The distinct and aggressive pathogenesis of arthritis in children with Down syndrome

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# Table of Contents

Declaration and Statement of Plagiarism ........................................................................................................ ii  
Scientific Abstract ........................................................................................................................................ xi  
Lay Abstract .................................................................................................................................................. xiii  
Acknowledgments ........................................................................................................................................ xvi  
Published Abstracts ....................................................................................................................................... xx  
Abbreviations .............................................................................................................................................. xxiii  

1.1 History of Juvenile Idiopathic Arthritis ................................................................................................... 2  
1.2 Classification of JIA ................................................................................................................................. 2  
1.3 Epidemiology/ prevalence of JIA .............................................................................................................. 4  
1.4 Genetic factors in JIA .............................................................................................................................. 4  
1.5 Environmental factors of JIA .................................................................................................................. 6  
1.6 Comorbidities of JIA .............................................................................................................................. 7  
1.7 Treatment for JIA ................................................................................................................................... 8  
1.8 History of Down syndrome ..................................................................................................................... 8  
1.9 Comorbidities of DS ............................................................................................................................... 10  
1.10 Genotype/ phenotype of DS ................................................................................................................ 10  
1.11 History of Down syndrome-associated arthritis .................................................................................. 12  
1.12 Clinical differences between DA and JIA ............................................................................................. 13  
1.13 Comorbidities of DA ............................................................................................................................ 14  
1.14 Normal synovium ................................................................................................................................... 14  
1.15 Inflamed synovium ............................................................................................................................... 15  
1.16 Angiogenesis ......................................................................................................................................... 21  
1.17 Leukocyte transendothelial cell migration: adhesion molecules and chemokines ......................... 23  
1.18 Innate immune system .......................................................................................................................... 26  
1.19 The adaptive immune system ............................................................................................................. 27  
1.20 T cells .................................................................................................................................................... 28  
1.21 T cells in IA and DS ............................................................................................................................... 29  
1.22 B cells .................................................................................................................................................... 33  
1.23 B cells in DS, JIA, and DA .................................................................................................................. 34  
1.24 Monocytes ............................................................................................................................................ 36  
1.25 Synovial fibroblasts ............................................................................................................................... 38  
1.26 Cytokines .............................................................................................................................................. 39  
1.27 Cellular metabolism .............................................................................................................................. 41  
1.28 Impaired metabolic pathway ................................................................................................................ 43  
1.29 Metabolic alterations in the joint ......................................................................................................... 44  
1.30 Metabolic inhibition .............................................................................................................................. 46  
1.31 DA therapeutic options ....................................................................................................................... 48
3.3.9 Flow cytometry ................................................................. 132
3.3.10 Cytokine and chemokine measurements .................................................. 133
3.3.11 Cellular bioenergetic analysis ........................................................................ 134
3.3.12 Statistical analysis ....................................................................................... 135
3.4 Results .............................................................................................. 136
3.4.1 DA FLS-CM induces tube formation and migration capacity in HUVECs .................................................. 136
3.4.2 DA FLS-CM induces leukocyte adhesion through ICAM-1 expression on HUVECs .................................................. 139
3.4.3 DA FLS-CM induces CXCR3, CXCR5 and CD105 expression on HUVECs .................................................. 143
3.4.4 Effect of the DA joint microenvironment on the metabolic activity of HUVEC .................................................. 146
3.4.5 To examine the effect of TNFα, IL-17a and IFN-γ on HUVEC tube formation. .................................................. 149
3.4.6 TNF-α, IL-17a and IFN-γ induce HUVEC cytokine secretion .................................................. 150
3.4.7 The effect of TNF-α, IL-17a, and IFN-γ on adhesion molecule expression on HUVECs .................................................. 153
3.4.8 Effect of TNF-α, IL-17a, and IFN-γ on expression of CXCR3, CXCR4, CXCR5, CCR6, VEGFR, and decreases Claudin 5 on HUVECs .................................................................................. 156
3.4.9 No synergistic effects on HUVEC tube formation .................................................. 165
3.4.10 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces proinflammatory mediator secretion from HUVECs .................................................. 166
3.4.11 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces ICAM-1 and VCAM-1 expressions on HUVECs .................................................................................. 169
3.4.12 Cytokine synergistic effects on chemokine and angiogenic markers on HUVECs .................................................. 173
3.4.13 TNF-α and IL-17a or TNF-α and IFN-γ did not affect HUVEC cell metabolism .................................................. 183
3.5 Discussion .......................................................................................... 189
4.1 Introduction ...................................................................................... 202
4.2 Aims and Objectives ........................................................................... 208
4.3 Materials and methods ...................................................................... 209
4.3.1 Patient recruitment .................................................................................. 209
4.3.2 PBMC isolation ..................................................................................... 211
4.3.3 Cell stimulation ..................................................................................... 211
4.3.4 Flow cytometric analysis ...................................................................... 212
4.3.5 Statistical analysis .................................................................................. 214
4.4 Results .............................................................................................. 215
4.4.1 Increased cytokine production and higher polyfunctionality in DA compared to HC, JIA and T21 .................................................. 215
4.4.2 Increased CD4+, CD8+ and CD4- CD8- polyfunctionality in DA .................................................................................. 223
4.4.3 Increased frequency of chemokine receptor expression on T cell subpopulations in DA .................................................. 228
4.4.4 IFN-γ R1 expression on CD8 T cells higher in DA compared to HC, JIA and T21 .................................................. 233
4.4.5 Increased frequency of memory T cell subpopulations in DA .................................................................................. 236
4.4.6 Chemokine Receptor Expression on Memory T cells .................................................. 238
4.4.7 IFN-γ R1 expression across the different memory T cell subpopulations were similar .................................................. 246
4.4.8 Nonclassical monocytes express reduced CD86 in DA compared to T21 .................................................. 247
4.4.9 Classical monocytes are dominant in DA................................................................. 254
4.5 Discussion .................................................................................................................. 259
5.1 General Discussion .................................................................................................. 266
5.2 In Summary .............................................................................................................. 271
5.3 Future Direction ..................................................................................................... 273
Bibliography ..................................................................................................................... 279
List of Figures

Chapter 1

Figure 1. 1 Portrait of Sir Francis Ford’s Children Giving a Coin to a Beggar Boy ........................................ 2
Figure 1. 2 Photograph of a child with DS who has arthritis. ............................................................... 9
Figure 1. 3 The different subtypes between DA and JIA. ................................................................. 13
Figure 1. 4 Macroscopic images of a healthy control joint compared to an inflamed RA joint. ............ 17
Figure 1. 5 The healthy joint versus the inflamed joint or synovitis .................................................. 19
Figure 1. 6 Macroscopic and microscopic characteristics of an RA patient ........................................ 20
Figure 1. 7 Leukocyte adhesion cascade. ......................................................................................... 24
Figure 1. 8 Stages of the antibody-mediated immune response from B cells. .................................... 34
Figure 1. 9 General overview of the primary metabolic pathways. ....................................................... 43

Chapter 2

Figure 2. 1 TNF-α, IL-17a and IFN-γ induce IL-6 and MCP-1 secretion from DA FLS ..................... 66
Figure 2. 2 TNF-α, IL-17a and IFN-γ induce RANTES and IL-8 secretion from DA FLS ................. 67
Figure 2. 3 TNF-α, IL-17a and IFN-γ induce IL-6 and MCP-1 gene expression in DA FLS .......... 68
Figure 2. 4 TNF-α, IL-17a and IFN-γ induces RANTES and IL-8 gene expression in DA FLS ......... 69
Figure 2. 5 TNF-α, IL-17a and IFN-γ induce MMP secretion in DA FLS ........................................... 70
Figure 2. 6 TNF-α, IL-17a and IFN-γ induce GLUT-1 and LDHA gene expression in DA FLS ......... 71
Figure 2. 7 TNF-α, IL-17a and IFN-γ induce HIF-1α, HK2 and PKM2 gene expression in DA FLS. ... 72
Figure 2. 8 TNF-α, IL-17a and IFN-γ induce the adhesive capacity of DA FLS .............................. 75
Figure 2. 9 Gating strategy for flow cytometric analysis of DA FLS ................................................... 76
Figure 2. 10 TNF-α, IL-17a and IFN-γ induce ICAM-1 expression in DA FLS ................................. 77
Figure 2. 11 TNF-α, IL-17a and IFN-γ induce VCAM-1 expression in DA FLS ............................... 78
Figure 2. 12 TNF-α or IFN-γ induces CXCR3 expression in DA FLS ............................................... 80
Figure 2. 13 TNF-α or IFN-γ induces CXCR4 expression in DA FLS ............................................... 81
Figure 2. 14 TNF-α or IFN-γ induces CXCR5 expression in DA FLS .................................................. 82
Figure 2. 15 TNF-α or IFN-γ induces CCR6 expression in DA FLS ................................................. 83
Figure 2. 16 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces DA FLS IL-6 and MCP-1 secretion ................................................................. 86
Figure 2. 17 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ differentially regulates DA FLS RANTES and IL-8 secretion. .................................................. 87
Figure 2. 18 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces IL-6 and MCP-1 gene expression of DA FLS. ......................................................... 88
Figure 2. 19 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces RANTES and IL-8 gene expression of DA FLS ........................................................................ 89
Figure 2. 20 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ differentially regulates MMP secretion by DA FLS ........................................................................ 90
Figure 2. 21 TNF-α and IFN-γ synergy induces adhesion in DA FLS .............................................. 93
Figure 2. 22 TNF-α and IFN-γ synergy induces ICAM-1 expression in DA FLS .................................. 94
Figure 2. 23 TNF-α and IFN-γ synergy induces VCAM-1 expression in DA FLS ................................ 95
Figure 2. 24 No synergistic effects were observed for CXCR3 expression in DA FLS ....................... 98
Figure 2. 25 TNF-α and IFN-γ synergy induces CXCR4 expression in DA FLS .................................. 99
Figure 2. 26 TNF-α and IFN-γ synergy induces CXCR5 expression in DA FLS ................................. 100
Figure 2. 27 No synergistic effects were observed for CCR6 expression in DA FLS ....................... 101
Figure 2. 28 Synergy between TNF-α and IL-17a induces a glycolytic shift in DA FLS ................... 105
Figure 2. 29 Synergy between TNF-α and IL-17a induces a glycolytic shift in DA FLS ................... 106
Figure 2. 30 Synergy between TNF-α and IFN-γ induces a glycolytic shift in DA FLS ................... 107
Figure 2. 31 Synergy between TNF-α and IFN-γ induces a glycolytic shift in DA FLS ................... 108
Figure 3.1 DA FLS-CM induces tube formation and NOTCH 1IC HUVECs. ........................................ 137
Figure 3.2 DA FLS-CM induces migrative capacity of HUVECs. ..................................................... 138
Figure 3.3 DA FLS-CM induces adhesive mechanisms in HUVECs. .................................................. 140
Figure 3.4 HUVEC gating strategy. ..................................................................................................... 141
Figure 3.5 DA FLS-CM induces ICAM-1 expression in HUVECs. ......................................................... 142
Figure 3.6 The effect of DA FLS-CM on CCR3, CCR4 and CCR5 expression in HUVECs. .................. 144
Figure 3.7 The effect of DA FLS-CM on CCR6 and CD105 expression in HUVECs. ......................... 145
Figure 3.8 DA joint microenvironment does not alter metabolic activity of HUVECs. ..................... 147
Figure 3.9 DA joint microenvironment does not alter the glycolytic capacity of HUVECs. .............. 148
Figure 3.10 The effect of TNFα, IL-17a or IFN-γ on tube formation in HUVECs. .............................. 149
Figure 3.11 TNF-α, IL-17a and IFN-γ induce IL-6 and/or MCP-1 secretion from HUVECs. .......... 151
Figure 3.12 Effect of TNF-α, IL-17a and IFN-γ on RANTES and IL-8 secretion from HUVECs. ...... 152
Figure 3.13 TNF-α and IFN-γ induce ICAM-1 expression in HUVECs. .......................................... 154
Figure 3.14 TNF-α induces VCAM-1 expression in HUVECs. ............................................................. 155
Figure 3.15 TNF-α and IL-17a induce CXCR3 expression in HUVECs. .......................................... 158
Figure 3.16 TNF-α induces CXCR4 expression in HUVECs. .............................................................. 159
Figure 3.17 TNF-α induces CCR5 expression in HUVECs. ................................................................. 160
Figure 3.18 TNF-α induces CCR6 expression in HUVECs. ............................................................... 161
Figure 3.19 TNF-α decreases Claudin 5 expression in HUVECs. ...................................................... 162
Figure 3.20 TNF-α induces CD141 expression in HUVECs. ............................................................... 163
Figure 3.21 TNF-α, IL-17a or IFN-γ had no effect on VEGFR expression in HUVECs. .................... 164
Figure 3.22 No synergistic effects on HUVEC tube formation in response to cytokine stimulation. ....... 165
Figure 3.23 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induced IL-6 and MCP-1 secretion from HUVECs. .............................................................................................................. 167
Figure 3.24 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ on RANTES and IL-8 secretion from HUVECs. .................................................................................................................. 168
Figure 3.25 Synergy between TNF-α and IFN-γ induced ICAM-1 expression in HUVECs. .............. 171
Figure 3.26 Synergy between TNF-α and IFN-γ induced VCAM-1 expression in HUVECs. .......... 172
Figure 3.27 Synergy between TNF-α and IL-17a and TNF-α and IFN-γ had differential effects on expression in HUVECs. .............................................................................................................. 176
Figure 3.28 Synergy between TNF-α and IFN-γ induced CXCR4 expression in HUVECs. ............. 177
Figure 3.29 Synergy between TNF-α and IFN-γ induced CXCR5 expression in HUVECs. .............. 178
Figure 3.30 Synergy between TNF-α and IFN-γ induced CCR6 expression in HUVECs. ............... 179
Figure 3.31 TNF-α and IL-17a or IFN-γ did not affect Claudin 5 expression in HUVECs. ............... 180
Figure 3.32 TNF-α and IL-17a or IFN-γ did not affect CD141 expression in HUVECs ................... 181
Figure 3.33 TNF-α and IL-17a or IFN-γ did not affect VEGFR expression in HUVECs ............... 182
Figure 3.34 TNF-α and IL-17a did not affect the metabolic capacity of HUVECs. ....................... 185
Figure 3.35 TNF-α and IL-17a did not affect the glycolytic capacity of HUVECs ....................... 186
Figure 3.36 TNF-α and IFN-γ did not affect the metabolic capacity of HUVECs. ....................... 187
Figure 3.37 The effects of TNF-α and IFN-γ cytokine synergy on the glycolytic capacity of HUVECs ........ 188
Figure 3.38 Summary of Chapter 3. .................................................................................................. 200

Chapter 3

Figure 2. 32 Synergy between TNF-α and IL-17a or IFN-γ induces GLUT-1 and LDHA gene expression in DA FLSs............................................................................................................................................. 109
Figure 2. 33 TNF-α alone and in combination with IL-17a or IFN-γ induces HIF1α, HK2 and PKM2 gene expression in DA FLSs .................................................................................................................. 110
Figure 2. 34 An overview of Chapter 2 focusing on TNF-α and IFN-γ ........................................ 121
Chapter 4

Figure 4. 1 Gating strategy for Th panel. .......................................................... 218
Figure 4. 2 Increased CD4+ and CD8+ T cell subpopulations in DA peripheral blood. ........................................... 219
Figure 4. 3 Increased proinflammatory cytokine expression from patients with DA peripheral blood CD4+ T cells. .......................................................... 220
Figure 4. 4 Increased proinflammatory cytokine expression from patients with DA peripheral blood CD8+ T cells. .......................................................... 221
Figure 4. 5 Increased proinflammatory cytokine expression from patients with DA peripheral blood CD4- CD8- T cells .......................................................... 222
Figure 4. 6 Snapshot of all possible 256 combinations of cytokines using Boolean gating. .................................... 225
Figure 4. 7 Children with DA have increased polyfunctional CD4+, CD8+ and CD4- CD8- T cells. ........................................... 226
Figure 4. 8 Children with DA have increased coexpression of proinflammatory cytokines. ........................................... 227
Figure 4. 9 Gating strategy for chemokine panel. .................................................. 229
Figure 4. 10 CCR1 and CCR2 expressions on CD4+, CD8+ and CD4- CD8- T cells in DA differ. ................................ 230
Figure 4. 11 CCR4 and CCR5 expressions on CD4+, CD8+ and CD4- CD8- T cells in DA differ. ................................ 231
Figure 4. 12 CXCR3 and CXCR6 expressions on T cells in DA differ. .......................................................... 232
Figure 4. 13 Gating strategy for IFN-γ R1 panel. ................................................... 234
Figure 4. 14 IFN-γ R1 expressions on CD8+ T cells populations in DA are increased. ........................................... 235
Figure 4. 15 Memory T cell subpopulations differ in DA compared to HC, JIA and T21. ........................................... 237
Figure 4. 16 CCR1 expression on different memory T cells populations in DA differ ........................................... 240
Figure 4. 17 CCR2 expression on different memory T cells populations in DA differ ........................................... 241
Figure 4. 18 CCR4 expression on different memory T cells populations in DA differ ........................................... 242
Figure 4. 19 CCR5 expression on different memory T cells populations in DA differ ........................................... 243
Figure 4. 20 CXCR3 expression on different memory T cells populations in DA differ ........................................... 244
Figure 4. 21 CXCR6 expressions on different memory T cells populations in DA differ ........................................... 245
Figure 4. 22 IFN-γ R1 expression on different memory T cell subpopulations are similar in JIA, T21 and DA. .......................................................... 246
Figure 4. 23 Gating strategy for monocyte panel .................................................... 249
Figure 4. 24 Frequencies of monocyte subsets are similar in JIA, T21 and DA. .................................................... 250
Figure 4. 25 Frequency of nonclassical monocytes expressing CD86 is reduced in DA compared to T21. ............ 251
Figure 4. 26 Frequency of intermediate monocytes expressing CD86 is reduced in DA compared to T21. ............ 252
Figure 4. 27 Frequency of classical monocytes expressing CD15 is reduced in DA compared to T21. ......... 253
Figure 4. 28 Cluster 1 and 3 prominent in DA and is different compared to JIA and T21. ................................. 256
Figure 4. 29 Cluster 1 and 3 prominent in DA and is different compared to JIA and T21. ................................. 257
Figure 4. 30 Cluster 1, 2, 3 and 4 prominent in DA, JIA and T21 .......................................................... 258
Figure 4. 31 Summary of Chapter 4. ......................................................................... 263

Chapter 5

Figure 5. 1 Summary of the aggressive stromal cell phenotypes in the DA microenvironment and aggressive immune cell phenotypes in the periphery.......................................................... 272
Figure 5. 2 Primary DA FLS were primed with IFN-γ for 24 hrs, before stimulation with TNF-α alone and in combination with IFN-γ for a further 24 hrs.......................................................... 273
Figure 5. 3 2-DG reduces the production of IL-6, MCP-1 and RANTES and reduces the expression of ICAM-1 from DA FLS. .......................................................... 276
Figure 5. 4 2-DG reduces the expression of CCR6, CXCR3, CXCR4, CXCR5 and switches the metabolic profile of DA FLS to glycolysis.................................................... 277
List of Tables

Chapter 1

Table 1. 1 ILAR classification of seven JIA subtypes .................................................................................. 3
Table 1. 2 Significant CD4+ T cell subsets in humans and the related diseases ........................................... 29
Table 1. 3 Accumulated data of T cell frequencies of children with DS ......................................................... 31

Chapter 2

Table 2. 1 DA patient demographic, clinical, laboratory features and treatments prior sampling and at time
of sampling .................................................................................................................................................. 57
Table 2. 2 Extracellular antibodies used for DA FLS flow cytometric staining ........................................... 59
Table 2. 3 Designed primer sequences used for real-time PCR analysis ...................................................... 61

Chapter 3

Table 3. 1 DA patient demographic, clinical, laboratory features and treatments prior sampling and at time
of sampling .................................................................................................................................................. 129
Table 3. 2 Antibodies used for EC cultured with 33% DA FLS-CM .............................................................. 133
Table 3. 3 Antibodies used for ECs stimulated with T cell-derived cytokines .............................................. 133

Chapter 4

Table 4. 1 HC demographics that includes age and gender .......................................................................... 209
Table 4. 2 JIA patients’ demographic, clinical, laboratory features and treatments prior sampling and at
time of sampling ......................................................................................................................................... 210
Table 4. 3 T21 patients’ demographics that includes age and gender ............................................................ 210
Table 4. 4 DA patients’ demographic, clinical, laboratory features and treatments prior sampling and at
time of sampling ......................................................................................................................................... 211
Table 4. 5 Th cell antibody panel * indicates intracellular stains ................................................................. 212
Table 4. 6 Chemokine receptor antibody panel ............................................................................................ 213
Table 4. 7 IFN receptor antibody panel ....................................................................................................... 213
Table 4. 8 Monocyte antibody panel ........................................................................................................... 213
Scientific Abstract

Juvenile idiopathic arthritis (JIA) is the most common form of inflammatory arthritis (IA) in children. However, recently we described a more aggressive, erosive inflammatory form of arthritis of little-known aetiology that occurs 20 times more frequently in children with Down syndrome (DS). The early diagnosis and treatment of IA in children is critical for long-term joint health and normal growth. However, the diagnosis of IA in children with DS, termed, Down syndrome-associated arthritis (DA), is extremely challenging. As a result, it is rarely recognised at onset, and is under-diagnosed. We have previously shown that polyfunctional T cells coexpressing TNF-α, IFN-γ and IL-17a are enriched in children with DA compared to DS or JIA, reflective of the more erosive disease observed clinically. There remains a significant lack of knowledge on the underlying mechanisms of disease pathogenesis. Therefore, the aims of this thesis are to further examine the pathogenic function of immune and stromal cells in DA and investigate their contribution to shaping the inflammatory response in this autoimmune disease.

