak, Chabaud et al. 1999; Nistala, Moncrieffe et al. 2008).

TNF α production was significantly increased by treatment with rIL-1/23 from KIR3DL2+ CD4+ T cells from AS and RA patients and HLA-B27- healthy controls. In addition rIL-1/23 significantly increased production of TNF α from KIR3DL2- CD4+ T cells from all groups investigated. The suggestion that KIR3DL2+ CD4+ T cells in AS patients are polyfunctional is in agreement with work by Jandus *et al* (Jandus 2008) who demonstrated elevated numbers of

circulating Th17 cells in the peripheral blood of patients with AS, with up to 87% producing both IL-17 and TNF- α , and up to 20% of these cells simultaneously producing IL-2.

The addition of rIL-1/23 significantly increased IL-22 production by KIR3DL2+ CD4+ T cells from healthy controls and RA patients. IL-22 has been shown to be induced by IL-23 in autoimmune-pathogenic CD4+ T cells in a time- and dose-dependent manner in a study in EAE (Kreymborg, Etzensperger et al. 2007). Here the authors showed that CNS-invading encephalitogenic Th17 cells co-expressed both IL-17 and IL-22.

IL-6 was one of only two cytokines whose production was significantly increased by KIR3DL2+ CD4+ T cells as compared to KIR3DL2- CD4+ T cells from AS patients. Treatment with rIL-23/IL-1 significantly increased production of IL-6 by KIR3DL2+ CD4+ T cells from HLA-B27+ and HLA-B27- healthy controls. Interestingly a study into colitis has shown that IL-23 promotes memory-activated T cell production of IL-6 and IL-17 and blockade of both IL-6 and IL-17 ameliorated IBD in these mice (Yen, Cheung et al. 2006).

GM-CSF production was increased following treatment with rIL-1/23 treatment of KIR3DL2+ CD4+ T cells from both RA patients and HLA-B27- healthy controls. Although typically associated with production by macrophages and monocytes, Th17 cells have been shown to produce GM-CSF with one study showing IL-23 stimulated production of GM-CSF by Th17 cells, which induced a positive feedback loop whereby GM-CSF secreted by Th17 cells stimulated further production of IL-23 by antigen-presenting cells (El-Behi, Ciric et al. 2011). ROR γ t has been identified as the factor that drives production of GM-CSF in T cells (Codarri, Gyulveszi et al. 2011). This study also marked these GM-CSF producing T cells as the pathogenic subset, since T cells specifically lacking GM-CSF failed to initiate neuroinflammation despite expression of IL-

17A or IFN- γ , whereas GM-CSF secretion by *Ifng*(-/-)*Il17a*(-/-) T cells was sufficient to induce EAE.

Treatment with rIL-1/23 significantly increased the amount of IL-10 production by KIR3DL2- CD4+ T cells from RA patients but not by KIR3DL2+ CD4+ T cells. Recently IL-23 has been shown to suppress IL-10 production in IBD, with overexpression of IL-23 in the intestinal mucosa suppressing the production of IL-10, thus weakening the defensive barrier by reducing the production of IgA in the gut (Liu, Feng et al. 2012).

Production of IL-5 was not increased by the addition of rIL-1/23 in any of the groups investigated. This is perhaps not unexpected since rIL-1/23 are Th17 polarizing cytokines and IL-5 is a cytokine associated with Th2 cells, where IL-4 acts to polarize naïve T cells towards a Th2 phenotype. It is proposed that Th2 cells are more rigid in terms of plasticity as compared to both Th1 and Th17 cells. This stabilization of Th2 cells is thought to be the result of early extinction of IL-12 signaling (Szabo Immunity 1995).

In summary, I have shown using ICS that KIR3DL2+ CD4+ T cells from AS patients produce more IL-17 than KIR3DL2+ CD4+ T cells from HLA-B27- healthy controls. Moreover, using multiplex bead analysis, I have shown that following anti-CD2/3/28 stimulation for 5 days KIR3DL2+ CD4+ T cells from inflammatory arthritis patients produce significantly higher amounts of IL-17 compared to KIR3DL2- CD4+ T cells from the same patients. There was also significantly greater production of IL-6 by KIR3DL2+ CD4+ T cells compared to KIR3DL2- CD4+ T cells from AS patients and HLA-B27+ healthy controls. Moreover, treatment with rIL-1/23 significantly increased amounts of IL-17 by KIR3DL2+ CD4+ T cells in AS patients but not RA patients and healthy controls. Although numbers used in this explorative study were small, and therefore may be underpowered to detect significant differences, the results suggest that

KIR3DL2+ CD4+ T cells are an interesting subset to investigate and would warrant further investigation, especially with regards to the effect of rIL-1/23 on IL-17 production.

This data is consistent with the theory that AS is a Th17 or a Th17/1 driven disease. This is supported by numerous studies showing that IL-17 is elevated in serum and synovial fluid from SpA patients (Wendling, Cedoz et al. 2007; Melis, Vandooren et al. 2009) and that circulating Th17 cells are elevated in AS patients (Jandus 2008; Shen, Goodall et al. 2009; Limon-Camacho, Vargas-Rojas et al. 2012). Of note the magnitude of the IL-17 responses is greater in this work compared to previous studies and although difficult to make direct comparisons due to experimental differences, one major difference is that previous studies have looks at total CD4+ T cells, where as I have focused on cytokine production by KIR3DL2+ CD4+ T cells. The theory that AS is a Th17 or a Th17/1 driven disease is also supported by genetic studies into AS which have found associations of AS with genes involved in the IL-17/23 axis, including *IL23R*, *JAK2* and *IL12B* (Burton, Clayton et al. 2007; Chen, Zhang et al. 2010; Evans, Spencer et al. 2011).

Chapter 5: Effects of activation on KIR3DL2 expression by CD4+ T cells

5.1. Introduction

KIR expression is primarily associated with NK cells, so a key question is: “under what circumstances is KIR3DL2 expression upregulated on CD4+ T cells?” Expression of the NK receptor CD94/NKG2A on CD8+ T cells is observed following TCR engagement and is clonally determined (Jabri, Selby et al. 2002). Less is known about the determinants of NK receptors expression on CD4+ T cells. One study found that T cells derived from cord blood or thymus did not express NK receptors, but T cells expressing NK receptors were present in all the peripheral tissues analyzed, which included the spleen, lymph nodes and tonsils (Mingari, Ponte et al. 1997). Interestingly the size of the KIR-expressing CD8+ T cell population increases with age, reaching up to 30% of CD8+ T cells in elderly individuals (Anfossi, Pascal et al. 2001). The number of CD4+ T cells expressing KIR in peripheral blood has also been shown to increase with age (van Bergen, Thompson et al. 2004).

I wanted to test the hypothesis that activation of CD4+ T cells could increase expression of and/or lead to *de novo* expression of KIR3DL2 on CD4+ T cells. ReA, a subtype of SpA, occurs after infection with Gram-negative intracellular bacteria including *Salmonella*, *Shigella*, *Campylobacter* and *Chlamydia trachomatis*. However despite extensive research no causative infectious agent has yet been identified in the development of AS. In lieu of a specific infectious agent to stimulate CD4+ T cells, I investigated whether activation of CD4+ T cells could lead to the expression of KIR3DL2 on CD4+ T cells. I used different methods of stimulation to

determine the effect of activation on expression of KIR3DL2, and these included *Staphylococcal enterotoxin B* (SEB), anti-CD2/3/28 beads, and PMA and ionomycin stimulation.

5.2. Aim

The overall aim of this chapter was to investigate whether activation induced and/or increased KIR3DL2 expression on CD4⁺ T cells. To achieve this I had three objectives.

- To determine whether activation increases KIR3DL2 expression on CD4⁺ T cells and whether this is greater expression of KIR3DL2 by CD4⁺ T cells from AS patients compared to healthy controls and RA patients after activation.
- To determine whether KIR3DL2 expression on CD4⁺ T cells and/or IL-17 production could be inhibited by SEB stimulation in the presence of DX31, a KIR3DL2 specific antibody.
- To determine whether activation induces *de novo* KIR3DL2 expression on naïve CD4⁺ T cells, and whether this is higher for AS patients compared to HLA-B27⁻ healthy controls.

5.3. Results

5.3.1. KIR3DL2 expression is increased by SEB stimulation of PBMC

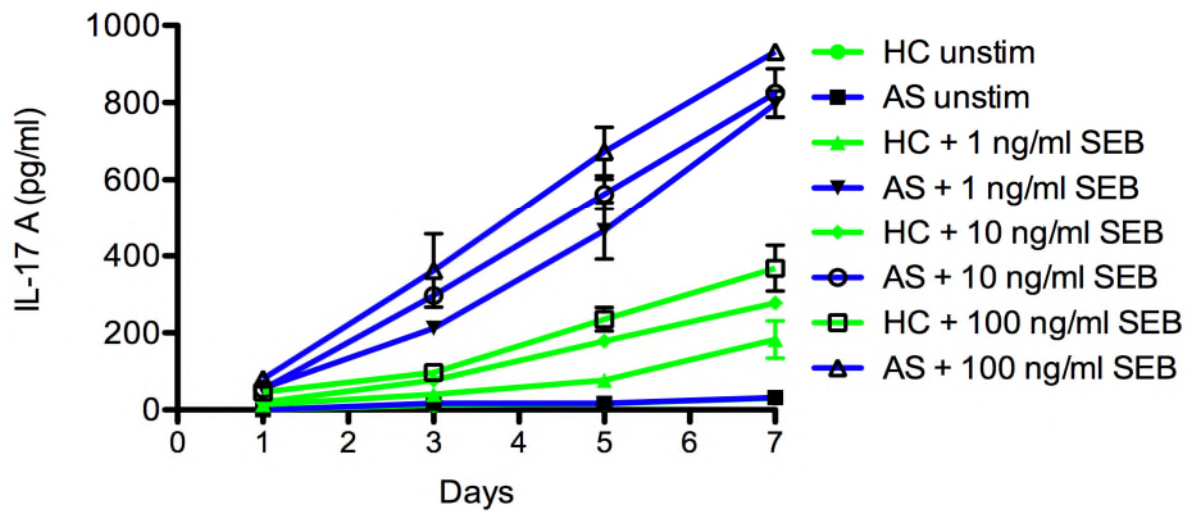
The factors leading to upregulation of KIR3DL2 on CD4⁺ T cells remain unknown. To address this I asked whether activation of CD4⁺ T cells could cause upregulation of KIR3DL2. To determine the concentration of SEB to be used I performed a titration using PBMC from one AS patient and one HLA-B27⁻ healthy control. I used SEB stimulation at between 0 and 100ng/ml, over a 7 day period, measuring IL-17 production by ELISA. The results are shown in Figure 5.1.A. From this titration I choose to look at cytokine production after 5 days stimulation with 100ng/ml SEB.

SEB is a superantigen which cross-links the TCR and HLA-class II, without the need for peptide, therefore allowing non-specific T cell activation. SEB activates only a proportion of T cells, more specifically those expressing TCR-V β -3, -12, -13.2, -14, -17 or -20 (Krakauer 1999). In order to rule out the possibility that SEB was preferentially activating one study group I FACS stained PBMC to investigate expression of these specific TCR-V β s on CD4⁺ T cells. Figure 5.1.B shows that there is no difference in the expression of these TCR-V β on CD4⁺ T cells from inflammatory arthritis patients and healthy controls.

Figure 5.2 shows representative FACS staining from one HLA-B27⁻ healthy control and one AS patient, showing KIR3DL2 expression on live CD4⁺ T cells after 5 days with or without SEB stimulation. Without stimulation 5.3% of CD4⁺ T cells from the AS patient express KIR3DL2, compared to 0.1% of CD4⁺ T cells from the healthy control. Following SEB stimulation the expression of KIR3DL2 on CD4⁺ T cells from the AS patient was greater than that seen on CD4⁺ T cells from the healthy control. In total 15.1% of CD4⁺ T cells express KIR3DL2 after 5 days

SEB stimulation in the AS patient sample, compared to 6% of CD4+ T cell from the HLA-B27-healthy control.

A



B

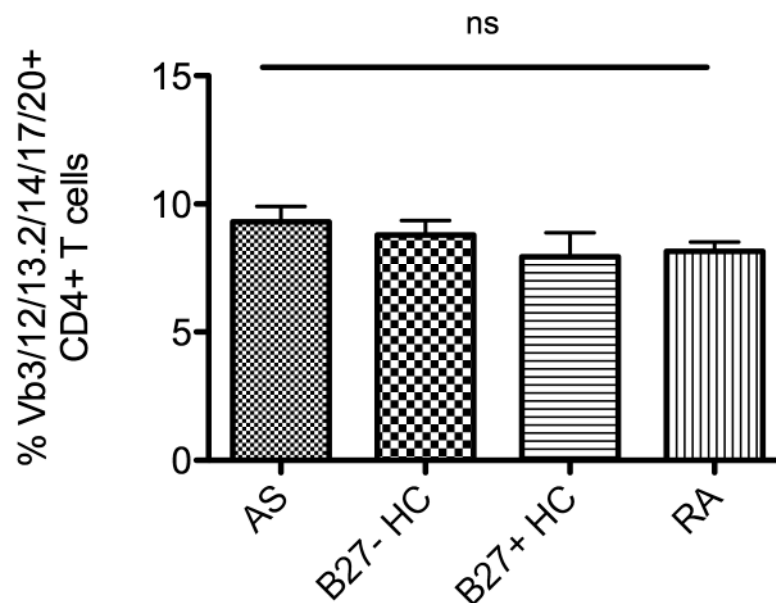


Figure 5.1: Assay optimization for KIR3DL2 expression on CD4+ T cells after activation with SEB. (A) SEB titration to determine concentration of SEB to be used for PBMC stimulation and number of days of stimulation. IL-17 production by PBMC of one healthy control and one AS patient, without SEB stimulation or with 1-100ng/ml SEB stimulation, was measured by ELISA. (B) FACS staining for TCR-V β -3, -12, -13.2, -14, -17 and -20 on CD4+ T cells from AS patients (n=12), HLA-B27- healthy controls (n=14), HLA-B27+ healthy controls (n=8) and RA patients (n=7).

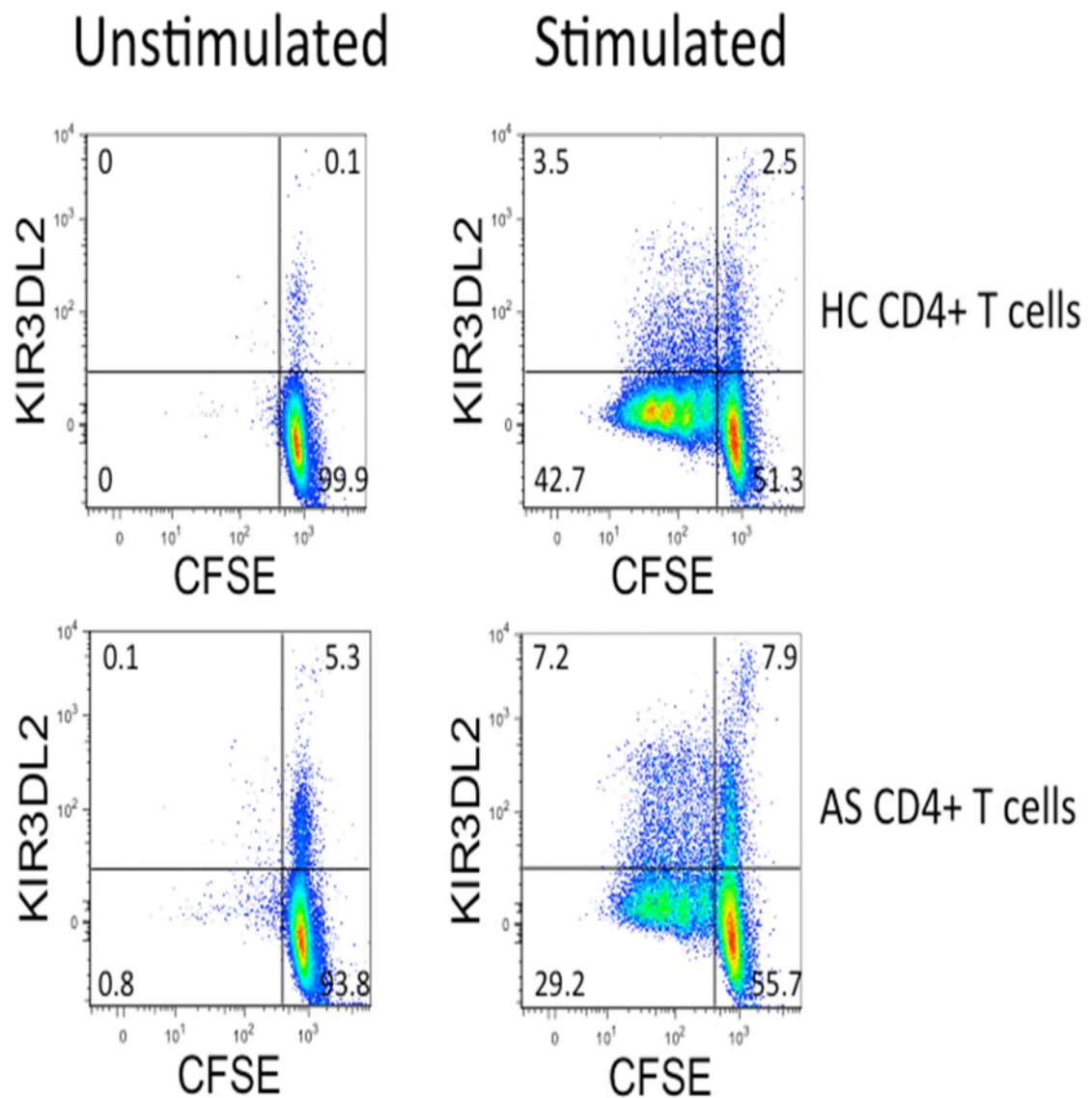


Figure 5.2. A higher percentage of CD4+ T cells from an AS patient express KIR3DL2, before and after stimulation with SEB, than CD4+ T cells from a HLA-B27- healthy control. Representative FACS staining showing the percentage KIR3DL2 expression on CFSE labeled live CD4+ T cells after 5 days culture of one healthy control and one AS patient PBMC, with or without SEB stimulation.

Figure 5.3 shows the percentage of CD4+ T cells expressing KIR3DL2 after 5 days, with or without SEB stimulation, from all the patients and healthy controls investigated. SEB stimulation led to an increase in the percentage of KIR3DL2+ CD4+ T cells in all groups investigated. In Figure 5.4.A we see that although there is no significant difference in the percentage of CD4+ T cells expressing KIR3DL2 without stimulation, after SEB stimulation a significantly higher percentage of CD4+ T cells from AS patients expressed KIR3DL2 compared to CD4+ T cells from HLA-B27- healthy controls, as shown in Figure 5.4.B. PBMC were CFSE labeled with the intention of analyzing KIR3DL2 expression on proliferating as compared to non-proliferating CD4+ T cells, however since CFSE staining was not consistent it was not possible to carry out this analysis. However the CFSE labeling does show, as seen in Figure 5.2, that KIR3DL2 expression is present on both proliferating and non-proliferating live CD4+ T cells after 5 days activation with SEB.

I analyzed the same data studying the absolute numbers of KIR3DL2+ CD4+ T cells, using Flow-Count Fluorespheres (Beckman Coulter), as detailed in section 2.2.8.1. This was carried out to exclude the possibility that relative changes in cell numbers were occurring, for example as a result of the depletion of other cell populations during the course of the experiment. Figure 5.5 shows the absolute number of CD4+ T cells expressing KIR3DL2, with or without SEB stimulation for 5 days. SEB stimulation led to an increase in the number of KIR3DL2+ CD4+ T cells, in all groups investigated. In Figure 5.6 we see that there is no significant difference in the numbers of CD4+ T cells expressing KIR3DL2 with or without SEB stimulation from AS patients compared to healthy controls and RA patients.

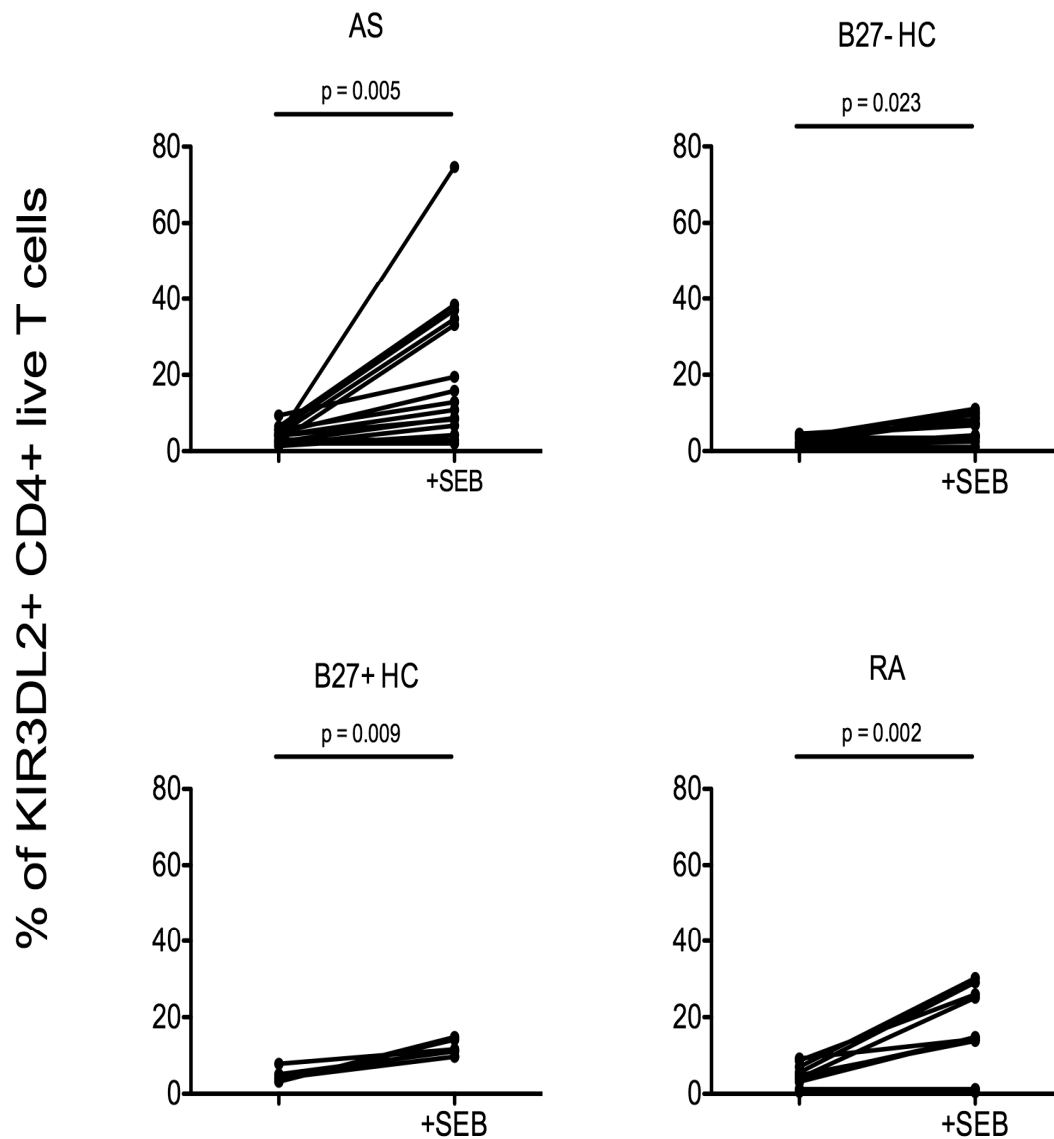


Figure 5.3: A higher percentage of CD4+ T cells express KIR3DL2 following stimulation with SEB. FACS staining after 5 days culture of PBMC from AS patients (n=15), HLA-B27- (n=13) and HLA-B27+ (n=6) healthy controls and RA patients (n=11), with or without SEB stimulation. A two-tailed student t test was used for statistical analysis.

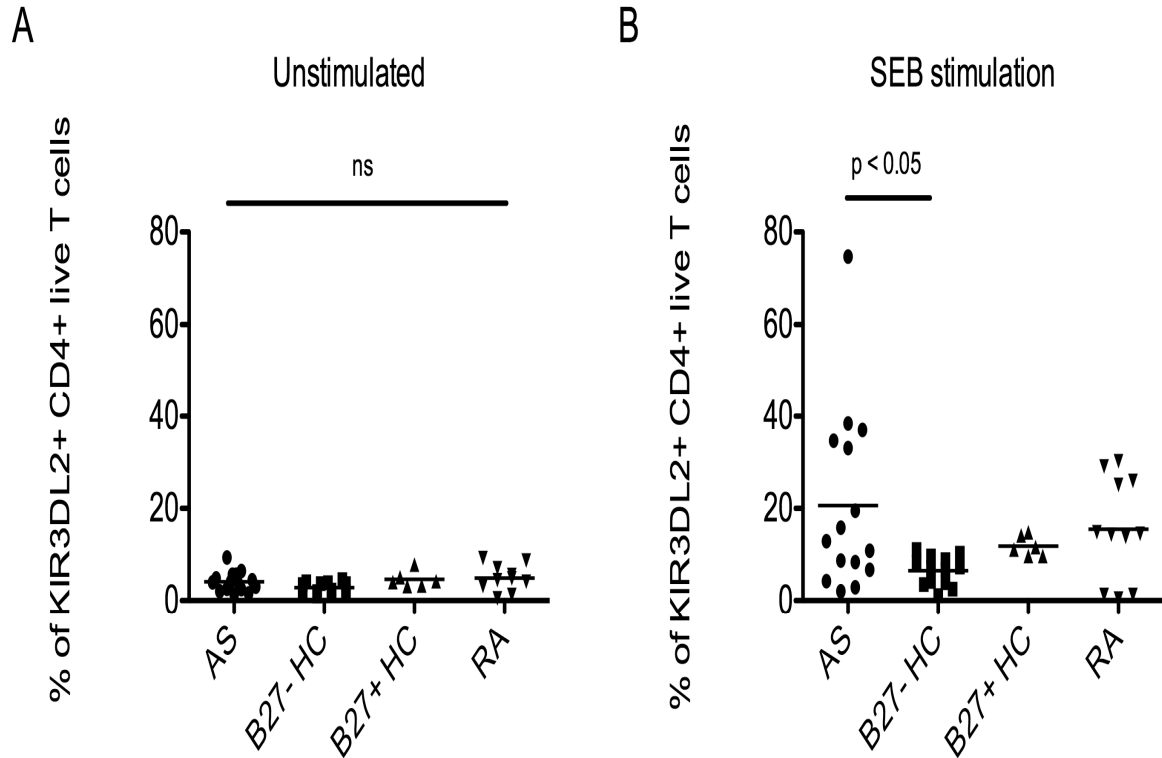


Figure 5.4: A higher percentage of CD4+ T cells from AS patients express KIR3DL2 following stimulation with SEB, compared to HLA-B27- healthy controls. FACS staining after 5 days culture of PBMC from AS patients (n=15), HLA-B27- (n=13) and HLA-B27+ (n=6) healthy controls and RA patients (n=11), without (A) or with (B) SEB stimulation. A one-way ANOVA was used for statistical analysis. Note data is drawn from Figure 5.3.