In Chapter 2, we isolated, for the first time, primary synovial fibroblast (FLS) from the joints of children with DA and examined the effect of T cell-associated cytokines on DA FLS function. TNF-α, IFN-γ and IL-17a alone induced the expression of proinflammatory mediators, metabolic markers and key matrix degrading enzymes in DA FLS. This was paralleled by a significant increase in leukocyte-DA FLS adhesion, which was associated with induced expression of key adhesion molecules and chemokine receptors. We identified that both IL-17a and IFN-γ either potentiate the effect of or synergized with TNF-α to differentially modulate pathogenic function of DA FLS, an effect that was particularly prominent for IFN-γ. Cytokine combinations also induced a shift in Ox-Phos in DA FLS. This led to an increase in the ECAR:OCR ratio, a reduction in the ATP synthesis and max metabolic profile of DA FLS where a switch to a glycolytic profile, with concomitant inhibition of respiratory capacity, in addition to potentiated/synergistic induction of key metabolic markers including GLUT-1, LDHA, HK2 and PKM2. These data demonstrate that key cytokine interactions enhance the aggressive phenotype of DA FLS, particularly TNF-α and IFN-γ, with possible implications for current therapeutic approaches.

The inflamed joint microenvironment is complex due to cell-cell interactions and the secretion of proinflammatory mediators. One of the primary events across all forms of IA, including DA, is dysfunctional neo-angiogenesis. Therefore, in Chapter 3, we examined the effect of conditioned media from primary DA FLS (reflecting the joint microenvironment) on endothelial cell (EC) function, to assess whether soluble mediators that are secreted from DA FLS induce a pathogenic phenotype in ECs. We demonstrated that the DA joint microenvironment promotes EC tube formation, migratory capabilities and leukocyte-EC adhesion, effects that were paralleled by increases in adhesion molecules and chemokine receptor expression. Furthermore, we examined the effect of TNF-α, IL-17a and IFN-γ alone and in combination on EC function. IFN-γ and/or IL-17a potentiated/synergistically induced the effect of TNF-α on the secretion of proinflammatory cytokines. Furthermore, similar to that observed in DA FLS, IFN-γ potentiated/synergistically induced the effect of TNF-α on ICAM-1, and on chemokine receptor expression of CXCR4, CXCR5 and CCR6, associated by a decrease in Claudin 5. Collectively, these data suggest that the DA joint microenvironment induces a pathogenic EC phenotype further facilitating immune cell influx into the joint, which in turn further exacerbates joint inflammation.
In Chapter 4, we demonstrated that children with DA have impaired immune cell frequencies and function. Higher CD8+ T cell frequency and reduced CD4+ T cell frequency was observed. An enrichment of CD4+, CD8+ and CD4- CD8- polyfunctional T cells was also demonstrated in DA, which simultaneously produce TNF-α, IFN-γ, IL-2, GM-CSF and IL-22, however significance was particularly associated with coexpression of TNF-α and IFN-γ. Furthermore, we demonstrated increased frequencies of effector memory and central memory T cells in DA, in contrast to a decrease in naïve T cells. In addition, enhanced homing mechanisms were evident by the observed differential expression of CCR1, CCR2, CCR4 and CXCR3 across the T cell subsets was also demonstrated. Finally, using tSNE analysis, we demonstrated that DA monocytes displayed phenotypically classical CD11c, CD14, CD38 and CD86 with low CD16, CD80, VISTA, PD-1, BTLA and CD15. JIA clusters also displayed a classical monocyte phenotype, in contrast, T21 displayed a non-classical monocyte phenotype. This is consistent with previous studies in adult forms of IA and suggests that monocytes in the systemic circulation of DA and JIA are primed for heightened pro-inflammatory responses.

Overall, this thesis provides greater insight into the pathogenic immune cell mechanisms that are distinct to DA. This along with the stromal cell responses provides further understanding of potential therapeutic strategies that could be effectively targeted in the treatment of DA, particularly TNF-α and IFN-γ signalling. Better understanding of this disease will make biologically and therapeutically meaningful changes for these vulnerable children living with this debilitating disease.
Lay Abstract

Juvenile idiopathic arthritis (JIA) is the most common form of inflammatory arthritis (IA) in children. However, recently we described a more aggressive form of IA in children with Down syndrome (DS), termed Down syndrome associated Arthritis (DA). DA is 20 times more common than JIA, occurring at a rate of 1/50 in children with DS. The early diagnosis and treatment of IA in children is critical for long-term joint health and normal growth. However, the diagnosis of IA in children with DS is extremely challenging. As a result, it is rarely recognised at onset, and is under-diagnosed. Therefore, by the time these children attend a Rheumatology Clinic, they may already have significant joint damage. While many children with DA respond to current treatments, a significant proportion do not respond or have adverse side effect. Therefore, new treatment strategies along with better approaches for early diagnosis are required so that these children can be treated early with the right treatment from the onset, which ultimately will lead to improved quality of life. However, little is known about the proteins, genes and cells that are involved in driving inflammation in the inflamed joint from children with DA. Therefore, this thesis examined immune cells in the blood from children with DA, in addition to specific cells in the joint tissue called synovial fibroblasts (which are the cell type that invade the cartilage and bone) and endothelial cells which allow bad immune cells to enter the joint from the circulation.

Initially we demonstrated that three key proinflammatory proteins induce the synovial fibroblasts to become more invasive, which facilitates their ability to breakdown cartilage and bone within the joint. Furthermore, these proteins activate endothelial cells which allows the immune cells to enter the joint. These immune cells do not function properly and produce more proinflammatory proteins that further enhance the inflammatory response in the joint. This causes the joint tissue to swell resulting in more pain and subsequent disability if not treated early. Interestingly, two of these proteins acted together in their ability to induce inflammation, and this has implications for treatment strategies as targeting both may produce better outcomes. We next identified that synovial fibroblasts and endothelial cells altered the way they use energy within the cell, a process that allows them to maintain their pathogenic inflammatory state. We showed that blockade of these energy pathways, led to resolution of inflammation, therefore there is potential to develop medicines that target both these specific dysfunctional pathways. Finally, we examined immune cells in the circulation of children with DA. We demonstrated that a specific immune cell called a T cell is more activated in children with DA compared to JIA or healthy controls. Indeed, these particular T cells produce more proinflammatory proteins simultaneously, and are known therefore to be more potent in driving the inflammatory response but are also more resistant to treatment. Interestingly, the two most dominant proinflammatory proteins being produced by these T cells are the same two proteins that together drive synovial fibroblast and endothelial cells’ function.

Thus, the research in this thesis represents a significant advancement in our understanding of the disease and identifies distinct immune-synovial cell dysregulation in the pathogenesis of DA. In particular, these data have potential implications for combination therapy or manipulation of metabolic pathways for the treatment of DA. Further studies and support for this condition, will lead to improved diagnostic and prognostic outcomes for these vulnerable children. It also demonstrates the importance of translational research, and the involvement of patients with research, thus we would
like to thank the children and their parents as this research could not have been performed without them.
“If you find something you are passionate about, you’ve got to try. Even if something doesn’t quite work out, disappointment is a temporary thing. Regret lasts forever.”

Gemma Chan

“I intend to make my own way in the world.”

Jo March

“Every time someone steps up and says who they are, the world becomes a better, more interesting place.”

Captain Raymond Holt
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Hand on my heart this was the most challenging and rewarding time of my life. I’ve learnt so much not only about my project but also about myself. I haven’t laughed (and cried) as much as I have in the last 3 years.

Ursula, you are a powerhouse and a compassionate leader breaking the glass ceiling! I usually make life decisions viscerally, so I knew instantly I wanted to be part of your team as soon as I left the interview. This intuition really cemented over our love of Honey Truffle’s sausage rolls and the importance of science communication. Sometimes, it felt like you were more than my mentor – when I came barging into your office with a crisis (no matter how big or small) you remained empathetic. All talks about contingency plans/experiments, fibros, coupled with the shock I don’t share your fondness for Love Island (very aware Love is Blind is not far off) along with some occasional tears I can easily say you’ve been the most incredible boss. Through your guidance and relentless support, you gave me the confidence to be the researcher I am today. Thank you for giving me this opportunity and for your faith in me. I hope I did you justice in the science world. …Dare I say I may have cracked the code on your thought process? My project wouldn’t have existed if it wasn’t for the generous donations from patients and their families. I hope this research will help you and future patients live the life every child deserves. Dr Órla Killeen, Dr Emma MacDermott, Dr Ronan Mullan, Dr Charlene Foley, Prof. Doug Veale, Prof. Eleanor Molloy, Emma, Derek, and everyone who helped… Thank you for your invaluable collaboration in CHI at Crumlin, Tallaght and St Vincent’s.

To be working with such incredibly kind, smart, and funny humans who understand and let your true self come to light is remarkable. Brianne and Órla (can’t forget the fáda), you are the two most jovial people I’ve ever met. Our chats about everything and anything as well as the universal “why are my donors doing opposite things?” kept me going. No-one will understand the struggles of being a single mother to millions of cells. You are both so kind I was delighted when Órla got an offer and I managed to convince Brianne that doing a PhD with us was much better than going back to the US. You should really consider setting up a GoFundMe for your future bakery side hustle. Boop. Órla, I’ll miss the little side stares we give each other when things are overwhelming and that end of day wind-down. You’re so open and caring, at times it felt like you were the older one. I’m very grateful we managed to squeeze in some international conferences together. To the then post-docs now PIs! Mary and Vivi – my dissertation would be a vast space if it wasn’t for your help with the experiments, reviewing, being impromptu supervisors, and everything in between. So much of your time was poured into this, thank you for stepping up to the challenge and for all the hugs and laughs. I’d say you’d need a few nights to relax. The grumpy grandad, how can I forget Conor? We started this journey at the same time so it’s only fitting you have a mental blueprint of all the places in TBSI where I’ve had minor meltdowns. I’ll miss your no-nonsense nature and how you would put on your headphones when the rest of us start to talk about things that are “too girly”. Adam, helping me get the ECs over the finish line. Achilleas, those mammoth immune cell panels.
And to the rest of my lab-mates – Success, Alyssa (the eagle eye detective skills and your gorgeous eye shadows), Megan (a special thanks for enduring my chapters and continuing
Storytime), Aenea, Keelin, Niamh, Aisling, Seán “56 plants” Dixon, Siobhán, Hannah, Barry, Aoife, Andreea, Jean and Dumitru thank you for everything and for making my time here so glorious.

To the home crew and my oldest friends. From the leaving cert, celebrating milestones and achievements, doggy outings, to the tradition that is the annual Christmas dinner it’s heart-warming to know we’re still together after all this time. There are too many of you to name but I do want to give a special mention to Liam, Elaine (I guess you’re ok) and Jess who have witnessed and soothed a lot of the growing pains. What makes our friendship extra sparkly is that while you all have multiple children (fur babies count), you still make the time to check in and to be honest, I don’t think we’ve changed that much. Liam, you get an extra point as you’re one of the few outside of the lab who know the full title of my dissertation.

Felice, my tough love and forever dear friend. Crazy to think our friendship started all those years ago in Lan Kwai Fong. The first night we met you weren’t drinking because you were on meds, I thought “Wow, this woman has some serious self-control”. The inadequacies of our LDR lessened when I moved back to Dublin (you were already home in London), and it became easier to incessantly bombard each other with the smallest details of our everyday lives. I think we both believe in context being key when it comes to catching up – you still wear the crown for queen of voice notes coming in hot at over 15 minutes long. Doing a PhD yourself, you understand the ups and downs of research peppered with everything else life throws at you. You know exactly the kind of person you are, what you deserve and show no shame in owning it. The only falling out we ever had was over Dua Lipa - a fair testament to how ridiculously stubborn and how wonderful we are together. To this day, I’m still shook you thought Ireland was an hour behind the UK.

There are few people in life you meet and instantly click – for me, that person is Tory. I can’t remember where exactly in New York we met but remembered hearing so much about you. From our 1400km Brooklyn to Savannah road trip to galivanting in London and more recently the boujee Dubai and Maldives escapades there are too many stories and memories. The most recent one that lives in my mind rent-free was where we spent a good 20 minutes laughing/ fighting for air after the extremely generous staff brought Tabitha to life then driving her back to the villa. You are the biggest empath with an even bigger heart. I was always wildly in awe of your strength, creative prowess, unabashed fight for women’s rights and how openly you speak your mind. Even though you’re in a completely different industry you still grasped how hard this was and were able to recall all the debacles and details of my experiments! Your letters and thoughtful care packages are a reminder that while there is a literal ocean separating us, I am so glad our lives crossed paths. It’s very rare to know someone with the same sense of humour but I can attest that yours is a shade or two darker, yet oddly enough, more wholesome than mine. While we don’t see each other enough when we do it’s as if nothing’s changed. But most of all, I’m glad to have found a friend who uses the same type of expressions as much as I do.
Louise, by far, one of the purest people I know: tactful with everything you say and do I’m lucky to have a friend like you. We’ve come a long way since Maynooth, but I’m still flabbergasted by how quickly you developed a country accent considering we grew up across the road from each other. Claire Hutton-Williams, another one of my tough love friends. It’s been more than ten years since King’s yet we’re still part of each other’s lives. I do enjoy your insights about life when things are particularly difficult. Like Felice, I am speechless you also thought Ireland was an hour behind (is this an English thing?).

To my Flaming Cheetohs: Aaron and Shaakya. I don’t think I could have survived the last few years without you. Ugly Betty nights galore as well as broadening my horizons in terms of vegan eats - I can never look at seitan chicken popcorn again (RIP). All our road trips and our adventure in London which we will need more of since Aaron abandoned us for UCL. The constant “which tattoo/ piercing should I get?” are sorely missed. Shaakya, (the wittier Teasearcher) where do I begin?! You deserve only the best, with all your quirky mannerisms you always make me chuckle. I think we understand each other so well it’s a bit hard to describe our bond. Whenever I was stressed, you listened, whenever an adventure was to be had you had your boots on ready to go and you’re 100% the biggest food enabler. One day, you’ll finish an entire meal. We still have our wetsuits so maybe we should go for one last sea swim before it gets too Baltic. I cannot wait till you finish yours so we can be the map-scratchers we’re destined to be.

To my siblings. Veronica, the trendsetter, always doing things long before it became fashionable. Our sisterhood is summed up nicely here: “Sibling relationships are weird. I’ll give you my kidney, but you can’t have my charger.” You’ve always marched to the beat of your own drum and never let anyone else doubt who you are. Ponson, the bravest person I know. The giggles and IG food reels kept me going. We’ll find a tin box of the Japanese hard sweets below market price. Haru and Hunter – all the cuddles and cuteness.

Finally, my two biggest cheerleaders: Mam and Dad. This is for you. You’ve always supported my decisions no matter how wild they are. 媽咪, the queen of positivity and goofiness. I think you have some sort of internal radar that bleeps if my nutrition levels are low: you’ll automatically come into my room armed with a plateful of cut fruit, herbal teas, and some new skincare hack you saw on YouTube. Your kind nature and quick wit always made me laugh and you knew how to comfort me when things were tough and you’re definitely the most selfless person around, constantly putting our needs before yours, making sure we have all we needed and wanted. 老豆, the retired life suits you and has made you funnier but one of the ways you show your love is through food. When I was stressed or upset, you would automatically roll up your sleeves and cook one of my favourites (there are so many) without a second thought. You taught me to stand up for myself and for those who can’t and to never give up, no matter what other people think. On that note, I will say we should probably stop making bets with each other considering how you haven’t won yet. I have a feeling you’d like saying that I have a PhD more than I will. Your unquestioning belief in me has led me to take on all the adventures I’ve had so far. Thank you both for letting me be an absolute diva over the last few months and never
making a big deal out of it, for shaping me into the woman I am today and for sacrificing so much so that me, Veronica and Jonson can have everything.

**Honourable mentions:** Brooklyn 99 (especially Gina and Captain Holt), Prof. Derek Doherty, Cicaplast Balm, Euphoria, Mendeley, Graphpad (all about the significance and aesthetics), Lyon’s tea, Alice, Genevieve Beauty and Graham Norton (a man of many talents).
*Increased T cell polyfunctionality and cytokine synergy drive an aggressive synovial fibroblast phenotype in children with Down syndrome-associated arthritis.

**Serena Foo**, Adam Dignam, Mary Canavan, Viviana Marzaioli, Achilleas Floudas, Ronan Mullan, Charlene Foley, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. *Irish Society for Rheumatology (ISR) Dublin (Sep 2023) (oral presentation)*

*Young Investigator of the Year (2023)*

**The effect of the Down syndrome-associated Arthritis (DA) joint microenvironment on endothelial cell function**

**Serena Foo, Adam Dignam**, Niamh Kenny, Mary Canavan, Viviana Marzaioli, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. *European Workshop for Rheumatology Research (EWRR) Dublin (March 2023) (poster presentation)*

**Cytokine synergy perilously drives the aggressive fibroblast phenotype of arthritis in children with Down Syndrome**

**Serena Foo**, Mary Canavan, Viviana Marzaioli, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. *EWRR Dublin (March 2023) (poster presentation)*

**Cytokine synergy enhances synovial fibroblast activation in children with Down’s syndrome-associated arthritis**

**Serena Foo**, Mary Canavan, Viviana Marzaioli, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. *European Alliance of Associations for Rheumatology (EULAR), Copenhagen (June 2022) (poster presentation)*

**Cytokine synergy enhances synovial fibroblast activation in children with Down’s syndrome-associated arthritis**

**Serena Foo**, Mary Canavan, Viviana Marzaioli, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. *EWRR Vienna (May 2022) (poster presentation)*
Cytokine synergy and glycolytic activity used to promote aggressive phenotype in FLS of children with Down syndrome-associated arthritis

Serena Foo, Mary Canavan, Viviana Marzaioli, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. UCD SVHG Translational Medicine Symposium, Dublin (Nov 2021) (poster presentation)

Cytokine synergy and glycolysis used to promote aggressive phenotype in fibroblasts of children with Down’s syndrome-associated arthritis

Serena Foo, Mary Canavan, Achilleas Floudas, Aisling O’ Brien, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. TBSI Host Pathogen Conference, Dublin (Nov 2021) (poster presentation)

Altered T Cell Responses, and Synergistic Regulation of Synovial Fibroblasts Function in Children with Down’s Syndrome-Associated Arthritis

Serena Foo, Achilleas Floudas, Mary Canavan, Aisling O’ Brien, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. American College of Rheumatology (Nov 2021) (poster presentation)

*Cytokine synergy and glycolytic activity used to promote aggressive phenotype in FLS of children with Down syndrome-associated arthritis

Serena Foo, Mary Canavan, Achilleas Floudas, Aisling O’ Brien, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. ISR (Oct 2021) (poster presentation)

*2nd prize poster winner

*Cytokine synergy used to promote aggressive phenotype in fibroblasts in children with Down syndrome-associated arthritis

Serena Foo, Mary Canavan, Achilleas Floudas, Aisling O’ Brien, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. European Congress of Immunology (Sep 2021) (poster presentation)

*Guided poster tour
Increased T cell responses, metabolic activity and fibroblast invasive capacity in children with Down's syndrome-associated arthritis compared to juvenile idiopathic arthritis

Serena Foo, Achilleas Floudas, Mary Canavan, Aisling O’ Brien, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. EULAR (June 2021) (poster presentation)

*Guided poster tour

Aggressive synovial invasiveness and metabolism in children with Down syndrome-associated arthritis

Serena Foo, Achilleas Floudas, Mary Canavan, Aisling O’ Brien, Ronan Mullan, Doug Veale, Emma McDermott, Orla Killeen, Ursula Fearon. IMF (May 2021) (YouTube presentation)

*Shortlisted best presentation

Increased T cell responses, metabolic activity and FLS invasive capacity in children with DA compared to JIA

Serena Foo, Achilleas Floudas, Mary Canavan, Sharon Ansboro, Ronan Mullan, Doug Veale, Emma McDermott, Derek Deely, Charlene Foley, Orla Killeen, Ursula Fearon. UCD Research Symposium (Dec 2020) (poster presentation)

Increased T cell responses, metabolic activity and invasive capacity of fibroblasts in children with DA compared to JIA

Serena Foo, Achilleas Floudas, Mary Canavan, Sharon Ansboro, Ronan Mullan, Doug Veale, Emma McDermott, Derek Deely, Charlene Foley, Orla Killeen, Ursula Fearon. ISR (Sep 2020) (poster presentation)

Conference Bursary granted:

Awarded EULAR Registration Bursary 2021, virtual conference
Abbreviations

3PO 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one
ADAMTS1 A Disintegrin-Like And Metalloprotease (Reprolysin Type) With Thrombospondin Type 1 Motif
AICAR 5-aminomidazole-4-carboxamide ribonucleotide
ANA antinuclear antibody
Ang angiopeotin
APC antigen presenting cell
A-SAA acute-phase protein serum amyloid A
ASPN Asporin
ATP adenosine triphosphate
BAFF B cell activating factor
BTLA B and T lymphocyte attenuator
C complement
CD cluster of differentiation
cDNA complementary DNA
CHI Children’s Health Ireland
Chr21 chromosome 21
CIA collagen-induced arthritis models
CM Conditioned Media
COVID-19 coronavirus disease
csDMARDs Conventional synthetic disease-modifying anti-rheumatic drugs
Ct comparative threshold cycle
CXC Chemokine motif
DA Down syndrome-associated arthritis
DAF decay accelerating factor
DC dendritic cell
DLL4 Delta-like ligand 4
DMARD disease modifying anti-rheumatic drug
DNA deoxyribonucleic acid
DS Down syndrome
EC endothelial cell
ECAR extracellular acidification rate
ECM extracellular matrix
ELF3 E74-like factor 3
ERA Enthesitis-related arthritis
ETC electron transport chain
FADH Flavin adenine dinucleotide
FAP fibroblast activation protein
FBS foetal bovine serum
FCCP trifluorocarbonylcyanide phenylhydrazone
FGF Fibroblast Growth Factor
FLS fibroblast-like synoviocytes
Flt-1 Vascular Endothelial Growth Factor Receptor-1
FMO Fluorescence Minus One Control
G6PD glucose-6-phosphate
G6PI  glucose 6-phosphate isomerase
GAGs  glycosaminoglycans
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
GLUT  glucose transporter
GM-CSF  granulocyte-macrophage colony stimulating factor
GRO  Growth-regulated oncogene
HC  healthy control
hEGF  human epidermal growth factor
HIF  hypoxia-inducible factor
HK2  Hexokinase 2
HLA  human leukocyte antigen
HMGB1  high mobility group box chromosomal protein 1
HPF  high-power fields
HUVECs  Human umbilical vein endothelial cells
IA  Inflammatory arthritis
IBD  Inflammatory bowel disease
ICAM  intracellular adhesion molecule
ICD  intracellular domain
IDO  indoleamine 2,3-dioxygenase
IFN  interferon
Ig  immunoglobulin
IL  interleukin
ILAR  International League of Associations for Rheumatology
Jak  Janus kinase
JIA  Juvenile idiopathic arthritis
JNK  c-Jun N-terminal kinase
kDa  kilo Daltons
L  ligand
LDHA  lactate dehydrogenase
LPS  lipopolysaccharide
MAPK  mitogen-activated protein kinase
MAS  Macrophage activation syndrome
Mcl-1  myeloid cell leukaemia-1
MCP-1  monocyte chemoattract protein-1
MCT4  monocarboxylate transporter 4
MFI  Median Fluorescence Intensity
MHC  major histocompatibility complex
mi  micro
MIP-1  macrophage inflammatory protein-1
MMP  metalloproteinase
MS  Multiple sclerosis
mTOR  mammalian target of rapamycin
MTX  Methotrexate
NADH  nicotinamide adenine dinucleotide
NADPH  Nicotinamide adenine dinucleotide phosphate
NCAM  neural cellular adhesion molecule
NCPR  National Centre for Paediatric Rheumatology
NFκB  Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK   NFκB-inducing kinase
NK    natural killer
NSAID non-steroidal anti-inflammatory drugs
OA    osteoarthritis
OCR   Oxygen consumption rate
oligoJIA Oligoarticular JIA
OPN   osteopontin
OSM   oncostatin M
OxPhos oxidative phosphorylation
PAMPs Pathogen Associated Molecular Patterns
PBMC  Peripheral blood mononuclear cells
PBS   phosphate-buffered saline
PD-1  programmed cell death protein 1
PDGF  platelet-derived growth factor
PDH   pyruvate dehydrogenase
PFA   paraformaldehyde
PFKFB3 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3
PKM2  Pyruvate kinase M2
PolyJIA Polyarticular JIA
POSTN Periostin
PPP   pentose phosphate pathway
PRG4  Proteoglycan 4
PRIME pre-inflammatory mesenchymal
PRRs  pathogen-recognition receptors
PsJIA Psoriatic JIA
R    receptor
RA    Rheumatoid arthritis
RANK  receptor activator of NFκB
Regulated upon Activation, Normal T Cell Expressed and Presumably
Secreted
RANTES
RF    rheumatoid factor
RIPA  Radio-Immunoprecipitation Assay
RNA   Ribonucleic acid
RNaseq RNA sequencing
ROS   reactive oxygen species
RORC  RAR-related orphan receptor C
RT    room temperature
S1P   sphingosine 1 - phosphate
SA    septic arthritis
scRNAseq single cell RNA sequencing
SDS-PAGE sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SF    synovial fluid
sJIA  Systemic-onset JIA
SLC   synovial lining cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SVUH</td>
<td>St Vincent’s University Hospital</td>
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<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
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<td>T21</td>
<td>Trisomy 21</td>
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<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<td>TCZ</td>
<td>Tocilizumab</td>
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<tr>
<td>T&lt;sub&gt;EMRA&lt;/sub&gt;</td>
<td>terminally differentiated</td>
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<td>Tfh</td>
<td>follicular helper T cells</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<td>T helper</td>
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<td>Type 1 helper T</td>
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<td>Th3</td>
<td>Type 3 helper T</td>
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<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>Tph</td>
<td>peripheral helper T cells</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<td>TREC</td>
<td>T cell receptor excision circle</td>
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<td>Treg</td>
<td>regulatory T cells</td>
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<tr>
<td>TREM-1</td>
<td>triggering receptor expressed on myeloid cells 1</td>
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<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
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<tr>
<td>TUH</td>
<td>Tallaght University Hospital</td>
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<tr>
<td>UDPGD</td>
<td>uridine diphosphoglucose dehydrogenase</td>
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<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VISTA</td>
<td>V domain-containing Ig suppressor of T-cell activation</td>
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<td>lambda</td>
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CHAPTER ONE

General Introduction
1.1 History of Juvenile Idiopathic Arthritis

Juvenile idiopathic arthritis (JIA) is the most prevalent paediatric rheumatic disease that occurs before a child’s 16th birthday and persists for at least six weeks (Martini et al., 2019). The word ‘idiopathic’ refers to the unknown cause of a disorder thus the aetiology remains unclear. However, the discovery of prehistoric skeletons with this particular type of chronic arthritis dates back to Viking times in 900AD (Lewis, 1998). In 1545, the first English reference to ‘rheumatism’ in children was described (Phaire, 1545). Interestingly, Cornil reported an autopsy of a 28-year-old woman who was diagnosed with polyarticular arthritis at 12 years old. This showed evidence of ankylosis of some joints and synovial proliferation with notable cartilage destruction in other joints (Cornil, 1864).

Towards the end of the 19th century, there were several small case studies documented, thus it was no longer considered rare (Bouchet, 1875; West, 1881; Barlow, 1883). These reports also showed several different symptoms were associated with JIA possibly due to different forms of the arthritis. While not the first to write about the disease, Still defined the original description of childhood arthritis and was the first to suggest that childhood arthritis is different to adult arthritis (Still, 1897).

Figure 1. Portrait of Sir Francis Ford’s Children Giving a Coin to a Beggar Boy, by Sir William Beechey (circa 1793). This is an early portrayal of JIA and may predate the first medical description. Photo from Tate, London, UK (2015).

1.2 Classification of JIA

The term ‘JIA’ was defined by the International League of Associations for Rheumatology (ILAR). ILAR further classified JIA into seven mutually exclusive subtypes based on the
disease presentation and persists for more than six weeks (Shih et al., 2019). Each subtype is clinically distinct in terms of epidemiology, symptoms, complications, and prognosis.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Definition</th>
<th>Exclusion Criteria</th>
<th>% of all JIA subtypes</th>
</tr>
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<tbody>
<tr>
<td>Enthesitis-related arthritis (ERA)</td>
<td>Arthritis and enthesitis or arthritis or enthesitis plus at least 2 of the following: Sacroiliac tenderness or inflammatory spinal pain HLA-B27 positivity Family history in at least one first degree relative of ankylosing spondylitis, ERA, sacroiliitis with IBD, reactive arthritis or acute anterior uveitis Acute anterior uveitis Onset of arthritis in a male older than 6 years</td>
<td>a, b, c</td>
<td>10-20%</td>
</tr>
<tr>
<td>Oligoarticular JIA (OligoJIA)</td>
<td>Arthritis that affects ≤ 4 joints during the first 6 months of disease. There are 2 subclassifications: Persistent oligoJIA – less than or equal to 4 joints affected. Extended oligoJIA – more than 4 joints affected after the first 6 months</td>
<td>a, b, c, d, e</td>
<td>Up to 50%</td>
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<tr>
<td>Psoriatic JIA (PsJIA)</td>
<td>Arthritis and psoriasis or arthritis and at least 2 of the following: Dactylitis Nail changes Family history in at least one first degree relative of psoriasis 50% antinuclear antibodies (ANA) positive 10% of patients develop uveitis</td>
<td>b, c, d, e</td>
<td>5-10%</td>
</tr>
<tr>
<td>RF negative Polyarticular JIA (RF- polyJIA)</td>
<td>Arthritis that affects ≥5 joints within the first 6 months of disease. Rheumatoid Factor (RF) negative 40% ANA positive 10-14% of patients develop uveitis</td>
<td>a, b, c, d, e</td>
<td>20%</td>
</tr>
<tr>
<td>RF positive Polyarticular JIA (RF+ polyJIA)</td>
<td>Arthritis that affects ≥5 joints within the first 6 months of disease. RF positive Linked to Rheumatoid nodules</td>
<td>a, c, d, e</td>
<td>≤ 5%</td>
</tr>
<tr>
<td>Systemic-onset JIA (SJIA)</td>
<td>Arthritis with or preceded by daily fever of at least 2 weeks’ duration, that persists daily for at least 3 days, and accompanied by one or more of the following. Temporary, non-fixed, erythematous rash (salmon-pink) Generalised lymphadenopathy Hepatomegaly and/or splenomegaly</td>
<td>a, b, d, e</td>
<td>5-10%</td>
</tr>
<tr>
<td>Undifferentiated arthritis</td>
<td>Arthritis that does not fulfil inclusion criteria for any category or is excluded by fulfilling criteria for more than one category.</td>
<td></td>
<td>10-20%</td>
</tr>
</tbody>
</table>

**Table 1. ILAR classification of seven JIA subtypes.** This table was generated using the following sources: (Mellins et al. 2011; Petty et al., 2015; Ekelund et al., 2017; Gmuca et al., 2017).
1.3 Epidemiology/ prevalence of JIA

The European prevalence of JIA is 1 in 1000 children (Hayward et al., 2009; Thierry et al., 2013). Globally, there is a major disparity of JIA prevalence between regions and ethnicities. The first epidemiological studies performed in Western Europe and Northern America identified how difficult it was to strictly define these numbers. Consequently, the results suggested that JIA prevalence was between 7-148 per 100,000 (Laaksonen, 1966; Saurenmann et al., 2007).

Comparison of these studies is difficult due to the use of different diagnostic criteria and study methods. These widely disparate rates may reflect genetic susceptibilities, environmental factors, and possibly other unknown traits. Currently, 1 in 10,000 Irish children are diagnosed with JIA (Arthritis Ireland, 2023).

1.4 Genetic factors in JIA

JIA is a complex disease, the cause of which is most likely a combination of both genetic and environmental influences. Ellis et al. (2010) describe an augmented prevalence of JIA in twins and siblings of JIA probands. Concordance rates in monozygotic twins are predicted at 25-40%. However, the proportion of both twins affected by JIA are higher than the expected rate in monozygotic twins. These findings highlight the importance for shared genetic composition. In sibling pairs who have JIA and are not twins, there is a significant correlation between the siblings regarding age at onset of disease, disease duration, with a prevalence rate that is 15-30 times that of the general population (Ellis et al., 2010). The twin studies identify the importance of genetic attributes in JIA. Since monozygotic twins are genetically identical and if the chance of developing JIA was based strictly on genetics it would be thought that monozygotic twins would also share the same risk of developing JIA. As stated previously, the concordance rates are below 50% which suggests that both genetics and environmental influences contribute to the development of JIA. The strongest genetic associations with childhood arthritis are the genes that lie on the major histocompatibility complex (MHC), the human leukocyte antigen (HLA) complex (Edmonds, 1973).
“The antigen-presenting molecules encoded by the MHC class I and class II genes are cell-
surface glycoproteins that bind intracellular and extracellular peptides, respectively. The human MHC is located on chromosome 6 and contains more than 200 genes. The human MHC-encoded glycoproteins are known as HLA and are specialized in presentation of short peptides to T cells and play a key role in the body’s immune defence.”

(Mosaad, 2015).

Extensive and well-defined associations with HLA alleles in JIA have been examined for over 25 years. The most well-characterised JIA associations are the genes of the MHC class I and class 2 loci. Class I loci encompass:

- HLA-A
- HLA-B
- HLA-C

The first known documentation of the link between HLA alleles and JIA is HLA-B27 which is associated with ERA. The HLA-A2 allele (HLA-A*0201) is associated with a few JIA subtypes, most notably in oligo JIA and early-age onset JIA (Brunner, 1993; Murray et al., 1996). Hollenbach et al. (2011) identified that HLA-C*0202 correlates with persistent oligo JIA (Hollenbach et al., 2011).

Modern developments in biomedical technology have advanced our understanding of the genetic influences in JIA. Specifically, the HLA region in addition to PTPN22 and PTPN2 and non-HLA loci such as LACC1 are now known to be JIA risk loci (Thompson et al., 2010; Nigrovic et al., 2019).

Many other genes have been implicated in JIA depending on the specific endotype. These include toll-like receptor (TLR)-4, NLRP3, CXCR4 for ERA (Weiss, 2016), PRR9_LOR, ILDR1_CD86, WDFY4, PTH1R, RNF215, AHI1_LINC00271, Janus kinase (JAK) 1, LINC00951, HBP1 for oligoJIA (McIntosh et al., 2017), MHC Class II and III, LOC284661 (De Silvestri et
in addition to HLA-DRB1 amino acid position 13 for RF+ polyJIA (Hinks et al., 2017).

1.5 Environmental factors of JIA
Breast milk has been shown to provide immunological advantages for the growing baby. This specific nutrition source for new-borns contains a vast array of immune-active molecules that give protection against infections early in life, in addition to forming appropriate mucosal immune responses. Protective factors include soluble immunoglobulin (Ig)A, IgG, interleukin (IL)-10, Transforming Growth Factor-β (TGF-β), and defensins (Hennet et al., 2016). Furthermore, imprinting events also occur during the first months of life suggesting that breast milk is crucial in influencing potential long-term health benefits (Borba et al., 2017). Mason et al. (1995) demonstrated that breastfeeding had a protective effect against JIA (Mason et al., 1995). Although, some other studies found no significant difference between breastfeeding in children with JIA and the healthy controls (HC) (Kasapcopur et al., 1998; Koker et al., 2022). In contrast, Hyrich et al. (2015) reported that breastfed children had a lower median age at onset of arthritis and lower pain at presentation. Furthermore, breastfed children were shown to be more likely associated with the ERA or psJIA pathotypes (Hyrich et al., 2015). Consistent with Hyrich’s study, another group showed that children who were breastfed for less than four months had an increased risk of developing JIA (Kindgren et al., 2017).

Breast milk has natural prebiotics and probiotics and is known to be advantageous to infants’ gut microbiome leading to elevated levels of commensal Bifidobacteria and lower levels of potentially pathogenic commensal bacteria (Bridgman et al., 2017). Numerous studies have suggested that the microbiome composition in children with JIA differs from healthy children. Tejesvi et al. (2016) examined the microbiome profiles of 30 children with JIA to HC and demonstrated that a specific bacterium, phylum Firmcutes, was significantly lower in children with JIA, with increased levels of Bacteroidetes observed in JIA compared to HC (Tejesvi et al., 2016).

Previous reports suggest that maternal smoking is not associated with the development of JIA (Shenoi et al., 2015; Horton et al., 2019). On the contrary, when compared to adult arthritis, personal smoking is a major risk factor for rheumatoid arthritis (RA) (Wieczorek et al., 2022).
Another element to consider is the hygiene hypothesis. When living with older siblings one is more likely to be exposed to the bacteria and infections. In turn, these infections may protect younger siblings from autoimmune diseases by priming their immune system (Horton et al., 2019).

There is some evidence to suggest that streptococcal infection may induce JIA or cause spikes in disease activity (Ellis et al., 2010). In a study of 173 children with arthritis, only 9% of the children with JIA tested positive for streptococcal infection. In contrast, another study that investigated all JIA cases treated across a ten-year duration found an elevated level of co-occurrence of disease flares with streptococcal infection: 22% of JIA patients had two or more disease flares with streptococcal infection (Barash et al., 2007). Thus, these studies suggest that *Streptococcus* may influence the course of the disease progression (Barash et al., 2007).

### 1.6 Comorbidities of JIA

JIA may be associated with a myriad of complications – uveitis being the most frequent followed by atopic dermatitis and rhinitis. Pain disorders, as well as depression and anxiety, were identified as the next most common complications of JIA. Other comorbidities may include allergic asthma, amyloidosis, anaemia, hypertension atopic dermatitis, autoimmune thyroiditis, and fibromyalgia (Horneff et al., 2022) - this analysis was conducted in a German population. Analysis of a UK cohort demonstrated varicella infection was the most common complication followed by uveitis and tuberculosis (Kearsley-Fleet et al., 2022). Regardless of geographical location, Type1 Diabetes Mellitus (T1D) irrefutably occurs more frequently in children with JIA compared to the healthy population (Schenck et al., 2018; Lee et al., 2019).

Macrophage activation syndrome (MAS) is a potentially fatal complication of sJIA. This is caused by overactive and expansive T cells and macrophages displaying hemophagocytic activity (Schulert et al., 2018). Some of the severe clinical complications that may arise are pericarditis and myocarditis (Hakim et al., 2017). In terms of MAS detection, Mizuta et al. (2019) showed that elevated levels of serum CXCL9 were observed in sJIA-associated MAS.
(Mizuta et al., 2019). However, the main detection of MAS is through C-reactive protein (CRP) as a peripheral biomarker (Di Benedetto et al., 2020).

1.7 Treatment for JIA
Methotrexate (MTX) is a disease modifying anti-rheumatic drug (DMARD) most commonly prescribed first-line treatment for JIA. It may be taken orally or by subcutaneous injection for RF+ patients, in addition, most patients are given non-steroidal anti-inflammatory drugs (NSAID) at least once during their treatment course (Davies et al., 2016). Biologic therapy, mainly tumour necrosis factor (TNF) inhibitors, are also used in combination with MTX (Berthold et al., 2019). For patients who have adverse side effects in response to MTX or who do not benefit from MTX, the next line of treatment is anti-TNF therapy which has been shown to be very effective in reducing disease activity and preventing disease progression (Mistry et al., 2019). Tocilizumab is a recombinant monoclonal antibody that binds to the IL-6 receptor. This targeted biological therapy was effective and well tolerated in children with sJIA or polyJIA over two years of age (Machado et al., 2017). More recently, tofacitinib (a Jak-signal transducing and activator of transcription (STAT) inhibitor) taken orally has also proved to be effective, with results demonstrating 1 in 2 JIA patients achieving a complete response and 1 in 3 had a partial response (Kostik et al., 2022).

1.8 History of Down syndrome
Down Syndrome (DS) is a disorder caused by a triplication of chromosome 21 (chr21) hence its synonym: Trisomy 21 (T21). Quite remarkably, the earliest evidence of DS dates to 500BC. Bernal and Briceno described how the ancient culture of Tumaco-La Tolita (which lies in between Colombia and Ecuador) had pottery with faces depicting that of children with DS (Bernal et al., 2006). Despite the fact DS was portrayed in ancient ceramics, it was first clinically recognised by Dr John Langdon Down in 1866 who it is named after.
In 1959, the chromosome triplication was described by Dr Jerome Lejeune (Akhtar et al., 2018). It is characterised by the presence of an extra full or partial chr21, but the molecular mechanisms underlying the genetic disorder is still unclear. Three forms of DS have been described (i) T21 which occurs in 95% of cases (Hassold et al., 2000; Perkins, 2017), (ii) chromosomal translocations which occurs in ~3-4% of cases (Antonarakis et al., 2004; Flores-Ramírez et al., 2015), and finally (iii) mosaicism which occurs in less than 2% of cases (Antonarakis et al., 2004; Flores-Ramírez et al., 2015). DS is the most common genetic disorder with a global incidence rate of 1 in 1000 while the Irish incidence rate is 1 in 444 – the highest rate in Europe (Down syndrome Ireland, 2023). Prenatal diagnosis is now possible without invasive measures. Cell-free prenatal screening and parallel sequencing of maternal plasma cell-free deoxyribonuclease (DNA) is insightful for parents who could be a carrier of genetic translocation or for a female who is of high risk (Chitty et al., 2015). The earlier, invasive alternatives included amniocentesis whereby a sample of the amniotic fluid was taken for assessment (Alfirevic et al., 2017) or chorionic villus sampling where the chorionic villi is removed from the placenta for examination (Lim et
al., 2019). Postnatal diagnosis at birth is possible where the baby is examined for body habitus and physiognomic features that is usually followed by muscular hypotonia. When DS is suspected post-birth a karyotype genetic test is performed to confirm the diagnosis (Bull, 2020).

1.9 Comorbidities of DS

DS has a multitude of complexities that include a broad spectrum of intellectual disability, speech, memory and learning impairment (Baburamani et al., 2019). Approximately half of individuals with DS experience cardiovascular problems such as hypertension, coronary heart disease and heart failure (Startin et al., 2020). They also suffer from higher rates of gastrointestinal tract, eye and ear issues compared to children without DS (Jackson et al., 2019; Startin et al., 2020; Chicoine et al., 2021).

Cognitively speaking, DS is linked to language (Martin et al., 2009), cognition (Silverman, 2007) and memory (Jarrold et al., 2000) impairment. Additionally, a significant 95.7% of the cohort develop dementia before the age of 68 (McCarron et al., 2017). Other difficulties include microcephaly (the baby’s head is smaller compared to babies of the same sex and age), autism and congenital heart abnormalities (Startin et al., 2020). Respiratory issues such as pneumonia and bronchitis arise (Chicoine et al., 2021) and could be associated with a higher risk of developing dysphagia (difficulties in swallowing) (Jackson et al., 2019).

Furthermore, children with DS have an increased propensity to develop certain cancers such as leukaemia (Bercovich et al., 2008).

With regard to autoimmunity, there is an increased association of individuals with DS and development of autoimmune diseases including thyroid dysfunction (Startin et al., 2020), coeliac disease (Du et al., 2018) and T1D (Dierssen et al., 2020).

1.10 Genotype/ phenotype of DS

As highlighted above, DS is caused by the triplication of a full or partial chr21 and it is also the most common chromosomal disorder globally. Despite the many advances in
understanding this syndrome, the molecular mechanisms underlying the pathogenesis of DS still needs to be elucidated. With 48 million nucleotides making up for 1-1.5% of the human genome, chr21 is the smallest human autosome (Chernus et al., 2019). There are over 400 genes located on chr21. DS is typically caused by a cell division error (non-disjunction) that results in an embryo with three copies of chr21 in every cell. This type of DS is called T21 and accounts for roughly 94% of cases (Epstein, 2001).