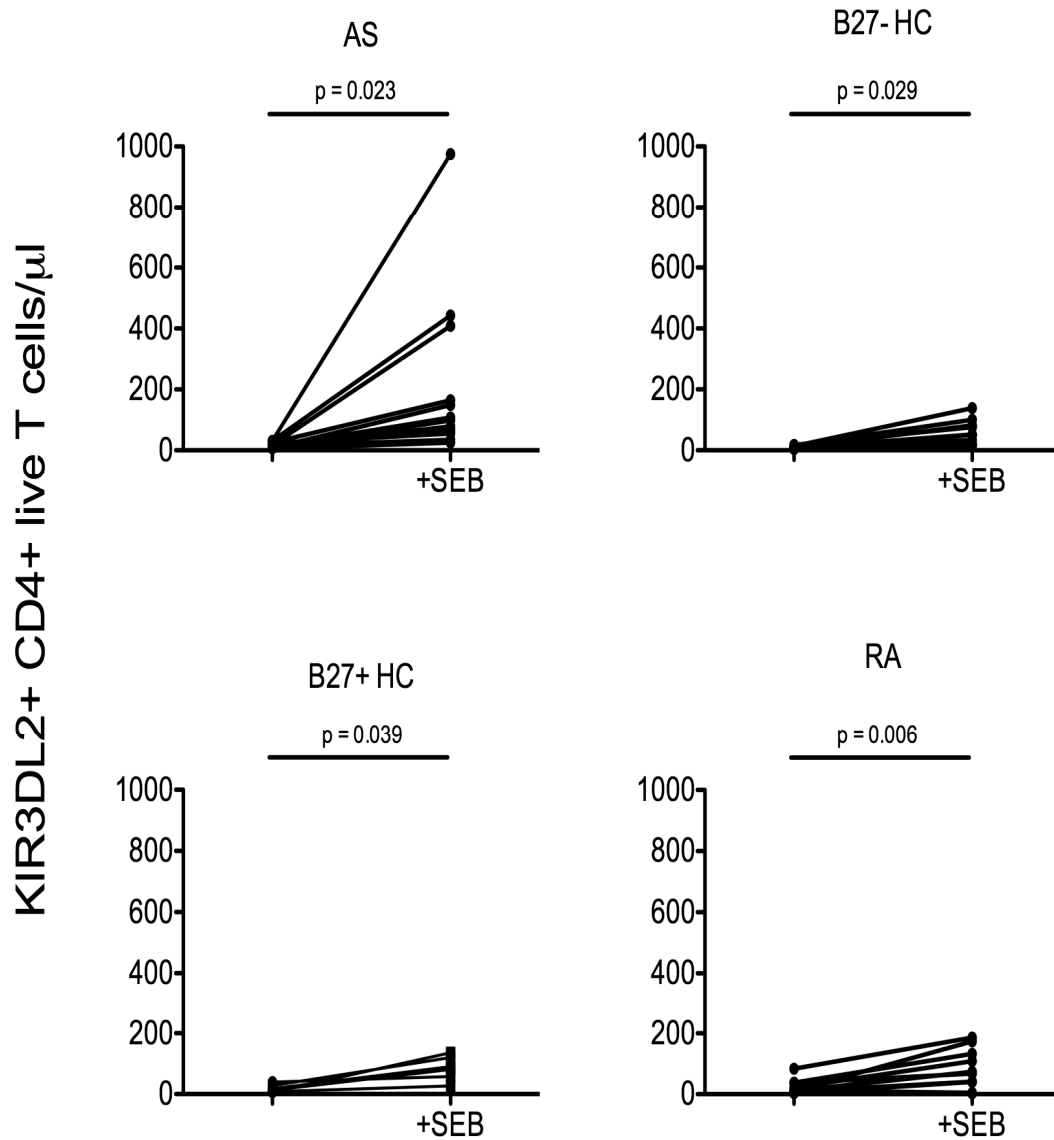


Figure 5.5: Elevated numbers of CD4+ T cells express KIR3DL2 following stimulation with SEB. FACS staining after 5 days culture of PBMC from AS patients (n=13), HLA-B27- (n=11) and HLA-B27+ (n=6) healthy controls and RA patients (n=11), with or without SEB stimulation. Flow-Count Fluorespheres were used to determine the number of KIR3DL2+ CD4+ T cells/μl following cell resuspension in FACS fix. A two-tailed student t test was used for statistical analysis.

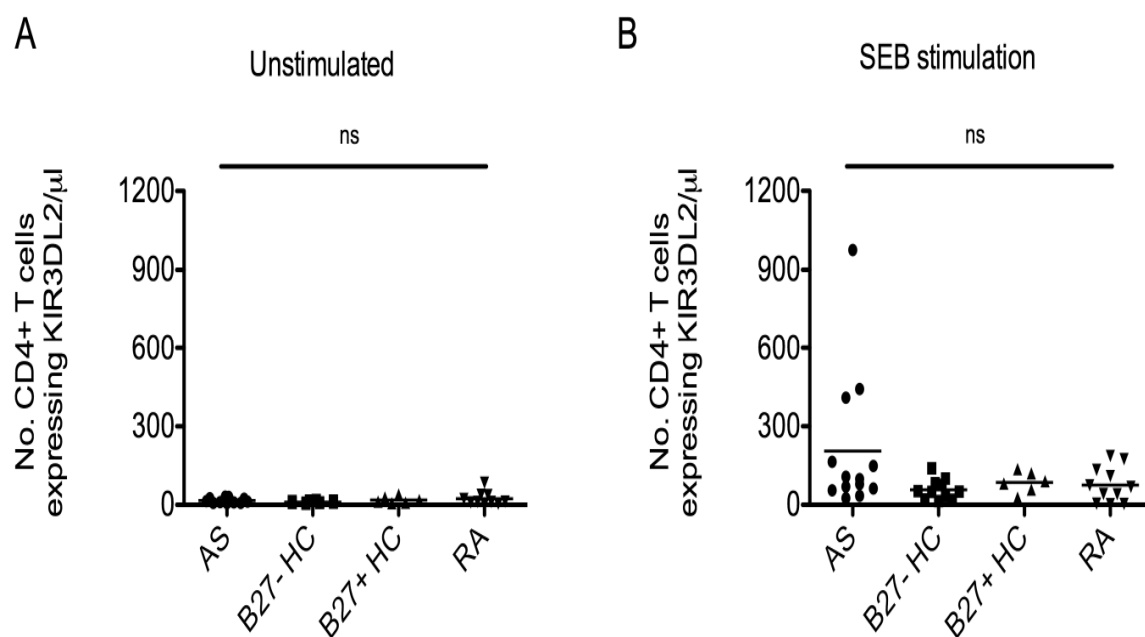


Figure 5.6: No significant difference in the numbers of CD4+ T cells expressing KIR3DL2 before or after SEB stimulation. FACS staining after 5 days culture of PBMC from AS patients (n=13), HLA-B27- (n=11) and HLA-B27+ (n=6) healthy controls and RA patients (n=11), without (A) or with (B) SEB stimulation. A one-way ANOVA was used for statistical analysis. Note data is drawn from Figure 5.5.

When the experiment was repeated but FACS staining for a panel of other KIRs, including KIR3DL1, KIR2DL1 and KIR2DL2, was performed there is no significant increase in expression of these other KIRs on CD4+ T cells after SEB stimulation of PBMC from AS patients or HLA-B27- healthy controls (Figure 5.7).

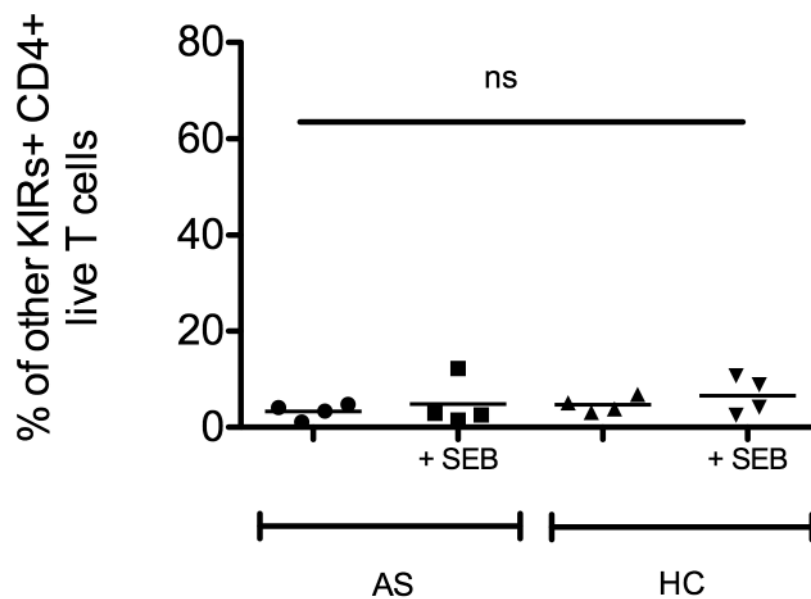


Figure 5.7: No significant difference in the expression of other KIRs on CD4+ T cells from AS patients and HLA-B27- healthy controls, before and after stimulation with SEB. Percentage expression KIR3DL1, KIR2DL1 and KIR2DL2 after 5 days culture PBMC from and AS patients (n=4) or HLA-B27- healthy controls (n=4), with or without SEB stimulation. A one-way ANOVA was used for statistical analysis.

5.3.2. IL-17 production is upregulated by SEB stimulation of PBMC and is greater for AS patients

In addition to KIR3DL2 expression I wanted to investigate cytokine production following SEB activation of PBMC. To do this I performed ELISAs, as described in section 2.2.11, on cultured supernatants after 5 days stimulation with SEB and compared this to supernatants from PBMC cultured without SEB. Figure 5.8 shows that there was a significant increase in IL-17 production by PBMC after SEB activation in all groups investigated. After co-culture with SEB, PBMC from AS patients produced nearly 10 times as much IL-17, increasing from an average of 67 to 589pg/ml. There was a similar fold increase in IL-17 production by PBMC from HLA-B27+ healthy controls, with an increase from an average of 2.5pg/ml without SEB to 206pg/ml after culture with SEB. IL-17 production from RA patients and HLA-B27- healthy controls also increased after SEB activation. It should be noted that using ELISA as a method of detection does not allow discrimination of the specific cell type producing IL-17.

Figure 5.9.A shows that there is no difference in the production of IL-17, by PBMC without SEB stimulation, between any of the groups investigated. Of note two AS patients produce over 200pg/ml IL-17 without stimulation, and this is not observed in any of the other groups investigated. Following stimulation with SEB PBMC from AS patients produce significantly more IL-17 than HLA-B27+ healthy controls, as shown in Figure 5.9.B.

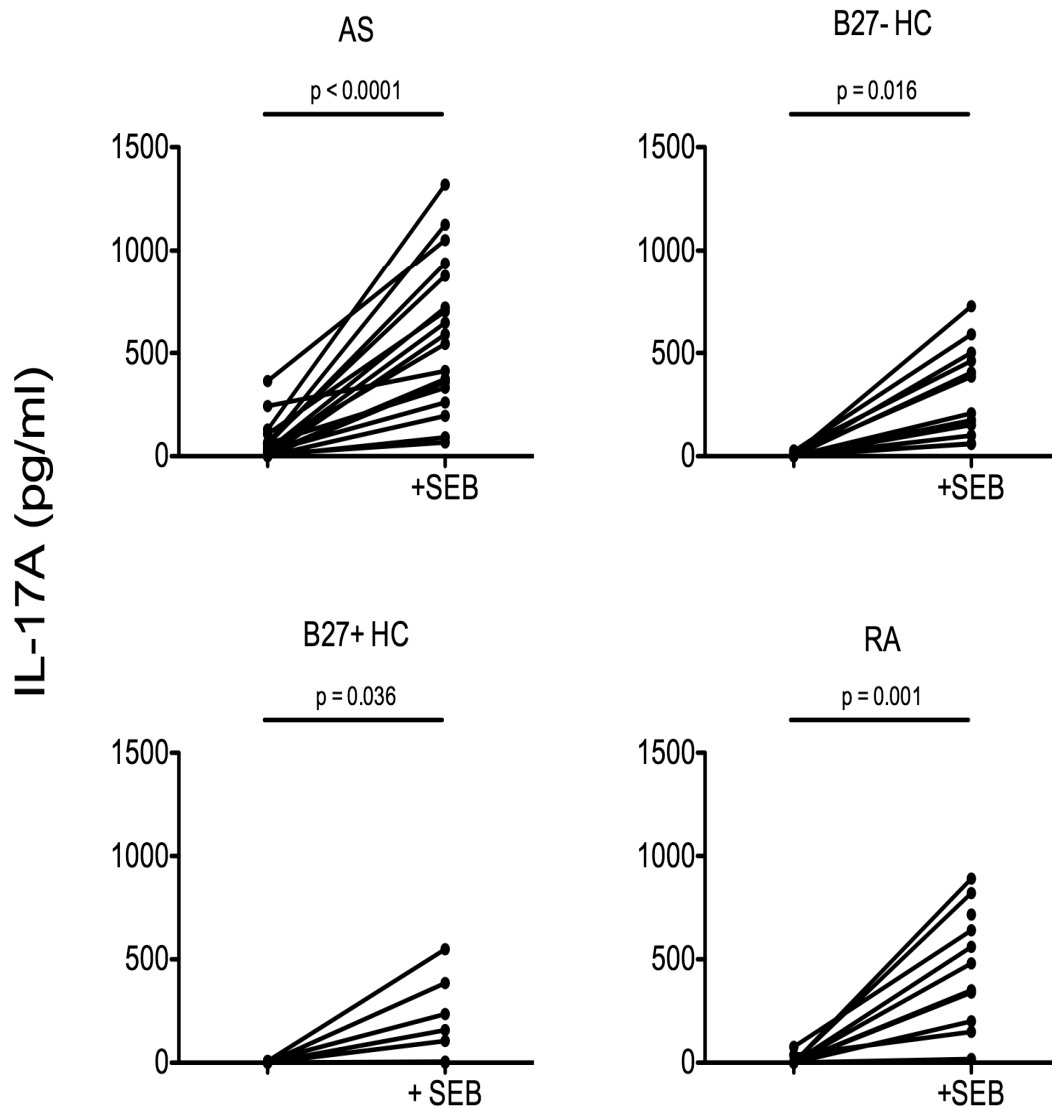


Figure 5.8: Significantly greater production of IL-17 by PBMC after stimulation with SEB. Production of IL-17, measured by ELISA of cultured supernatants, after 5 days culture of PBMC from AS patients (n=18), HLA-B27- (n=12) and HLA-B27+ (n=7) healthy controls and RA patients (n=11), with or without SEB stimulation. A two-tailed student t was used for statistical analysis.

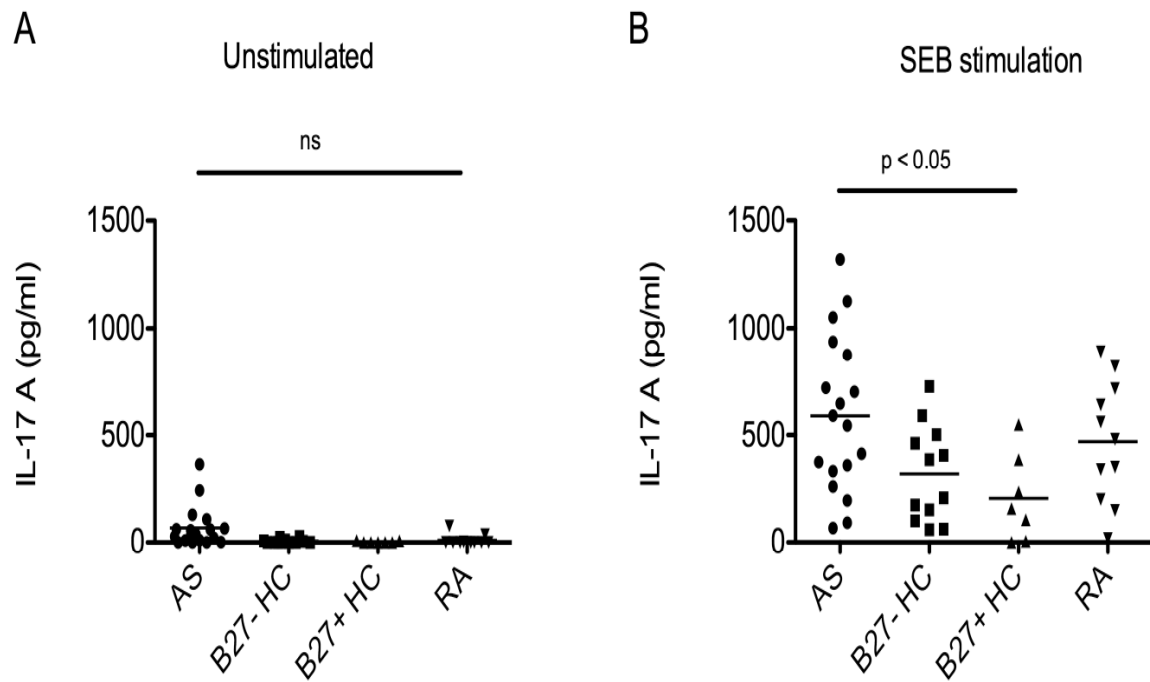


Figure 5.9: PBMC from AS patients produce significantly more IL-17 than PBMC from HLA-B27+ healthy controls following stimulation with SEB. ELISA of supernatants after 5 days culture of PBMC from AS patients (n=18), HLA-B27- (n=12) and HLA-B27+ (n=7) healthy controls and RA patients (n=11), without (A) or with (B) SEB stimulation. A one-way ANOVA was used for statistical analysis. Note data is drawn from Figure 5.8.

5.3.3. TNF α production is upregulated by SEB stimulation of PBMC, but does not differ between inflammatory arthritis patients and healthy controls

TNF α production following SEB stimulation was also investigated. Figure 5.10 shows that there was a significant increase in production of TNF α after SEB activation by PBMC from all groups investigated, as measured by ELISA. When comparing patient and controls groups, as shown in Figure 5.11, there was no significant difference in the production of TNF α by PBMC between any of the groups investigated, either with or without SEB stimulation.

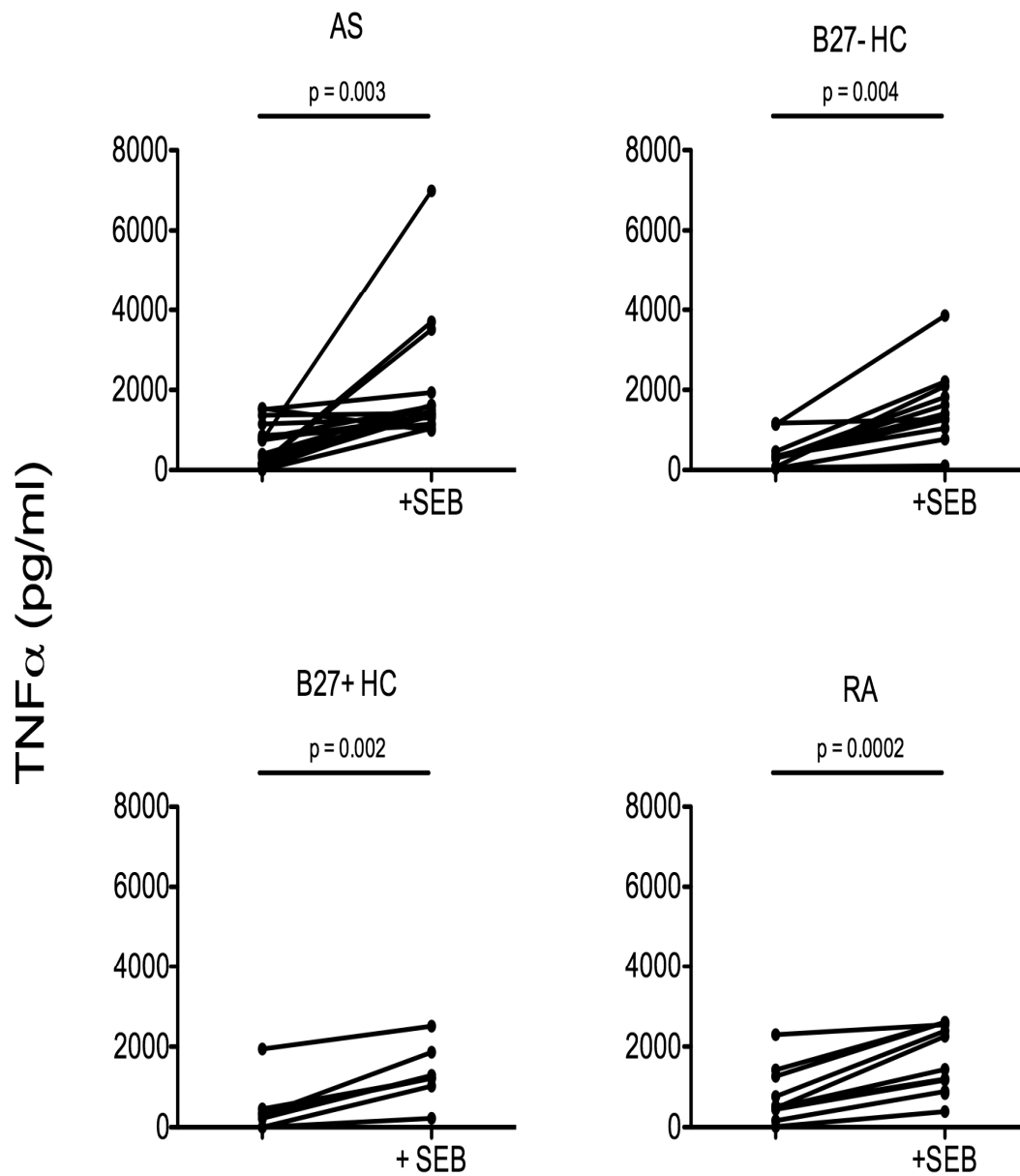


Figure 5.10: Significantly higher production of TNFα by PBMC after stimulation with SEB. Production of TNFα, measured by ELISA of cultured supernatants, after 5 days culture of PBMC from AS patients (n=18), HLA-B27- (n=12) and HLA-B27+ (n=7) healthy controls and RA patients (n=11), with or without SEB stimulation. A two-tailed student t was used for statistical analysis.

5.3.4. KIR3DL2 expression is upregulated by anti-CD2/3/28 bead stimulation of CD4+ T cells

Anti-CD2/3/28 beads were used as an alternative method of stimulation to confirm whether activation could upregulate expression of KIR3DL2 on CD4+ T cells. CD4+ T cells from HLA-B27- healthy controls and AS patients were separated using a CD4+ T cell isolation kit (Miltenyi Biotech), as described in section 2.2.6.1. An aliquot of CD4+ T cells were FACS stained for KIR3DL2 expression on the day the experiment was set up (day 0) and the remaining CD4+ T cells were stimulated for 5 days with anti-CD2/3/28 beads. 1:1 and 1:10 bead-to-cell ratios were used to investigate whether different strengths of T cell activation would differentially effect KIR3DL2 expression.

Figure 5.12 shows representative FACS staining after 5 days stimulation of CD4+ T cells from one HLA-B27- healthy control with anti-CD2/3/28 beads at either a 1:1 or a 1:10 bead-to-cell ratio, compared to KIR3DL2 expression on CD4+ T cells without anti-CD2/3/28 stimulation. Anti-CD2/3/28 bead stimulation increases KIR3DL2 expression on CD4+ T cells, with a greater strength of stimulation (1:1 v 1:10) causing greater expression of KIR3DL2.

Following 5 days stimulation with anti-CD2/3/28 beads at a 1:1 bead-to-cell ratio, a higher percentage of CD4+ T cells from both AS patients and HLA-B27- healthy controls express KIR3DL2 compared to no stimulation, as shown in Figure 5.13.A. In both AS patients and HLA-B27- healthy controls stimulation with a 1:1 bead-to-cell ratio significantly increased the percentage of CD4+ T cells expressing KIR3DL2 as compared to stimulation with a 1:10 bead-to-cell ratio. There was no difference between the percentage of CD4+ T cells from AS patients and HLA-B27- healthy controls expressing KIR3DL2 after anti-CD2/3/28 beads at a 1:1 or 1:10 bead-to-cell ratio, as shown in Figure 5.13.B.