Approximately 3-4% of cases are caused by chromosomal translocations. This type of DS occurs when a section of chr21 is transferred to another chromosome (normally, chromosome 14 or 15) and can also be inherited. When the translocated chromosome is inherited along with the two usual copies of chr21, DS will occur (Flores-Ramírez et al., 2015). The sex of the parent who is the carrier of the rearranged chromosome determines if the translocation will be passed on. A maternal carrier yields a risk of 12% compared to 3% for paternal carriers (Flores-Ramírez et al., 2015).

Lastly, the third and rarest form of DS which accounts for 2% of cases is termed mosaicism. While full T21 means an extra chr21 in all cells, mosaic DS defines that the extra chromosome is only present in some cells. This is caused by irregular cell division after fertilization and creates one set of cells that possess normal chromosomes and another set with T21 (Flores-Ramírez et al., 2015).

While T21 yields the duplication of more than 400 genes (Pérez-Villarreal et al., 2022), there are also chr21 genes which are downregulated as well as dysregulated genes found on other chromosomes (Letourneau et al., 2014).

There are two different hypotheses which could explain the genetic foundation of DS and the link of different genotypes with the phenotypes. The first is gene dosage imbalance where there is a heightened concentration or number of chr21 resulting in more gene expansion (Baburamani et al., 2019). The second theory is called amplified development instability – the presence of the extra chr21 disrupts the homeostasis of the entire genome (Vilardell et al., 2011).

Several groups have performed gene expression studies to decipher which of the two hypotheses described above are the main mechanisms that define the DS phenotype. These experiments included different methodological approaches with no definitive
result for either hypothesis (Volk et al., 2013; Zhao et al., 2016; Liu et al., 2017). Thus it is possible that DS phenotype is caused by both theories (Antonarakis et al., 2001). Interestingly Vilardell et al. (2011) and Guedj et al. (2016) used meta-analysis and reported that most of the dysregulated genes in DS are not located on chr21 (Vilardell et al., 2011; Guedj et al., 2016). However, chr21 has the largest number of overexpressed genes – this indicates that trisomic genes significantly affect alterations in global gene expression. Consistent with these studies, the highest number of differentially expressed genes map to the 21q11-21q22.3 chromosomal location (Vilardell et al., 2011; Guedj et al., 2016).

The physical characteristics that define DS include a flat nose bridge, midfacial hypoplasia and a tendency to protrude the tongue (Epstein, 1986). Another prominent feature is their short stature (Sherman et al., 2022) and hypotonia (Boutot et al., 2018).

1.11 History of Down syndrome-associated arthritis
Children with DS are now known to have greater risk of developing inflammatory arthritis (IA). Until very recently, there was minimal information regarding this type of IA apart from two separate case studies in 1982 (Herring et al., 1982; Sherk et al., 1982). Yancey’s group were the first to describe the JIA-like arthropathy in detail when they reported a case series with seven children and used the term “arthropathy of Down Syndrome” (Yancey et al., 1984). Until recently, the largest case series reported involved nine children with Down syndrome-associated arthritis (DA) (Olson et al., 1990).

Over the last 7 years, our clinical collaborators at Children’s Health Ireland (CHI) at Crumlin have identified the world’s largest cohort of children with DA (Foley et al., 2019) based on a nationwide musculoskeletal screening assessment in Ireland. Of 503 children under 21 years of age screened with DS, Foley et al. (2019) demonstrated a prevalence rate of IA in children with DS of 1 in 50, significantly greater than that previously documented (Olson et al., 1990; Padmakumar et al., 2002; Juj et al., 2009) – these studies had major limitations as their cohort range is n=9-87.

A significant delay in diagnosis of DA was observed. Most children presented with polyarticular (poly) RF- arthritis with a predominance in the small joints of the hands and
wrists. Interestingly, 75% of the cases were identified due to this nationwide approach (Foley et al., 2019). This shows how common DA is, yet it is usually under or misdiagnosed and unfortunately remains underreported in many cases.

1.12 Clinical differences between DA and JIA
In the study by Foley et al. (2019) they describe a significantly higher prevalence rate of 1 in 50 in DA compared to 1 in 1000 in JIA, rendering DA to be significantly more prevalent than JIA (Foley et al., 2019). The same study demonstrated a significant delay in diagnosis, on average 1.7 years later compared to their JIA counterparts. Due to the linguistic problems children with DS have, they are often incapable of clearly communicating that they are in pain.

While JIA has seven different clinical subtypes (as described in Table 1.1) DA has three main clinical subtypes, including the major subtype (poly RF-) which accounts for 81% of children with DA, followed by the second most dominant subtype (psoriatic) (16%) and the third subtype (oligoarthritis) (3%) (Foley et al., 2019).

Figure 1.3 The different subtypes between DA (A-DS) and JIA. DA has one main subtype: Poly RF-, followed by psoriatic and oligo. JIA has seven distinct subtypes: Extended oligo, Oligo, Psoriatic, Poly RF-, sJIA and ERA. Taken from Foley et al. (2019).

Additionally, Foley et al. (2019) documented several musculoskeletal anomalies for the two cohorts – DA scored higher in all subcategories, with significantly higher scores observed for erosive disease and soft tissue swelling compared to JIA. Despite the higher
erosive disease associated with DA, erythrocyte sediment rate (ESR) and CRP levels were lower in DA compared to JIA, making it harder to diagnose DA based on systemic inflammatory markers (Foley et al., 2019; Foley et al., 2019).

1.13 Comorbidities of DA

DA is a major comorbidity of DS.

When it comes to infection, one important difference to note between JIA and DA is that children with JIA have a higher risk of developing uveitis compared to their DA counterparts (Jones et al., 2019). Weight gain or obesity and possibly scoliosis was also linked to DA (Foley et al., 2019; Nicek et al., 2020).

1.14 Normal synovium

The normal synovial joint is a specialised structure known as a diarthrodial joint, that allows the frictionless and stable movement of two opposing bones. The articular cartilage lining opposing bone heads are separated by joint space lined by synovial membrane. The normal synovium is a membranous structure that lines the joint capsule of the diarthrodial joint, the smooth thin intimal lining layer, 0.5-5mm thick, maintains both the volume and composition of the synovial fluid (SF) which is critical for lubrication of the joint space (Smith, 2012; Hui et al., 2012). The synovium can be sub-divided into two main regions:

- the intima (or synovial lining layer) and
- the sub-intima (or sub-lining layer).

The lining layer is comprised of synovial lining cells (SLCs) that are divided into two cell types:

- Type A macrophage-like synoviocytes and
- Type B fibroblast-like synoviocytes (FLS)

(Barland et al., 1962; Veale et al., 2017).
The lining layer comprises of ~20% type A macrophages and 80% type B FLS. FLS are stromal cells that secrete key proteins including collagens, fibronectin, laminin, and proteoglycans required for the extracellular matrix (ECM), while the macrophages are involved in clearing cellular debris. The sub-lining layer is relatively acellular in the normal joint and composed mainly of loose connective tissue, fibrous collagenase tissue, adipose tissue which is nourished by an embedded microvascular vascular network (Walsh et al., 2001; Smith, 2012; Fearon et al., 2021). This microvascular layer is present in the sub-lining, but absent in the lining layer, and supplies molecules, nutrients and oxygen that are required for cell function and tissue integrity. In the normal synovium, these vessels are mature and stable, with an intact pericyte layer (Kennedy et al., 2010). The O₂ level of the normal synovium is ~7% (Ng et al., 2010). The sub-lining layer also comprises of mesenchymal stem cells, which have the capacity, depending on environmental cues, to differentiate into cell/tissue types including bone, cartilage and fat in vitro thus suggesting the ability to continually renew and maintain structure and integrity of the tissue structures within the joint (Bentley et al., 1975; De Bari et al., 2001; Sakaguchi et al., 2005; Fearon et al., 2021). As the synovial joint is continuously subjected to minor trauma and weight-bearing stresses, the ability to undergo constant synthesis and remodelling of bone/cartilage and removal of intra-articular debris by the lining layer macrophages is critically important for normal joint function (Iwanaga et al., 2000). Finally, to protect the normal joint and for normal function, the synovial membrane secretes hyaluronic acid and lubricin. This allows free movement of the cartilage surfaces that counteract the daily frictional forces that cartilage is subjected to, particularly in weight-bearing joints (Kemble et al., 2021).

1.15 Inflamed synovium

Synovial joint inflammation is one of the most distinctive components of inflammatory rheumatic diseases and is a central feature to DA and JIA. It is where joint destruction and immune dysregulation occurs. While the initial trigger involved in driving the inflammatory response remains undefined, one of the earliest events to occur is neo-angiogenesis: the formation of new blood vessels (Shen et al., 2020). Furthermore, the newly formed blood vessels are impaired which lead to endothelial cell (EC) activation and
sprouting, creating an ideal environment for promoting immune cell recruitment (Fearon et al., 2019). This facilitates the infiltration of immune cells such as B cells, T cells, monocytes, dendritic cells (DCs), natural killer (NK) cells and mast cells into the synovium (Pillinger et al., 1995; Tak et al., 2000; Fearon et al., 2003; McGarry et al., 2017; Orr et al., 2017; Fearon, et al., 2021). Depending on the environmental cues within the synovium, the infiltrating monocytes differentiate into macrophages and DCs, further contributing to the inflammatory response (Yu et al., 2017; Rana et al., 2018). In turn, these cells release proinflammatory mediators which activate FLS, further potentiating the degradation of the adjacent cartilage and bone (Guo et al., 2020; Makkar et al., 2020). The profile of the proteins released include proinflammatory cytokines and chemokines released by monocytes, macrophages, DC, T cells, B cells and stromal cells (Raza et al., 2005; Kim et al., 2020). These mediators entail IL-1β, IL-6, IL-10, TNF-α, IL-8, IL-17a, Interferon-gamma (IFN-γ), monocyte chemoattract protein-1 (MCP-1), chemokines, vascular endothelial growth factor (VEGF), growth factors, albumins and globulins to name a few (Lettesjö et al., 1998; Raza et al., 2005; Hui et al., 2012). Qu et al. (1994) suggested that local proliferation of synoviocytes occurs in the synovial lining which greatly contributes to hyperplasia in the rheumatoid synovium (Qu et al., 1994). In addition, Firestein et al. (1995) demonstrated that macrophages and some FLS are apoptotic in situ but in IA transform from a dormant state to an “imprinted aggressor” phase resistant to apoptosis (Firestein et al., 1995; Bottini et al., 2013; Ospelt, 2017; Karami et al., 2020; Kemble et al., 2021). This results in the synovial lining layer becoming hyperplastic where the cell depth increases from 2-3 cells thick to 8-10 cells in depth, leading to a tumour-like ‘pannus formation’. This ‘tumour-like pannus’ induces cartilage damage and joint degradation (Youssef et al., 1998; Bartok et al., 2010; Smollen et al., 2016; Veale et al., 2017; Fearon et al., 2021).
FLS produce proinflammatory mediators which include cytokines and chemokines, in addition to matrix degrading enzymes such as matrix metalloproteases (MMPs), cathepsins, aggregans and collagenases (Masoumi et al., 2020). These factors exacerbate bone and cartilage damage causing further chronic pain and disability. This inflammatory microenvironment also drives cell differentiation including the differentiation of monocytes into osteoclasts – the only cell type known to reabsorb bone which leads to further impairment and bone erosion (O’Brien et al., 2016). In addition, migrating FLS express podoplanin but do not express cluster of differentiation (CD)248 which is a sublining marker (Croft et al., 2016). However, it is now known that FLS are plastic and respond to microenvironmental stimuli that may dictate the position within the synovium and their function (Kemble et al., 2021).

Recent single cell ribonuclease acid (RNA) sequencing (scRNAseq) data highlights the complex microenvironment of the inflamed joint. Interestingly, enrichment in T cell activation and differentiation pathways occur before the onset of RA. Recent studies have shown that a switch in protective to pathogenic polyfunctional T cells was not only observed in RA but also in subjects ‘at-risk’ of developing RA, thus alteration in pathogenic mechanisms pre-date that of clinical manifestations of disease (Floudas et al., 2022). Another report demonstrated that synovial plasma cells may not stem from local memory B cell pool due to the different lambda (λ) and kappa (κ) Ig light chain usage (Floudas et
A study by Croft et al. (2019) highlight that FLS from both experimental arthritis mouse models and in the RA synovium have an increased expression of fibroblast activation protein alpha (FAPα) (Croft et al., 2019). Furthermore, scRNAseq data shows that within the FAPα population there are two unique FLS subsets: FAPα+ Thy (CD90)+ FLS found in the synovial sub-lining and FAPα+ Thy- FLS which are localised to the synovial lining (Croft et al., 2019).

Furthermore, a recent study by Micheroli et al. (2022) identified four main RA FLS clusters: (i) Proteoglycan 4 (PRG4+) lining FLS: CD55, MMP3, PRG4 and Thy-, (ii) Chemokine motif (CXC) ligand (L) (CXCL12)+ sub-lining FLS: CXCL12, C-C motif (CC) L2, A Disintegrin-Like And Metalloprotease (Reprolysin Type) With Thrombospondin Type 1 Motif (ADAMTS1) and Thy^low^, (iii) CXCL14+ sub-lining FLS: CXCL14, complement (c) 3, CD34, Asporin (ASPN), Thy1 and (iv) Periostin (POSTN)+ sub-lining FLS: POSTN, collagen genes, Thy1. Specifically, this study identified that CXCL12+ and POSTN+ FLS were mainly found in the fibroid RA pathotype while PRG4+ FLS were prominent in the myeloid RA pathotype (Micheroli et al., 2022).

Interestingly, Notch3 and Notch target genes are notably increased in RA FLS, Notch3 signalling has been shown to dictate the differentiation of sub-lining Thy1+ FLS thereby shaping FLS identity and pathogenic function within the synovium (Wei et al., 2020).
Figure 1.5 The healthy joint versus the inflamed joint or synovitis. In rheumatic diseases, increased neo-angiogenesis facilitates the influx of immune cells which in turn induces synovial membrane hyperplasia. The invading expanding synovial pannus migrates towards and invades articular cartilage and bone (made with BioRender).

Most of the above synovial studies have focused on adult forms of IA. In the context of IA in children (JIA), studies have shown that while there are the usual T and B cell synovial infiltrates (Finnegan et al., 2011), there are changes in B cell subpopulations, differentiation and homeostasis in the JIA SF (Moura et al., 2022). Similar to RA, hypoxia is prevalent in the JIA synovial environment (Bosco et al., 2009) where the JIA inflamed synovium is hallmarked by a lack of oxygen and induced activation of hypoxia-inducible factor 1α (HIF-1α) and HIF-2α in joint monocytes (Bosco et al., 2009). As such, HIF-1α was demonstrated to induce the transcription of glycolytic enzymes and drive proinflammatory IL-1β production (Tannahill et al., 2013). The hypoxic environment induces CCL20 in JIA synovial monocytes suggesting that hypoxia and immune cell infiltration have a positive relationship (Wu et al., 2021). These monocytes also produce high levels of proangiogenic VEGF and osteopontin (OPN) which are required for neovascularization, cell migration and monocyte recruitment (Bosco et al., 2009). In the oligoJIA hypoxic joint, synovial macrophages show increased expression of the triggering receptor expressed on myeloid cells 1 (TREM-1). TREM-1 is an hypoxia-inducible gene and promotes proinflammatory reprogramming in this inflamed microenvironment (Raggi et
Additionally, proinflammatory high mobility group box chromosomal protein 1 (HMGB1) is increased in the JIA synovium (Schierbeck et al., 2013), expression of which is induced in FLS under hypoxic conditions (Hamada et al., 2008). In turn, HMGB1 interacts with lipopolysaccharide (LPS), IL-1α or IL-1β potentiating the induction of proinflammatory mediators including TNF, IL-6, IL-8 and MMP-3 in RA FLS and osteoarthritis (OA) FLS (Wähämaa et al., 2011).

However, in the context of DA, our group is the only study to have examined the synovium of children with DA. Although numbers were small, our previous work demonstrated that the lining layer thickness is increased in DA compared to JIA. In addition we demonstrated an increase in number of blood vessels and immune cells including CD3+ T cells and CD68+ macrophages (Foley et al., 2020). This reflects the clinical aggressive phenotype we observed in DA (Foley et al., 2019). DA was found to have a higher restrictive joint count and scored higher in erosive tissue and soft tissue swelling compared to JIA (Foley et al., 2019; Foley et al., 2019, 2020).

![Figure 1. 6 Macroscopic and microscopic characteristics of an DA patient.](image)

Microscopic images of the synovium HC compared to DA synovium, demonstrating increased numbers of CD3+ T cells, CD20+ B cells and CD68+ macrophages. Taken from Foley et al. (2020).

Many studies have shown that the SF cellular and soluble mediators reflect the pathogenic cellular phenotype in the joint. Recent studies using advanced RNAseq, and single cell analysis has shown that the inflamed joint is much more complex than previously thought, displaying many different subpopulations for each cell type, which may differ across pathotypes and compartments within the joint. For instance, while both RA SF and synovium have high frequency of CD68+ macrophages the subpopulations differ: CD163+ CD206+ macrophages are dominant in the synovium while CD163- CD206- macrophages
are dominant in the SF (Hanlon et al., 2020). In contrast, T cell subtype analysis are similar in synovium compared to SF (Floudas et al., 2022), suggesting that phenotypes for specific cell types may be site-specific. Inflammation increases the permeability of the synovium, rendering it unable to identify which proteins should enter or leave (Kushner et al., 1971; Pejovic et al., 1995; Mccarty et al., 2011; Aguilar-Cazares et al., 2019). Interestingly, Culemann et al. (2019) demonstrated a distinct population of CX3CR1+ tissue-resident macrophages protects the healthy joint by forming an internal immunological barrier in the lining layer of the synovium, thus it protects the joint from site-specific insults (Culemann et al., 2019). While it is known that FLS produce hyaluronic acid to lubricate the joint, studies have shown that during inflammation the molecular weight of hyaluronic acid is reduced and is almost absent in arthritic joints (Sato et al., 1988; Hui et al., 2012).

### 1.16 Angiogenesis

Angiogenesis is a fundamental process that contributes to normal physiological events such as embryogenesis, growth and tissue repair, but is significantly dysregulated in IA pathogenesis (Walsh et al., 2001; Balogh et al., 2019). The process of new capillaries sprouting from existing blood vessels is called ‘sprouting angiogenesis’ and is one of the earliest events to occur in IA (Fearon et al., 2003). The early development of a rich vascular network provides nutrients required for the expansion of resident cells and facilitates the infiltration of inflammatory leukocytes into the joint to promote disease progression and bone destruction (Szekanecz et al., 2005, 2007; Lainer-Carr et al., 2007; Kennedy et al., 2010; MacDonald et al., 2018; Balogh et al., 2019).

ECs are maintained in a quiescent state until exposed to proangiogenic mediators leading to EC differentiation, proliferation, migration, tip-stalk cell selection and sprout elongation to form new vascular networks (Geudens et al., 2011; Potente et al., 2011). One of the key proangiogenic mediators is VEGF and is thought to be the main angiogenic ‘on switch’ (Claesson-Welsh, 2016). Studies have also observed differential expression of many other angiogenic factors in IA including angiopoietin (Ang)1, Ang2, basic Fibroblast Growth Factor (bFGF), TNF-α, IL-8, TGF-β, IL-18 and IL-1β (Creamer et al., 1997; Fearon et al., 1999; Fraser et al., 2001; Fearon et al., 2003; Kennedy et al., 2010; Szekanecz et al., 2019).
VEGF is secreted by several cell types e.g. neutrophils, platelets, macrophages, DCs, activated T cells, pericytes, and FLS (Lapeyre-Prost et al., 2017). Interestingly, many of the FLS activation pathways promote VEGF synthesis including: TNF-α, IL-17, TLR stimulation, CD40 ligation, Jak-STAT and hypoxia (Elshabrawy et al., 2015; Aguilar-Cazares et al., 2019; He et al., 2020). Additionally, chemokines such as MCP-1 and CX3CL1 direct immune cells to the site of injury that can further perpetuate neovascularization thus inadvertently drive angiogenesis (Tas et al., 2016). Hypoxia is a key feature of IA: the high bioenergetic demand of inflammation and proliferation of cells in the synovial membrane results in an altered metabolic and higher bioenergetic turnover in inflamed joints (Ding et al., 2020). Additionally, hypoxia drives the development of reactive oxygen species (ROS) which has also been shown to alter proteins involved in angiogenesis, EC differentiation, proliferation and migration (Cantatore et al., 2017). Due to the changes in oxygen levels in the inflamed joint microenvironment, HIFs are activated and transcribe genes that are involved in inflammation, angiogenesis and metabolism (Guo et al., 2020) creating a vicious cycle that further potentiates the inflammatory response in the joint.

VEGF, Ang2 (Gao et al., 2013) and FGF all activate previously quiescent ECs. In turn, ECs produce catabolic enzymes such as MMPs which degrade the basement membrane (Elshabrawy et al., 2015) resulting in migration of ECs away from their parent vessel. This leads to immature blood vessels within the joint that are also dysfunctional, resulting in defective blood flow, inadequate nutrient and oxygen supply to the expanding pannus thereby leading to a hypoxic microenvironment (Kennedy et al., 2010; Ng et al., 2010; Biniecka et al., 2014; Fearon et al., 2016). Additionally, proangiogenic factors, e.g. neural cellular adhesion molecule (NCAM), Ang1, platelet-derived growth factor (PDGF) and Notch signalling stabilize blood vessels and facilitate maturation (Lainer-Carr et al., 2007; Gerhardt et al., 2008; Fromm et al., 2019). Pericytes are then incorporated onto the newly formed basement membrane to assist the blood flow process (Koch i2007). These pericytes are contractile cells that are found on capillaries and are blood flow regulators (Attwell et al., 2016). Interestingly, Kennedy et al. (2010) demonstrated a mixture of mature vasculature with pericytes and immature vasculature without pericytes in the synovium of IA (Kennedy et al., 2010). Thus, synovial vessels go through an everchanging
process of simultaneous angiogenesis, maturation and regression which relies on growth factors and MMPs in the surrounding microenvironment (Veale *et al.*, 2017).

**1.17 Leukocyte transendothelial cell migration: adhesion molecules and chemokines**

In IA, the dysregulated vasculature and EC activation facilitate leukocyte infiltration into the inflamed synovium. The order of leukocyte adhesive interactions with ECs is known as the ‘leukocyte extravasation cascade.’ This sequential process encompasses a number of adhesive events leading to cell tethering, rolling, activation, adhesion, crawling and transendothelial cell migration (Raza *et al.*, 2006; Schnoor *et al.*, 2015).

After infection or tissue damage, cytokines and other danger signals are released. Consequently, weak adhesion or “rolling” occurs. This happens within two hours following infection or tissue damage. The endothelium is triggered causing the expression of P- or E-selectins (capture receptors), adhesion molecules, such as intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, and surface-presentation of lipids or chemokines (Zhang *et al.*, 2011). Next, marginated leukocytes are captured, roll along the endothelial surface until they receive a chemokine-mediated signal that results in inside-out signalling and integrin activation (α₄β₁-integrin; α₄β₁-integrin) (Ley *et al.*, 2007). This stabilizes the adhesive interactions as well as cytoskeletal rearrangement in the leukocyte inducing the leukocyte to crawl over the endothelium and into the subendothelial space which comprises of the basement membrane and stromal compartment (Ley *et al.*, 2007). This entire process is highly controlled by stromal-derived signals and haemodynamic forces of the flowing blood (McGettrick *et al.*, 2012).
Figure 1. Leukocyte adhesion cascade. A step-by-step process of leukocyte migration into the endothelium. 1. Endothelial activation and leukocyte margination. 2. Leukocyte capture and rolling via integrins and selectins. 3. Leukocytes become activated and firm adhesion to the membrane occurs via integrins, chemokines and adhesion molecules. 4. Leukocytes migrate through the endothelium. 5. Leukocytes finally migrate through the basement membrane and tissue. Taken from Manning et al. (2021).

Chemokines (or chemotactic cytokines) are members of the chemoattract cytokine family and mediate leukocyte activation and accumulation at sites of inflammation. They are categorised into four supergene families based on cysteine residue location. In 2000, the classification system was redefined as chemokines are now recognised as chemokine receptor ligands (CXCL, CCL, CL and CX3CL) and the corresponding receptors (CXCR, CCR, CR, CX3CR) (Bacon et al., 2002). In IA, chemokines have a critical role in the recruitment of immune cells to the site of inflammation, which significantly contribute to the inflammatory process and pannus formation thus targeting chemokines have been an attractive potential therapeutic strategy for IA (Loos et al., 2006; Elemam et al., 2020). Th1 cell migration to the synovium has been shown to be critical in RA pathogenesis and is considered an early event. CXCR3 and CCR5 are required for Th1 cell migration to the synovium where elevated expression of these ligands has been demonstrated – CXCL9, CXCL10, CXCL11 denotes the ligand for CXCR3 while CCL3, CCL4, CCL5 are the ligands for CCR5 (Qin et al., 1998; Wedderburn et al., 2000; García-López et al., 2001; Patel et al., 2001; Yang et al., 2002; Mohan et al., 2007; Hou et al., 2020). Evidence from Yang et al. (2002) showed that blocking CCR5 prevents T cell trafficking leading to reduced synovitis (Yang et al., 2002). For leukocyte chemotaxis to occur, chemokines bind to negatively-
charged glycosaminoglycans molecules (GAGs) (Filer et al., 2008). Therefore, studying the chemokine receptor expression in the context of synovial inflammation has identified mechanisms that mediate the infiltration of immune cells to the joint. Firstly, CCR7 has been shown to aid the guidance of DC and T cells to the inflamed synovium, with blockade of CCR7-CCL21 interactions resulting in the inhibition of DC/T cell migration (Van Raemdonck et al., 2020). In addition, a subgroup of T helper (Th) cells expressing CCR6 has been detected in the peripheral blood, SF and inflamed synovial tissue of RA patients (Hirota et al., 2007) and have been shown to further expand and polarize into Th1, Th17 and Th22 cells, which are associated with progression of RA disease (Paulissen et al., 2015; Floudas et al., 2022).

Other chemokines such as CXCL12 and its receptor CXCR4 play a crucial role in leukocyte recruitment. Naïve T cells express CXCR4 but this is not the case for highly differentiated peripheral CD45RO+ T cells (Buckley et al., 2000). Its corresponding ligand, CXCL12, is expressed abundantly on ECs at the sites of T cell accumulation (Buckley et al., 2000; Nanki et al., 2000; Pablos et al., 2003). Buckley et al. (2000) also observed that stromal-derived TGF-β brought about the increase of CXCR4 receptors on T cells in the synovial cavity (Buckley et al., 2000). Thus communication between chemokine and cytokine networks plays a key role in the promotion and retention of T cells (Nanki et al., 2000).

As highlighted earlier, previous studies have shown increased expression of CCL20 on synovial monocytes in JIA (Wu et al., 2021), higher expression of which facilitated the influx of Th17 cells to the site of inflammation via CCR6. Additionally, JIA FLS have been shown to secrete CXCL1, CXCL9 and CXCL6 further potentiating leukocyte extravasation into the joint. JIA SF contains numerous other chemokines including CXCL5, CXCL10, CXCL13, CX3CL1, CCL18, which are known for mediating the recruitment of T cells and monocytes (Brescia et al., 2017). Furthermore, CCL26 and CCL1 have been shown to be released by T cells and facilitate the recruitment of monocytes, NK cells, immature B cells and DCs (Brescia et al., 2017). Analysis of children with JIA and lung disease demonstrated increased levels of both CXCL9 and CXCL10 in their bronchoalveolar lavage fluid (Schulert et al., 2019). Furthermore, other groups have analysed chemokines as a potential salivary biomarker for monitoring the immune system, however levels were comparable between JIA and HC (Collin et al., 2022).
Several studies have noted that children with DS have impaired neutrophil chemotaxis (Licastro et al., 1990a; Novo et al., 1993). Chemokines are not directly involved in DS, however studies have noted that increased levels of IL-6 induces CCL2 and CCL20 in the serum of children with DS compared to HC (Mattos et al., 2018) while CCR6 is secreted by γδ T cells (Park et al., 2021), albeit in the periphery, who’s frequency is higher in children with DS than HC (Huggard et al., 2020).

1.18 Innate immune system

The innate immune system is an evolutionary conserved system which acts as our first line of defence against invading pathogens. Importantly, the innate immune system is rapidly activated (within minutes) and can clear pathogens without the formation of any immunological memory. When innate effectors cells such as macrophages or DCs encounter a foreign antigen, they recognise conserved molecular patterns uniquely found on pathogens termed Pathogen Associated Molecular Patterns (PAMPs) via germline encoded pathogen-recognition receptors (PRRs). The recognition results in innate immune cell activation and the secretion of proinflammatory cytokines and chemokines (Iwasaki et al., 2015). Ultimately this leads to neutrophil recruitment and the production of broadly specific effector molecules with the goal of removing the infectious agent. If the pathogen continues to grow and innate effector mechanisms are unable to remove the pathogen, specialised antigen presenting cells (APCs), DCs, travel to the lymph node where they present the peptides from the invading antigen on MHC molecules to naïve T cells. This process abridges the innate and the adaptive immune system and leads to the generation of antigen specific T and B cells (Gierut et al., 2010).

Given the diversity in JIA subtypes, it is therefore plausible that a heterogenous group of innate immune factors may drive specific JIA subtypes. Indeed, according to Kessel et al. (2017), sJIA is induced by the innate immune system such as IL-1β-activating IL-17a overexpression by γδ T cells (Kessel et al., 2017). Additionally, neutrophils have been shown to trigger polyJIA (Jarvis et al., 2006). Furthermore, a proinflammatoty cytokine signature consisting of both innate and adaptive cytokines has been reported in JIA plasma and SF consisting of high expression of TNF-α, macrophage migration inhibitory
factor (MIF), macrophage inflammatory protein (CCL3), macrophage-derived chemokine (CCL22), monokine induced by IFN-γ (CXCL9), monocyte chemoattractant protein-1 (CCL2) and IFN-γ-induced protein-10 (CXCL10) (de Jager et al., 2007). Furthermore, examination of patients with different JIA subtypes significantly higher concentrations of plasma CCL11, CXCL10 and CCL2 were observed in oligoJIA compared to sJIA, while patients with sJIA demonstrated higher level of IL-1, IL-6 and IL-18 in serum (de Jager et al., 2007; Vastert et al., 2009).

In addition to JIA, innate immune dysfunction has also been reported in DS. Bloemers et al. (2010) documented significant differences in innate immune cells in DS compared to individuals without DS. Decreased absolute leukocyte, monocyte and granulocyte counts with a concomitant increase in NK cells and proinflammatory CD14\textsuperscript{dim} CD16\textsuperscript{+} monocytes have been shown in DS (Bloemers et al., 2010; Huggard et al., 2020). Furthermore, Licastro et al. (1990) also reported decreased neutrophil phagocytic activity in children with DS compared to HC suggesting there may be innate defects in neutrophil functionality in DS (Licastro et al., 1990b).

1.19 The adaptive immune system

The adaptive immune system, while paramount in mediating host protection to infectious agents, has also been implicated in the pathology of many autoimmune diseases. The adaptive immune system is predominantly regulated by the interplay of two main cell types:

- T cells (cell-mediated immunity) and
- B cells (antibody-mediated immunity)

The key differences between the adaptive and innate immune system are specificity and memory. These unique traits of adaptive immunity guarantees it to differentiate between different pathogens (specificity) and quickly responding with force upon reinfection (memory) (Omata et al., 2022).

Lymphocytes are distinctive cells of the adaptive immune system and comprises of subgroups that have different purposes and protein products. Early B cell development
occurs in the bone marrow where it creates B lymphocytes. T lymphocytes stem from the bone marrow and migrate to the thymus where they mature. After the maturation stage in the generative lymphoid organs, lymphocytes circulate in the blood to secondary lymphoid organs such as lymph nodes, spleen and regional lymphoid tissues. Mature T cells then leave the thymus while immature B cells leave the bone marrow and proceed to the secondary lymphoid organs where they finish their maturation process. Interestingly, naïve lymphocytes may encounter foreign antigens here or return to the blood via lymphatic drainage and recirculate through other secondary lymphoid organs (Van Den Broek et al., 2018).

1.20 T cells

T cells are important drivers of cell-mediated adaptive immunity thus defending the host against invading microbes. T cells are categorised into different subgroups according to their phenotype and effector functions. Cytotoxic T cells (CD8+ T cells) express the CD8 surface marker and recognise foreign antigen proteins or self-antigens expressed on the surface of APCs using MHC class I molecules. These CD8+ T cells have potent cytotoxic capabilities and are involved in the killing of virally infected, damaged, or transformed cells. Th cells (CD4+ T cells) are distinguished by CD4 surface expression which recognise foreign and self-antigens presented using MHC class II molecules on APCs (Broere et al., 2019).

T cell plasticity is defined as a T cell that can switch between different T cell phenotypes and carry out the functions associated with that phenotype (Geginat et al., 2014). Presently, there are a minimum of nine distinct CD4+ T cell subsets which are either proinflammatory (e.g. Th1 and Th17 cells) or anti-inflammatory (e.g. regulatory T cells (Treg) and Treg type 1 (Tr1) cells (Russ et al., 2013; Ohl et al., 2018; Zhu et al., 2020).
Table 1. 2 Significant CD4+ T cell subsets in humans and the related diseases (adapted from Petty, 2016).

<table>
<thead>
<tr>
<th>Th subset</th>
<th>Gate keeper and transcription factor</th>
<th>Cytokines</th>
<th>Function</th>
<th>Related diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>T-bet, STAT-4</td>
<td>IFN-γ, IL-12, TNF-α</td>
<td>Protection against microorganism delayed type hypersensitivity</td>
<td>Autoimmune diseases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th2</td>
<td>GATA-3</td>
<td>IL-4, IL-5, IL-13</td>
<td>Protection against parasites, allergy, B cell help, class switching</td>
<td>Allergy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGF-β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th3</td>
<td>Unidentified</td>
<td>IL-6</td>
<td>Mucosal tolerance</td>
<td>Unidentified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGF-β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th17</td>
<td>RAR-related orphan receptor C (RORC)</td>
<td>IL-17</td>
<td>Provide immunity to several extracellular pathogens e.g. Candida</td>
<td>Autoimmune diseases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(IL-6, TGF-β)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treg</td>
<td>Forkhead box P3 (FOXP3)</td>
<td>Consumption of IL-2/ IL-10, TGF-β</td>
<td>Modulating inflammation</td>
<td>Autoimmune diseases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tr1</td>
<td>Unidentified</td>
<td>IL-21</td>
<td>Modulating inflammation</td>
<td>Diabetes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isotype switching and B cell memory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tfh (follicular helper T cells)</td>
<td>B cell lymphoma 6 (Bcl6)</td>
<td>IL-9</td>
<td>Several inflammatory diseases</td>
<td>Psoriasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α, Granzyme B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th9</td>
<td>Unidentified</td>
<td>IL-22</td>
<td>Protection against extracellular pathogens</td>
<td>Several inflammatory diseases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protection and repair</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th22</td>
<td>Unidentified</td>
<td>IL-22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.21 T cells in IA and DS

In the context of JIA, it is known that proinflammatory Th1 and Th17 cells in the blood are augmented in JIA compared to HC (Omoyinmi et al., 2012). The Th1 cytokines induced include IFN-γ and IL-22. Th1-secreted IFN-γ is involved in both the innate and adaptive immune system whereby it can activate monocytes and stimulate HC II expression (Udalova et al., 2016). On the other hand, IL-22 is required for protection against infections of extracellular pathogens (Ouyang, 2010). Additionally, Th17 cells produce more proinflammatory IL-17a, TNF-α and granulocyte-macrophage colony stimulating factor (GM-CSF). In IA, Th17 cells have been shown to be highly plastic and transient, with their phenotype dependent on environmental cues. Th17 cells produce IL-17a and IFN-γ while ex-Th17 cells can produce IFN-γ and lose the ability to secrete IL-17a. Therefore, ex-Th17 cells can resemble Th1 cells (Gaffen et al., 2014; Basdeo et al., 2017; Kotake et al., 2017). Previous work in adult forms of arthritis have demonstrated accumulation of Th1, Th17 and ex-Th17 cells in the RA and PsA synovium (Basdeo et al., 2017; Wade et al., 2019). Studies have shown these cells can be polyfunctional in nature and demonstrated that
polyfunctional T cells that simultaneously secrete numerous pro-inflammatory cytokines are associated with disease activity (Basdeo et al., 2017; Wade et al., 2018). Indeed, studies have shown that polyfunctional cytokine-producing T cells and not mono-cytokine-producing T cells are also associated with therapeutic response (Wade et al., 2018). Furthermore, to add to their pathogenic nature polyfunctional T cells are highly resistant to Treg suppression (Basdeo et al., 2015, 2017). In the context of DA, our group has shown a marked increase in T cell functional plasticity in the periphery, evident by the observed increased frequency of polyfunctional CD8+ Th cells, CD161+ Th cells and CD8− Th cells co-expressing TNF-α, IFN-γ and IL-17a compared to HC, DS and JIA (Foley et al., 2020). Furthermore, significant expansion of CXCR3+ CCR6+ (Th1/Th17) Tfh cells and CXCR3+ CCR6+ Tph cells was observed in DA compared to DS. These effects were also paralleled by a decrease in CXCR3− CCR6− (Th2) Tfh cells (Foley et al., 2020).

Various groups have indicated the important balance between Th17 and Treg cells (de Kleer et al., 2004; Ruprecht et al., 2005). At the site of inflammation, the two cell subpopulations have an interdependent relationship where amplified levels of Th17 cells is positively associated with disease severity (Nistala et al., 2008). Interestingly, a study by Henderson et al. (2020) demonstrated an accumulation of IL-17a-producing Tregs cells which accumulate throughout JIA disease progression – whereby Treg cells, in the context of increased inflammation, began to differentiate to an IL-17-like effector population (Henderson et al., 2020). CD4+ CD25+ Treg cells are suppressive by releasing IL-10 and combating inflammatory responses to promote homeostatic regulation (Sakaguchi et al., 1995). FoxP3+ is among one of the most studied Treg variants and is marked by this transcription factor. Counterintuitively, JIA patients tend to have more Treg cells in sites of inflammation compared to the periphery (de Kleer et al., 2004; Ruprecht et al., 2005) suggesting that these cells are defective or incompetent. As described by Henderson et al. (2020), these Tregs could begin to resemble proinflammatory Th17 cells by producing IL-17a aiding the inflammation as opposed to suppressing it (Henderson et al., 2020).

However, deducing Treg cells to just being anti-inflammatory is limiting as FoxP3+ T cells have been shown to have the proinflammatory properties of Th1, Th2 and Th17 cells (Campbell et al., 2011). Interestingly, analysis of T cells in JIA SF revealed how Th1-related markers were expressed by CD4+ (including Tregs), CD8+ and γδ T cells where the CD4+
subpopulation was positively linked with disease severity. Even though these Tregs are Th1-skewed, they kept their regulatory gene signature (Julé et al., 2021).

<table>
<thead>
<tr>
<th>Lymphocyte subpopulations</th>
<th>Frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells</td>
<td>Lower (abc, %)</td>
<td>(De Hingh et al., 2005)</td>
</tr>
<tr>
<td>CD4+ CD45+ RA cells</td>
<td>Lower (%)</td>
<td>(Murphy and Epstein, 1992)</td>
</tr>
<tr>
<td>CD4+ Th cells</td>
<td>Lower (abc, %)</td>
<td>(De Hingh et al., 2005)</td>
</tr>
<tr>
<td>CD4+/CD8+ ratio</td>
<td>Inverted ratio</td>
<td>(Cuadrado and Barrena, 1996)</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>Lower or normal (abc, %)</td>
<td>(De Hingh et al., 2005)</td>
</tr>
<tr>
<td>CD8+ CD57+ cells</td>
<td>Higher (%)</td>
<td>(Cossarizza et al., 1990)</td>
</tr>
<tr>
<td>NK cells</td>
<td>Lower (abc)</td>
<td>(De Hingh et al., 2005)</td>
</tr>
<tr>
<td>T cells</td>
<td>Lower or normal (abc, %)</td>
<td>(De Hingh et al., 2005)</td>
</tr>
<tr>
<td>TCR-αβ+ T cells</td>
<td>Lower (%)</td>
<td>(Murphy and Epstein, 1992)</td>
</tr>
<tr>
<td>Th1/Th2 ratio</td>
<td>Higher</td>
<td>(Franciotta et al., 2006)</td>
</tr>
<tr>
<td>IgA</td>
<td>Higher or normal for &gt; 6 years</td>
<td>(Nespoli et al., 1993)</td>
</tr>
<tr>
<td>IgG</td>
<td>Higher for &gt; 6 years</td>
<td>(Franciotta et al., 2006)</td>
</tr>
<tr>
<td>IgG1</td>
<td>Higher or normal</td>
<td>(Barradas et al., 2002)</td>
</tr>
<tr>
<td>IgG2</td>
<td>Lower or normal</td>
<td>(Barradas et al., 2002)</td>
</tr>
<tr>
<td>IgG3</td>
<td>Higher or normal</td>
<td>(Barradas et al., 2002)</td>
</tr>
<tr>
<td>IgG4</td>
<td>Lower or normal</td>
<td>(Barradas et al., 2002)</td>
</tr>
<tr>
<td>IgM</td>
<td>Lower for &gt; 6 years</td>
<td>(Ugazio et al., 1990)</td>
</tr>
<tr>
<td>Response to vaccination</td>
<td></td>
<td></td>
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<tr>
<td>Hepatitis A</td>
<td>Normal</td>
<td>(Ferreira et al., 2004)</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>Lower or normal</td>
<td>(Garcia Bengoechea and Cortes, 1990)</td>
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<tr>
<td>Influenza</td>
<td>Lower</td>
<td>(Philip et al., 1986)</td>
</tr>
<tr>
<td>Pertussis</td>
<td>Lower</td>
<td>(LiVolti et al., 1996)</td>
</tr>
<tr>
<td>Pneumococcal</td>
<td>Lower or normal</td>
<td>(Costa-Carvalho et al., 2006)</td>
</tr>
<tr>
<td>Polio (oral)</td>
<td>Lower</td>
<td>(McKay et al., 1978)</td>
</tr>
<tr>
<td>Tetanus</td>
<td>Lower</td>
<td>(Philip et al., 1986)</td>
</tr>
</tbody>
</table>

**Table 1.** 3 Abc = absolute counts, % = relative counts. Accumulated data for children with DS from sources in table.
According to Levin et al. (1979), infants and children with DS have a smaller thymus suggesting accelerated thymic involution in utero (Levin et al., 1979). This abnormality could imply that T cells are the fundamental issue in DS. However, children who have undergone partial or whole thymectomy cardiac surgery due to their congenital heart disease demonstrate quick and irreversible changes in T cell numbers. In comparison to children with DS, these children do not have a higher percentage of infections and autoimmune diseases (Eysteinsdottir et al., 2004). Children with DS have a reduced frequency of naïve T cells with impaired thymus development and function compared to children without DS (Kusters et al., 2010; Bloemers et al., 2011; Marcovecchio et al., 2019). Additionally, children with DS have a lower proportion of phenotypically mature thymocytes that show increased levels of αβ-form of the T cell receptor (TCR-αβ) and associated CD3 molecule, TNF-α and IFN-γ compared to children without DS. Heightened expression of these cytokines could mean that there is impaired cytokine production in DS which could explain the irregular thymic structure and thymocyte maturation (Murphy et al., 1992).