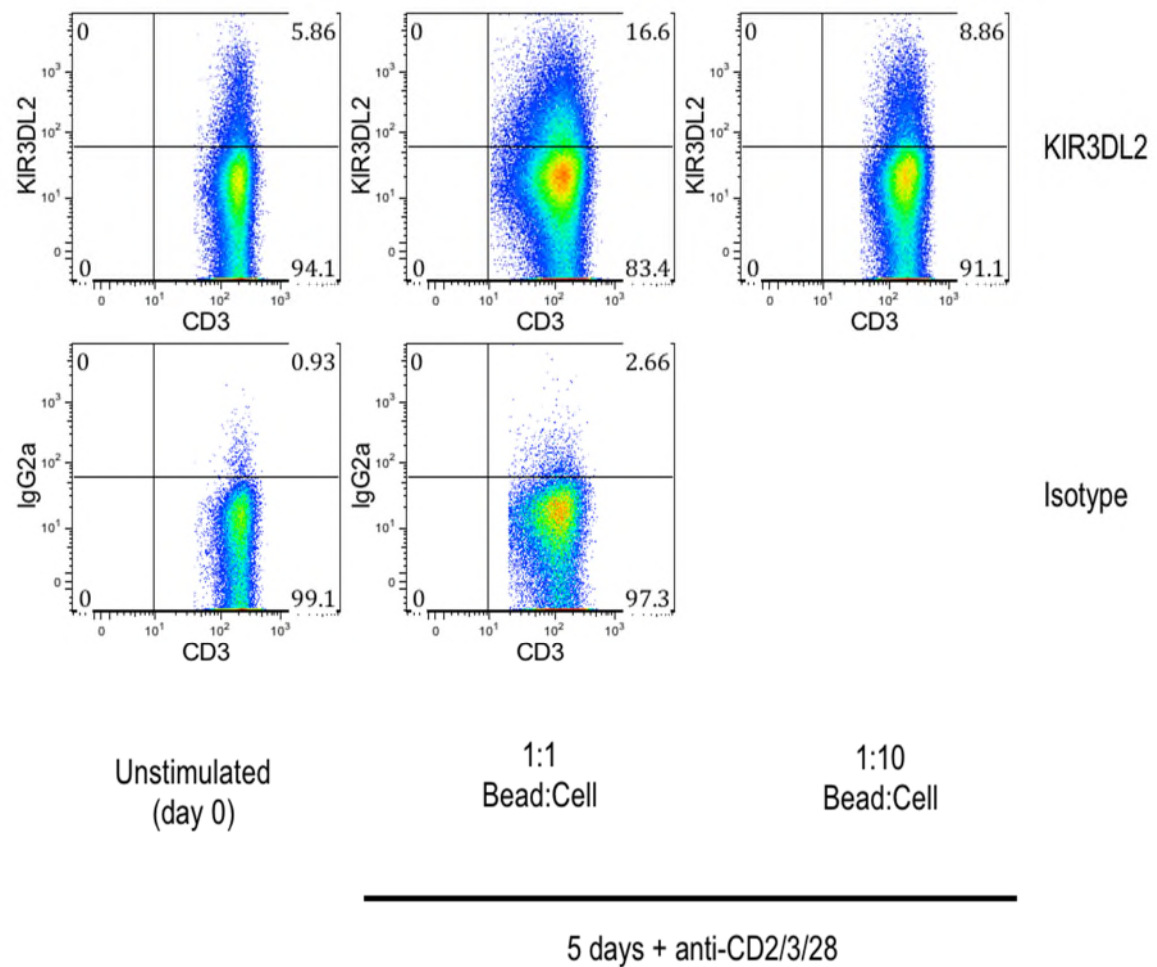


Figure 5.12: A higher percentage of CD4+ T cells from a HLA-B27- healthy control express KIR3DL2 after stimulation with anti-CD2/3/28 beads at a bead:cell ratio of 1:1, than after stimulation with anti-CD2/3/28 beads at a bead:cell ratio of 1:10. Representative FACS staining showing percentage KIR3DL2 expression after 5 days culture of CD4+ T cells from a HLA-B27- healthy control, with anti-CD2/3/28 beads at a bead:cell ratio of 1:1 (middle) or 1:10 (right) or without anti-CD2/3/28 bead stimulation (left).

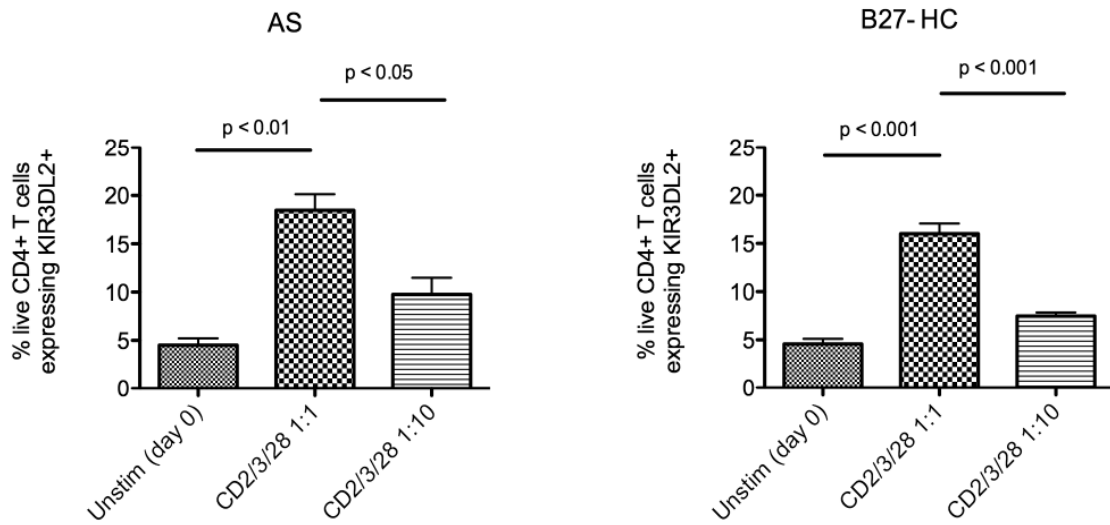
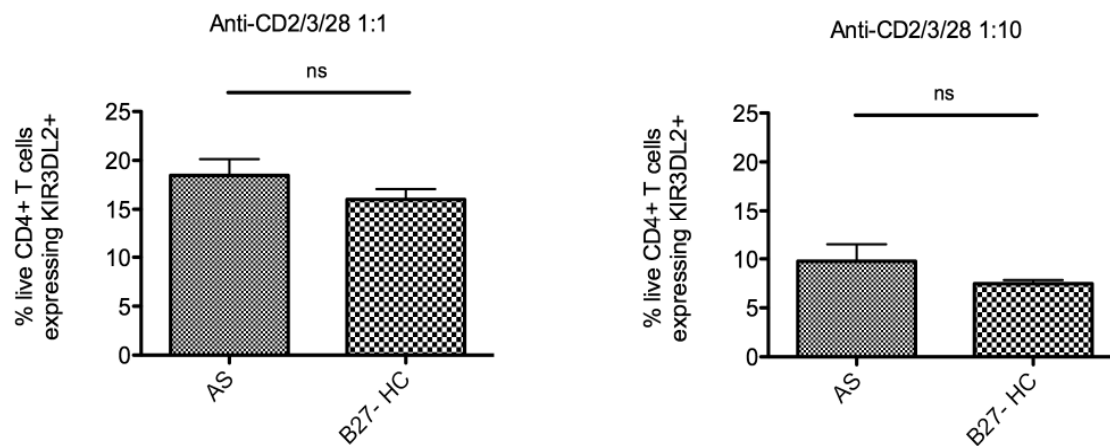
A**B**

Figure 5.13: A higher percentage of CD4+ T cells express KIR3DL2 following stimulation with anti-CD2/3/28 beads, but this does not differ between AS patients and HLA-B27- healthy controls. (A) CD4+ T cells from AS patients (n=3) and HLA-B27- healthy controls (n=3), were cultured for 5 days with anti-CD2/3/28 beads at a 1:1 or 1:10 bead-to-cell ratio, before FACS staining for KIR3DL2 expression on live CD4+ T cells. Shown in comparison to KIR3DL2 expression on CD4+ T cells from the same individuals at day 0, without anti-CD2/3/28 stimulation. A one-way ANOVA was used for statistical analysis. (B) Comparison of KIR3DL2+ CD4+ T cells after 5 days stimulation with anti-CD2/3/28 beads at a 1:1 and 1:10 bead-to-cell ratio. An unpaired t test was used for statistical analysis. Bars represent mean values ($\pm$ SD). Note data in Figure 5.13.B is drawn from Figure 5.13.A.

5.3.5. Increased KIR3DL2 expression on CD4+ T cells after SEB stimulation is not inhibited by SEB stimulation in the presence of DX31

Having observed an increase in KIR3DL2 expression on CD4+ T cells after activation I wanted to investigate whether SEB stimulation in the presence of DX31, a KIR3DL2 specific antibody, could inhibit this KIR3DL2 upregulation. DX31 or control antibody (IgG2a) at 50µg/ml was added to SEB activated PBMC and cultured for 5-days. A further 5µg of DX31 or control antibody was added after 3 days incubation. Figure 5.14 shows representative FACS staining from one AS patient and one HLA-B27- healthy control after 5 days stimulation of PBMC with or without SEB and with DX31, an anti-KIR3DL2 antibody, or a control antibody. SEB activation increases KIR3DL2 expression on CD4+ T cells from 3.2% to 3.7% in the AS patients and from 3.7% to 8.1% in the healthy control. Following co-culture with DX31 KIR3DL2 expression was 3.1% in the AS patient and 6.7% in the healthy control.

Figure 5.15 shows the percentage of CD4+ T cells expressing KIR3DL2 before and after SEB stimulation for 5 days, with or without DX31 or a control antibody, from all the healthy controls and inflammatory arthritis patients investigated. Co-culture with DX31 did not significantly alter the percentage of CD4+ T cells expressing KIR3DL2, in any of the conditions, or in any of the groups investigated. Similarly in Figure 5.16, co-culture with DX31 did not significantly alter the number of CD4+ T cells expressing KIR3DL2 in any of the groups investigated following stimulation with SEB.

In Figure 5.17 we see that co-culture with DX31 does not inhibit the production of IL-17. As before it is worth noting that using ELISA as a method of detection does not allow discrimination of the specific cell type producing the IL-17.

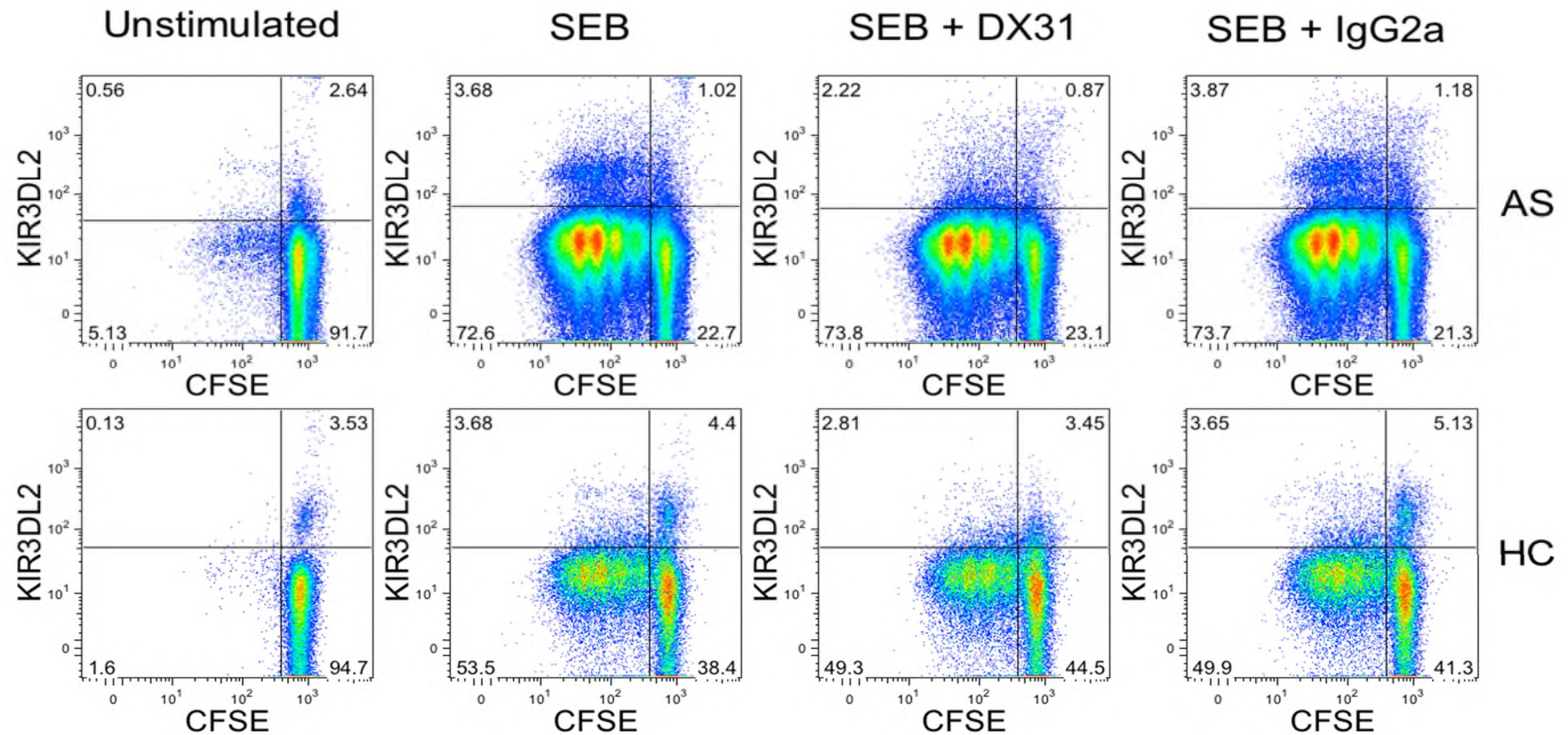


Figure 5.14: PBMC from a healthy control and an AS patient were stimulated for 5 days in the presence of SEB alone or with SEB and a KIR3DL2 specific antibody (DX31) or a control antibody (IgG2a). Representative FACS staining shows the percentage KIR3DL2 expression on CFSE labeled live CD4⁺ T cells after 5 days culture of one healthy control (HC) and one AS patient PBMC.

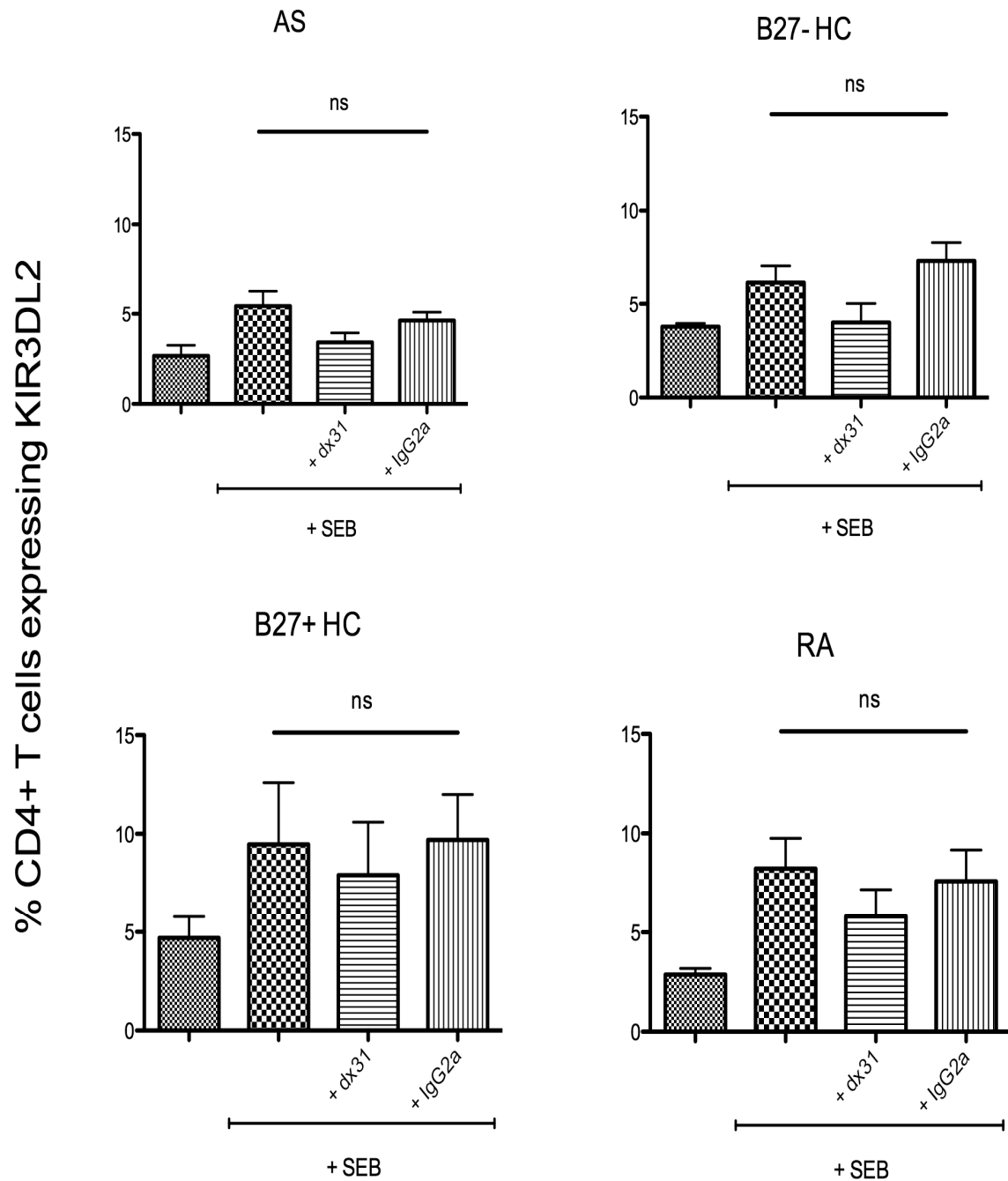


Figure 5.15: Increased KIR3DL2 expression on CD4+ T cells is not inhibited by SEB stimulation in the presence of DX31. Percentage KIR3DL2 expression after 5 days culture of PBMC from AS patients (n=4), HLA-B27- (n=4) and HLA-B27+ (n=3) healthy controls and RA patients (n=3) with or without SEB stimulation. A one-way ANOVA was used for statistical analysis. Bars represent mean values ($\pm$ SD).

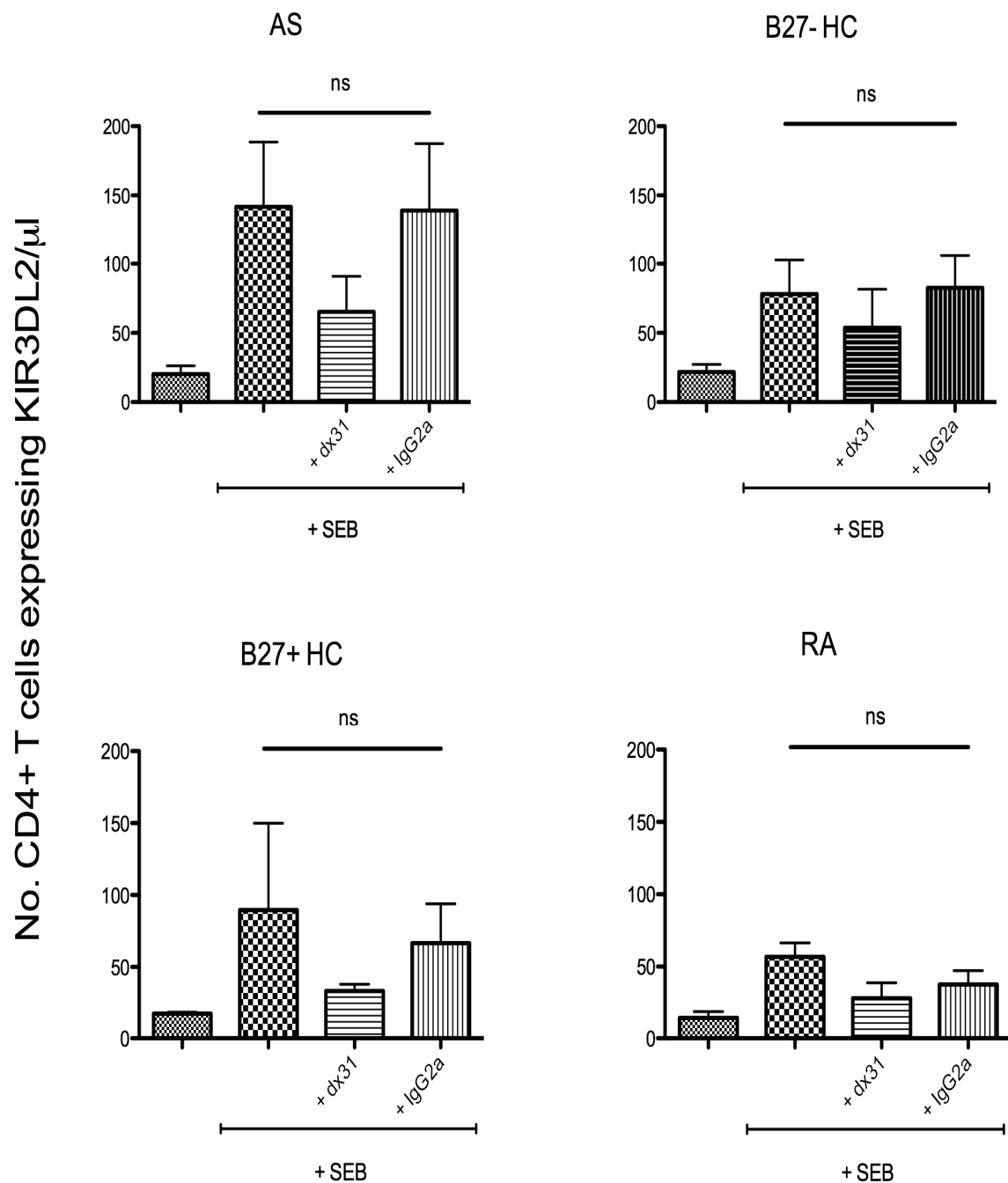


Figure 5.16: Increased KIR3DL2 expression on CD4+ T cells is not inhibited by SEB stimulation in the presence of DX31. Percentage KIR3DL2 expression after 5 days culture of PBMC from AS patients (n=4), HLA-B27- (n=4) and HLA-B27+ (n=3) healthy controls and RA patients (n=3) with or without SEB stimulation. A one-way ANOVA was used for statistical analysis. Bars represent mean values ($\pm$ SD).

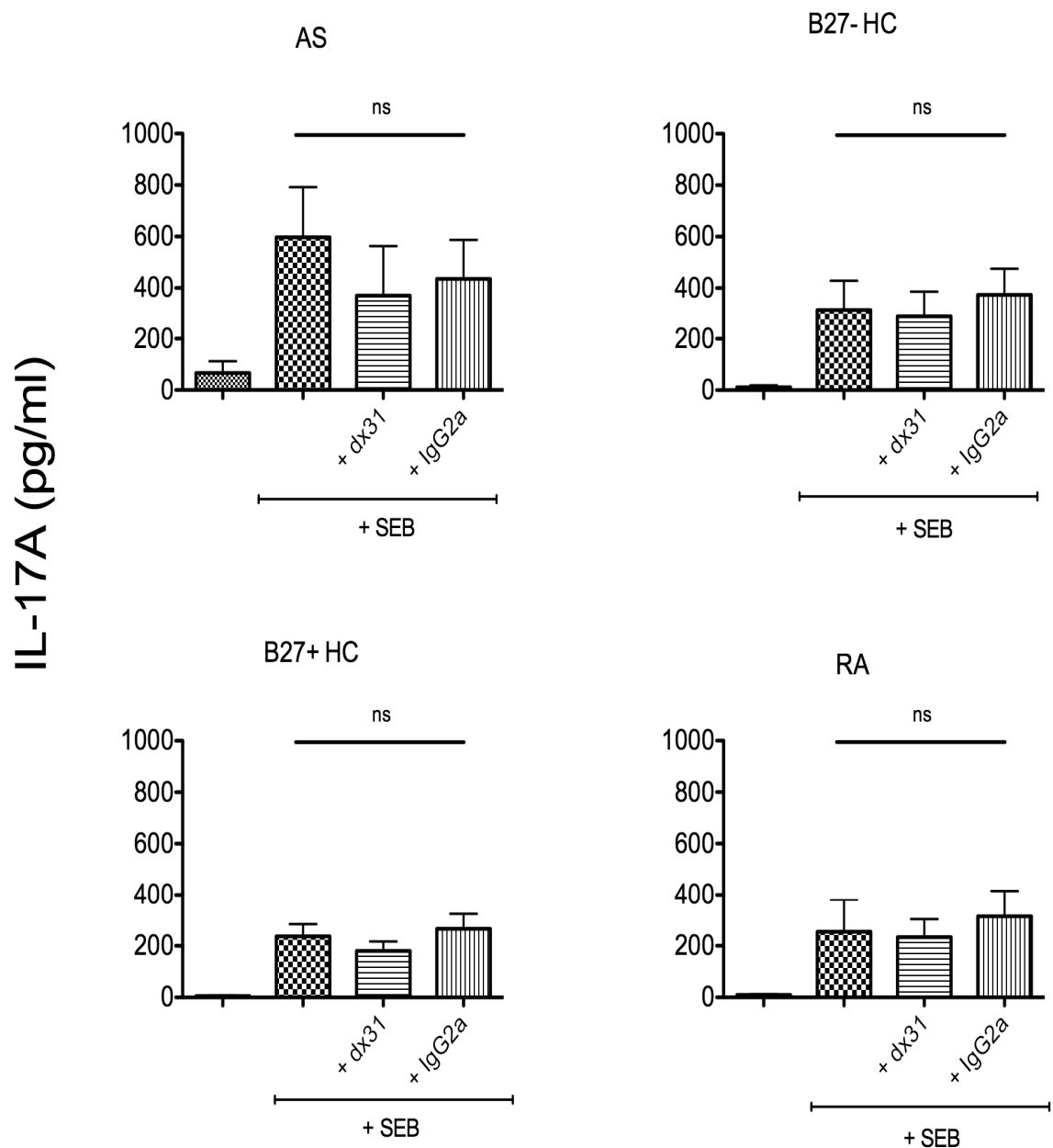


Figure 5.17: Increased IL-17 production by SEB-stimulated PBMC is not inhibited by SEB stimulation in the presence of DX31. IL-17A production after 5 days culture of PBMC from AS patients (n=4), HLA-B27- (n=4) and HLA-B27+ (n=3) healthy controls and RA patients (n=3) with or without SEB stimulation. A one-way ANOVA was used for statistical analysis. Bars represent mean values ($\pm$ SD).

5.3.6. IL-17 production by PBMC is inhibited in the presence of an anti-HLA-B27 dimer antibody

HD6 is a chimeric monoclonal antibody, comprising a human Fab₂ and a murine IgG1 Fc portion, that recognises recombinant and cell surface HLA-B27 dimers and HLA-B27 free heavy chains (Payeli, Kollnberger et al. 2012). 5µg/ml of HD6 or control antibody (IgG1) was added to SEB activated PBMC and cultured for 5-days. A further 5µg of HD6 or control antibody was added after 3 days incubation. Cultured supernatants were collected and IL-17 production was measured by ELISA. Figure 5.18 shows that SEB stimulation increases IL-17 production, with a greater increase in AS patients compared to HLA-B27- healthy controls. The increase in IL-17 production after SEB stimulation significantly decreases following incubation of AS patient PBMC with HD6.

In Figure 5.19 we see that the addition of HD6 does not reduce the production of TNFα after SEB stimulation, either in AS patients or HLA-B27- healthy controls.