Interestingly, a higher frequency of peripheral T cells that express the alternative receptor, γδ – TCR, has been documented along with reduced numbers of CD4+ CD45RA+ naïve cells (this includes cells that have recently emigrated from the thymus) and higher numbers of memory cells (Murphy and Epstein, 1992). Bloemers et al. (2011) used T cell receptor excision circle (TREC) counts to approximate recent thymic emigrants. Unsurprisingly, a dramatic reduction in the percentage of signal joint TREC+ peripheral blood cells are reported in children with DS when compared to children without DS (Bloemers et al., 2011). The same study also documented lower thymic outputs enforcing the narrative that children with DS have a dysfunctional thymus development. Altogether, these premature clinical manifestations are linked to senescence – healthy older individuals have lower naïve Th cells and CD8+ T cells along with reduced TREC+ peripheral blood cells. Several reports suggest an increase in the number of central and effector memory Th cells and effector memory and terminally differentiated CD8+ T cells (Junge et al., 2007; Sansoni et al., 2008). In contrast, results from Kusters et al. (2010) imply that individuals with DS have normal memory counts as well as reduced naïve cells. This suggests the presence of defective T cells as opposed to early ageing as the study observed
the absence of the normal expansion of naïve Th cells (CD4+ CD45RA+) and cytotoxic T cells (CD8+ CD45RA+ CD27+) during the first few years of the individual with DS’s life (Kusters et al., 2010). In terms of Tregs, these cells are suppressed and there is higher thymic oxidative stress in DS which could contribute to their propensity to develop autoimmune diseases (Marcovecchio et al., 2019; Huggard et al., 2020).

However, very little is known about T cells in the context of DA. Our previous work describes how T cells in DA have heightened plasticity. A significant increase in CD8-, CD8+ and CD161+ polyfunctionality T cells which simultaneously produced TNF-α, IFN-γ, IL-17a and GM-CSF were demonstrated in DA compared to DS, JIA and HC, which was also associated with a decrease in frequency of Tregs (Foley et al., 2020). Furthermore, the same study demonstrated increased frequencies of CXCR3+ and CCR6+ Tfh and Tph cells compared to HC, JIA and T21. The increased chemokine expression could be indicative of enhanced recruitment of immune cells to the site of inflammation in DA, but also defines that plasticity of Tfh and Tph further emphasising the complexity of the joint environment (Foley et al., 2020). Significant changes to the synovial pathology and inflammation in DA when compared to JIA were also observed (Foley et al., 2020). T cell polyfunctionality has been implicated in RA and PsA, where frequency is associated with increased pathogenic function, resistance to Treg cell suppression and associated with disease activity and response (Basdeo et al., 2017; Wade et al., 2018; Floudas et al., 2022).

Additional studies by other groups have demonstrated that individuals with DS have dysregulated T cell responses which are skewed towards an autoimmune phenotype. Specifically, Araya et al. (2019) report an enrichment of T cells subsets which express higher levels of markers of activation, senescence and overproduce cytokines linked to autoimmunity (e.g., TNF-α). They also report increased differentiation of Th1 cells towards Th17 cells concomitant with an overproduction of the IL-17a and IL-22 (Araya et al., 2019).

**1.22 B cells**

The ability of B cells to produce antigen-specific, shielding antibodies and their function as powerful APCs which are required for T cell activation make them the driving force
behind the antibody immune response. Following activation, B cells differentiate into subsequent effector cells: mainly Ig-secreting plasma cells and memory B cells.

![Diagram of the antibody-mediated immune response from B cells](image)

**Figure 1. 8 Stages of the antibody-mediated immune response from B cells.** When a naïve B cell encounters an antigen, Th cells and other stimuli drive the activation of B cells. B cells then proliferate into various B cell subtypes that have different functions pending the IgG it produces. Taken from Abbas *et al.* (2015).

B cell activation is induced by antigen-specific recognition mediated by the surface Ig receptor of the cells. Antigens along with other stimulants (*e.g.*, Th cells) initiate the proliferation and differentiation of B cell clone. Offspring of that clone can then differentiate into plasma cells which create Ig isotypes, undergo affinity maturation or continue as memory B cells (Abbas *et al.*, 2015).

### 1.23 B cells in DS, JIA, and DA

Numerous reports describe how children with DS, and are over five years of age, have IgG and IgA hypergammaglobulinemia with increased levels of IgG1 and IgG3 and decreased levels of IgG2 as well as IgG4 (Burgio *et al.*, 1975; Avanzini, 1990; Nespoli *et al.*, 1993).

Conversely, there is conflicting data from several groups who have found notable B lymphocytopenia (an absence of B lymphocytes) in DS along with the absence of the usual expansion during the first year of their life (De Hingh *et al.*, 2005; Verstegen *et al.*, 2010).
Zizka et al. (2006) also document significantly lower numbers of B cells in the foetuses with DS (Zizka et al., 2006). Two plausible reasons can explain these irregularities: an intrinsic B cell defect or dysfunctional Th cells which could bring about uncontrolled B cell activation and proliferation. Perhaps the fact that both hypergammaglobulinemia and B lymphocytopenia exist in DS cements the latter hypothesis to be true in addition to the suggestion that antibody responses may be oligoclonal and/ or insubstantial in DS (Verstegen et al., 2010). The same study also demonstrated lower CD27+ CD21<sup>high</sup> and CD23+ cells which mirror common variable immunodeficiency – this implication could mean there is defective peripheral B cell maturation.

Th1 cells secrete mediators, such as IFN-γ, IL-2 and TNF-α, which activate cytotoxic T cell responses. Th2 cells, on the other hand, produce cytokines e.g., IL-4. IL-5, IL-6 and IL-10, which induce the antibody response by B cells and facilitates the formation of IgG2 and IgG4. Adults with DS have significantly increased numbers of CD4+ and CD8+ T cells that produce IFN-γ along with an increased Th1/Th2 ratio (Franciotta et al., 2006); which is possibly associated with the observed upregulation of IgG1 and IgG3 levels, in contrast to lower levels of IgG2 and IgG4 observed in DS. This reinforces the concept that Th cell function is compromised.

Children with DS are prone to be immunodeficient and develop recurrent ear-nose-throat as well as pulmonary airway infections during their early childhood. Consistent infection is associated with children with DS being more susceptible to developing an autoimmune diseases (De Vries, 2006). While antibody depletion could be the cause for these recurrent infections, macroglossia, hypotonia and modified upper airways are all common features of DS it is likely antibody depletion contributes to this.

In contrast, the major shift towards autoimmunity and lymphoproliferation is an indication of immune dysregulation. Limited T cell numbers and function disrupts the tolerogenic balance resulting in both immunodeficiency and immune dysregulation (Liston et al., 2008).

Due to the genetic predisposition of DS, the immune system of these children is usually compromised from birth, and it is possible that the immunological differences are not solely caused by senescence. As described previously, abnormal thymus and T cell
development and activity, potential intrinsic B cell defects in children with DS (Levin et al., 1979; Franciotta et al., 2006; Zizka et al., 2006; Bloemers et al., 2011) should be considered as primary reasons why they are prone to autoimmunity such as coeliac disease and T1D.

While studies have shown differences in B cells subsets and activation in JIA and DS compared to HC (Corcione et al., 2009; Carsetti et al., 2015; Zhao et al., 2018; Moura et al., 2022), little is known about B cells in the context of DA. Indeed, our group is the only study to date that has examined B cell frequency and activation in children with DA (Foley et al., 2020). In this study, we demonstrated that the frequency of B cells in DA is significantly reduced when compared to JIA, DS and HC. Interestingly there was a significant decrease in memory (CD27+) B cells in T21 compared to HC, however frequency was restored in children with DA. Furthermore, peripheral blood IgM-only (IgM+IgD-CD27+) germinal centre-like B cells were significantly increased within the switched memory compartment in DA compared to HC and JIA. Frequency of transitional B cells was also decreased in DA compared to HC and T21. Finally, DA had the highest number of IgM-only producing CD27+ IgD- memory B cells compared to the other groups (Foley et al., 2020).

1.24 Monocytes
Monocytes are bone marrow-derived cells of innate immune system. Through direct responses, they have a strong role in anti-microbial immunity including phagocytosis and production of proinflammatory cytokines (Cormican et al., 2020) as well as adaptive immune function, including antigen presentation (Jakubzick et al., 2017). They are extremely plastic thus they can differentiate into other key immune cells, including macrophages, DCs, and osteoclasts - all of which further drive inflammation and the immune response (Rana et al., 2018).

Over the last ten years, three main monocyte subsets have been identified. Based on the expression of CD14 and CD16 they are classical monocytes (CD14\textsuperscript{high} CD16-), intermediate monocytes (CD14+ CD16+) and non-classical monocytes (CD14- CD16\textsuperscript{high}) (Ziegler-Heitbrock et al., 2010). Numerous reports show that these subsets are transcriptionally
distinct (Ancuta et al., 2009; Zhao et al., 2009; Wong et al., 2011) and may have unique roles, with the classical subset involved in anti-microbial response, the intermediate subset in inflammation and antigen presentation, and the nonclassical subset in antiviral responses (Kapellos et al., 2019). New evidence suggests the existence of “regulatory” monocytes, and have been shown to have aid neutrophils and macrophage in the resolution of inflammation (Murray, 2018; Varga et al., 2018).

Monocytes are involved in the initiation of RA inflammation and positively correlate with disease activity and response to treatment (Luo et al., 2018). The intermediate monocytes are enriched in RA patients, and produce proinflammatory cytokines e.g. TNF-α, IL-1β and IL-6. Likewise, they are positively associated with disease activity (Rossol et al., 2012; Klimek et al., 2014; Yang et al., 2014; Tsukamoto et al., 2017; Ruiz-Limon et al., 2019).

Work from our group has shown that monocytes from RA patients are primed to differentiate into macrophages and DCs (Marzaioli et al., 2020; McGarry et al., 2021) as well as displaying higher metabolic and inflammatory features in comparison to HC. In addition, we and others have shown that monocyte priming precedes clinical manifestations of disease (Coulthard et al., 2012; McGarry et al., 2021).

Comparing JIA and septic arthritis (SA) as another form of childhood arthritis, the numbers of intermediate and nonclassical monocytes in both SF and the periphery were higher in JIA compared to SA (Cren et al., 2020) highlighting the involvement of monocytes in the pathogenesis of JIA. The understanding of monocytes has expanded to displaying a proinflammatory M1 or anti-inflammatory M2 phenotype determined by cytokine stimuli. Interestingly, children with oligoJIA have been shown to have polarized SF monocytes with a M1 (IFN-γ-initiated)/ M2 (IL-4-initiated)-like pattern meaning these children have a mixture of both pro and anti-inflammatory traits (Schmidt et al., 2020). In the context of sJIA, these patients were demonstrated to have plastic macrophages with an IFN-γ response (Schulert et al., 2021). This is particularly important since one of the comorbidities of sJIA is MAS (Section 1.6). The same study observed that sJIA has an overexpression of the IFN regulator, tripartite motif containing 8 (TRIM8), that discerns peripheral monocytes from bone marrow macrophages (Schulert et al., 2021).
While total monocytes in DS were found to be decreased, there was an increase in the number of nonclassical monocytes compared to HC (Bloemers et al., 2010). Additionally, these monocytes had higher TLR-4 expression paired with a higher TLR-2 expression on intermediate and nonclassical monocytes suggesting a proinflammatory phenotype (Huggard et al., 2020) which is different to the plastic monocytes observed in JIA. Analysis of monocyte functionality in DS demonstrated impaired migratory capacity compared to HC (Barroeta et al., 1983).

1.25 Synovial fibroblasts

In 1983, Fassbender first described the synovial fibroblasts as a key cell type which forms the characteristic pannus of RA (Fassbender et al., 1983). During inflammation, the synovial lining layer undergoes hyperplasia, with expansion of FLS populations in the joint, forming a thickened pannus which can directly invade and destroy adjacent cartilage and subchondral bone (Mor et al., 2005). One key feature of FLS is their invasive phenotype favouring tissue destruction, evident in studies that demonstrated that RA FLS but not OA or dermal FLS, could adhere to and invade the implanted cartilage. The authors also showed that these effects were mediated through increased expression of several adhesion molecules and integrins (Muller-Ladner et al., 1996). Furthermore, an elegant study by Lefevre et al. (2009) demonstrated that implanted RA FLS actively migrate and invade into contralaterally implanted naïve cartilage, supporting the concept of how destructive arthritis spreads between joints (Lefèvre et al., 2009). FLS act directly on the cartilage through secretion of MMPs, cathepsins and proinflammatory cytokines (Pap et al., 2000; Müller-Ladner et al., 2002) and act indirectly on bone by regulating osteoclastogenesis (Takayanagi et al., 2000). Recently, FLS were shown to migrate to non-inflamed regions through pre-inflammatory mesenchymal (PRIME) cells. Moreover, FLS are a heterogenous group which modulate different pathogenic mechanisms involved in the inflammatory response in RA (Kemble et al., 2021).

Similar to RA FLS, JIA FLS secrete proinflammatory mediators and degrading enzymes, such as MMP-1, MMP-3, IL-6, MCP-1, RANTES, CXCL1 and CXCL6. However, they have been shown to lack the expression of the modulatory signals of IL-10 and tissue inhibitor of metalloproteinases 2 (Agarwal et al., 2008; Brescia et al., 2017). Additionally, Maggi et
al. (2016) demonstrated that Th1 cells can stimulate the expression of VCAM-1 on JIA FLS which consequently recruit and retain leukocytes at the site of inflammation. This highlights the reciprocal relationship between T cells and FLS (Maggi et al., 2016). Previous work from Simonds et al. (2022) described heterogeneity in JIA FLS where it was demonstrated that FLS begin to resemble dysfunctional chondrocytes with increased disease severity (Simonds et al., 2022) - an effect associated with an upregulated expression of bone morphogenetic protein 4 (Simonds et al., 2021). Moreover, chondrocyte-like FLS have unique genetic fingerprints that are distinct across the different JIA subtypes (Simonds et al., 2022).

Currently, there are no studies on FLS function in DA.

1.26 Cytokines

Cytokines are small proteins secreted by various cells in the innate and adaptive immune system, in addition to stromal cells. They are soluble or membrane-bound with molecular weights from 8 to 50 kilo Daltons (kDa) whose main function is to transmit signals between cells (Zhang et al., 2007). These signals range from inducing cell replication, survival and death to tissue repair (Choy et al., 2001; McInnes et al., 2007). Therefore pleiotropism (biologically multifunctional) is one of the major cytokine traits. Cytokines are categorised by their biological function and not by their structure or amino acid sequence motifs (Dinarello, 2000) and are critical for nearly every biological process. They are involved in embryonic development, modifications in cognitive function and senescence. Formidable as they are, these proteins are rapidly becoming the gold target for diagnostic, prognostic and therapeutic intervention in disease (Dinarello, 2007; Friedrich et al., 2019; Lambrecht et al., 2019; Propper et al., 2022).

The first study that elucidated different involvement in RA was from Buchan et al. (1988), where they demonstrated higher levels of IL-1 and TNF-α in the sera of RA patients (Buchan et al., 1988). In particular, TNF-α is known to be a ‘master regulator’, directing several pathological mechanisms spanning from endothelial activation and angiogenesis, cytokine expression, stromal cell and osteoclast activation (Choy et al., 2001; McInnes et al., 2016). Due to the potency of TNF-α and its hierarchy in the regulation of the
inflammatory response in IA, TNF-α inhibitors were developed as a treatment for IA and have proven to be highly effective (Thalayasingam et al., 2011; Udalova et al., 2016; Nanchahal et al., 2022). One such TNF-α inhibitor, etanercept, disrupts VCAM-1 upregulation that is usually induced by Th1-derived cytokines (Maggi et al., 2016). In JIA, TNF-α activates innate immune cells such as monocytes and neutrophils, induces EC adhesion molecules, destroys the cartilage and suppresses Tregs (Ombrello et al., 2017; Prince et al., 2020) and is one of the first line treatments for JIA (Prince et al., 2020; Zaripova et al., 2021). Meta analysis demonstrates that peripheral TNF-α is higher in DS than in HC (Zhang et al., 2017). Only one study was conducted for cytokine assessment in DA where increased expression of TNF-α from polyfunctional T cells was shown for DA compared to HC, JIA and T21 (Foley et al., 2020).

IL-17a has a plethora of activities including promoting the production of cytokines and chemokines and inducing bone resorption by stimulating receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (RANK) ligand (Chabaud et al., 1999; Sato et al., 2006; Taams, 2020). In JIA, IL-17a secreted from NK cells, Th17 cells and CD8+ T cells facilitates the release of chemokines, activates FLS, degrades cartilage and stimulates osteoclastogenesis (Nistala et al., 2008; Paroli et al., 2022). In DS, higher IL-17a production from CD4+ T cells compared to HC has been observed, in addition to increased serum levels of soluble IL-17a (Araya et al., 2019) with some speculating γδ T cells also secrete IL-17a (Huggard et al., 2020). For DA, increased expression of IL-17a from polyfunctional T cells compared to HC, JIA and T21 was observed (Foley et al., 2020).

IFN-γ is the only member of Type II IFNs. It has a plenitude of roles in both the innate and adaptive immune system where it activates monocytes and stimulates MHC II expression (Udalova et al., 2016). In JIA, IFN-γ activates macrophages, differentiates Th1 cells while inhibiting Th2 cells, amplifies adhesions molecule expression on ECs and attracts Th17 cells (Lee et al., 2019; Schulert et al., 2021; Wu et al., 2021). In terms of DS, peripheral IFN-γ levels are raised compared to HC (Zhang et al., 2017), with an increased dosage of IFN receptors also observed (Sullivan et al., 2016; Powers et al., 2019). Genomic studies have demonstrated that TNF stimulation of FLS changes their expression of crucial genes including TNFAIP3 and IFNAR1 (Ge et al., 2021) indicating an IFN gene signature in DS. For
DA, increased expression of IFN-γ from polyfunctional T cells compared to HC, JIA and T21 was observed (Foley et al., 2020).

While the name GM-CSF suggests the sole function of this cytokine is to stimulate myeloid cell proliferation, GM-CSF is required for a broad range of processes such as chemotaxis, cell adhesion and proinflammatory cytokine expression (Avci et al., 2016). Perhaps, their most prominent characteristic in RA is the differentiation of Th17 cells as well as macrophages (El-Behi et al., 2011). In JIA, while the role of GM-CSF is not that well understood, Th17 plasticity may affect GM-CSF-expressing T cells since this subpopulation was enriched in SF compared to the periphery (Piper et al., 2014). Furthermore, children with DS showed elevated GM-CSF levels in sera compared to HC (Huggard et al., 2020). Additionally, in DA, increased expression of GM-CSF from polyfunctional T cells compared to HC, JIA and T21 was observed (Foley et al., 2020).

1.27 Cellular metabolism

Broadly speaking, the definition of cellular metabolism is the total biochemical processes in a cell which consists of thousands of reactions and metabolites. Cellular metabolism includes pathways involving fundamental nutrients such as fatty acids, carbohydrates, glucose, and amino acids required for energy homeostasis and catalysing macromolecules (Deberardinis et al., 2012). An emerging body of evidence over the last 15 years has demonstrated that metabolic alteration is at the forefront of immune and stromal cell regulation in autoimmune disease. These include IA, with numerous studies demonstrating a switch in the bioenergetic profile of cells from a resting regulatory phenotype to one that is highly metabolically active (Weyand et al., 2017, 2020; Pucino et al., 2020; McGarry et al., 2021; Municio et al., 2021; Wang et al., 2021; Fearon et al., 2022; Robinson et al., 2022).

The two primary metabolic pathways are glycolysis and oxidative phosphorylation (OxPhos). Glucose metabolism involves the stepwise degradation of glucose via glycolysis, aerobic oxidation, and pentose phosphate pathways (PPP) to generate energy, in the form of adenosine triphosphate (ATP), and a variety of metabolic substrates. Upon entry into the cell, glucose is phosphorylated into glucose-6-phosphate (G6PD) in an irreversible manner. Subsequently, the glucose molecules are oxidised through a chain of anaerobic
events yielding two molecules of pyruvate and the release of two ATP molecules. In the presence of oxygen, pyruvate is decarboxylated by pyruvate dehydrogenase (PDH) where it will enter the tricarboxylic acid (TCA) cycle in the mitochondrial matrix to generate electron carriers: nicotinamide adenine dinucleotide (NADH) and Flavin adenine dinucleotide-2 (FADH$_2$). The electron transport chain (ETC) is comprised of five large protein complexes embedded in the inner mitochondrial membrane – Complex I to IV and ATP synthase (Complex V). The ETC moves electrons across these five complexes resulting in an electrochemical proton gradient to produce ATP via OxPhos (Chaban et al., 2014; Fearon et al., 2019).

If there is a lack of oxygen, the pyruvate molecules (which originated from glycolysis) are diverted from the TCA cycle and converted to lactate via lactate dehydrogenase (LDHA) in the cytosol. While glycolysis is an inefficient way to produce ATP compared to OxPhos, anaerobic glycolysis produces energy rapidly and particularly when the cell lacks oxygen or exposed to environmental stress (Senyilmaz et al., 2015). Glycolytic intermediate metabolites can merge in parallel pathways including biosynthetic growth pathways needed for anabolic growth. As such, glycolysis can also result in the reduction of NAD$^+$ to NADH (a crucial cofactor). NADH is typically oxidised into NAD$^+$ to preserve glycolytic flux leading to the reduction of pyruvate to lactate (O’Neill et al., 2016).

There are numerous metabolic pathways utilised by cells which allow cells to generate sufficient energy for their function. The PPP occurs in the cytosol and permits the redirection of glycolytic intermediates to make amino acid and nucleotide precursors – these are critical for cell proliferation and growth. Fatty acid synthesis results in the generation of lipids in cells and is associated with mammalian target of rapamycin (mTOR) signalling. The fatty acid oxidation pathway triggers the mitochondrial conversion of fatty acids into acetyl co-A, NADH and other cofactors into energy reserves. Additionally, amino acid metabolism is critical as numerous metabolic pathways utilise amino acids as substrates. Indeed, these pathways interlink. For instance, nicotinamide adenine dinucleotide phosphate (NADPH) produced in the PPP and citrate from the TCA cycle are processed during fatty acid synthesis while fatty acid oxidation promotes the ETC by generating NADH and FADH$_2$. Eventually, amino acid metabolism merges into the TCA
cycle and the amino acid, glutamine, is utilised as a source of citrate which is required for fatty acid synthesis (O’Neill et al., 2016).