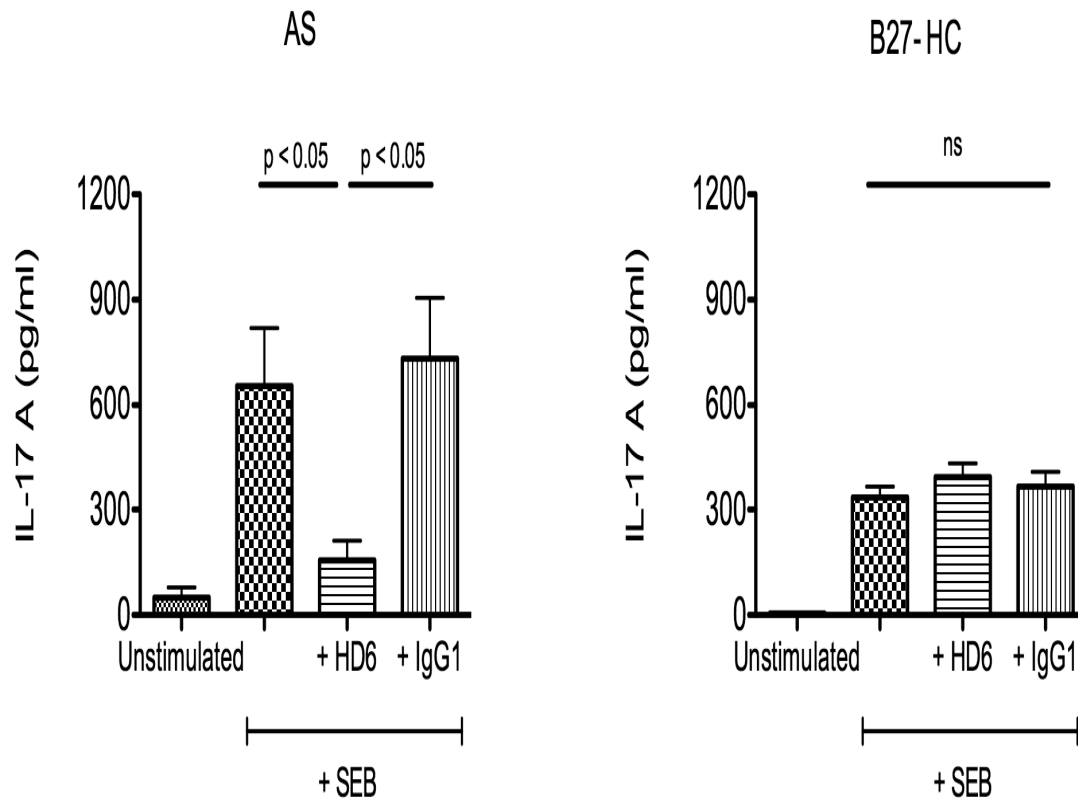


Figure 5.18: Blocking HD6 significantly reduces production of IL-17 by PBMC from AS patients after stimulation with SEB. Production of IL-17, measured by ELISA of cultured supernatants, after 5 days culture of PBMC from AS patients (n=5) and HLA-B27- healthy controls (n=3), with or without SEB stimulation. A one-way ANOVA was used for statistical analysis. Bars represent mean values ($\pm$ SD).

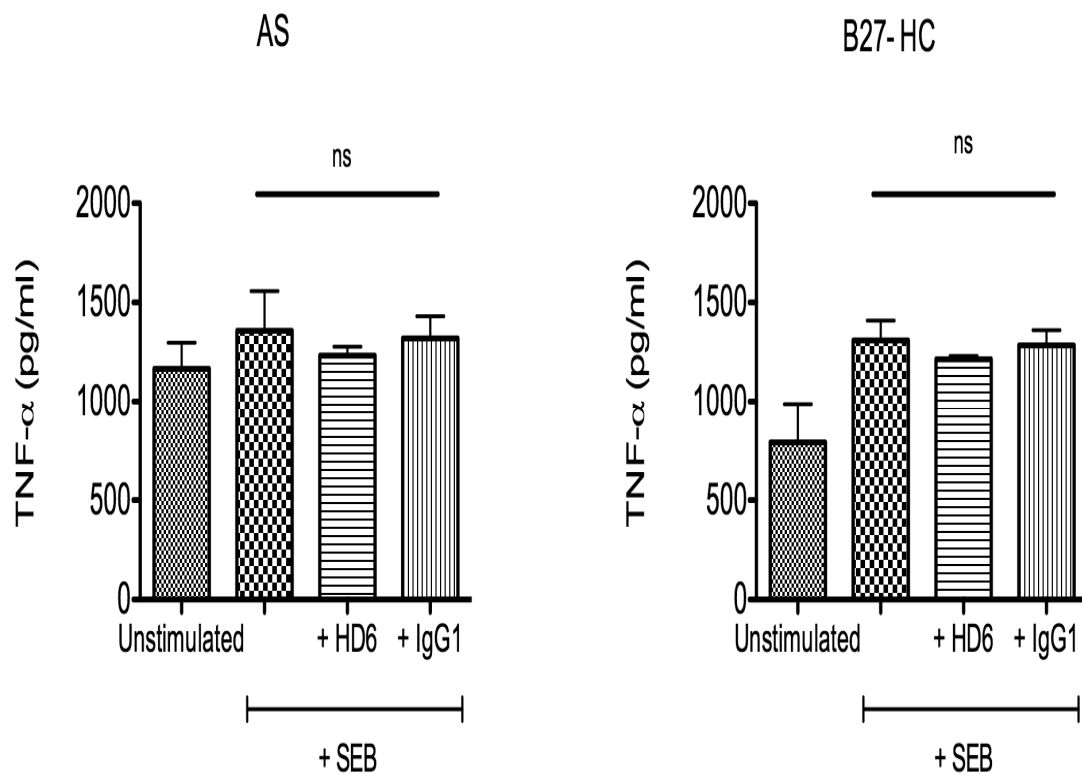
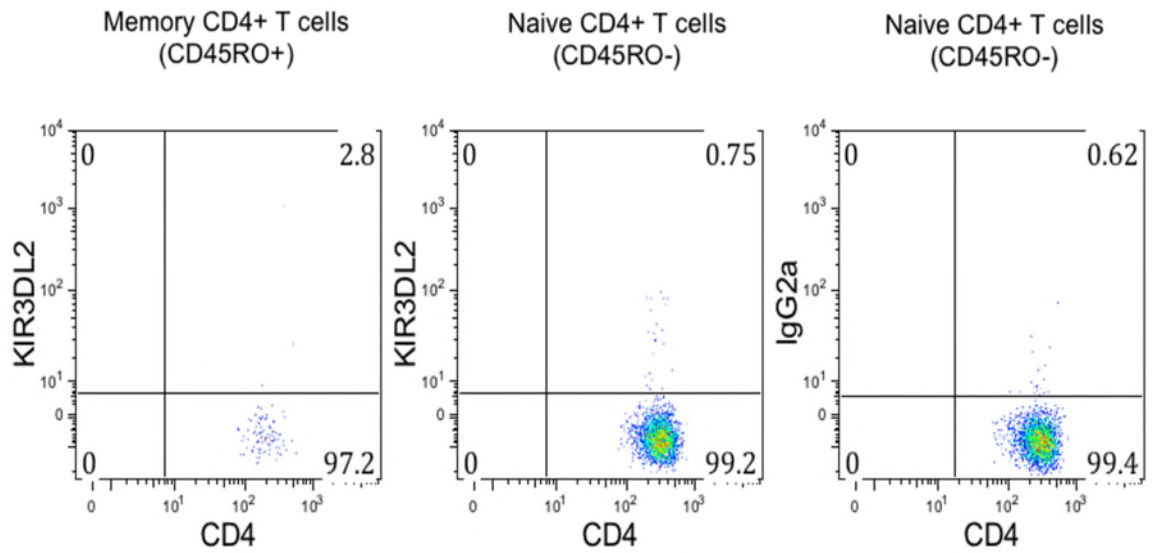


Figure 5.19: Blocking with HD6 does not significantly alter production of TNF α by PBMC after stimulation with SEB. Production of TNF α , measured by ELISA of cultured supernatants, after 5 days culture of PBMC from AS patients (n=5) and HLA-B27- healthy controls (n=3), with or without SEB stimulation. A one-way ANOVA was used for statistical analysis. Bars represent mean values ($\pm$ SD).

5.3.7. KIR3DL2 is induced on naïve CD4+ T cells following activation

Having shown that KIR3DL2 expression is increased on PBMC and CD4+ T cells by activation I wanted to explore whether activation could induce *de novo* expression of KIR3DL2 on KIR3DL2- CD4+ T cells. Since T cells derived from cord blood do not express NK receptors (Mingari, Ponte et al. 1997) I looked specifically at induction of KIR3DL2 on naïve CD4+ T cells. Figure 5.20.A confirms that KIR3DL2 is absent on naïve CD45RO- CD4+ T cells from a cord blood sample. KIR3DL2 is also absent on naïve CD45RO- CD4+ T cells from a healthy adult control sample (Figure 5.20.B). I obtained naïve CD4+ T cells using a naïve CD4+ T cell isolation kit (Miltenyi Biotech), as described in section 2.2.6.2.

A



B

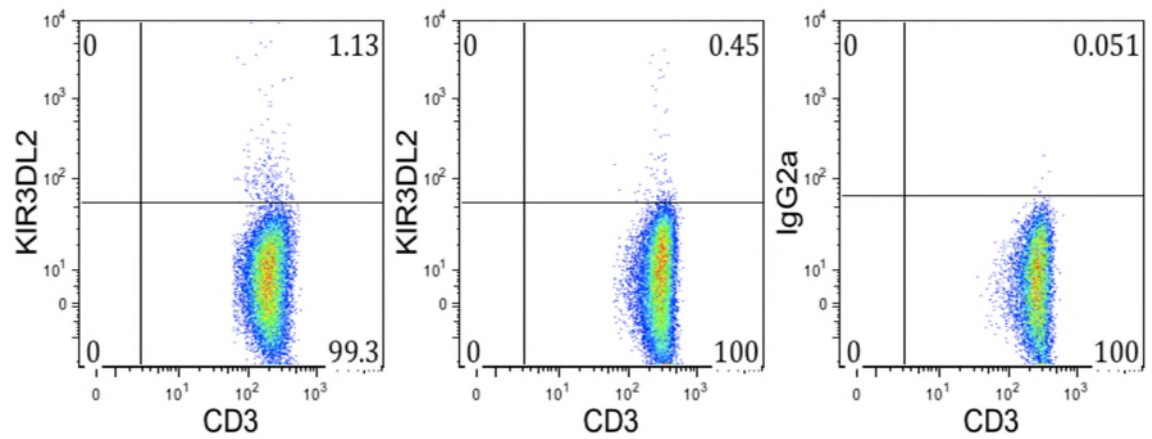


Figure 5.20: KIR3DL2 expression is restricted to memory CD4+ T cells. FACS staining of a cord blood sample (A) and a leukocyte cone sample (B) show that KIR3DL2+ CD4+ T cells are located within the memory CD4+ T cell population (left) but not the naïve CD4+ T cell population (center), as compared to isotype control staining (right).

Figure 5.21 shows that, after 6 hours activation with PMA and ionomycin, KIR3DL2- CD4+ T cells from an AS patient begin to express KIR3DL2. Following PMA and ionomycin stimulation cells become activated, as shown by expression of CD69, and KIR3DL2 expression is seen on CD69+ CD4+ T cells. In contrast those CD4+ T cells that are not activated by PMA and ionomycin remain KIR3DL2-.

To see whether activation of CD4+ T cells by PMA and ionomycin led to upregulation of another KIR I looked at expression of KIR3DL1, following 6 hours stimulation with PMA and ionomycin of naïve CD4+ T cells. In Figure 5.21 we see that following 6 hours of stimulation, although cells become activated, as seen by expression on CD69, the expression of KIR3DL1 remains below 1.2%.

When looking at all of the data in Figure 5.22.A, we see that PMA and ionomycin stimulation significantly increases expression of KIR3DL2 on naïve CD4+ T cells from both AS patients and HLA-B27- healthy controls. Stimulation with PMA and ionomycin did not significantly alter the expression of KIR3DL1, as shown in Figure 5.22.B. There was no significant difference between the percentages of CD4+ T cells expressing KIR3DL2 after 6 hours stimulation with PMA and ionomycin between AS patients and HLA-B27- healthy controls, as shown in Figure 5.20.C.

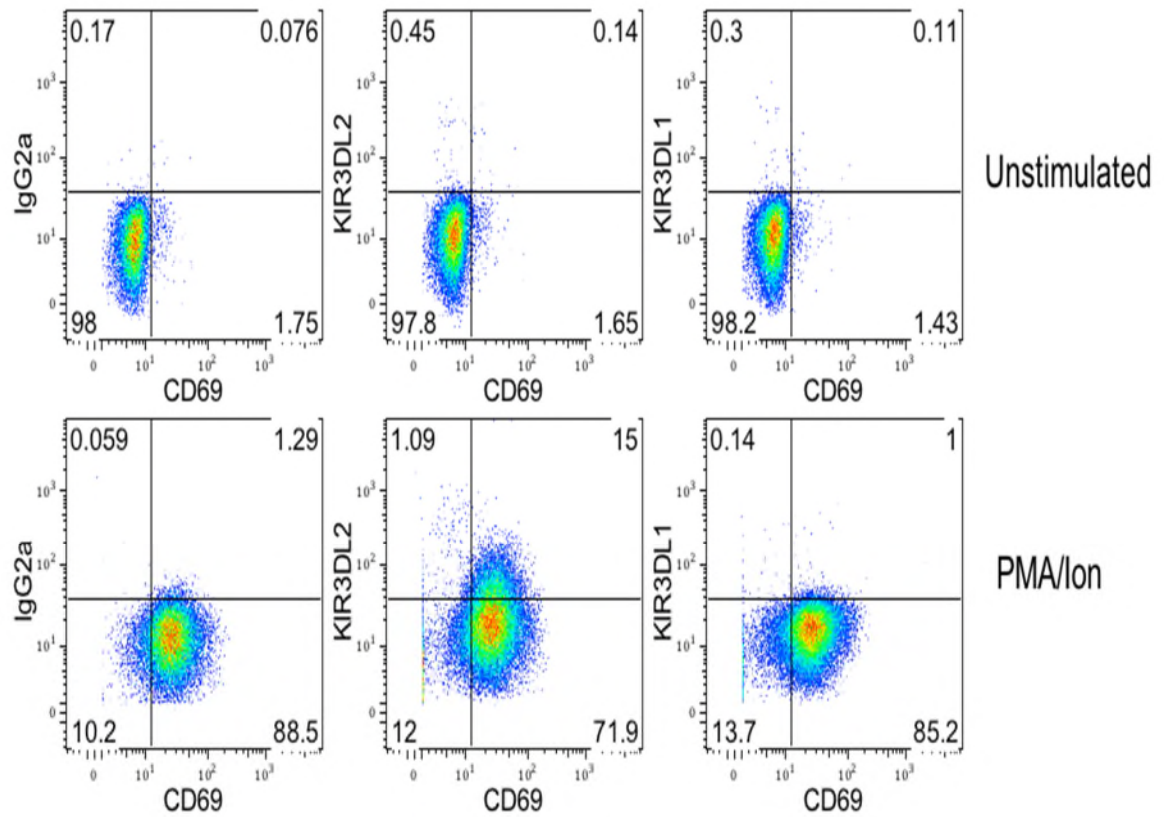


Figure 5.21: KIR3DL2 expression is induced by CD4⁺ T cells by PMA and ionomycin stimulation. FACS staining of KIR3DL2- CD4⁺ T cells from an AS patient shows that KIR3DL2 expression is induced on naïve CD4⁺ T cells that have become activated (middle) but KIR3DL1 is not upregulated after activation of CD4⁺ T cells (right).

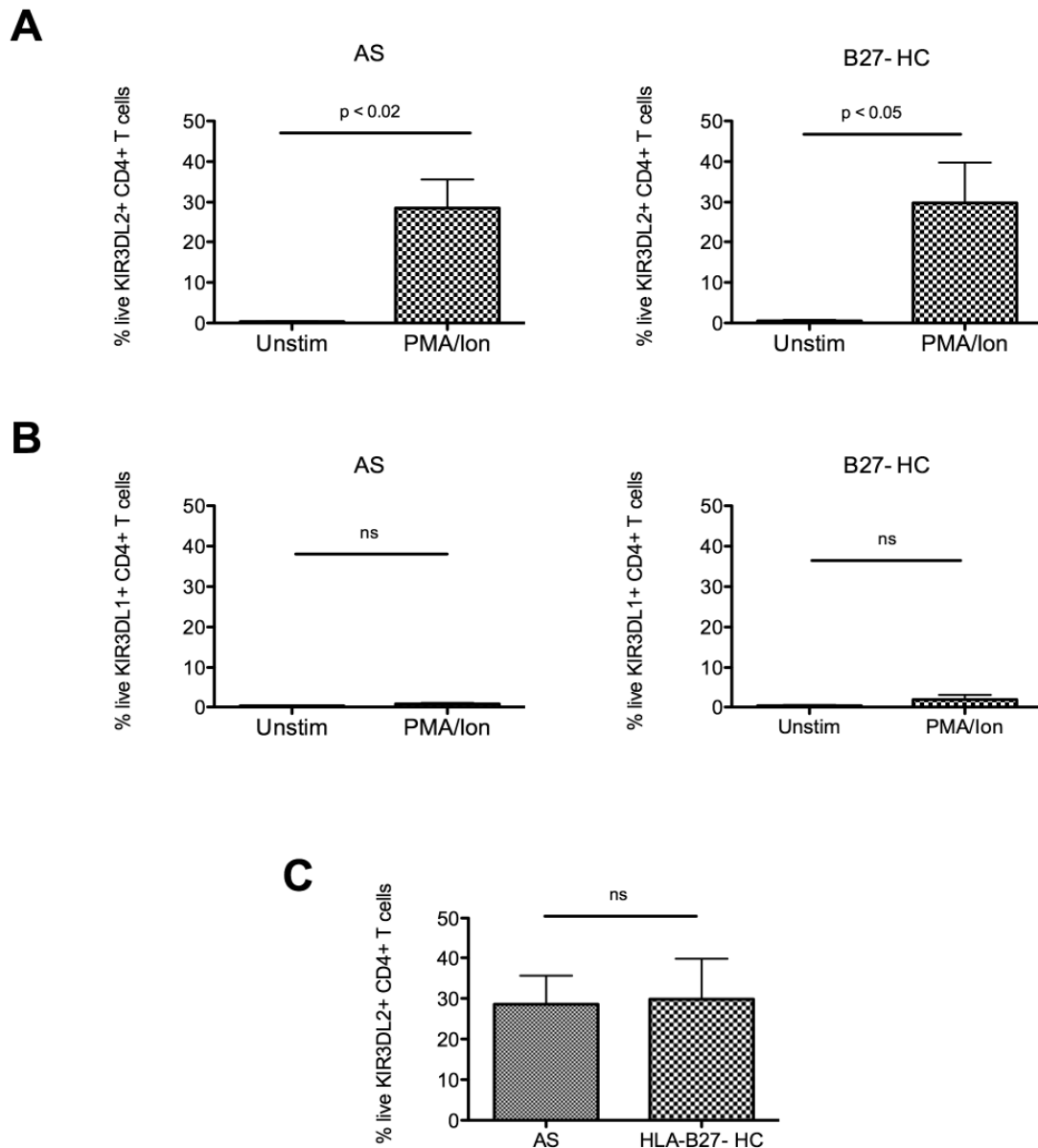


Figure 5.22: KIR3DL2 expression is induced by CD4+ T cells by PMA and ionomycin stimulation but does not significantly differ between AS patients and HLA-B27- healthy controls. FACS staining of KIR3DL2- CD4+ T cells from AS patients (n=3) and HLA-B27- healthy controls (n=3) shows that KIR3DL2 is induced on naïve CD4+ T cells that have become activated by PMA and ionomycin (A) but that KIR3DL1 is not upregulated after activation of KIR3DL2- CD4+ T cells (B). A paired student t test was used for statistical analysis. FACS staining of CD4+ T cells from AS patients (n=3) and HLA-B27- healthy controls (n=3) after 6 hours stimulation with PMA and ionomycin shows that there is no significant difference in KIR3DL2 expression between AS patients and healthy controls (C). An unpaired students t test was used for statistical analysis. Bars represent mean values ($\pm$ SD).

5.4. Discussion

The primary aim of this chapter was to investigate whether activation induced and/or increased KIR3DL2 expression on CD4⁺ T cells. In chapter 3 I showed that there is an increase in the percentage of KIR3DL2⁺ CD4⁺ T cells in the peripheral blood of AS patients and HLA-B27⁺ healthy controls compared to HLA-B27⁻ healthy controls and RA patients. In this chapter FACS staining was carried out directly *ex vivo*, so I wanted to investigate whether activation *in vitro* further increased the percentage of KIR3DL2⁺ CD4⁺ T cells in inflammatory arthritis patients and healthy controls. I studied activation of PBMC, CD4⁺ T cells and naïve CD4⁺ T cells with different types of stimulation, including SEB, anti-CD2/3/28 beads and PMA and ionomycin. The primary aim was subdivided into three objectives, which have been discussed in detail below.

1) To determine whether activation increases KIR3DL2 expression on CD4⁺ T cells and whether this is greater expression of KIR3DL2 by CD4⁺ T cells from AS patients compared to healthy controls and RA patients after activation.

I have shown that activation of PBMC from inflammatory arthritis patients and healthy controls with SEB significantly increases both the absolute number and the percentage of KIR3DL2⁺ CD4⁺ T cells in all groups investigated. Furthermore SEB stimulates a significantly higher percentage of CD4⁺ T cells from AS patients to express KIR3DL2 compared to HLA-B27⁻ healthy controls. I also show that activation of PBMC with SEB increases IL-17 and TNF α production, with significantly higher IL-17 production by PBMC from AS patients compared to HLA-B27⁺ healthy controls. It should be noted that since whole PBMC were stimulated in these experiments, IL-17 and TNF α production is not restricted to KIR3DL2⁺ CD4⁺ T cells.

As an alternative method of stimulation I used anti-CD2/3/28 beads to stimulate CD4⁺ T cells. I used two different ratios of CD4⁺ T cell-to-anti-CD2/3/28 beads, to investigate the effect of different strengths of activation. Stimulation of CD4⁺ T cells with anti-CD2/3/28 beads at the higher ratio (1:1) significantly increases the percentage of CD4⁺ T cells expressing KIR3DL2 compared to a no stimulation and to stimulation at the lower ratio (1:10) in both AS patients and HLA-B27- healthy controls. There was no significant difference in the percentages of CD4⁺ T cells that express KIR3DL2 after anti-CD2/3/28 bead stimulation when comparing AS patients and HLA-B27- healthy controls, at either bead-to-cell ratio. These data suggest that activation upregulates KIR3DL2 expression on CD4⁺ T cells regardless of HLA-B27 status.

There is evidence to suggest that clonal T cells in the elderly express a variety of NK receptors including CD16, CD56, CD57, CD94 and KIRs (Miyaji, Watanabe et al. 1997; McNerlan, Rea et al. 1998; Tarazona, DelaRosa et al. 2000; Snyder, Muegge et al. 2002). Expansions of T cells expressing NK receptors are not only associated with the elderly but are a hallmark of many conditions associated with chronic stimulation of the immune system, including viral infection, cancer and chronic inflammatory conditions such as rheumatoid arthritis (Yen, Moore et al. 2001; Snyder, Muegge et al. 2002). This would support the suggestion that KIR3DL2 expression on CD4⁺ T cells, which is raised in AS patients compared to HLA-B27- healthy controls, is a result of chronic activation of these cells in disease.

The mechanism for NK receptor expression on T cells remains unknown although there is evidence that it can accompany the loss of CD28 (Tarazona, DelaRosa et al. 2000). CD28 is constitutively expressed on CD4⁺ T cells, where it acts as a costimulatory molecule for activation of T cells. Antigen-exposed T cells can downregulate CD28 (Vallejo, Brandes et al.

1999). Moreover, under conditions of chronic antigen-stimulation CD28 expression can be completely lost as a result of the inactivation of *CD28* gene transcription (Vallejo, Schirmer et al. 2000; Vallejo, Weyand et al. 2001). In this present study I have not investigated CD28 expression by KIR3DL2+ CD4+ T cells, either directly *ex vivo* or after activation *in vitro*, although work previously carried out in our lab has shown that KIR3DL2+ CD4+ T cells, in both peripheral blood and synovial fluid, had low or absent CD28 expression (Chan, Kollnberger et al. 2005).

Analysis of V β usage by T cells expressing KIRs has shown a restricted TCR-V β usage in healthy donors, with KIR+ T cells expressing just one or two TCR-V β s, although the TCR-V β used were found to be different in different individuals (Mingari, Schiavetti et al. 1996). Furthermore, each V β family analyzed exhibited identical rearrangements. This would support the hypothesis that KIR-expressing T cells may represent oligoclonal or monoclonal T-cell expansions, reflecting chronic, antigen-driven stimulation. Modulation of KIR expression by TCR activation is supported by a further study showing that in the absence of TCR engagement, KIRs expression on CD8+ T cells was downregulated (Huard and Karlsson 2000). Interestingly this lower KIR expression was unable to inhibit T cell function. In contrast TCR signaling not only sustained KIR expression but re-induced functional levels of KIR expression, with the level of IFN γ production being restored. Flow cytometry was used to determine both cell-surface and total KIR2D staining, showing both were decreased after co-culture with APCs, suggesting that the down-regulation could not be explained by an intracellular accumulation, but rather KIR2D was being degraded.

Activation has also been shown to increase expression of the NK receptor CD94/NKG2A on $\gamma\delta$ T cells after activation (Boullier, Poquet et al. 1998). In this study peripheral blood CD94-V γ 9V δ 2 cells were found to express an intracellular pool of CD94/NKG2A that moved to the

cell surface following activation with phosphoantigens or IL-2. However, intracellular CD94/NKG2A complexes were undetectable in CD94⁻ thymus or peripheral blood leukocyte-derived mature V δ 2 T cell clones, and surface induction was not observed following phosphoantigen activation of these T cell clones.