Figure 1. General overview of the primary metabolic pathways. Glucose transporters help glucose enter the glycolytic pathway. Hexokinase 2 (HK2) converts glucose into G6PD. Pyruvate kinase M2 (PKM2) aids glycolysis by generating pyruvate from glucose. Energy is now created in the form of ATP. Pyruvate can now be converted into lactate and exit the cell or decarboxylated via PDH and thus become converted to Acetyl CoA, this now enters the tricarboxylic acid (TCA) cycle. This cycle produces NADH and FADH$_2$ which feed into the ETC and results in 36 ATP molecules. Glycolysis is linked to the PPP via G6PD to make ribose, NADPH and amino acids. Amino acid metabolism can also feed into the TCA cycle to promote ATP production via ETC. Citrate, a TCA intermediate, promotes fatty acid synthesis and fatty acid oxidation further promotes TCA cycle by making Acetyl CoA. Succinate and citrate can act as immunometabolites. Citrate, can be converted to itaconate through the enzyme, immune responsive gene 1 (IRG1). Taken from Hanlon et al. (2022).

1.28 Impaired metabolic pathway
The increased metabolic demand of the expanding synovial pannus requires a high metabolic turnover to meet the energy needed for the activated pathogenic immune and stromal cells. This leads to an hypoxic joint microenvironment (Fearon et al., 2022). Consequently, this microenvironment further promotes abnormal cell function and synovial invasiveness within the RA joint. These elevated metabolic demands, and the secretion of metabolic intermediates drive atypical cell function and synovial invasiveness
that ultimately result in a self-destructive cycle in the joint (Weyand et al., 2017; Fearon et al., 2019; Certo et al., 2020).

Hypoxia occurs when there is insufficient oxygen levels that causes inflammatory cells to activate pathogenic mechanisms and is significantly involved in the pathogenesis of RA (Fearon et al., 2016). Specifically, the synovial joint is profoundly hypoxic with pO$_2$ levels in vivo as little as 0.46% (Ng et al., 2010). Studies showed an inverse correlation between joint hypoxia and upregulated synovitis, immature and defective vasculature and immune cell infiltration (Kennedy et al., 2010; Ng et al., 2010; Biniecka et al., 2016). This hypoxic microenvironment in the joint also resulted in complex bi-directional interactions between key proinflammatory signalling pathways including NFκB, Jak-STAT, PI3K-AKT and Notch-1 (Oliver et al., 2009; Gao et al., 2012, 2015; Li et al., 2013).

Low pO$_2$ levels stabilize the transcription factor, HIF-1α, which subsequently, translocates to the nucleus where it dimerizes with HIF-1β and key co-factors facilitating binding to promoter regions on DNA, transcribing hypoxia inducible genes (Tannahill et al., 2013; Fearon et al., 2016). HIF-1α drives glycolysis by regulating genes responsible for several glycolytic transporters and enzymes while LDHA is purposefully more effective in response to HIF-1α (Majmundar et al., 2010). LDHA can convert pyruvate to lactate creating an acidic environment that perpetuates cell proliferation and invasiveness. Corroborating with previous reports, augmented lactate levels are inversely linked with lower glucose levels found in the synovial fluid of RA patients as well as synovial lactic acidosis correlating with the severity of synovial inflammation (Naughton et al., 1993; Ciurtin et al., 2006; Hitchon et al., 2009). In turn, this acidic microenvironment can further induce cellular invasiveness in the synovial joint, inducing cell mutations, in addition to inhibiting DNA repair and apoptotic mechanisms inducing pathogenic functions in immune and stromal cells (Gatenby et al., 2004; Chang et al., 2011; Biniecka et al., 2016; Certo et al., 2020).

1.29 Metabolic alterations in the joint

In the RA synovium, increased metabolic activity has been observed where differential expression of glucose transporters, GLUT-1 and GLUT-4, has been observed (Gallagher et al., 2020), in addition to increased expression of glyceraldehyde 3-phosphate
dehydrogenase (GAPDH) and PKM2 (Biniecka et al., 2016). Moreover, glycolytic enzymes and their by-products can have a secondary role of being autoantigens driving cell proliferation, neoangiogenesis and pannus formation (Lindy et al., 1971; Bae et al., 2012; Kim et al., 2014; McGarry et al., 2017; Pucino et al., 2017; Bustamante et al., 2018). Additionally, increased levels of the metabolite, succinate, is released from SF macrophages and accumulates in RA joints compared to HC (Littlewood-Evans et al., 2016). This in turn, induces GPR91 (receptor for extracellular succinate), stabilizes HIF-1α and strengthens IL-1β production creating a feedback loop (Tannahill et al., 2013; Littlewood-Evans et al., 2016). It has been speculated that GPR91 is involved in sensing immunological danger (Rubic et al., 2008).

Exposure of ECs to hypoxia, oxidative stress or key proinflammatory/angiogenic mediators, induces EC tube formation, leukocyte adhesion and invasion (Akhavani et al., 2009; Biniecka, et al., 2016; Hanlon et al., 2019). ECs also respond to a glycolytic flux required for tip cell migration and growth towards the angiogenic stimuli during neoangiogenesis (Cruys et al., 2016; Leung et al., 2022). Silencing of glucose 6-phosphate isomerase (G6PI) which has been shown to be increased on synovial EC leads to inhibition of hypoxia-induced angiogenesis (Lu et al., 2017). Low PO2 levels in the RA microenvironment is linked to blood vessel immaturity as well as EC oxidative stress (Kennedy et al., 2010; Biniecka et al., 2011) with inhibition of glycolytic enzyme PFKFB3 reducing EC function and activation of key transcriptional signalling pathways (Biniecka et al., 2016). ECs also secrete metabolites including lactate, nitric oxide, indoleamine 2,3-dioxygenase (IDO) and sphingosine-1-phosphate (S1P) that, in turn, regulates T cell metabolic reprogramming (Certo et al., 2021).

RA FLS function is associated with a change in their metabolic landscape including alterations in glycolysis, OxPhos and amino acid metabolism compared to OA FLS (Kim et al., 2014; Biniecka et al., 2016; Bustamante et al., 2017, 2018). Both glucose and glutamine metabolism are required by RA FLS for anabolic processes of lipids and nucleic acids, evident by studies demonstrated that the absence of either glucose or glutamine inhibits RA FLS invasive function (Ahn et al., 2016; Biniecka et al., 2016; Garcia-Carbonell et al., 2016; Bustamante et al., 2017). Moreover, a glutamine-deficit environment leads to lower
proliferation and invasiveness from RA FLS (Garcia-Carbonell et al., 2016). Petrasca et al. (2020) demonstrated that the coculture of CD4+ T cells and RA FLS led to an increased production of TNF-α, IFN-γ and IL-17a in T cells, and promoted the invasive capacity of RA FLS - effects that were coupled with an increase in glycolysis (Petrasca et al., 2020). Numerous studies have also shown that stimulation of RA FLS with TNF-α, TLR-2 ligands or hypoxia, leads to mitochondrial impairment and differential changes in mitochondrial gene expression linked with apoptosis and redox balance (Gao et al., 2015; Biniecka et al., 2016; McGarry et al., 2018). Thus, the microenvironment of the inflamed joint including proinflammatory mediators, oxidative stress and low PO2 levels, dictates the metabolic profile of RA FLS in favour of glycolysis (Ng et al., 2010; Balogh et al., 2018) an effect that is evident by the observed increase of in key glycolytic enzymes, HK2, PFKFB3, PKM2 and GLUT-1 (Biniecka et al., 2016; McGarry et al., 2017; Bustamante et al., 2018; Hanlon et al., 2019). Additionally, TNF signalling facilitates the mTOR pathway to promote IFN-γ-mediated actions in RA FLS through specific amino acid availability (Karonitsch et al., 2018).

### 1.30 Metabolic inhibition

Many of our current treatment strategies in the field of rheumatology can alter the metabolic profile of immune and stromal cells, however this has mainly been shown in the context of RA. Glucocorticoids modulate numerous genes associated with metabolic pathways including glycolysis, autophagy and mTOR pathways (Takahashi et al., 2017; Yang et al., 2018; Min et al., 2020). Conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs), which include MTX, are known to be anti-metabolic by inhibiting the synthesis of purine and pyrimidine and subsequent nucleotide formation which is essential for cellular function (Chan et al., 2002). MTX blocks the enzyme, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), which stops RANKL-mediated differentiation of osteoclasts (Wang et al., 2020), with other studies also showing that direct blockade of AICAR decreases FLS and T cell function in RA (Gallagher et al., 2020; Petrasca et al., 2020). Numerous studies have also shown that current biologic agents can alter metabolic pathways (Gao et al., 2015; Biniecka et al., 2016; McGarry et al., 2018; Canavan et al., 2020). TNF inhibitors decrease metabolic activity as observed by PET/MRI imaging and decreased synovial expression of GLUT-1 and key glycolytic enzymes in the
synovium (Biniecka et al., 2016). Tofacitinib, a Jak1 and Jak3 inhibitor, induces a quiescent metabolic phenotype in RA FLS, and inhibits glycolytic/proinflammatory pathways in RA synovial explants (McGarry et al., 2018). Tofacitinib has also been shown to suppress proinflammatory responses and OxPhos in RA FLS and macrophages that were stimulated with the Covid-19-Spike protein (Palasiewicz et al., 2021). Furthermore, while STAT3 blockade inhibits Notch signalling in ECs which is critical for EC tip-stalks cell selection and thus a functional vessel, STAT3 blockade also inhibits HIF1 and Notch 1 signalling in RA FLS resulting in reduced invasive capacity (Gao et al., 2013). A recent study also demonstrated that while Canakinumab, Secukinumab and Tocilizumab had no effect on RA FLS metabolic/inflammatory function following coculture with Th cell-conditioned media, Tofacitinib and the glycolytic inhibitors, 3-BrPA and FX11, supressed this RA FLS response (Kvacskay et al., 2021). Furthermore, functional analysis of RA FLS from patients with resolving synovitis compared to early disease demonstrated significant metabolic changes in mitochondrial function suggesting that altering these may suppress the RA FLS invasive function (Falconer et al., 2021).

Exploring metabolic inhibitors further, another study demonstrated that the pathogenic activities of RA FLS and human dermal microvascular EC were inhibited by a glycolytic inhibitor, 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (Biniecka et al., 2016). LDHA Blockade with GSK2837808A restored the ratio of ATP/adenosine monophosphate in activated OA FLS and in addition to restoring effector T cell function even with lactate present (Angelin et al., 2017; Min et al., 2020) while TNF inhibitors block PKM2 and prevents the proinflammatory pathways of RA FLS (Biniecka et al., 2016). Additionally, 3-BrPA and FX11 decreases the glycolytic and inflammatory response from RA FLS (Kvacskay et al., 2021) while GLS1 inhibits RA FLS proliferation (Takahashi et al., 2017). Another inhibitor of interest is metformin which is currently used in clinical practice for treating diabetes and has been shown to inhibit mTOR activity thus suppressing proinflammatory mediator production (Yan et al., 2015). Furthermore, silencing GPR91, a succinate receptor, led to preventing macrophage and DC activation as well as T cell expansion (Rubic et al., 2008; Saraiva et al., 2018).
1.31 DA therapeutic options
Adalimumab, a TNF-inhibitor, is the first line therapy for patients with DA (Jones et al., 2019, 2021). Previously, MTX was administered subcutaneously to patients with DA, however more than half of these children stopped treatment due to intolerance (Jones et al., 2019). Additionally, therapies for JIA that have been used to treat DA include NSAIDs, steroids, other DMARDs as well as biologic therapies (Jones, 2022) but they were not as effective as TNF-inhibitors.

Since T21 causes overactivation of IFN and Jak signalling (Sullivan et al., 2016) a small molecule biologic such as JAK inhibitor e.g. tofacitinib may be an attractive option (Jones, 2022). Other biologic therapies include abatacept targeting costimulatory molecules and APCs and tocilizumab targeting IL-6 receptor (Jones et al., 2019, 2021). It has been reported that 40% to 60% of DA patients using biologics encountered at least one change in biologic therapy due to ineffectiveness of the therapy (Jones et al., 2019, 2021). Patients with DA have been shown to have higher DMARD adverse effects (93%) compared to JIA patients (25%). The use of steroids are usually avoided due to unwanted side effects such as obesity and comorbidities linked with DS (Nicek et al., 2020).

1.32 Gaps in current knowledge of DA
DA is a significantly underreported and thus an underdiagnosed disease. Important advances in understanding and identifying the disease at a clinical level have been made in the clinical setting (Foley et al., 2019). However, there is a significant lack of knowledge regarding the underlying mechanisms of disease that promote the aggressive phenotype of DA compared to other forms of childhood rheumatic diseases including JIA. While biologic DMARDs have been shown to be effective in children with DA, a significant proportion have sub-optimal responses, no response or have adverse side effects. Better understanding of the underlying pathogenic mechanisms of disease would accelerate better therapeutic options and the right treatment from disease onset for children with DA (Foley et al., 2019; Foley et al., 2020). Inflammatory arthritis, including DA, is characterized by synovial neo-angiogenesis, which facilitates immune cell infiltration to the joint. In turn, immune cells release pro-inflammatory mediators that activate synovial FLS to invade and destroy cartilage and bone, leading to functional disability. While
studies in adult forms of arthritis have demonstrated reciprocal interactions between T cell-derived cytokines and synovial FLS, in addition to their role in driving angiogenic responses (Maggi et al., 2016; Petrasca et al., 2020) studies are lacking in DA. Indeed, no study, to date, has examined DA FLS. Importantly, cells and inflammatory mediators in disease do not act alone, with many studies showing cytokine synergy is a prominent pathogenic feature in autoimmune diseases including T1D and IA (Moran et al., 2011, Qiao et al., 2013; Shinjo et al., 2016; Wang et al., 2021; Hanlon et al., 2020, Kandhaya-Pillai et al., 2022). Therefore, to better understand the pathogenic mechanisms that drive DA, in this project we will examine (i) the effect T cell-derived cytokines synergy on DA FLS function, (ii) the effect of the joint microenvironment on angiogenic mechanisms and (iii) T cell plasticity. Better understanding of these mechanisms will contribute to development of more tailored treatment strategies for children living with this debilitating disease.

1.33 Overall aim and objectives

The overall aim of this thesis is to determine the role immune and stromal cells play in driving pro-inflammatory responses that contribute to the inflammation observed at the clinical level for this aggressive form of inflammatory arthritis in children with DA.
Results

CHAPTER TWO

Cytokine synergy drives the aggressive phenotype of DA Synovial Fibroblasts
2.1 Introduction

In the healthy synovium, FLS are the main stromal cells and are involved in maintaining joint homeostasis. They secrete ECM molecules into the SF which lubricate and protect the joint (Ospelt, 2017). Interestingly, these ECM molecules (including collagens, proteoglycans and glycoproteins) have been shown in vitro to allow nutrient exchange between the synovial fluid and synovial membrane (Buckley et al., 2021). FLS reside in the synovium and can be classified into sublining (CD90+ or Thy1+) or lining (CD90- or Thy1-) FLS (Wei et al., 2020) as well as both expressing type IV and V collagen (Bartok et al., 2010). FLS also express induced levels of VCAM-1, uridine diphosphoglucose dehydrogenase (UDPGD) and decay accelerating factor (DAF) (Edwards, 2000). Behavioural modifications in FLS are key to the pathogenic destruction of the joint in IA. An immunological hallmark of RA FLS is their enhanced migratory and invasive capacity which significantly contributes to synovial hyperplasia (Mousavi et al., 2021). However, as highlighted above, studies have identified numerous FLS subtypes in the inflamed joint with different functions defined by their invasive capacity or immune effector function (Pap et al., 2020; Wei et al., 2020; Kemble et al., 2021). In addition, anatomical location also impacts the phenotype of synovial FLS, with an elegant study by Frank-Bertoncelj et al. (2017), demonstrating that FLS function may also depend on positional epigenetic driven diversity (Frank-Bertoncelj et al., 2017). More recent studies from our own group identified 11 distinct FLS clusters in the RA and PsA synovium, frequency of which differs between the two arthropathies. In particular, Cluster 11, which are the Fap+ Thy1+ FLS subtype, were dominant in RA, in contrast to Cluster 1, the FAP- Thy1- FLS, which were dominant in PsA (Floudas et al., 2022). Pathway enrichment analysis identified several pathways that were differentially regulated across the clusters including ECM breakdown, focal adhesion, and invasive capacity.

FLS transform from a dormant state to an “imprinted aggressor” resistant to apoptosis phenotype (Bottini et al., 2013; Karami et al., 2020). Numerous reports have hypothesised why FLS may become impervious to apoptosis. According to Kim et al. (2018), RA FLS have increased expression of myeloid cell leukaemia-1 (Mcl-1) which makes these aggressive stromal cells resistant to Fas-induced apoptosis, in addition to the activation of death receptors TNF-related apoptosis-inducing ligand (TRAIL) (Kim et al., 2018). IL-17 has also
been implicated in anti-apoptotic mechanisms where it has been shown to induce mitochondrial abnormality and autophagosome formation in RA FLS, indicative of apoptosis-resistant mechanisms (Kim et al., 2017). Meanwhile, overexpression of the miRNA, miR-26a-5p, drives cell invasion and enhances the apoptotic-resistant capacity of RA FLS (Huang et al., 2019).

When FLS become activated in IA, the key molecules secreted are the MMPs (MMP-1, MMP-2, MMP-3, and MMP-9) which degrade the ECM allowing FLS invasion of the adjacent cartilage and bone. For pannus formation, FLS reorganize the ECM components so that the synovium is restructured into different microenvironmental niches (Chang et al., 2011). FLS secrete proinflammatory mediators (IL-6, MCP-1, IL-8, RANTES) that have a crucial role in arthritic diseases – an effect that is also dependent on alterations in metabolic pathways (Kennedy et al., 2010; Fromm et al., 2019; Gallagher et al., 2020). RA FLS are known to express higher levels of chemokine receptors including CXCR3, CXCR4, CXCR5 and CCR6 (Tang et al., 2017). As such, these chemokine receptors are involved in the homing of immune cells to the site of inflammation (Zhao et al., 2022). Facilitating and retaining leukocytes within the synovium is also supported by increased expression of the key adhesion molecules, ICAM-1 and VCAM-1 (Mullan et al., 2006; Maggi et al., 2016), regulation of which is activated by TNF-α, acute-phase protein serum amyloid A (A-SAA), IL-1β, oncostatin M (OSM) and the joint microenvironment (Fearon et al., 2006; Mullan et al., 2006; Fromm et al., 2019). FLS also secrete osteoclastogenesis activation molecules such as RANKL, a protein that specifically induces osteoclast formation and thus bone destruction. Osteoprotegerin, produced by osteoblasts, binds to RANKL and prevents osteoclastogenesis by inhibition of RANKL-driven signalling (Li et al., 2016).

Defective immunometabolism has been observed in FLS with significant changes in glycolysis, the PP, OxPhos and amino acid metabolism observed (Biniecka et al., 2016; Fearon et al., 2019, 2022; Gallagher et al., 2020; Davidson et al., 2021; Torres et al., 2022). Indeed, studies have shown that RA FLS are reliant on both glucose and glutamine metabolism, with in vitro studies showing that depletion of either of these molecules can inhibit RA FLS pathogenic function. Accumulation of metabolic intermediates in the inflamed joint has also been observed, which in turn can further stimulate synovial cells including FLS, EC and T cells, exacerbating the inflammatory response (Biniecka et al.,
Despite the increased vasculature observed at the early stages of disease pathogenesis, the highly metabolically active synovium and the dysfunctional vascular supply generates a hypoxic synovial microenvironment (Kennedy et al., 2010; Ng et al., 2010; Biniecka et al., 2016). Furthermore, not only do synovial pO\textsubscript{2} levels have an inverse correlation with synovitis, vascularity and T cell infiltrates, they also induce key proinflammatory signalling mediators including HIF-1\textalpha, NF-\kappaB, Notch and phosphorylated (p)-STAT (Oliver et al., 2009; Gao et al., 2013; Gao et al., 2015). Blockade of key glycolytic enzymes, including PFKFB3 (Biniecka et al., 2016) and HK2 (Bustamante et al., 2017) significantly decrease RA FLS pathogenic migratory and invasive capacities, through inhibition of key proinflammatory mediators and downstream transcriptional regulation (Fearon et al., 2022).

To date, no studies have examined the functional capacity of FLS from children with DA. Previous studies by our group demonstrated at an immunohistological level that there was an increase in cellular infiltrates in the synovium of children with DA compared to JIA (Foley et al., 2020). This was associated with an increase in the thickness of the lining layer region (10-15 cells thick) of the synovium, where subsets of the FLS reside. The increased lining layer thickness mirrors that of the associated increased erosive disease observed clinically in children with DA (Foley et al., 2019; Foley et al., 2019). This is in stark contrast to the healthy joint, where the synovial lining layer is one to two cells thick (Ospelt, 2017).

As highlighted in the main introduction (Section 1.25, Chapter 1), RNAseq analysis demonstrated that FLS isolated from different JIA subtypes displayed distinct molecular signatures. Specifically, LRRC15, GREM1 and GREM2 are overexpressed in persistent oligoJIA compared to pre-extension oligoJIA (Simonds et al., 2022). The same group noted that Th17 cells stimulate JIA FLS which in turn, activate osteoclasts through RANKL, recruit other immune cells and release MMPs (Margheri et al., 2019). Ligand stimulation for TLR-2, 3, 4, and 5 also triggers the production of IL-6, IL-8, MMP-1 and MMP-3 from JIA FLS. Other regulators of JIA FLS function are classical and nonclassical Th1 cells, effects that are mediated through TNF-\alpha-induced VCAM-1 hyperexpression-dependent pathways in JIA FLS (Margheri et al., 2019).