A further possibility is that cytokines may upregulate KIR expression or may play a role in the survival of KIR expressing cells. KIR expression on NK cells is dependent on IL-15 and starts after lineage commitment (Miller and McCullar 2001). Furthermore, IL-2 and IL-18 upregulated KIR2DL1 and KIR2DL2 on NK cells, as well as increasing the percentage of NK cells that express KIR2DL1 and KIR2DL2 (Chrul, Polakowska et al. 2006). Addition of IL-2 and IL-15 to human lymphocytes led to the expansion of NK and T cells expressing natural killer receptors, including KIR3DL1 (Dunne, Lynch et al. 2001). Cytokines, including IL-15, TGF- β and IL-12, have been implicated in the induction of CD94/NKG2A on adult human T cells (Mingari, Ponte et al. 1998; Bertone, Schiavetti et al. 1999; Derre, Corvaisier et al. 2002). However a further study of T cell clones found that after culture with various cytokines, including IL-12, IL-15, IL-18, as well as with anti-CD3 stimulation, there was no change in the patterns of transcription for any of the KIR receptors investigated. In contrast to my experiments this study only looked at stimulation with anti-CD3 and not the combined effects of stimulation with anti-CD2/3/28. With this combination of stimulation I was able to show an increase in KIR3DL2 expression on CD4⁺ T cells, however I did not specifically investigate the effects of cytokines. Since I have not looked at the contribution of specific cytokines it is possible that cytokines added the culture medium (where specified) may have an effect on upregulation of KIR3DL2. Furthermore, cytokines produced by cells upon activation could act either in a positive feedback mechanism, to maintain or further increase expression of KIR3DL2 expression by the same cells, or by bystander activation, to increase

and/or induce KIR3DL2 expression on other cells. This highlights the role of the inflammatory microenvironment in the induction of specific receptors.

2) To determine whether KIR3DL2 expression on CD4+ T cells and/or IL-17 production could be inhibited by SEB stimulation in the presence of DX31, a KIR3DL2 specific antibody.

Having shown that KIR3DL2 expression is increased on CD4+ T cells by activation, I wanted to investigate whether co-culture with DX31, a monoclonal antibody specific to KIR3DL2, could inhibit this increased KIR3DL2 expression. My results suggest that SEB stimulation in the presence of DX31 caused a small but not significant decrease in KIR3DL2 expression on CD4+ T cells. Particularly with regards to the number of CD4+ T cells expressing KIR3DL2, I saw much variation after treatment with DX31. In conclusion it is likely that the numbers used in the study are not sufficient to determine a significant effect. Further experiments are required to more fully answer the question of whether co-culture with a KIR3DL2 monoclonal antibody can significantly inhibit increased expression of KIR3DL2 induced by SEB activation.

The same is true for addressing the question of whether SEB stimulation in the presence of DX31 can effect IL-17 production, since my data shows that DX31 co-culture at the time of SEB stimulation of CD4+ T cells caused a small, but again, not significant decrease in IL-17 production. It is worth noting that these experiments are looking specifically at IL-17 from cultured supernatants after PBMC stimulation with SEB and not at IL-17 production by KIR3DL2+ CD4+ T cells, and it would be interesting to investigate the relative contribution from all the individual subsets, including NK cells, CD8+ T cells, $\delta\gamma$ T cells and innate lymphoid cells, which could be achieved by performing ICS.

Interestingly when HD6, a monoclonal antibody that has been shown to bind HLA-B27 dimers and free heavy chains (Payeli, Kollnberger et al. 2012), was used in similar experiments there was a significant decrease in IL-17 production by PBMC from AS patients but not HLA-B27- healthy controls. In chapter 4, I showed raised levels of IL-17, both directly *ex vivo* and in cultured supernatants from KIR3DL2+ CD4+ T cells. Several other studies have also shown raised IL-17 in peripheral blood, synovial fluid and cultured supernatant from AS patients (Wendling, Cedoz et al. 2007; Melis, Vandooren et al. 2009; Wang, Lin et al. 2009). Given the ability of HD6 to decrease IL-17 production by PBMC from AS patients, it makes this antibody an attractive candidate therapeutic reagent in the treatment of AS and other HLA-B27-associated spondyloarthropathies, and its potential as a therapeutic treatment is currently being investigated in the HLA-B27 transgenic rat model of disease.

3) To determine whether activation induces de novo KIR3DL2 expression on naïve CD4+ T cells, and whether this is higher for AS patients compared to HLA-B27- healthy controls.

I have shown that PMA and ionomycin stimulation induces KIR3DL2 expression on naïve CD4+ T cells. PMA and ionomycin stimulation did not increase expression of KIR3DL1. There was no difference between the percentages of CD4+ T cells expressing KIR3DL2 in AS patients or HLA-B27- healthy controls. This suggests, at least in the short term, that activation induces KIR3DL2 regardless of HLA type and that HLA-B27 has no role in initial KIR3DL2 expression. The presence of KIR transcripts has been detected in KIR- T cells, in addition to KIR+ T cells (Vely, Peyrat et al. 2001). Therefore it is conceivable that KIR transcripts are translated into protein and moved to the cell surface for expression, rapidly upon activation of KIR- T cells. This is supported by my findings that PMA and ionomycin stimulation of KIR3L2- CD4+ T cells induces surface expression of KIR3DL2 in just 6 hours, and this could be investigated by blocking transcription and translation.

One factor proposed to be involved in the induction of KIR expression is DNA methylation. Analyses of DNA methylation patterns of KIR genes in NK cell lines and cells revealed that a small CpG island surrounding the transcriptional start site of each KIR gene is consistently demethylated in expressed KIR and methylated in unexpressed KIR genes (Santourlidis, Trompeter et al. 2002). DNA-demethylating treatment led to induction of transcription of multiple KIR genes, within 8 hours of treatment, with *de novo* expression of KIRs on NK cell surface being detected within 48 hours of treatment. When looking at KIR induction by Jurkat cells the authors found that there was a slight induction of KIR3DL2 and a corresponding induction of *KIR3DL2* transcription, but none of the other KIRs investigated, following DNA-demethylating treatment. Furthermore, KIR expression was not detectable on the surface of a B cell line or any of the nonlymphoid cell lines investigated following DNA-demethylating treatment.

A further study suggests that KIR expression is also inducible on CD8⁺ T cells by CpG DNA demethylation (Li, Yu et al. 2009). This study looked at KIR2DL3 expression by CD8⁺ T cells, with the finding that with age CD8⁺ T cells developed a patchy and stochastic promoter demethylation, even in cells that did not express the *KIR2DL3*-encoded CD158b protein on their surface. This partial demethylation lowered the threshold for transcriptional activation, thus rendering CD8⁺ T cells more susceptible to express this particular KIR. They hypothesized that *de novo* expression of negative regulatory receptors may contribute to immune defects in the elderly. This is supported by work in T cells looking at the inhibition of DNA methylation, which increased KIR expression through effects on both promoter methylation and transcription factors (Liu, Kuick et al. 2009). Using KIR reporter constructs, both KIR2DL2 and KIR2DL4 expression were increased in demethylated T cells and required Ets1, Sp1 and AML sites, implying effects on transcription factors.

In summary I have shown that *de novo* KIR3DL2 expression occurs on KIR3DL2- CD4+ T cells after short-term activation with PMA and ionomycin. KIR3DL2 expression is also increased on CD4+ T cells after stimulation with SEB or anti-CD2/3/28 beads. I propose that chronic activation of the immune system in AS drives expression of KIR3DL2 on CD4+ T cells. Since KIR3DL2 is expressed on a number of cell types, including CD8+ T cells, $\gamma\delta$ T cells, NK cells and innate lymphoid cells, it is likely that activation of these different cell types would also induce expression of KIR3DL2 on these cells. *De novo* induction of KIR3DL2 expression occurred in both AS patients and HLA-B27- healthy controls to a similar extent, suggesting that activation leads to upregulation of KIR3DL2 expression on CD4+ T cells regardless of HLA-B27 status. However after 5 days SEB stimulation a significantly higher percentage of CD4+ T cells from AS patients expressed KIR3DL2 compared to CD4+ T cells from HLA-B27- healthy controls, therefore it is possible that maintained expression of KIR3DL2 is related to expression of HLA-B27 dimers or free heavy chains and this will be addressed in chapter 6.

Chapter 6: Effects of naïve CD4+ T cell co-culture with HLA-B27-expressing cell lines on CD4+ T cell development and differentiation

6.1. Introduction

KIR3DL2 binds to HLA-B27 dimers and free heavy chains but not to conventionally folded HLA-B27 (Kollnberger, Bird et al. 2002). HLA-B27 has the tendency to misfold in the ER owing to the fact its heavy chains form more slowly than other HLA-class I molecules (Mear, Schreiber et al. 1999; Antoniou, Ford et al. 2004). Moreover, HLA-B27 forms complexes with peptide more weakly than other HLA-class I molecules are able to, since HLA-B27 is less dependant on tapasin, thus HLA-B27 molecules have a greater propensity to fall apart at the cell surface (Peh, Burrows et al. 1998). This potentially leads to the formation of more free heavy chains at the cell surface, in addition to HLA-B27 dimers, for interaction with KIR3DL2.

Since up to 94% of AS patients are HLA-B27+ (Brewerton, Hart et al. 1973; Caffrey and James 1973) a pivotal question to address to explore the link between HLA-B27 and AS pathogenesis is: “what is the interaction of KIR3DL2 and HLA-B27 doing to CD4+ T cells?” I wanted to test the hypothesis that co-culture of CD4+ T cells with HLA-B27-expressing cell lines would differentially effect outcome, in terms of KIR3DL2 expression and CD4+ T cell differentiation, as compared to co-culture with cells that lack HLA-B27 expression or expressed control HLA-class I molecules. To this end I used LBL.721.220 (220) or LBL.721.221 (221) cells, which lack expression of HLA-class I, or LBL.721.220.B27 (220.B27) cells or LBL.721.220.B7/B8 (220.B7/B8) cells, which are transfected or lentivirally-transduced to express HLA-B27 or control HLA-class I molecules. These cells were co-

cultured with naïve CD4⁺ T cells, in the presence of SEB, and KIR3DL2 expression, differentiation and proliferation was investigated.

6.2. Aim

The overall aim of this chapter was to investigate the effect that co-culturing naïve CD4+ T cells with HLA-B27-expressing cell lines has on CD4+ T cell development and differentiation. To achieve this I subdivided the overall aim into three objectives.

- To determine whether co-culture of naïve CD4+ T cells with HLA-B27-expressing cell lines affected expression of KIR3DL2 on CD4+ T cells, as compared to co-culture with control cell lines.
- To determine whether co-culture of naïve CD4+ T cells with HLA-B27-expressing cell lines affected CD4+ T cell differentiation, as compared to co-culture with control cell lines.
- To determine whether co-culture of naïve CD4+ T cells with HLA-B27-expressing cell lines affected CD4+ T cell survival, as compared to co-culture with control cell lines.

6.3. Results

6.3.1. Co-culture of naïve CD4⁺ T cells and HLA-B27-expressing cells partially inhibits CD45RO acquisition

In the previous chapter I showed that activation was able to induce *de novo* expression of KIR3DL2 on naïve CD4⁺ T cells. I subsequently wanted to explore what effects co-culture of CD4⁺ T cells with HLA-B27-expressing cell lines was having on CD4⁺ T cell development and differentiation. I used a naïve CD4⁺ T cell isolation kit to separate naïve CD4⁺ T cells from AS patients and healthy controls, which allows separation of untouched CD45RO⁻ CD4⁺ T cells, as described in section 2.2.6.2. Figure 6.1.A. shows that the typical purity of the naïve CD4⁺ T cells population after magnetic separation is over 96%. Naïve CD4⁺ T cells were stimulated for 5 days with SEB and co-culture with cell lines expressing HLA-B27 or control HLA-class I molecules.

Figure 6.1.B. shows that after activation with SEB and 5 days co-culture of naïve CD4⁺ T cells from an AS patient and a HLA-B27⁻ healthy control, with 220.B8 or 220.B27 cells, a proportion of the cells have moved from the naïve CD45RO⁻ CD4⁺ T cell compartment to become CD45RO⁺ CD4⁺ T cells. In both the AS patient and healthy control the percentage of T cells that become CD45RO⁺ is highest after co-culture with 220.B8 cells, with 86.8% and 87.6% becoming CD45RO⁺ respectively. In contrast fewer CD4⁺ T cells become CDRO⁺ after co-culture with 220.B27 cells, with less than 50% of CD4⁺ T cells becoming CD45RO⁺. In Figure 6.1.C. we see the same experiment but with naïve CD4⁺ T cells from a leukocyte cone, following SEB stimulation and co-culture with either 220, 220.B7 or 220.B27 cells. After co-culture with 220 or 220.B7 cells, 79.8% and 82.9% of CD4⁺ T cells become CD45RO⁺ respectively, compared to 61.6% of CD4⁺ T cells that gained CD45RO expression after co-culture with 220.B27 cells.

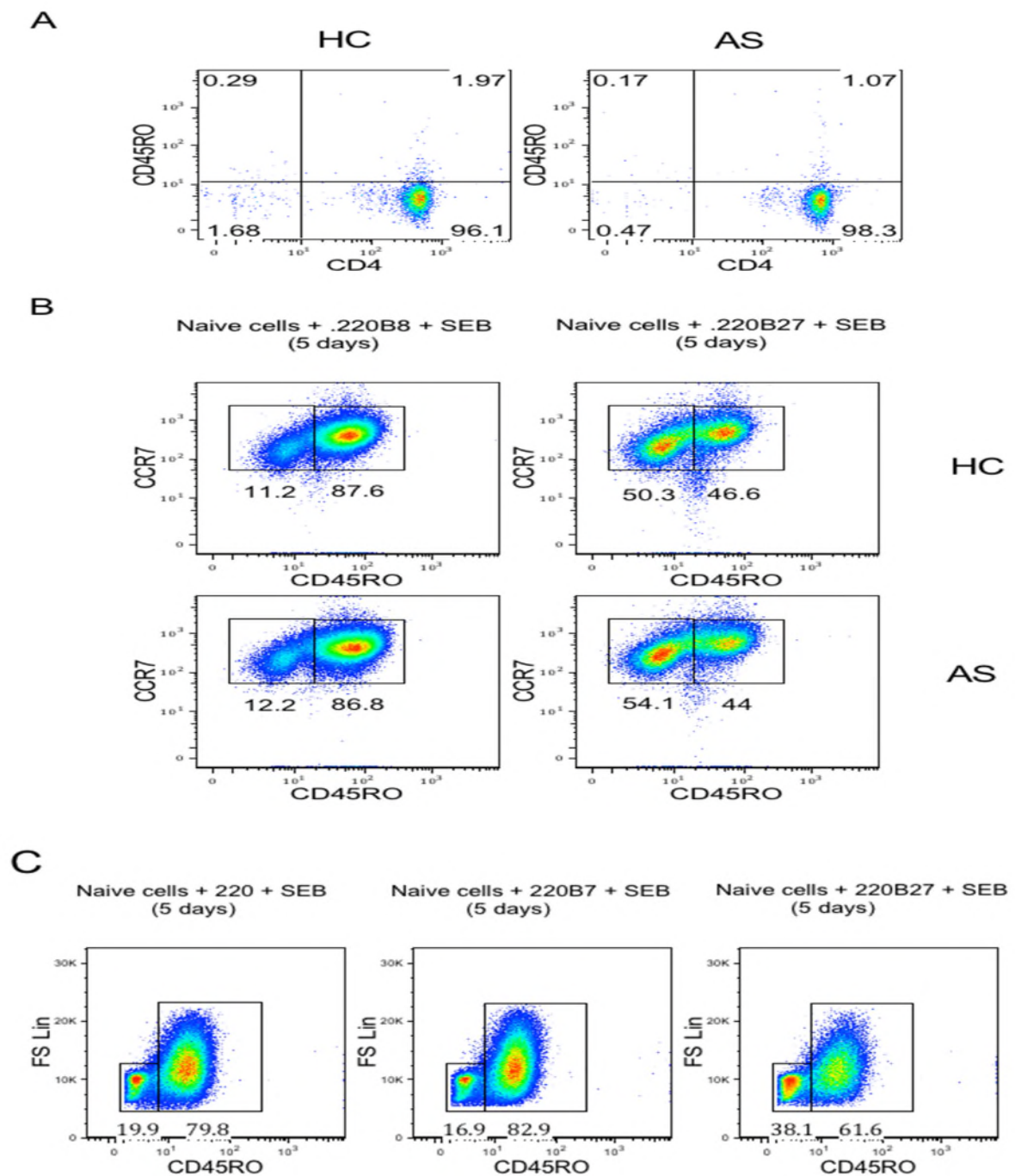


Figure 6.1: Co-culture of naïve CD4⁺ T cells and HLA-B27-expressing cells partially inhibits CD45RO acquisition. (A) FACS staining of magnetic bead-separated naïve CD4⁺ T cells isolated from a healthy control (HC) and an AS patient, showing purity of naïve CD4⁺ T cell. (B) Naïve CD4⁺ T cells from an AS patient and a HLA-B27⁻ healthy control were co-cultured with 220.B8, or 220.B27 irradiated cells, in the presence of SEB, for 5 days, before FACS staining for CD45RO and CCR7 on live CD4⁺ T cells. (C) Naïve CD4⁺ T cells from leukocyte cone were co-cultured with 220, 220.B8, or 220.B27 irradiated cells, in the presence of SEB, for 5 days, before FACS staining for CD45RO on live CD4⁺ T cells.

6.3.2. Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells increases KIR3DL2 expression

As described in section 6.3.1. I used peripheral blood samples from an AS patient and a healthy control as a source of naïve CD4+ T cells. Cells were stimulated with SEB and co-cultured with 220.B27 or 220.B8 cells for 5 days. Cells were then FACS stained for CD45RO, CD4 and a live/dead marker to look at naïve or memory CD4+ T cells, and stained with DX31 antibody to look at KIR3DL2 expression.

Figure 6.2 and 6.3 shows that the expression of KIR3DL2 on CD45RO+ CD4+ T cells is upregulated and that this upregulation is even greater after co-culture with 220.B27 cells compare to 220.B8 cells. There is also expression of KIR3DL2 on CD4+ T cells that have remained CD45RO-, again with greatest KIR3DL2 expression after co-culture of naïve CD4+ T cells with 220.B27 cells compare to 220.B8 cells. However KIR3DL2 expression is lower on the cells that remain CD45RO- as compared to those which have become CD45RO+, after co-culture with either 220.B27 or 220.B8 cells. Although we see the same effect for the HLA-B27 negative healthy control as the AS patient, the upregulation of KIR3DL2 is greater on CD4+ T cells from the AS patient.

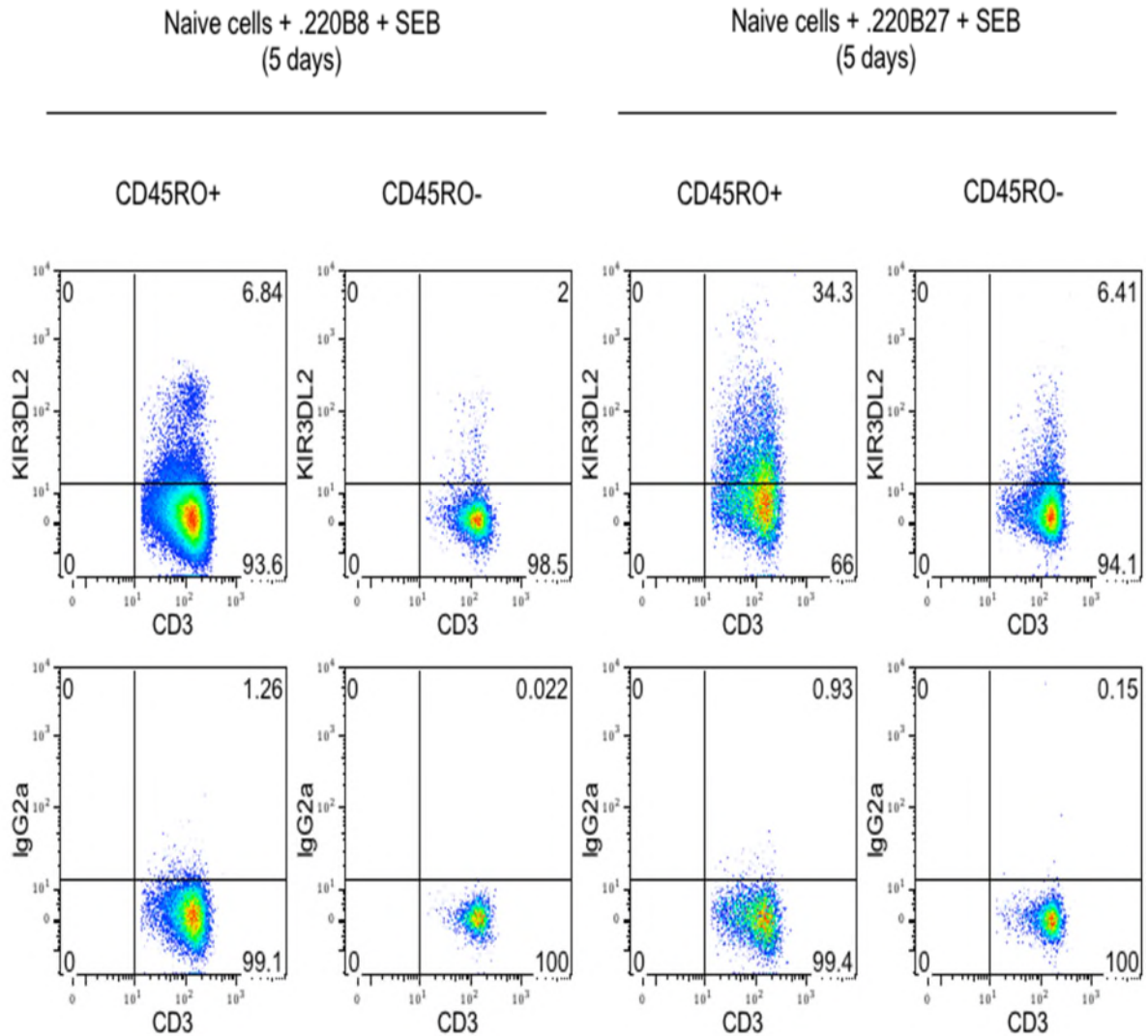


Figure 6.2: KIR3DL2 expression, induced by naïve CD4+ T cells from a HLA-B27- healthy control, is greater after co-culture with 220.B27 compared to .220B8 cells. Naïve CD4+ T cells from a healthy control were stimulated for 5 days with SEB and co-cultured with 220.B8 (left) or 220.B27 (right). FACS staining shows KIR3DL2 expression after 5 days gated on live CD4+ T cells (top panel) compared to staining with an isotype control (bottom panel).

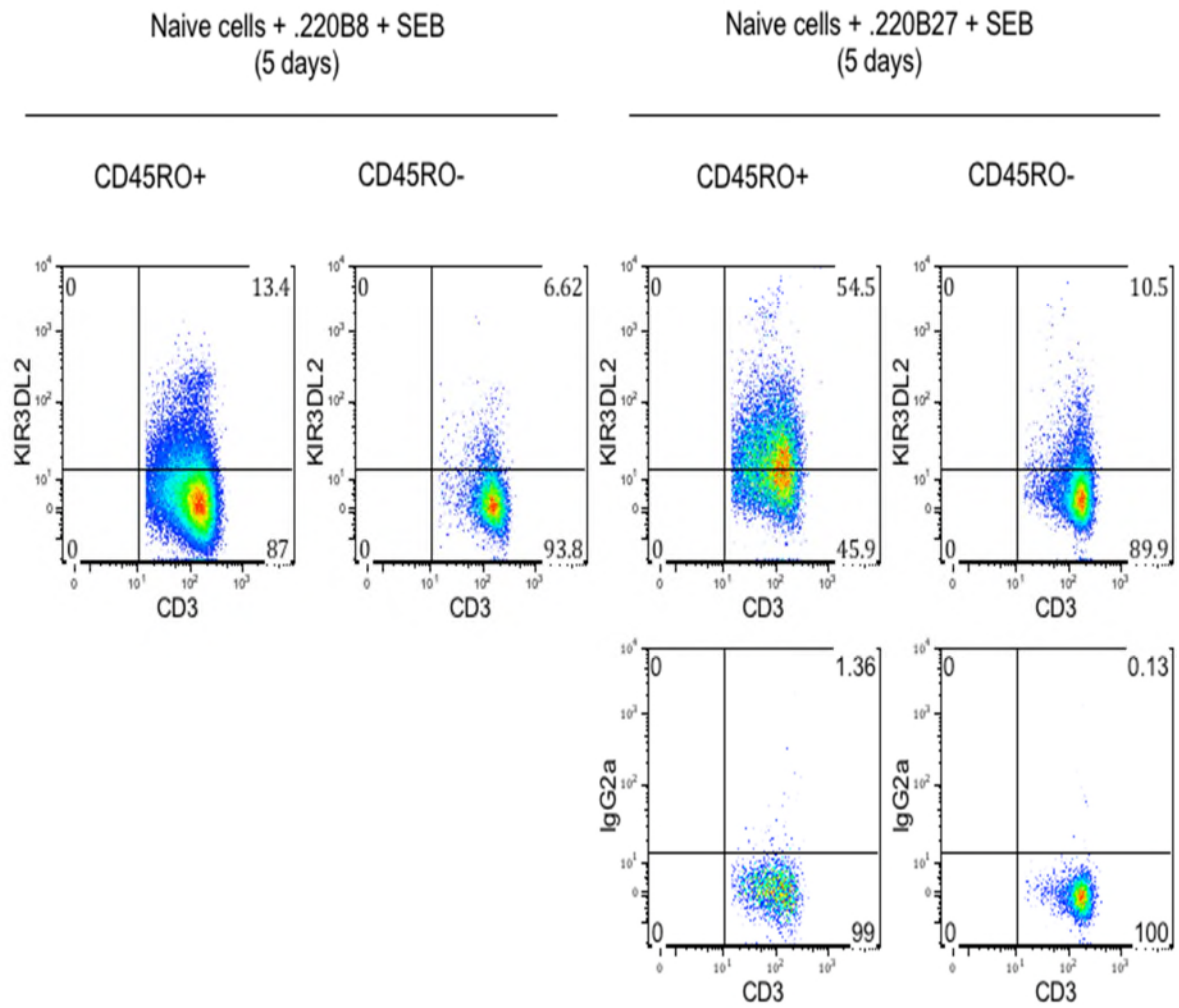


Figure 6.3: KIR3DL2 expression, induced by naïve CD4+ T cells from an AS patient, is greater after co-culture with 220.B27 compared to 220.B8 cells. Naïve CD4+ T cells from an AS patient were stimulated for 5 days with SEB and co-cultured with 220.B8 (left) or 220.B27 (right). FACS staining shows KIR3DL2 expression after 5 days gated on live CD4+ T cells (top panel) compared to staining with an isotype control (bottom panel).

Since naïve CD4⁺ T cells make up around half of CD4⁺ T cells in peripheral blood adults (D'Arena, Musto et al. 1998) I sought other sources of naïve CD4⁺ T cells. One such source was cord blood, since it is enriched for naïve T cells (D'Arena, Musto et al. 1998). Figure 6.4 shows that after 5 days stimulation with SEB and co-culture with either 220.B8 or 220.B27 cells greatest KIR3DL2 expression occurs after co-culture with 220.B27 cells, particularly on CD4⁺ T cells that acquire expression of CD45RO. After co-culture with 220.B27 cells 43% of CD4⁺ T cells that acquire CD45RO expression co-expressed KIR3DL2, compared to 5% after co-culture with 220.B8 cells. Greater KIR3DL2 expression is also seen on CD4⁺ T cells that remain CD45RO⁻ after co-culture with 220.B27 cells, as compared to the same cells after co-culture with 220.B8 cells, however KIR3DL2 expression is lower than that seen compared to CD4⁺ T cells that have become CD45RO⁺.

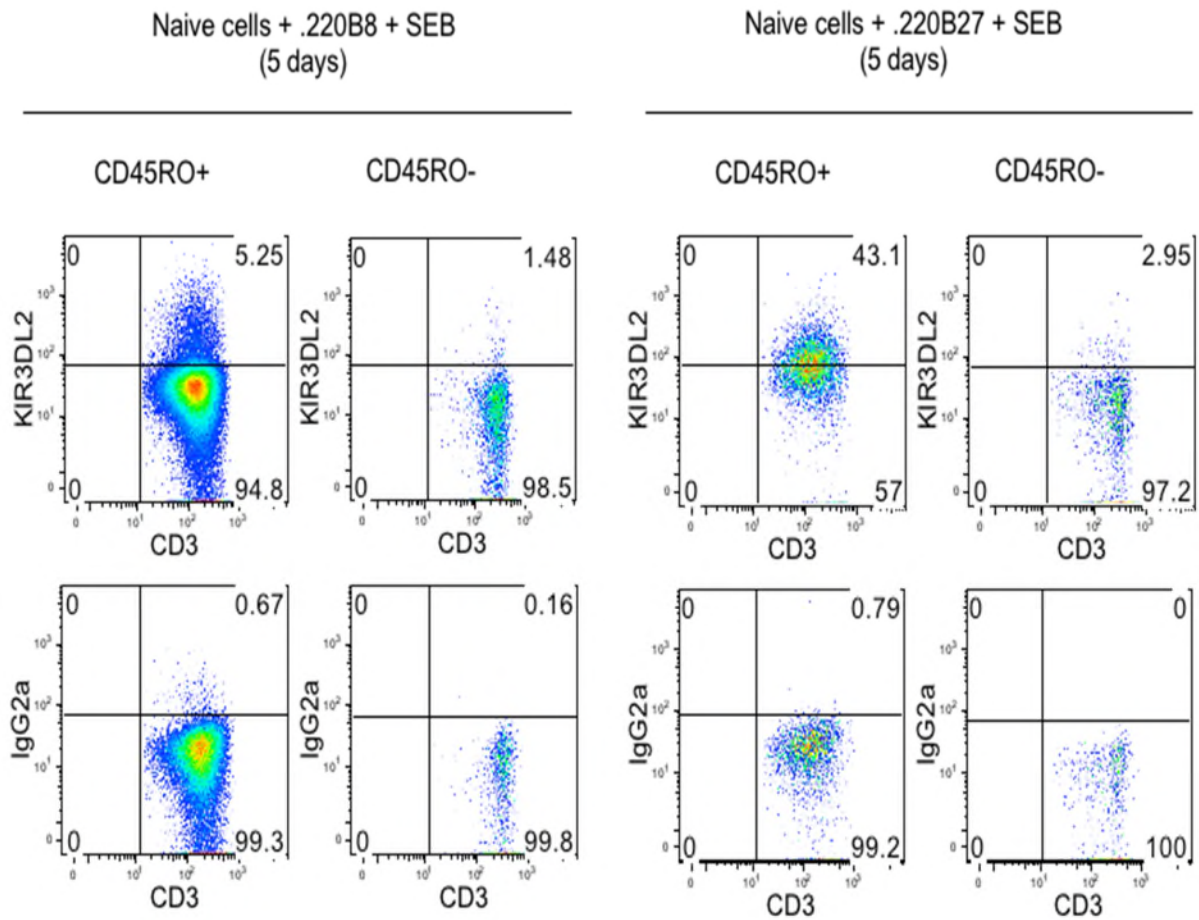


Figure 6.4: KIR3DL2 expression, induced by naïve CD4+ T cells from a cord blood sample, is greater after co-culture with 220.B27 compared to 220.B8 cells. Naïve CD4+ T cells from an AS patient were stimulated for 5 days with SEB and co-cultured with 220.B8 (left) or 220.B27 (right). FACS staining shows KIR3DL2 expression after 5 days gated on live CD4+ T cells (top panel) compared to staining with an isotype control (bottom panel).

As a further source of naïve CD4⁺ T cells I used a leukocyte cone, since several hundred million PBMC can be obtained from one sample. Figure 6.5 shows that greatest KIR3DL2 expression is seen after co-culture with 220.B27 cells, specifically on CD4⁺ T cells that acquire expression of CD45RO. After co-culture with 220.B27 cells 23.4% of those CD4⁺ T cells that now express CD45RO also express KIR3DL2, compared to 13.4% after co-culture with 220.B7 cells. Greater KIR3DL2 expression is also seen on CD4⁺ T cells that remain CD45RO⁻ after co-culture with 220.B27 cells, compared to the same cells after co-culture with 220.B8 cells, however KIR3DL2 expression is lower than that seen compared to CD4⁺ T cells that have become CD45RO⁺.

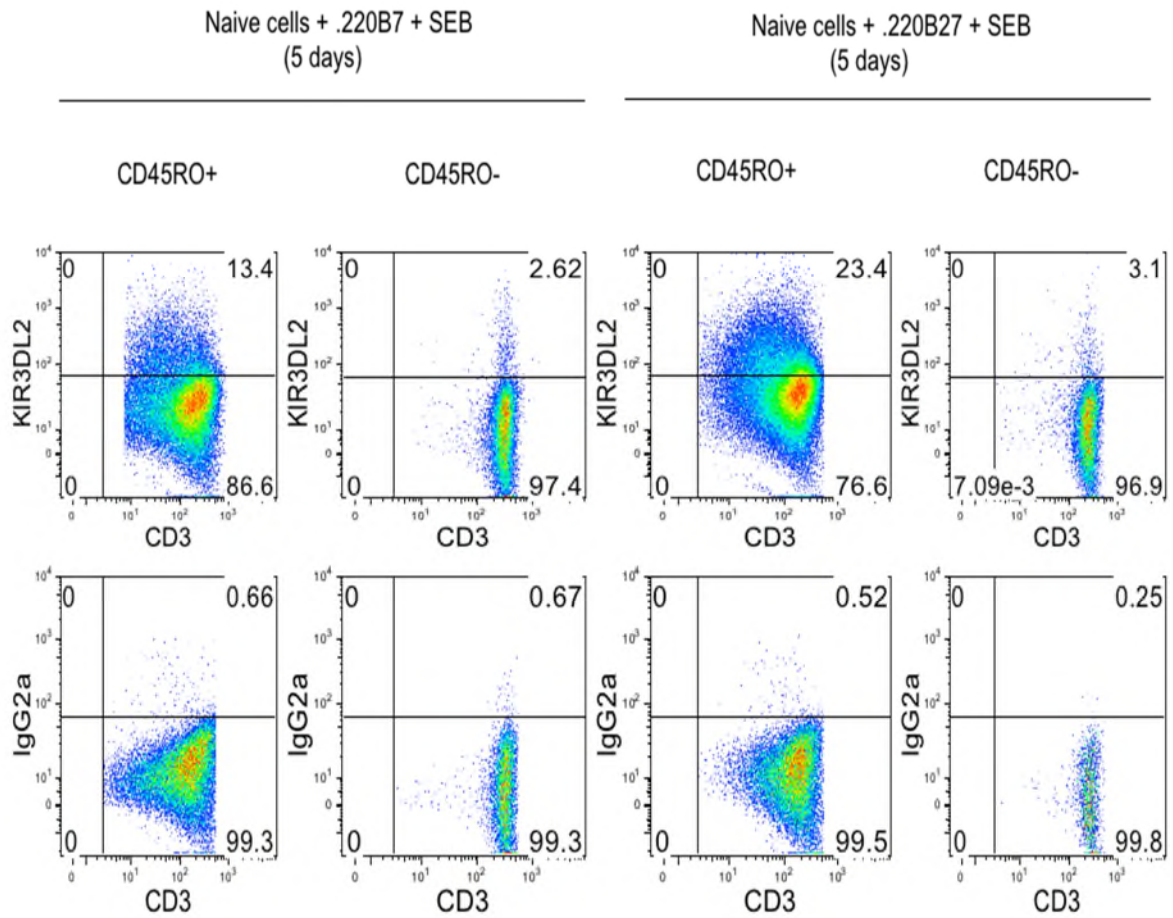


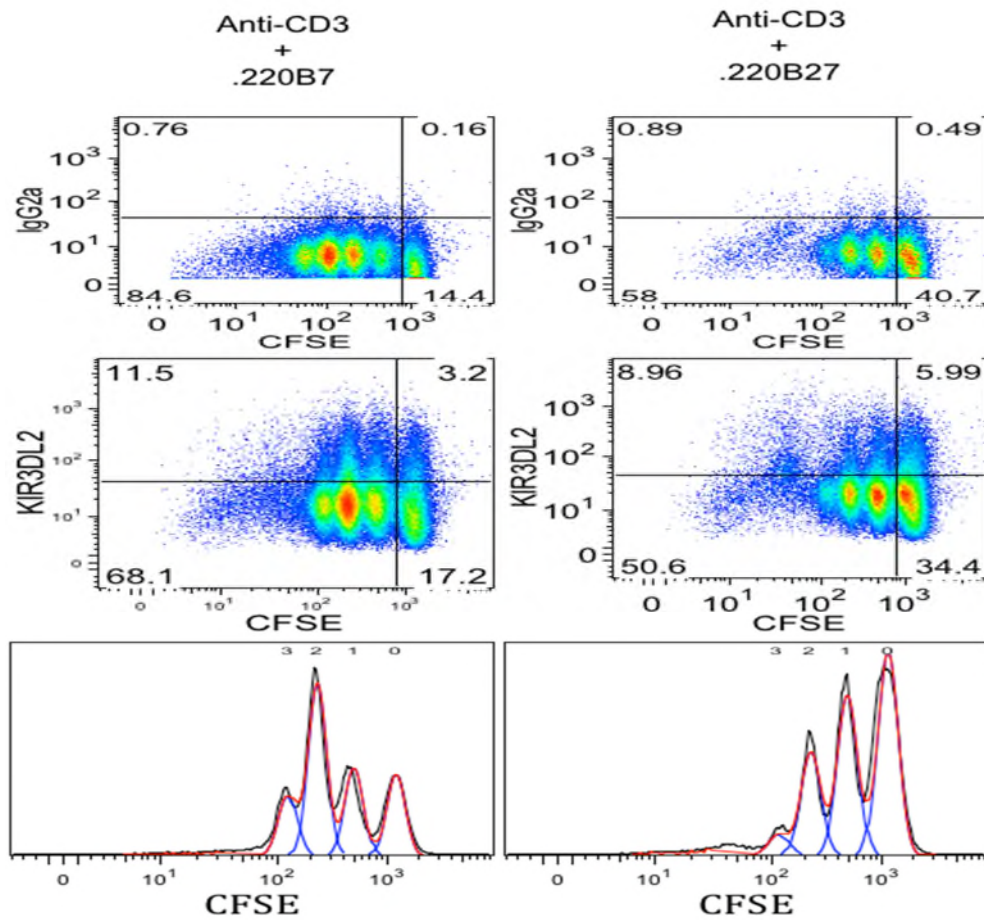
Figure 6.5: KIR3DL2 expression, induced by naïve CD4+ T cells from a leukocyte cone, is greater after co-culture with 220.B27 compared to 220.B7 cells. Naïve CD4+ T cells from an AS patient were stimulated for 5 days with SEB and co-cultured with 220.B7 (left) or 220.B27 (right). FACS staining shows KIR3DL2 expression after 5 days gated on live CD4+ T cells (top panel) compared to staining with an isotype control (bottom panel).

6.3.3. Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells partially inhibits proliferation

I next addressed the question of whether cell proliferation explained the increase in KIR3DL2 expression after co-culture with HLA-B27-expressing cells. Naïve CD4+ T cells were labeled with CFSE and stimulated for 3 days with anti-CD3 (OKT3), for pan-T cell activation, and co-cultured with 220.B7 or 220.B27 cells. After 5 days cells were FACS stained to investigate KIR3DL2 expression on, and proliferation by live CD4+ T cells. Figure 6.6 shows that after 3 days anti-CD3 stimulation and co-culture with 220.B7 more CD4+ T cells have proliferated (79.6% CFSE+) as compared to co-culture with 220.B27 cells (59.6% CFSE+). Proliferation analysis with FlowJo software reveals that a higher percentage of cells have divided after co-culture with 220.B7 cells. Furthermore, both the division index and proliferation index are higher after co-culture with 220.B7 cells compared to after co-culture with 220.B27 cells. The division index is the average number of cell divisions for all the cells in the starting population and the proliferation index is the average number of cell divisions of just the responding cells (i.e. those that have undergone at least one division).

The experiment was also carried out using 221 or 221.B27 cells. Proliferation and KIR3DL2 expression were analyzed after 8 days co-culture and stimulation with anti-CD3. As seen in Figure 6.7 after 8 days stimulation and co-culture with 221 cells all CD4+ T cells have proliferated, compare to 73.7% after co-culture with 221.B27 cells. Following co-culture with 221.B27 KIR expression was 7.29%, compare to 6.83% after co-culture with 221 cells.

A

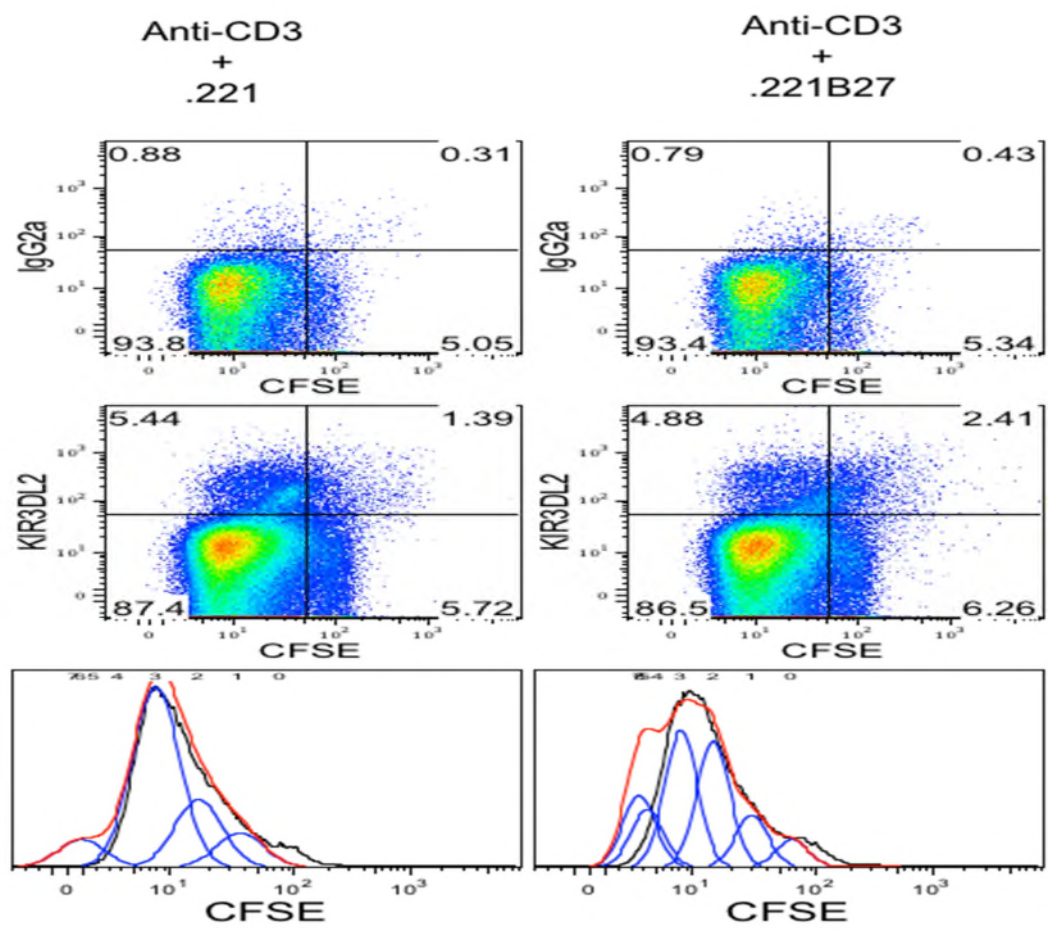


C

	Anti-CD3 + 220.B7	Anti-CD3 + 220.B27
Percentage divided	53.5	35.9
Division Index	0.86	0.46
Proliferation Index	1.61	1.29

Figure 6.6: Co-culture of naïve CD4⁺ T cells and 220.B27 cells partially blocks naïve CD4⁺ T cell proliferation. CFSE labeled naïve CD4⁺ T cells isolated from a leukocyte cone were co-cultured with 220.B7 or 220.B27 cells, in the presence of anti-CD3, for 3 days. (A) FACS staining of KIR3DL2 after gating on live CD4⁺ T cells. (B) Proliferation analysis using FlowJo software on FACS staining from Figure 6.6.A. (C) Table showing proliferation analysis statistics using FlowJo software.

A



C

	Anti-CD3 + 221	Anti-CD3 + 221.B27
Percentage divided	100	73.7
Division Index	2.12	1.47
Proliferation Index	2.12	1.99

Figure 6.7: Co-culture of naïve CD4+ T cells and 221.B27 cells partially blocks naïve CD4+ T cell proliferation. CFSE labeled naïve CD4+ T cells isolated from a leukocyte cone were co-cultured with 221 or 221.B27 cells, in the presence of anti-CD3, for 8 days. (A) FACS staining of KIR3DL2 after gating on live CD4+ T cells. (B) Proliferation analysis using FlowJo software on FACS staining from Figure 6.6.A. (C) Table showing proliferation analysis statistics using FlowJo software.

6.3.4. Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells increases RORC and T-bet expression

To address the question of what co-culture of naïve CD4+ T cells and HLA-B27-expressing cells is doing to CD4+ T cell differentiation I looked at the expression of transcription factors associated with Th1 and Th17 cells, in a time course experiment, by qPCR. Naïve CD4+ T cells were isolated by magnetic bead separation, and stimulated with SEB and co-cultured for 5 or 8 days with cells expressing HLA-B27 (220.B27) or lacking HLA expression (220) or expressing a control HLA molecule (220.B7). Following co-culture dead cells were removed with a dead cell removal kit and mRNA was extracted. qPCR was performed on cDNA synthesized from mRNA samples, as detailed in section 2.2.13. Data were analyzed with the $\Delta\Delta CT$ method, with fold changes compared to day 0 shown, and is representative of three experiments.

Figure 6.8.A shows that T-bet expression significantly increases after co-culture for 5 days with 220, 220.B7 and 220.B27 cells. Peak expression is seen at day 5, with expression decreasing by day 8 as compared to day 5 after co-culture with all cell lines used. On day 5 there is lower expression of T-bet after co-culture with 220.B27 compared to 220 cells but this is reversed by day 8 (Figure 6.8.B).

Figure 6.9.A shows that RORC expression significantly increases after co-culture for 8 days with 220, 220.B7 and 220.B27 cells. There is also an increase from 5 to 8 days after co-culture with 220 and 220.B27 cells. On both day 5 and day 8 there is significantly greater expression of RORC after co-culture with 220.B27 cells compared to 220 cells (Figure 6.9.B). There is also greater expression of RORC after 8 days co-culture with 220.B27 cells compared to 220.B7 cells.

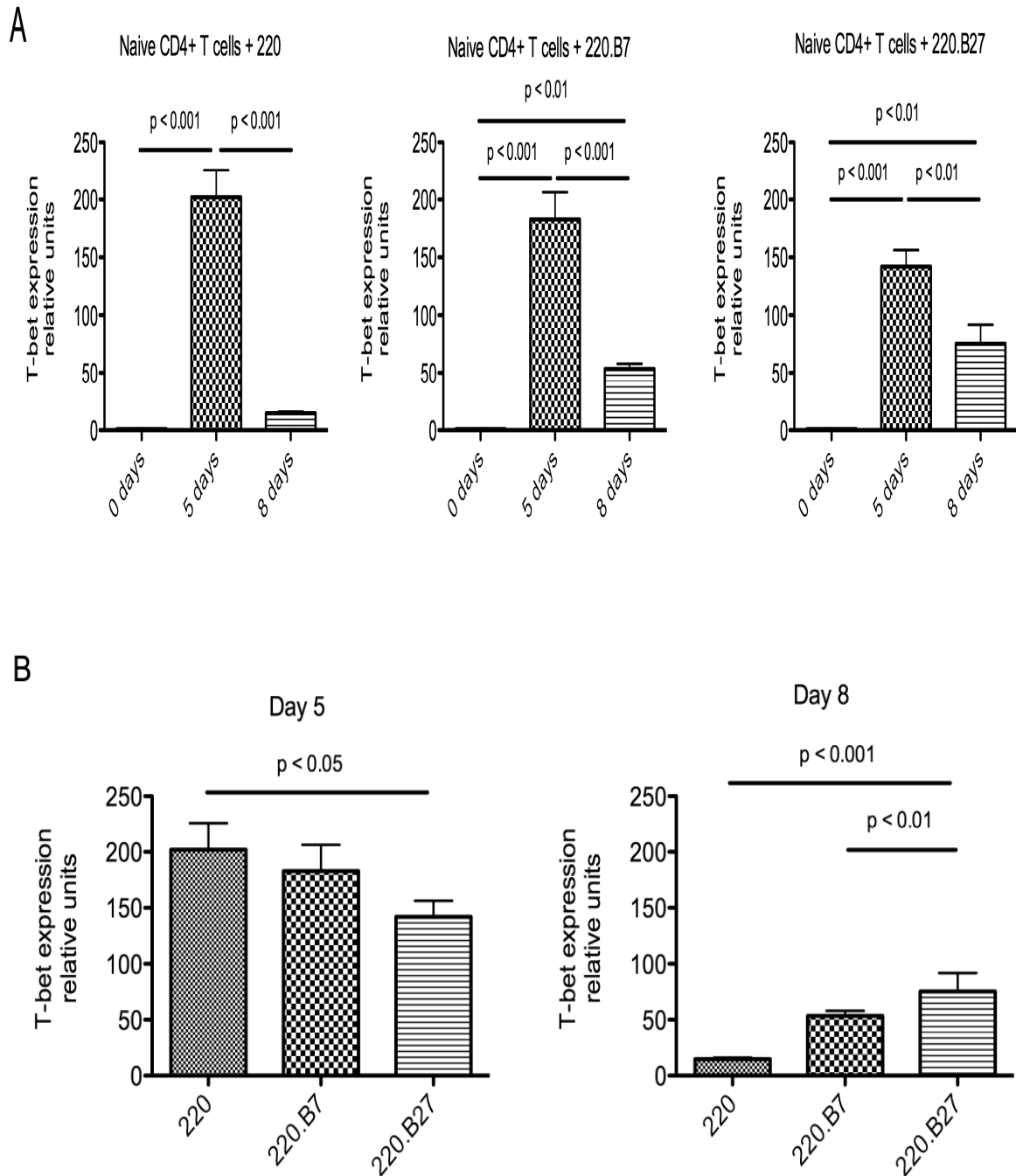
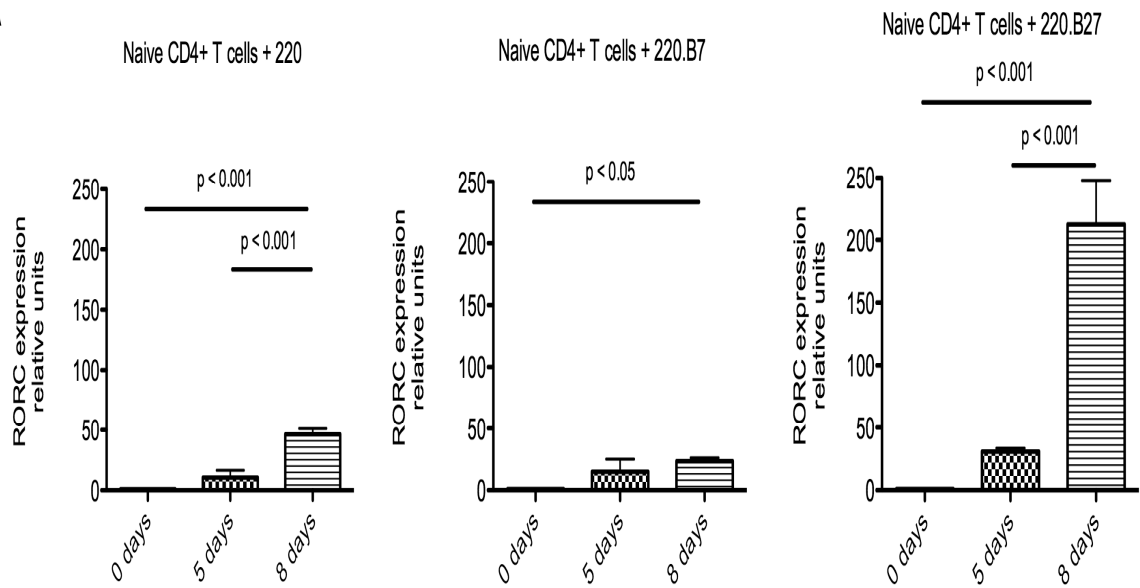


Figure 6.8: Co-culture of naïve CD4+ T cells with 220.B27 cells for 8 days increase expression of T-bet, compared to co-culture with 220 or 220.B7 cells. Naïve CD4+ T cells isolated from peripheral blood from a healthy control were co-cultured with 220, 220-B27 or 220-B7 cells, with SEB, for 5 or 8 days. Comparison of T-bet expression with 220 (left), 220.B7 (middle) or 221.B27 (right) cells (A) or the same cell lines after 5 (left) or 8 (right) days co-culture (B), normalized to CD3. Bars represent mean values ($\pm$ SD). Data was analyzed using the $\Delta\Delta$ CT method. A one-way ANOVA was used for statistical analysis. Note data in Figure 6.8.B is drawn from Figure 6.8.A.

A



B

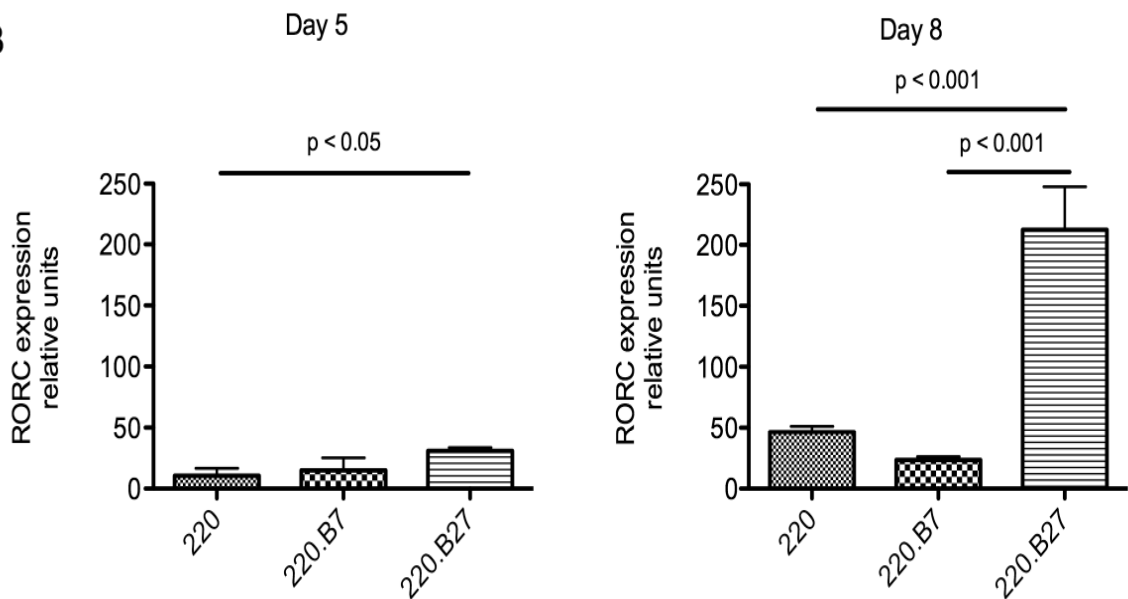


Figure 6.9: Co-culture of naïve CD4+ T cells with 220.B27 cells for 8 days increases expression of RORC, compared to co-culture with 220 or 220.B7 cells. Naïve CD4+ T cells isolated from peripheral blood from a healthy control were co-cultured with 220, 220-B27 or 220-B7 cells, with SEB, for 5 or 8 days. Comparison of RORC expression with 220 (left), 220.B7 (middle) or 221.B27 (right) cells (A) or the same cell lines after 5 (left) or 8 (right) days co-culture (B), normalized to CD3. Bars represent mean values ($\pm$ SD). Data was analyzed using the $\Delta\Delta$ CT method. A one-way ANOVA was used for statistical analysis. Note data in Figure 6.9.B is drawn from Figure 6.9.A.

6.3.5. Co-culture of naïve CD4+ T cells and HLA-B27-expressing cells increases Bcl-2 expression

To address the question of what co-culture of naïve CD4+ T cells and HLA-B27-expressing cells is doing to CD4+ T cell survival expression of Bcl-2, an anti-apoptotic molecule, was investigated in a time course experiment, by qPCR. Naïve CD4+ T cells were isolated by magnetic bead separation, stimulated with SEB and co-cultured for between 6 hours and 7 days with cells expressing HLA-B27 (221.B27) or lacking HLA-class I expression (221). A dead cell removal kit was used to remove dead cells following the specified time. mRNA was collected at 6 hours, 24 hours and 7 days, converted to cDNA, and qPCR analysis was performed. Data analysis was performed using the $\Delta\Delta CT$ method, with fold changes compared to day 0 shown.

Figure 6.10.A shows that after co-culture with 221 or 221B27 cells expression of Bcl-2 increases after both 6 and 24 hours, as compared to 0 hours. Expression of Bcl-2 further increase between 6 and 24 hours after co-culture with 221.B27 cells. This increase in expression is transient, with expression of Bcl-2 decreasing by 7 days as compared to 6 and 24 hours after co-culture with 221 or 221.B27 cells. Figure 6.10.B shows that after 24 hours co-culture of naïve CD4+ T cells with 221.B27 cells there is significantly greater expression of Bcl-2 than after co-culture with 221 cells.

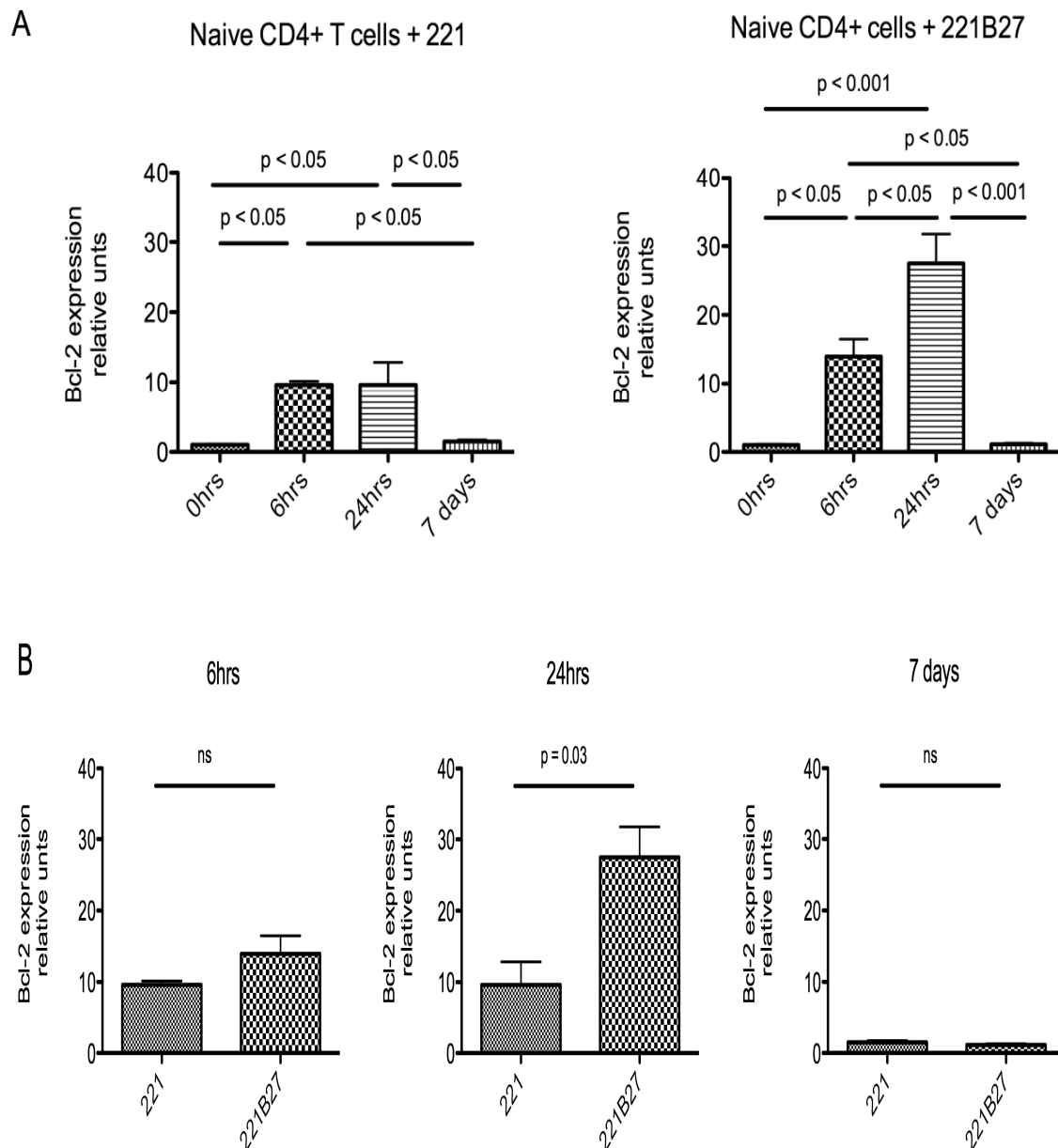


Figure 6.10: Greater expression of Bcl-2 after 24 hours co-culture of naïve CD4+ T cells with 221.B27 cells compared to 221 cells. FACS sorted KIR3DL2+ CD4+ T cells isolated from peripheral blood from a healthy control were co-cultured with 221 or 221.B27 cells, in the presence of SEB, for up to 7 days (A) A one-way ANOVA was used for statistical analysis. Comparison of co-culture of naïve CD4+ T cells with 221 and 221.B27 cells at 6 hours, 24 hours or 7 days (B), normalized to CD3. Bars represent mean values ($\pm$ SD). Data was analyzed using the $\Delta\Delta$ CT method. A two-tailed student t-test was used for statistical analysis. Note data in Figure 6.10.B is drawn from Figure 6.10.A.

6.4. Discussion

The primary aim of this chapter was to investigate the potential effect of KIR3DL2 ligation by HLA-B27 on CD4⁺ T cell development and differentiation, using a co-culture system. In the previous chapter I showed that activation with SEB or anti-CD2/3/28 beads increased KIR3DL2 expression on CD4⁺ T cells, and that activation with PMA and ionomycin induced *de novo* expression of KIR3DL2 on naïve CD4⁺ T cells. SEB stimulation was further utilized in this chapter to investigate the combined effect of CD4⁺ T cell activation and co-culture of naïve CD4⁺ T cells with HLA-B27-expressing cells, compared to co-culture with cell lines lacking HLA-class I expression or expressing control HLA-class I molecules. The primary aim was subdivided into three objectives, which will be discussed in detail below.

- 1) *To determine whether co-culture of naïve CD4⁺ T cells with HLA-B27-expressing cell lines affected expression of KIR3DL2 on CD4⁺ T cells, as compared to co-culture with control cell lines.*

I have shown that co-culture of naïve CD4⁺ T cells with HLA-B27-expressing cell lines (220.B27) increases KIR3DL2 expression above that of KIR3DL2 expression after co-culture with cell lines expressing control HLA-class I molecules (220.B7 or 220.B8). Since KIR3DL2 is not expressed on naïve CD4⁺ T cells, but increases upon activation, as shown in chapter 5, in this system it is likely that KIR3DL2 expression is initially induced by activation with SEB, and subsequent interaction with HLA-B27 acts to maintain or further increase KIR3DL2 expression on CD4⁺ T cells. KIR3DL2 expression is seen both on CD45RO⁻ and CD45RO⁺ CD4⁺ T cells following 5 days co-culture with HLA-class I expressing cells lines, however there is greatest KIR3DL2 expression on CD4⁺ T cells that have become CD45RO⁺.

Signaling through ITIM-containing receptors mediates recruitment of SH2 domain-containing phosphatases SHIP, SHP-1 or SHP-2. Following receptor ligation the tyrosine residue in the ITIM is phosphorylated, usually by Src family kinases (Binstadt, Brumbaugh et al. 1996). Following its recruitment SHIP dephosphorylates various phosphoinositol lipids, which are important for recruitment of Pleckstrin Homology (PH)-domain to the plasma membrane, thus disrupting recruitment of molecules involved in cellular activation (Lemmon and Ferguson 2000). A further way in which ITIM-containing receptors function is by selectively inhibiting activating receptors in their close vicinity. For example SHP-1 dephosphorylates Src family kinases, Lck and Lyn, and Syk family kinases, Syk and ZAP-70, thus interfering with recruitment of molecules that are usually activated upon signaling through ITAM-containing receptors (Olcese, Lang et al. 1996; Plas, Johnson et al. 1996; Dustin, Plas et al. 1999; Somani, Yuen et al. 2001).

When considering functional consequences of signaling through different receptors it is worth noting that some receptors considered as activating receptors contain ITIM-like sequences, whilst not all receptors containing ITIM motifs act solely as inhibitory receptors. TREM-like transcript (TLT)-1 is a co-stimulatory molecule that contains an ITIM, which is required for SHP-2 binding and co-stimulatory function, raising the possibility that SHP-2, or another ITIM associated molecule is involved in cell activation (Barrow, Astoul et al. 2004). Conversely signal regulatory protein- α (SIRP α) contains ITIM motifs but does not act solely as an inhibitory receptor. SIRP α , expressed on myeloid cells and neurons, inhibits macrophage phagocytosis, and dendritic cell maturation and activation following ligation with CD47 (Latour, Tanaka et al. 2001; Yamao, Noguchi et al. 2002). However ligation with CD47 is also required for transmigration of monocytes across endothelium, whilst SIRP α cross-linking on macrophages lead to nitric oxide production (Adams, van der Laan et al. 1998; de

Vries, Hendriks et al. 2002). Therefore it is possible that signaling through KIR3DL2 is activating various signaling pathways whilst inhibiting other pathways.

Ligation of inhibitory KIRs on CD8+ T cells has been shown to promote cytokine production, with ligation of KIR3DL2 on CD8+ T cells triggering cytotoxicity and IFN γ production (Dorothee, Echchakir et al. 2003). Ligation of other KIR family members has also been associated with cytokine production. One such example is of KIR2DL4 ligation leading to increased IFN γ production in an NK-like cell line (Kikuchi-Maki, Yusa et al. 2003). KIR2DL4 is interesting since it contains features of both activating and inhibitory KIR family members, with a basic arginine residue in the transmembrane domain, which allows association with the Fc ϵ RI γ transmembrane adaptor protein, and a single ITIM in the cytoplasmic domain. In a more recent study of KIR2DL4 it was shown that overexpression of the E3 ubiquitin ligase, Triad3A, selectively abrogated the cytokine-producing function of KIR2DL4 (Miah, Purdy et al. 2011). KIR2DL4 endocytosis was vital for stimulation sustained NF- κ B signaling and cytokine production, and Triad3A expression diminished localization of internalized receptor in early endosomes. Further studies have shown that production of TNF α and IFN γ , as well as cytotoxic activity, can be down regulated by co-ligation of TCRs and inhibitory KIRs (D'Andrea, Chang et al. 1996; De Maria, Ferraris et al. 1997).

KIR3DL2 is the only KIR family member that is expressed as a dimer, thought to be mediated by an interaction between two unpaired cysteines in the stem region at positions 302 and 336 (Pende, Biassoni et al. 1996). One possibility is that dimerization of KIR3DL2 may increase the avidity of its interaction with HLA-B27 free heavy chain, supported by the observation that inhibiting the interaction of KIR3DL2 and HLA-B27 by the heavy chain-specific monoclonal antibody HC10 (Kollnberger, Chan et al. 2007). Of interest there is also

increased expression of HC10-reactive heavy chains on monocytes from patients with psoriatic arthritis (Lan, Tsai et al. 2004).

2) To determine whether co-culture of naïve CD4+ T cells with HLA-B27-expressing cell lines affected CD4+ T cell differentiation, as compared to co-culture with control cell lines.

I have shown that co-culture of naïve CD4+ T cells with HLA-B27-expressing cell lines for 8 day increases RORC expression by naïve CD4+ T cells, compared to co-culture with control cell lines. This might suggest that the interaction between KIR3DL2 and HLA-B27 skews differentiation of naïve CD4+ T cells down a Th17 pathway. However, interaction of naïve CD4+ T cells with HLA-B27-expressing cell lines for 8 days also increases T-bet expression. Therefore it is likely that naïve CD4+ T cell differentiation is more complex than simply skewing down either a Th17 or a Th1 pathway, and further investigation is required to more thoroughly study this interaction and the subsequent effect on T cell subset differentiation. A study of Th17 and Th1 clones derived from the gut of patients with Crohn's disease demonstrated that whilst ROR γ t was expressed in Th17, but not in Th1 clones, T-bet could be detected in both Th1 and Th17 clones (Annunziato, Cosmi et al. 2007). Moreover, Th17/Th1 clones were found to express either ROR γ t and/or T-bet. Stimulation of Th17 clones in the presence of IL-12 down-regulated the expression of ROR γ t and the production of IL-17, but induced IFN γ production, showing the importance of the microenvironment in T cell subset plasticity.

There is evidence to suggest that the strength of TCR stimulation can effect CD4+ T cell subset differentiation, with a lower strength of T cell stimulation promoting Th17 responses (Purvis, Stoop et al. 2010). CD4+ T cells received either high or low strength stimulation via anti-CD3/28 beads or dendritic cells pulsed with superantigen in the presence of Th17

polarizing cytokines IL-1 β , TGF β and IL-23, with low strength stimulation promoting Th17 responses by enhancing both the relative proportion and total number of Th17 cells. My data shows that co-culture of naïve CD4⁺ T cells with HLA-B27-expressing cells upregulates the expression of RORC. Since KIR3DL2 is an inhibitory receptor one possibility is that signalling through this receptor is dampening down the signal strength through the TCR following T cell activation, thus favouring the differentiation of Th17 cells.

Interestingly co-culture of naïve CD4⁺ T cells with HLA-B27-expressing cell lines also results in a partial block in proliferation of naïve CD4⁺ T cells, with fewer CD4⁺ T cells dividing after co-culture with HLA-B27-expressing cell lines as compared to cell lines expressing a control HLA-class I molecule (220.B7) or lacking HLA-class I expression (220). In addition the percentage of cells acquiring CD45RO expression is lower after co-culture with 220.B27 cells as compared to 220.B7, 220.B8 or 220 cells. The decrease in the percentage of cell acquiring CD45RO expression after co-culture with HLA-B27 expressing cells lines could be a result of the partial block in proliferation observed after co-culture with HLA-B27 expressing cells lines. This provides further evidence that the interaction of KIR3DL2 with HLA-B27 is having an effect on differentiation of CD4⁺ T cells.

Inhibitory NK receptors are thought to form microclusters at NK cell immunological synapses upon receptor ligation (Treanor, Lanigan et al. 2006). The authors used fluorescence lifetime imaging (FLIM) to show that phosphorylated KIR was confined to microclusters within the aggregate of KIR, contrary to an expected homogeneous distribution of KIR signaling across the immune synapse. Although less is known about signalling through KIRs on T cells, a study of the inhibitory receptor KIR2DL2 showed that engagement of KIR2DL2 by its ligand HLA-Cw3 did not effect conjugate formation between KIR2DL2⁺ CD4⁺ T cells and superantigen-pulsed target cells or the development of mature immune synapses (Henel, Singh et al. 2006).

KIR2DL2 and HLA-Cw3 were firstly recruited to the peripheral supramolecular activation cluster (pSMAC), then moved to the central supramolecular activation cluster (cSMAC), co-localizing with CD3, after 30 minutes. KIR2DL2 subsequently moved to the central supramolecular activation cluster (cSMAC). This event led to dissociation of the TCR synapses, thereby reducing phosphorylated phospholipase C- γ 1, Vav1, and extracellular signal-regulated kinase 1/2 (ERK1/2) at 90 minutes following initial stimulation.

SHP-1 has been shown to effect intracellular calcium concentration, with KIR2DL2 being found to inhibit the influx of calcium from extracellular stores, as well as inhibiting calcium release from intracellular calcium stores (Blery, Delon et al. 1997). This is interesting since evidence suggests that Th17 cells have a distinct calcium signaling profile from Th1 and Th2 cells (Weber, Miller et al. 2008). Th1 cells were found to have the highest sustained levels of calcium mobilization over time, whilst Th2 have the lowest sustained calcium levels, with Th17 cells exhibiting an intermediate level. Thus, signaling through KIR3DL2 may potentially alter intracellular calcium concentration, which may in turn effect T cell subset differentiation.

In terms of further experiments it would be of interest to look at the effect of altering the strength of stimulation, both in terms of the concentration of SEB used to stimulate naïve CD4⁺ T cells, and also to look at the effect of altering the ratio of naïve CD4⁺ T cells to cell lines. In all experiments I used a ratio of 2:1 of naïve CD4⁺ T cells to cell lines. It would be interesting to see if reducing the ratio, for example to 1:10, had an effect on naïve CD4⁺ T cell differentiation and Th17 responses, in this system.

3) *To determine whether co-culture of naïve CD4+ T cells with HLA-B27-expressing cell lines affected CD4+ T cell survival, as compared to co-culture with control cell lines.*

Previous data from our lab showed that co-culture of CD4+ T cells with HLA-B27-expressing cell lines increased the percentage of viable KIR3DL2+ CD4+ T cells after stimulation with SEB, as compared control cell lines (Bowness, Ridley et al. 2011). Moreover, this increase in the percentage of viable cells significantly decreased after treatment with DX31, a KIR3DL2 specific antibody. To investigate the mechanism which might lead to this increase in the percentage of viable KIR3DL2+ CD4+ T cells I looked at expression of the anti-apoptotic molecule Bcl-2 after co-culture of naïve CD4+ T cells with HLA-B27-expressing cells or cell lacking HLA-class I expression. I show that co-culture of naïve CD4+ T cells with HLA-B27-expressing cells for 24 hours significantly increases expression of Bcl-2, therefore interaction of KIR3DL2 with HLA-B27 may result in the preferential survival of KIR3DL2+ CD4+ T cells.

Ligation of inhibitory KIRs on CD8+ T cells has been shown to promote cell survival by inhibiting AICD (Ugolini, Arpin et al. 2001). Using mice that were transgenic for KIR2DL3 and its cognate HLA-Cw3 ligand, the authors showed that engagement of KIR2DL3 selectively drove the *in vivo* accumulation of a subset of CD8+ T cells. Moreover, *in vitro*, recognition of MHC class I molecules by inhibitory KIRs on CD8+ T cells down-regulated AICD, suggesting that the expansion of KIR2DL3+ CD8+ T cells occurred as a consequence of increased T cell survival. This was supported by a further study where engagement of KIR2DL2 and KIR2DL3 by MHC ligands diminished early TCR signaling, as well as AICD (Arlettaz, Degermann et al. 2004). In another study Jurkat cells transfected to express KIR3DL1 were used to study the effect of KIR signaling. Following stimulation with PHA, PMA/ionomycin, or anti-CD3, AICD was inhibited in the Jurkat cells expressing KIR3DL1 (Chwae, Chang et al. 2002). This

inhibition was thought to be caused by blocking the induction of Fas ligand expression following stimulation.

Interestingly, STAT3 has been shown to enhance survival of activated T cells by upregulating CD134 and Bcl-2, whilst down-regulating FasL and Bad expression (Oh, Yu et al. 2011). Genetic polymorphisms in STAT3 have been associated with AS (Danoy, Pryce et al. 2010; Davidson, Liu et al. 2011). Given its role in the Th17 pathway and T cell survival, it would be interesting to look in future at phosphorylation of STAT3 following co-culture of CD4⁺ T cells with HLA-B27 expressing cells.

In summary I have shown that co-culture of naïve CD4⁺ T cells with HLA-B27-expressing cell lines increases KIR3DL2 expression. Since activation alone is sufficient to induce KIR3DL2 expression on naïve CD4⁺ T cells, as shown in chapter 5, it is possible that the subsequent interaction of KIR3DL2 with HLA-B27 acts to maintain or further increase KIR3DL2 expression in HLA-B27⁺ individuals. Co-culture of naïve CD4⁺ T cells with HLA-B27-expressing cell lines also increase RORC expression by naïve CD4⁺ T cells, which could skew CD4⁺ T cells down a Th17 pathway. Given the reports that a lower strength of stimulation skews CD4⁺ T cells down a Th17 pathway it is also possible that KIR3DL2 interaction with HLA-B27 is acting to damp down the strength of the TCR signal, thus pushing CD4⁺ T cells down a Th17 differentiation pathway. Co-culture of naïve CD4⁺ T cells with HLA-B27-expressing cell lines also increase Bcl-2 expression by CD4⁺ T cells, which could be a mechanism whereby these cells preferentially survive and accumulate in the peripheral blood of HLA-B27⁺ individuals.

Since CD4⁺ T cells express both activating and inhibitory KIRs, it is conceivable that the balance between the two may further play a role in differential regulation of immune

responses, for example by altering the threshold for activation, or promoting or leading to the termination of an immune response (Vilches and Parham 2002). KIR3DL2 is a framework KIR, meaning it expressed by all individuals, and as such it is of particular interest to study KIR3DL2 and its interaction with HLA-molecules, especially given that up to 94% of AS patients are HLA-B27+ (Brewerton, Hart et al. 1973; Caffrey and James 1973).

Chapter 7: Final discussion and future directions

The overall aims of my project were to determine the phenotype and function of KIR3DL2+ CD4+ T cells in AS. Following on from my initial findings I also investigated whether activation induced and/or increased KIR3DL2 expression by CD4+ T cells, and explored the effects of co-culturing naïve CD4+ T cells with HLA-B27-expressing cells.

One of the most significant finding of my work is that IL-17 production by KIR3DL2+ CD4+ T cells was significantly greater than by KIR3DL2- CD4+ T cells in inflammatory arthritis patients. Moreover, IL-23R is enriched on KIR3DL2+ CD4+ T cells, and KIR3DL2+ CD4+ T cells from AS patients produced more IL-17 in response to rIL-1/23. It would be of interest to carry out longitudinal studies to investigate whether cytokine production by KIR3DL2+ CD4+ T cells changes over time, particularly during periods of disease activity. A recent study in AS patients showed that the frequencies of Th17 cells significantly decreased in anti-TNF responders, but significantly increased in non-responders, and was accompanied by a reduction in the levels of TNF- α , IL-6, IL-17, and IL-23 in responders (Xueyi, Lina et al. 2012). It would be of interest to investigate cytokine production by KIR3DL2+ CD4+ T cells after treatment with different therapies to see differences in cytokine production before and after therapy.

IL-17 and IL-6 were the only cytokines that I studied that were produced in significantly greater amounts by KIR3DL2+ CD4+ T cells compared to KIR3DL2- CD4+ T cells from AS patients after 5 days stimulation with anti-CD2/3/28. Additional studies show raised levels of IL-17 and IL-6 in AS patients, in serum, synovial fluid and cultured supernatants (Singh,

Aggarwal et al. 2007; Wang, Lin et al. 2009; Londono, Romero-Sanchez et al. 2012). This suggest that both anti-IL-17 and anti-IL-6 are attractive potential targets for the treatment of AS.

IL-17 has effects on a wide range of target cells, including epithelial and endothelial cells, fibroblasts, keratinocytes, osteoblasts, monocytes and macrophages (reviewed in (Onishi and Gaffen 2010)). IL-17 acts on cells for production of cytokines, for example inducing stromal cells to produce IL-6 and IL-8 (Fossiez, Djossou et al. 1996). IL-6 itself is required for the differentiation of Th17 cells, thus Th17 cells may act in a positive feedback mechanism for further development of Th17 cells. IL-17 also acts for the recruitment of inflammatory cells, including neutrophils (Laan, Cui et al. 1999). IL-17-producing neutrophils have been observed in the facet joints of patients with AS (Appel, Maier et al. 2011), and synovium infiltration by macrophages and neutrophils in axial spondyloarthritis correlates with disease activity (Baeten, Kruithof et al. 2005).

Secukinumab, a fully humanized anti-IL-17-A monoclonal antibody, has demonstrated efficacy in proof of concept trials of psoriasis, RA and uveitis (Genovese, Van den Bosch et al. 2010; Hueber, Patel et al. 2010) and in a phase IIb clinical trial in RA (Genovese, Van den Bosch et al. 2010). Results from a phase II trial in moderate-to-severe AS showed promising results, with secukinumab resulting in a higher ASAS20 response than placebo at 6 weeks (61% v 17%) (oral abstract presented at the British Socitey for Rheumatology annual conference, 2012). However a proof-of-concept study of secukinumab in patients with moderate to severe Crohn's disease was unsuccessful with a greater rate of adverse events in the secukinumab treated group as compared to the placebo group (Hueber, Sands et al. 2012). Ixekizumab, a further anti-IL-17-A monoclonal antibody, has shown to be effective in the treatment of psoriasis (Leonardi, Matheson et al. 2012). An additional target for therapy

is the IL-17R. Brodalumab, an anti-IL-17RA monoclonal antibody, has shown to be effective in the treatment of psoriasis (Papp, Leonardi et al. 2012). Since this antibody is specific for the receptor, it is able to block binding of IL-17A, IL-17F, IL-17A/F and IL-17E (Russell, Kerkof et al. 2012).

Tocilizumab, an anti-IL-6R monoclonal antibody that binds both soluble and membrane bound IL-6R, has shown efficacy in RA and systemic-onset juvenile idiopathic arthritis (Yokota, Imagawa et al. 2008; Nishimoto, Miyasaka et al. 2009) and is now licensed for treatment of RA in Europe and the USA. However despite its success in RA a clinical trial with tocilizumab in AS patients who had failed treatment with NSAIDs but had not received anti-TNF α therapy was suspended when it failed to achieve efficacy (www.clinicaltrials.gov/show/NCT01209689).

Although up to 94% of patients with AS are HLA-B27+, many individuals who are HLA-B27+ remain healthy and it is interesting to speculate why this might be. I have shown similar percentages of CD4+ T cells from AS patients and HLA-B27+ healthy controls express KIR3DL2, and this is significantly higher than CD4+ T cells from HLA-B27- healthy controls. Following interaction with HLA-B27-expressing cell lines there is greater expression of KIR3DL2 on CD4+ T cells than after co-culture with cell lines expressing a control HLA-class I molecule. This suggests that expression of HLA-B27 and its subsequent interaction with KIR3DL2 increases and/or maintains KIR3DL2 expression on CD4+ T cells, and potentially on other cells that express KIR3DL2. This is the first description of an immunological phenomenon linking HLA-B27 and SpA, with the interaction of KIR3DL2 and HLA-B27 potentially predisposing HLA-B27+ individuals to disease.

I have identified further similarities between AS patients and HLA-B27+ healthy controls, with a significantly higher proportion of IL-1R+ CD4+ T cells expressing KIR3DL2, from AS patients and HLA-B27+ healthy controls, as compared to HLA-B27- healthy controls. The same is true for both CCR6+ CD4+ T cells expressing KIR3DL2 and CCR9+ CD4+ T cells expressing KIR3DL2. KIR3DL2+ CD4+ T cells from AS patients and HLA-B27+ healthy controls are enriched for IL-23R expression, with the KIR3DL2+ CD4+ T cell population accounting for 57% and 54% of the total number of IL-23R+ CD4+ T cells in AS patients and HLA-B27+ healthy controls, respectively. This is over double that seen in HLA-B27- healthy controls, although it does not reach statistical significance.

An interesting difference between AS patients and HLA-B27+ individuals is the response of KIR3DL2+ CD4+ T cells to treatment with rIL-1/23, with rIL-1/23 significantly increasing IL-17 production by KIR3DL2+ CD4+ T cells from AS patients but not by KIR3DL2+ CD4+ T cells from HLA-B27+ healthy controls. Of the AS patients I investigated there were two groups in terms of IL-17 production, with one group producing levels similar to controls and the other producing elevated levels of IL-17, after the addition of rIL-1/23. There are two regions of association within the IL-23R gene and in the secondary region of association two of the three patients shared the same SNPs, with KIR3DL2+ CD4+ T cells from these patients producing the higher levels of IL-17. In contrast the other patient had a different genotype and KIR3DL2+ CD4+ T cells from this patient produced much less IL-17 after the same treatment. Although the numbers are low this data suggests that it would be worthwhile investigating greater numbers of AS patients to see if there is a correlation between the genotype of the IL-23R and the function of KIR3DL2+ CD4+ T cells, in terms of IL-17 production.

In vivo there may be different levels of IL-23 and IL-1 in AS patients as compared to HLA-B27+ healthy controls. It has been described that AS patients have higher levels of IL-23 in serum and supernatants from cultured PBMC compared to healthy controls, however these studies do not state whether the healthy controls are HLA-B27- or HLA-B27+ (Wang, Lin et al. 2009; Mei, Pan et al. 2011). Dendritic cells and macrophages are thought to be the main producers of IL-23 (Oppmann, Lesley et al. 2000). Moreover, macrophages predominate in biopsy tissues from early spondylarthritis inflammatory lesions (Bollow, Fischer et al. 2000). In response to LPS, macrophages from AS patients secreted more IL-23, CXCL9, IL-10, IL-12p70, and TNF α than macrophages from HLA-B27- healthy controls, although studies comparing HLA-B27+ healthy controls are yet to be carried out (Zeng, Lindstrom et al. 2011).

A further potential difference is the level of HLA-B27 expression on cells from AS patients compared to HLA-B27+ healthy controls. Although not explored in this project it has been reported that expression of HLA-B27 mRNA in HLA-B27+ AS patients is significantly higher compared to unaffected HLA-B27+ family members and HLA-B27+ healthy controls (Liu, Yu et al. 2006). Moreover, expression of HLA-B27 mRNA correlated with clinical disease activity in patients with AS. Therefore, it is conceivable that the higher level of expression of HLA-B27 on cells from AS patients, and the different forms of HLA-B27, including HLA-B27 dimers and free heavy chains, may alter the interaction with KIR3DL2, potentially increasing KIR3DL2 expression and/or altering the level of cytokine production by cells expressing KIR3DL2.

I propose a model, as shown in Figure 7.1, to explain how KIR3DL2+ CD4+ T cells may be involved in the pathogenesis of AS. In the initial step I propose that activation causes KIR3DL2 expression to be induced by CD4+ T cells. This could be by some kind of bacterial infection and I have show that SEB can upregulate expression of KIR3DL2 on CD4+ T cells. I

also show *de novo* expression of KIR3DL2 on naïve CD4⁺ T cells following activation with PMA and ionomycin. It is likely that activation-induced KIR3DL2 upregulation is not limited to CD4⁺ T cells, since this receptor is expressed by a variety of different cells, including NK cells, CD8⁺ T cells, $\gamma\delta$ T cells and innate lymphoid cells (Ridley, Wong-Baeza, Keidel and Kollnberger, unpublished data). This step is dependent upon activation but is independent of HLA-type, since activation induces and increases KIR3DL2 expression on CD4⁺ T cells regardless of whether the individual is HLA-B27⁺ or HLA-B27⁻.

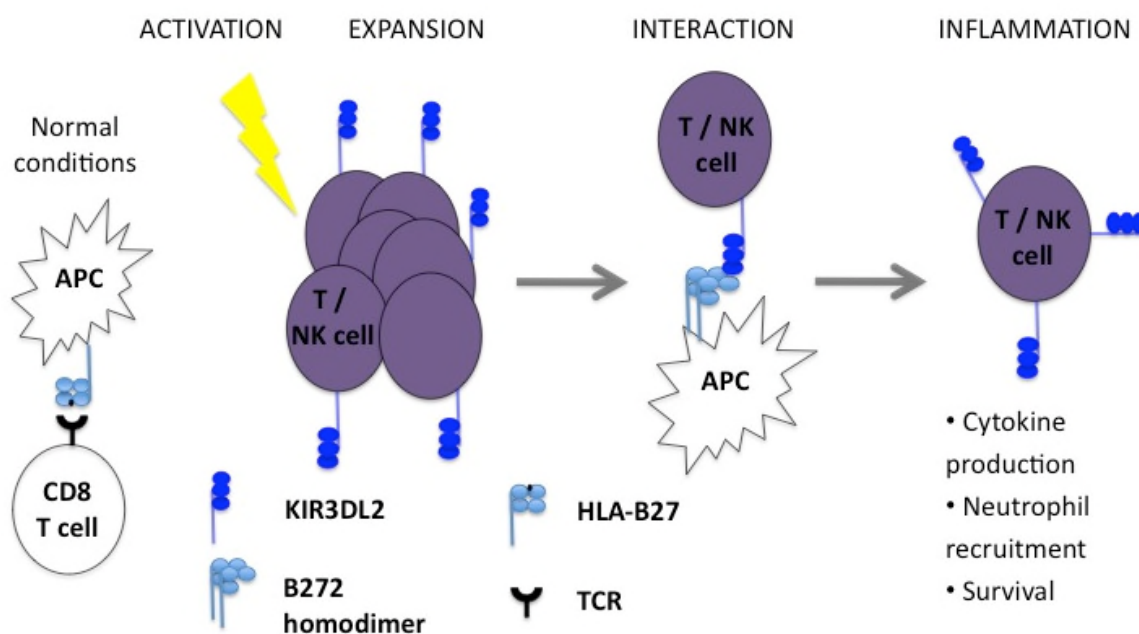


Figure 7.1: Model to explain KIR3DL2 and HLA-B27 interaction in the pathogenesis of AS. I propose a two-step model to explain how KIR3DL2+ CD4+ T cells are involved in the pathogenesis of AS. In the first step activation induces expression of KIR3DL2 on CD4+ T cells. Although the work in the thesis described responses by CD4+ T cells it is possible that activation may induce KIR3DL2 expression on other cells, including NK cells, CD8+ T cells and $\delta\gamma$ T cells. In the second step interaction occurs between KIR3DL2 and aberrantly folded forms of HLA-B27, which I propose leads to survival of these pro-inflammatory cytokine-producing CD4+ T cells.

Under normal conditions HLA-B27 heterotrimers present peptide to CD8+ T cells. In addition to classically folded heterotrimers HLA-B27 can exist as β_2m -free homodimers, which are able to interact with KIR3DL2 (Kollnberger, Bird et al. 2002). HLA-B27 free heavy chains have also been shown to interact with KIR3DL2 (Kollnberger, Bird et al. 2002). Therefore in

the second step I propose that in those individuals who are HLA-B27+ there is interaction of KIR3DL2+ CD4+ T cells with aberrantly folded HLA-B27. KIR3DL2 is also able to interact with HLA-A3 and HLA-A11, which are not associated with the development of AS (Dohring, Scheidegger et al. 1996; Hansasuta, Dong et al. 2004), however work from our lab has shown that KIR3DL2 binds to HLA-B27 dimers and free heavy chains more strongly than other HLA-class I molecules (Wong-Baeza, Ridley et al. 2013). I have shown that co-culture of naïve CD4+ T cells with HLA-B27-expressing cell lines leads to greater KIR3DL2 expression compared to co-culture of naïve CD4+ T cells with cell lines that express control HLA-class I molecules. Thus, the interaction between KIR3DL2 and HLA-B27 dimers or free heavy chains could act to maintain or increase KIR3DL2 expression. I have also shown that co-culture of naïve CD4+ T cells with HLA-B27-expressing cell lines increases RORC and T-bet expression, which may effect CD4+ T cell differentiation, pushing naïve CD4+ T cells down a Th17 or Th17/1 differentiation pathway.

In addition to upregulation of KIR3DL2 by activation, certain circumstances may also lead to the upregulation of aberrantly folded forms of HLA-B27. This could be as a result of bacterial infection, hypoxia or cellular stress. Work from our lab has demonstrated that HD6, an antibody raised against HLA-B27 dimers, binds to PBMC from SpA patients following low pH treatment (McHugh, in revision). This potentially highlights the importance of the microenvironment in expression of aberrantly folded forms of HLA-B27. Thus it is conceivable that expression of these forms of HLA-B27 are limited to certain anatomical sites, such as the sacroiliac, knee and hip joints, possibly explaining why these are the sites of inflammation in AS.

Following interaction of KIR3DL2 with HLA-B27 dimers and free heavy chains I propose there is preferential survival of these KIR3DL2+ CD4+ T cells that might otherwise die

through apoptosis. The expansion of CD4⁺ T cells after activation and the survival of KIR3DL2⁺ CD4⁺ T cells has been published (Bowness, Ridley et al. 2011). The greatest percentage of viable KIR3DL2⁺ CD4⁺ T cells was seen after co-culture of CD4⁺ T cells from AS patients with HLA-B27-expressing cells. Here I provide evidence showing upregulation of the anti-apoptotic molecule Bcl-2 following co-culture of naïve CD4⁺ T cells with HLA-B27-expressing cell lines. Further support to this theory comes from microarray data showing greater expression of a further anti-apoptotic molecule Bcl-x_L in FACS-sorted KIR3DL2⁺ CD4⁺ T cells compare to KIR3DL2⁻ CD4⁺ cells from AS patients (Wong-Baeza, unpublished data). Therefore, in my model those cells expressing KIR3DL2, which interact with aberrantly folded HLA-B27, upregulate the anti-apoptotic molecule Bcl-2, and preferentially survive over cells that lack KIR3DL2 expression. To confirm whether signalling through KIR3DL2 leads to upregulation of these molecules it will be necessary to look at mutated forms of KIR3DL2. Experiments transducing primary CD4⁺ T cells with wild type KIR3DL2 or mutated KIR3DL2 and comparing the phenotype and cytokine production of these CD4⁺ T cells after co-culture with cell lines expressing HLA-B27 or a control HLA-class I molecule, are ongoing.

It is interesting to speculate where in the body this initial activation of CD4⁺ T cells might be occurring. Given the crossover between AS and IBD, and that ReA is known to be initiated after infection with various Gram-negative intracellular bacteria, it is possible that bacterial infection in the gut might have provided the initial stimulation leading to the expression of KIR3DL2. Some preliminary data suggests that CD4⁺ T cells from gut biopsies are enriched for KIR3DL2 (Kollnberger, unpublished data). I have shown that KIR3DL2⁺ CD4⁺ T cells from peripheral blood are enriched for CCR9 expression, which might indicate that these cells have homed from the gut and subsequently moved into systemic circulation. Interestingly, it has been shown that lamina propria lymphocytes isolated from inflamed gut of IBD patients bind more efficiently to synovial vessels than cells isolated from uninfamed gut (Salmi and

Jalkanen 2001), thus it is possible that following activation in the gut KIR3DL2+ CD4+ T cells move into peripheral circulation and then home to joints. It would be of interest to carry out more detailed analysis of KIR3DL2+ CD4+ T cells from synovial fluid to draw comparisons with peripheral blood samples, although preliminary analysis in this project suggests that KIR3DL2+ CD4+ T cells from synovial fluid have greater expression of IL-23R than matched peripheral blood samples from SpA patients. Moreover, although difficult to track cells in humans, analysis of TCR clonality of KIR3DL2+ CD4+ T cells at different sites would provide further information.

Osteoimmunology is the study of the interaction between the immune system and the skeleton. This is particularly pertinent in AS since new bone formation leading to fusion of the vertebrae is a defining feature. However AS is a complex disease with areas of bone resorption in addition to areas of new bone formation. Bone remodeling involves a delicate balance between osteoclasts, which are involved in bone resorption, and osteoblasts, which are involved in new bone formation. Osteoclasts degrade bone matrix proteins by secreting proteolytic enzymes, such as cathepsin K (Gelb, Shi et al. 1996). A study using cathepsin K inhibitor-treated and cathepsin K-deficient dendritic cells revealed defective TLR9 signaling in dendritic cells in response to unmethylated CpG DNA, which in turn lead to attenuated induction of Th17 cells (Asagiri, Hirai et al. 2008). More specifically CpG DNA induced production of IL-6 and IL-23 was impaired, with a subsequent reduction in Th17 cells. A further study identified Th17 cells as an osteoclastogenic T cell subset (Sato, Suematsu et al. 2006). In this study LPS injection into the calvarial bone in wild type mice resulted in severe bone destruction associated with aberrant formation of osteoclasts. In contrast bone destruction was much less pronounced and osteoclast formation was significantly reduced in both the *Il17*^{-/-} mice and *Il23a*^{-/-} mice, suggesting that Th17 cells, expanded through IL-23, are involved in T cell-mediated osteoclastogenesis *in vivo*. The same study looked at the

synovial tissue from RA patients, showing mRNA expression of receptor activator of nuclear factor kappa-B ligand (RANKL) correlated with IL-23 (IL23A) mRNA expression, but not *IL-12 (IL12A)* mRNA expression, suggesting that IL-23 is an important determinant of arthritic bone destruction through the induction of RANKL.

Some preliminary data from our lab has shown that KIR3DL2+ CD4+ T cells are enriched for RANKL (Kollnberger, unpublished data). These KIR3DL2+ CD4+ T cells, or indeed any cells expressing KIR3DL2, could therefore potentially interact with RANK on osteoclasts. Signaling through RANK induces the transcription factor NFATc1 for terminal differentiation of osteoclasts (Takayanagi, Kim et al. 2002). I have shown that KIR3DL2 + CD4+ T cells are enriched for IL-17 production. Since IL-17 has been shown to induce RANKL expression on osteoclastogenesis-supporting mesenchymal cells, including osteoblasts and synovial fibroblasts (Kotake, Udagawa et al. 1999), it is possible that KIR3DL2+ CD4+ T cells could both directly and indirectly induce osteoclast differentiation.

My work supports targeting the interaction between KIR3DL2 and HLA-B27 in the treatment of AS, by targeting KIR3DL2 or aberrantly folded forms of HLA-B27. The monoclonal antibody HD6, has been shown to bind to recombinant HLA-B27 dimers and cells lines transfected with HLA-B27. I have shown that HD6 blocks SEB induced IL-17 production by PBMC from AS patients but had no effect on IL-17 production by PBMC from HLA-B27-healthy controls. This work suggests that HD6 has potential for use in the investigation and treatment of AS and other B27-associated spondyloarthritis, and this is currently being addressed in the HLA-B27 transgenic rat model.

In conclusion, work presented in this thesis shows that KIR3DL2+ CD4+ T cells are expanded in the peripheral blood of AS patients and HLA-B27+ healthy controls, and are enriched for

Th17 markers, including IL-23R, IL-1R, CCR6 and CD161. Interestingly, treatment with rIL-1/23 increases IL-17 production by KIR3DL2+ CD4+ T cells in AS patients but not RA patients and healthy controls. ICS reveals that KIR3DL2+ CD4+ T cells from AS patients and healthy controls are enriched for IL-17 production, as well as for dual production of IL-17 and IFN γ , consistent with the theory that AS is a Th17 or a Th17/1 driven disease. Activation of naïve CD4+ T cells induces *de novo* expression of KIR3DL2 and interaction with HLA-B27-expressing cells maintains and/or further increases KIR3DL2 expression and increases expression of Bcl-2. I propose a model to explain the role of KIR3DL2 and HLA-B27 interaction in the pathogenesis of AS, whereby interaction of KIR3DL2+ CD4+ T cells with aberrantly folded forms of HLA-B27 preferentially promotes the survival of pro-inflammatory cytokine producing KIR3DL2+ CD4+ T cells in AS.

Appendix

A-D: Heat maps showing cytokine production, as measure by multiplex bead analysis (IL-17A, IFN γ , IL-6, IL-10, GM-CSF, TNF α and IL-5) or ELISA (IL-22), by KIR3DL2- or KIR3DL2+ CD4+ T cells from AS patients (A) HLA-B27- (B) and HLA-B27+ healthy controls (C) and RA patients (D). Data for each individual patient or control is displayed (1-7 for each group) and levels of cytokine after anti-CD2/3/28 stimulation by KIR3DL2+ or KIR3DL2- CD4+ T cells, with or without rIL-1/23, is shown. For each heat map (A-D) values for each cytokine were ranked highest to lowest for each individual in all conditions and the highest seven values displayed in dark green, the next seven values displayed in mid-green, the next seven values displayed in light green and the lowest seven values displayed in white.

A AS	1				2				3				4				5				6				7			
Cytokine	KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-	
	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23
IL-17A	2513	48334	1484	6388	7374	45901	2310	13942	3897	8451	3761	11765	1264	8248	1030	4510	8113	49989	5722	50000	6543	15035	3899	14871	1248	3624	395	1632
IFN γ	8639	65921	4547	44503	8750	56800	3675	29652	50404	59564	12272	50381	6355	37366	5973	32327	34948	117608	24811	64919	41767	75601	33253	68118	11244	57871	4875	24077
IL-6	331	980	30	401	664	1147	20	217	585	1172	59	763	300	1309	79	789	1837	8071	95	867	385	668	19	545	503	1023	36	376
IL-10	419	424	164	197	1158	1198	784	749	773	589	890	802	260	299	415	353	1575	3432	493	449	437	401	474	422	791	933	232	359
GM-CSF	638	1289	462	761	682	842	740	752	1066	952	1077	1469	433	611	752	559	1818	2810	1353	2051	1301	1969	1472	1909	440	518	157	254
TNF α	233	1136	1014	2157	567	1134	545	934	1724	2077	885	1887	698	1288	1406	1446	2499	3415	1558	3425	498	1244	860	1278	2150	5034	574	1014
IL-5	288	329	227	205	519	825	429	657	184	176	367	232	579	452	450	325	589	990	414	365	326	364	250	294	251	324	222	136
IL-22	786	1956	563	1793	206	606	220	396	450	393	270	653	1482	4666	1350	2970	703	807	1238	1607	828	1297	521	617	2	159	28	276

B B27- HC	1				2				3				4				5				6				7			
Cytokine	KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-	
	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23
IL-17A	497	2301	1317	3641	4392	14585	1946	7004	2813	5059	2175	4361	273	321	214	256	468	616	450	660	4121	6963	670	866	1628	8932	1053	4842
IFN γ	7839	37633	6644	35880	21359	49287	13649	36378	12613	37487	6073	24961	9923	30360	6677	27177	5790	7138	1819	2027	5599	5524	360	437	13602	51517	6982	14563
IL-6	152	748	61	628	184	1650	442	1958	217	646	61	503	389	1079	55	634	303	1077	39	58	734	952	30	276	127	889	25	338
IL-10	774	769	636	738	93	94	512	571	460	456	561	500	537	726	325	327	109	103	60	107	384	750	97	141	234	596	216	216
GM-CSF	1473	2256	1635	2058	918	1708	2860	3186	642	1323	601	929	559	687	781	1201	474	538	598	684	873	644	137	193	1084	1506	713	915
TNF α	886	1795	674	1220	684	2318	1628	2094	315	829	145	643	396	700	616	1084	531	1081	471	568	1516	1938	733	995	340	2070	294	2281
IL-5	435	477	416	813	45	1264	1115	219	589	615	469	590	374	470	410	587	188	201	357	277	113	315	122	124	108	306	57	203
IL-22	1101	1218	665	1171	1	3	603	810	123	483	3	213	203	586	216	703	826	1053	1256	2	136	445	146	890	100	418	284	545

C B27+ HC	1				2				3				4				5				6				7			
Cytokine	KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-	
	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23
IL-17A	752	3819	611	2253	200	663	138	480	10300	10585	2131	13390	1605	3081	1316	4443	143	838	241	497	4082	22919	266	570	734	1084	431	478
IFN γ	10154	53807	2723	16858	10156	37645	1489	5630	54940	59487	13334	51367	50734	59914	46941	74062	874	8015	2561	4104	7033	9512	9307	27026	9053	10931	3888	4649
IL-6	414	761	15	92	390	807	28	240	344	362	33	292	642	1850	308	941	273	796	15	31	1013	1572	922	1530	471	490	30	195
IL-10	119	225	149	182	77	134	57	60	1175	944	868	596	746	863	777	1136	73	243	36	195	464	1890	128	990	550	1107	132	914
GM-CSF	265	1045	295	691	284	375	204	377	2825	2143	1082	1587	1265	2212	1451	1748	1690	982	1206	904	228	278	1308	1319	522	534	512	600
TNF α	243	523	179	274	151	210	41	99	2193	2106	541	2075	686	845	1387	1628	853	1443	219	708	788	2832	648	2142	1189	2200	568	1205
IL-5	123	105	162	228	131	202	132	271	720	780	853	682	516	563	401	358	363	414	189	114	523	252	241	208	779	751	217	243
IL-22	44	683	1	156	1	1	3	7	1046	2133	453	856	498	559	379	800	171	310	106	329	162	676	230	719	66	227	101	914

D RA	1				2				3				4				5				6				7			
Cytokine	KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-		KIR3DL2+		KIR3DL2-	
	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23	w/o rIL- 1/23	w rIL- 1/23
IL-17A	2140	5576	148	1001	3597	11190	1292	1604	1287	1462	983	1345	710	1493	252	411	3035	11166	1153	2842	1569	2669	1095	1535	1380	1450	1000	1380
IFN γ	3232	8924	2083	6597	10997	15716	3960	9276	4670	10048	2894	3445	28620	49056	10069	27840	24402	46589	11774	23681	20756	37928	13946	16398	6982	14221	3900	5109
IL-6	180	345	53	689	951	3788	110	450	160	576	163	456	75	691	18	147	54	153	26	67	1013	1131	105	843	289	486	40	228
IL-10	389	516	160	401	641	684	329	597	392	534	169	443	358	429	104	198	406	527	140	196	212	451	198	371	76	120	60	82
GM-CSF	119	246	277	336	664	844	720	1099	634	878	378	240	114	457	186	378	350	1050	140	420	1996	2032	1667	1720	1064	1786	1297	1334
TNF α	271	2903	254	1549	1216	1759	983	1787	135	1421	525	1216	349	1023	213	746	990	1960	396	1584	859	1364	629	1268	351	1168	191	1093
IL-5	162	203	572	700	279	648	313	224	208	950	325	584	180	379	254	255	314	151	917	791	205	172	144	185	373	122	513	260
IL-22	422	423	362	445	548	771	507	679	389	735	245	663	547	771	507	669	111	747	108	473	872	1289	338	933	617	1190	125	799

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