There is an abundance of data supporting the synergistic interplay between T cells and FLS in driving the proinflammatory response in RA (Paulissen et al., 2013; Mori et al., 2017;
Previous work from our group demonstrated that DA T cells display a polyfunctional phenotype (as described in Section 1.21, Chapter 1), with significantly higher frequencies of CD8-, CD8+, CD161+ Th cells coexpressing TNF-α, IL-17a, IFN-γ and GM-CSF observed in DA compared to HC, JIA and T21 children (Foley et al., 2020). Indeed, studies have shown that polyfunctional T cells are more pathogenic than single cytokine producing T cells, are resistant to Treg suppression and are associated with disease activity (Basdeo et al., 2017; Wade et al., 2018). We also previously demonstrated the expansion of CXCR3+ CCR6+ Tfh cells and CXCR3+ CCR6+ Tph cells in DA compared to T21, paralleled by a reduction in Treg numbers (Foley et al., 2020). Thus the increased polyfunctionality, with a concomitant decrease in frequency of Treg, is consistent with the more aggressive erosive disease observed in DA (Foley et al., 2019, 2020).

To date, no studies have examined the pathogenic phenotype of FLS isolated from children with DA. Based on the polyfunctional T cell signature previously described in DA (Foley et al., 2020), this chapter examines the effect of these T cell-derived cytokines (TNF-α, IL-17a, IFN-γ and GM-CSF) alone and in combination on DA FLS function.
2.2 Aims and Objectives

Overall aim: To examine the effect of T cell-derived cytokines on DA FLS pathogenic function

1. To determine the effects of TNF-α, IL-17a, IFN-γ and GM-CSF on DA FLS pro-inflammatory responses including cytokines, chemokines, and MMPs
2. To determine the effects of TNF-α, IL-17a, IFN-γ and GM-CSF on DA FLS leukocyte adhesion
3. To investigate the effects of cytokine synergy on DA FLS pro-inflammatory responses
4. To determine whether TNF-α, IL-17a and IFN-γ alter the bioenergetic profile of DA FLS
2.3 Materials and methods

2.3.1 Patient recruitment

This project was approved by the Ethics Research Committees of CHI (Children’s Health Ireland) at Crumlin and St Vincent’s University Hospital (SVUH). All families and participants received verbal and documented information on the study and written consent was obtained prior to recruitment. Children with DA were enrolled from the National Centre for Paediatric Rheumatology (NCPR) in CHI at Crumlin. Fully informed parental consent and participant acceptance were obtained for all individuals who provided blood and synovial tissue. DA synovial biopsies were obtained via ultrasound guided biopsies at CHI Crumlin.
<table>
<thead>
<tr>
<th>DA n=9</th>
<th>Age</th>
<th>Prior Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median (range) 10 (4-17)</td>
<td>NIL (inhibitor of inducible nitric oxide synthase) 3</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>NIL + NSAID 2</td>
</tr>
<tr>
<td></td>
<td>F = 5 M = 4</td>
<td>MTX + Etanercept 1</td>
</tr>
<tr>
<td>Active joint count</td>
<td></td>
<td>Adalimumab + Etanercept 2</td>
</tr>
<tr>
<td></td>
<td>n (range) 18 (0-7) unknown 2</td>
<td>Tocilizumab 1</td>
</tr>
<tr>
<td>ESR</td>
<td>Treatments at time of sample</td>
<td></td>
</tr>
<tr>
<td></td>
<td>high ESR (%) (11) median 9 unknown 0</td>
<td>NIL 3</td>
</tr>
<tr>
<td>CRP</td>
<td></td>
<td>NIL + NSAID 1</td>
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<tr>
<td></td>
<td>high CRP (%) (0) median &lt;5 unknown 0</td>
<td>NSAID 1</td>
</tr>
<tr>
<td>RF/ ACPA</td>
<td></td>
<td>Etanercept 1</td>
</tr>
<tr>
<td></td>
<td>Positive 0 Negative 9 Unknown 0</td>
<td>Tocilizumab 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Golimumab (TNF-inhibitor) 1</td>
</tr>
</tbody>
</table>

Table 2. 1 DA patient demographic, clinical, laboratory features and treatments prior sampling and at time of sampling.

2.3.2 Synovial biopsies-synovial fibroblast isolation

Synovial biopsies were obtained from ultrasound-guided biopsies. For primary synovial fibroblast isolation, synovial biopsies were digested with 1 mg/ml collagenase type 1 (Worthington Biochemical, Freehold, NJ, USA) in RPMI-1640 (Gibco-BRL, Paisley, UK) for 4hr at 37°C in humidified air with 5% CO₂. Dissociated cells were grown to confluence in RPMI 1640, 10% FBS (foetal bovine serum) (Gibco-BRL), 10 ml of 1 mmol/l HEPES (Gibco-BRL), penicillin (100 units/ml; Bioscience, UK), streptomycin (100 units/ml; Bioscience, UK) and fungizone (0.25 μg/ml; Bioscience, UK) (cRPMI) before passaging. Cells were used between passages 3–8.
2.3.3 Cytokine production

DA FLS were seeded into 6 well plates at a cell count of $1 \times 10^5$/ well and grown to confluence in cRPMI. DA FLS were then incubated with 1% FBS RPMI for 24hr prior to TNF-α (0.1ng/ml and 1ng/ml), IL-17a (20ng/ml and 50ng/ml), IFN-γ (10ng/ml and 50ng/ml) or GM-CSF (20ng/ml and 100ng/ml) stimulation for 24hr. For synergy experiments, cells were also cultured with TNF-α (0.1 ng/ml) in the presence or absence of IL-17a (50ng/ml), IFN-γ (50ng/ml) or GM-CSF (100ng/ml) for 24hr. Culture supernatants were harvested and stored at -20°C for further ELISA analysis.

2.3.4 Flow cytometric analysis of chemokines/adhesion molecules in DA FLS

DA FLS were seeded into 6 well plates at a cell count of $1 \times 10^5$/ well and grown to confluence in cRPMI. DA FLS were cultured with TNF-α (0.1 ng/ml and 1 ng/ml), IL-17a (20ng/ml and 50ng/ml) or IFN-γ (10ng/ml and 50ng/ml) for 24hr. For synergy experiments, cells were cultured with TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml) or IFN-γ (50ng/ml) for 24hr. Following culture, cells were gently detached with 500μl of Accutase (Sigma-Aldrich, Ireland) and incubated at 37°C for 5mins. Cells were then transferred to flow cytometry tubes and washed in ice cold phosphate-buffered saline (PBS) prior to incubation with Zombie NIR™ Fixable Viability Kit (BioSciences, UK) for 30mins at 4°C. An Fc receptor blocking step was performed by incubating the cells with TruStain FcX blocking solution (Biolegend, UK). Cells were then stained with fluorochrome-conjugated antibodies against specific extracellular markers (described in Table 2.1), vortexed then incubated in the dark at 4°C for 30mins. Samples were then washed in FACS Buffer (Dulbecco’s PBS without Mg2+ or Ca2+ (Sigma-Aldrich, Ireland), 1% heat-inactivated FBS (Sigma-Aldrich, Ireland) and 0.05% sodium azide (Sigma-Aldrich, Ireland, pH 7.4-7.6) (1% FBS RPMI) and centrifuged at 400g. Supernatants were removed, and the cells were fixed with 1% paraformaldehyde (PFA) for 10mins before at 4°C, washed and resuspended in 200μl of FACS Buffer. Samples were acquired using the LSRFortessa Flow Cytometer (BD, UK) and analysed using FlowJo software (BD, UK). To adjust for spectral overlap between detectors, compensation was applied using single stained compensation beads (BD, UK). Specific surface marker fluorescence gating was performed by comparison with an FMO (Fluorescence Minus One Control).
### Table 2. Extracellular antibodies used for DA FLS flow cytometric staining

<table>
<thead>
<tr>
<th>Marker</th>
<th>Other name</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR3</td>
<td>CD183</td>
<td>BV650</td>
<td>G025H7</td>
<td>Biolegend, UK</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CD184</td>
<td>Pe Dazzle 594</td>
<td>12G5</td>
<td></td>
</tr>
<tr>
<td>CXCR5</td>
<td>CD185</td>
<td>BV785</td>
<td>J252D4</td>
<td></td>
</tr>
<tr>
<td>CCR6</td>
<td>BN1</td>
<td>BV711</td>
<td>G034E3</td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td>CD106</td>
<td>PeCy5</td>
<td>STA</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>CD54</td>
<td>BV605</td>
<td>HA58</td>
<td>BD, UK</td>
</tr>
</tbody>
</table>

#### 2.3.5 Enzyme-linked immunosorbent assay (ELISA)

IL-6 and IL-8 levels in DA FLS cultured supernatants were determined using R&D DuoSet assays, while MCP-1 levels were determined by Invitrogen kits and RANTES levels assessed using Biolegend kits. IL-6 (min detection range of 9.4 pg/ml and standard range of 9.4-600 pg/ml) (R&D, UK), IL-8 (min detection range of 31.2 pg/ml and standard range of 31.2-2000 pg/ml) (R&D, UK), MCP-1 (min detection of 7pg/ml and standard range of 7-1000 pg/ml) (eBiosciences, USA), RANTES (min detection range of 3 pg/ml and standard range of 7.8-500 pg/ml) (Biolegend, UK). All ELISAs were performed according to the manufacturers’ instructions.

#### 2.3.6 Measurement of MMP-1, 3, and 9

To assess MMP-1, 3 and 9 levels, DA FLS were seeded at a density of 1 x 10⁵/well in a 6 well plate and grown to confluence in cRPMI. DA FLS were serum starved with 1% FBS RPMI for 24hr before stimulated with TNF-α (0.1 ng/ml and 1 ng/ml), IL-17a (20ng/ml and 50ng/ml) or IFN-γ (10ng/ml and 50ng/ml) for 24hr. For synergy experiments, cells were also incubated with TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml) or IFN-γ (50ng/ml) for 24hr. Following culture, supernatants were then harvested and stored at -20°C. MMP-1, 3 and 9 were measured by multiplex cytokine panel V-PLEX Pro-inflammatory assay (Meso Scale Discovery (MSD), USA) according to the manufacturer’s instructions. Electro-chemiluminiscence was measured using the MSD Sector Imager 2400. Cytokine standards ranged from 2 – 10,000 pg/ml.
2.3.7 Adhesion assay

DA FLS were seeded at a cell count of $1 \times 10^4$/ well in 48 well plates and grown to confluence in cRPMI. DA FLS were cultured in 1% FBS RPMI for 24hr and subsequently stimulated with TNF-α (0.1ng/ml and 1ng/ml), IL-17a (20ng/ml and 50ng/ml) and IFN-γ (10ng/ml and 50ng/ml) for a further 24hr. For synergy experiments, cells were also cultured with TNF-α (0.1 ng/ml) in the presence or absence of either IL-17a (50ng/ml) or IFN-γ (50ng/ml) for 24hr. Following culture, supernatants were removed and replaced with fresh media containing no cytokines. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation (Lymphoprep; Stemcell Technologies, Canada) according to the manufacturer’s recommendations. PBMC were then resuspended in PBS and $5 \times 10^5$ PBMC were added to each well containing DA FLS and incubated at 37°C with 5% CO₂ for 1hr. Following culture, supernatants were removed, and wells were washed with PBS x 3. Semi-quantification was performed by counting the number of PBMC adherent using phase-contrast microscopy (Leica, Germany) at 20X magnification. Quantification was assessed by counting the number of cells from three random high-power fields (HPF) for each well to assess the average number of adherent cells.

2.3.8 mRNA Extraction and cDNA Synthesis

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Germany) according to manufacturer’s instructions and stored at -80°C until required. To ensure that the integrity of RNA was sufficient for PCR assays, RNA quality was analysed using a NanoDrop 2000 spectrophotometer (ThermoFisher, USA). Samples with a 260:280 nm ratio of 1.8 and above were used in subsequent experiments. 100 ng total RNA was reverse-transcribed to complementary DNA (cDNA) using the high-capacity cDNA reverse transcription kit according to manufacturer’s instructions (Applied Biosystems, UK) and stored at -20°C until required.

2.3.9 Gene Expression Analysis

PCR reaction mixtures included 1μl cDNA, 10μM of specifically designed forward and reverse primers (described in Table 2.2), SYBR Green II Universal Master Mix (Thermo
Fisher Scientific, USA) and RNase-free water. To ensure target-specific quantification, samples without multiscrict reverse transcriptase were used as negative controls. Gene expression data were quantified by RT-PCR using the Quant Studio 5 Thermal Cycler (Applied Biosystem, Lewes, UK). All reactions/negative controls were performed in duplicate in a 96 well plate format. Thermal cycling conditions were applied as recommended by the manufacturer (Applied Biosystems, UK). Data were analysed using the comparative threshold cycle (Ct) method with normalisation to the expression of RPLPO as endogenous controls. Relative changes in gene expression were determined using the $2^{\Delta\Delta\text{Ct}}$ method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPLPO</td>
<td>5’ GCGTCTCTGGAATGACATCG 3’</td>
<td>5’ TCAGGGATTGCCACGCAGGG 3’</td>
</tr>
<tr>
<td>HPRT1</td>
<td>5’ ATGGACAGGACTGACCCTTG 3’</td>
<td>5’ GGCTAATGTGATGCGCCCT 3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’ CCCTGAGAAAGGACATGAA 3’</td>
<td>5’ CCTTTTGGCTTTTCACACATG 3’</td>
</tr>
<tr>
<td>IL-8</td>
<td>5’ TTGCAGCCTCTGATTC 3’</td>
<td>5’ TGGCAAAACTGCACCTTCAC 3’</td>
</tr>
<tr>
<td>RANTES</td>
<td>5’ CATCTGCTCCCATATCTC 3’</td>
<td>5’ ATGTAGGCAAAGCACGGGT 3’</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5’ GTCGCTACAGCAAGCACC 3’</td>
<td>5’ TGGTGAAGTTATAACAGCAAGTGA 3’</td>
</tr>
<tr>
<td>LDHA</td>
<td>5’ ATGGGATTTGCTGTGACTG 3’</td>
<td>5’ CAGAGACACGACAGCAATTC 3’</td>
</tr>
<tr>
<td>PKM2</td>
<td>5’ ATATTTTAGGAAACTCCGCGCCT 3’</td>
<td>5’ ATTCGGGATCACGGAATGTGG 3’</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>5’ GAACATTCTGTGCTTGTATTT 3’</td>
<td>5’ GCAATTCATCTGTGCTTTTGTCA 3’</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>5’ GCCGGCGGATTACGAGCTG 3’</td>
<td>5’ AGCATCTAAAGGAGCTGSCCA 3’</td>
</tr>
<tr>
<td>HK2</td>
<td>5’ TTGCGAGATTGGCTCGGACTTG 3’</td>
<td>5’ TTGCAGGATGCGACTTG 3’</td>
</tr>
</tbody>
</table>

Table 2. Designed primer sequences used for real-time PCR analysis

### 2.3.10 Cellular bioenergetic functional analysis

Oxygen consumption rate (OCR) defined oxygen phosphorylation and extracellular acidification rate (ECAR) defined glycolysis. These were examined using the Seahorse XFe96 analyzer (Seahorse Biosciences, USA). DA FLS were seeded at a density of $12 \times 10^3$ cells/well in a 96 well cell culture XFe microplate (Seahorse Biosciences, USA) and allowed to adhere overnight. For DA FLS, cells were stimulated with TNF-α (0.1ng/ml) in the presence or absence of IL-17α (50-ng/ml) or IFN-γ (10ng/ml and 50ng/ml) for 24hr. Cells were then washed with assay medium (unbuffered DMEM supplemented with 10mM
glucose, pH 7.4) before incubation with assay medium for 30min at 37°C in a non-CO₂ incubator. Basal oxidative phosphorylation/glycolysis was calculated by the average of three baseline OCR/ECAR measurements. This was acquired before injection of specific metabolic inhibitors; oligomycin (ATP-synthase inhibitor) (2μg/ml; Seahorse Biosciences, UK) trifluorocarbonylcyanide phenylhydrazone (FCCP) (mitochondrial uncoupler) (5μM; Seahorse Biosciences) and antimycin A (complex-III inhibitor) (2μM, Seahorse Biosciences). Oligomycin was injected to evaluate both the maximal glycolytic rate and ATP synthesis, determined by subtracting the amount of respiration left after oligomycin injection from baseline OCR. FCCP was injected to evaluate the maximal respiratory capacity (average of three measurements following injection). Maximal respiratory capacity was ascertained by subtracting the baseline OCR from FCCP-induced OCR and the respiratory reserve (baseline OCR subtracted from maximal respiratory capacity) as per Figure 2.3.1.

![Seahorse XF Cell Mito Stress Test Profile](image)

**Figure 2.3.1.** Representative Seahorse Cell Mito Stress test assay depicting measurable parameters.

### 2.3.11 Statistical analysis

Statistical analyses were performed on GraphPad Prism 9 software. One-way ANOVA (#) test or paired t-test (*) was used for analysis of non-parametric data. P- values of <0.05 (#p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001) were determined as statistically significant for Friedman One-way ANOVA while *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 were defined as statistically significant for Wilcoxon signed Rank paired te
2.4 Results

2.4.1 TNF-α, IL-17a and IFN-γ induce cytokine production, metabolic marker expression and MMPs production from DA FLS

To investigate the effects of T cell-derived proinflammatory mediators on DA FLS function, DA FLS were stimulated with increasing concentrations of TNF-α, IL-17a, IFN-γ and GM-CSF, and secretion of proinflammatory mediators assessed by ELISA. Following stimulation with TNF-α, we observed a significant dose-dependent increase of both IL-6 and MCP-1 secretion from DA FLS compared to basal (0.1ng/ml, \( p < 0.05 \); 1ng/ml, \( p < 0.001 \)) (Figure 2.1(a)(i)) and (b)(i)). We also demonstrated an increase in IL-6 secretion in response to IL-17a (50ng/ml, \( p < 0.01 \)) compared to basal with no effects observed for MCP-1 secretion (Figure 2.1(a)(ii) and (b)(ii)). Additionally, IFN-γ-treated DA FLS led to a significant increase in IL-6 secretion (10ng/ml, \( p < 0.05 \); 50ng/ml, \( p < 0.05 \)) paralleled by a significant increase in MCP-1 production (10ng/ml, \( p < 0.01 \); 50ng/ml, \( p < 0.05 \)) compared to basal (Figure 2.1(a)(iii) and (b)(iii)). While IL-6 and MCP-1 production increased in response to GM-CSF, no significant effects were observed (Figure 2.1(a)(iv) and (b)(iv)).

Furthermore, RANTES and IL-8 secretion were significantly upregulated in a dose-dependent manner when stimulated with TNF-α (0.1ng/ml, \( p < 0.05 \); 1ng/ml, \( p < 0.001 \)) (Figure 2.2 (a)(i) and (b)(i)). No effect was observed for RANTES in response to IL-17a (Figure 2.2(a)(ii)), however while IL-8 secretion was increased in a dose-dependent manner in response to IL-17a, this did not reach significance (20ng/ml, \( p = 0.07 \)) (Figure 2.2(b)(ii)). IFN-γ stimulation led to an increase in RANTES secretion compared to basal (10ng/ml, \( p < 0.05 \); 50ng/ml, \( p < 0.05 \)) (Figure 2.2(a)(iii)) with no effect observed for IL-8 secretion. No effects for RANTES and IL-8 were observed in response to GM-CSF stimulation (Figure 2.2(a)(iv) and (b)(iv)).

Next, we examined the effect of TNF-α, IL-17a and IFN-γ on gene expression. Since no effects were observed at the protein level in response to GM-CSF stimulation (Figure 2.1 and 2.2), GM-CSF was not examined. A dose-dependent increase in fold induction was observed when DA FLS were treated with TNF-α (0.1ng/ml, \( p = 0.06 \); 1ng/ml, \( p < 0.01 \)) and IL-17a (20ng/ml, \( p = 0.06 \); 50ng/ml, \( p < 0.01 \)) for IL-6 (Figure 2.3(a)(i – ii)). Similarly, MCP-1 was increased in a dose-dependent manner in response to TNF-α (0.1ng/ml, \( p = 0.06 \);
1ng/ml, p<0.01) and IL-17a (50ng/ml, p<0.05) (Figure 2.3(b)(i – ii)). Stimulation with IFN-γ led to an increase in fold induction of IL-6 (50ng/ml, p<0.05) (Figure 2.3(a)(iii)) and MCP-1 (50ng/ml, p<0.05) (Figure 2.3(b)(iii)). Similarly, TNF-α stimulation induced a dose-dependent increase in RANTES (0.1ng/ml, p<0.05; 1ng/ml, p<0.01) (Figure 2.4(a)(i)) and IL-8 expression (0.1ng/ml, p=0.06; 1ng/ml, p<0.05) (Figure 2.4(b)(i)). IL-17a (50ng/ml, p<0.05) significantly induced a fold induction in RANTES (Figure 2.4(a)(ii)) and IL-8 (Figure 2.4(b)(ii)) gene expression. IFN-γ stimulation also resulted in a significant fold induction in RANTES (10ng/ml, p<0.05; 50ng/ml, p<0.05) (Figure 2.4(a)(iii)) and IL-8 gene expression (50ng/ml, p<0.05) (Figure 2.4(b)(iii)).

MMPs are a critical component of the cartilage degradation process (Smolen et al. 2016), therefore we sought to investigate whether the T cell-derived cytokines regulated MMP secretion by DA FLS. Cells were cultured with increasing concentrations of TNF-α, IL-17a and IFN-γ for 24hr, supernatants were harvested, and an MSD multiplex assay was performed. We observed a dose-dependent increase in response to TNF-α for MMP-1 (0.1ng/ml, p<0.05; 1ng/ml, p<0.01) (Figure 2.5(a)(i)), MMP-3 (1ng/ml, p=0.06) (Figure 2.5(b)(i)), and MMP-9 (0.1ng/ml, p<0.05; 1ng/ml, p<0.05) (Figure 2.5(c)(i)). MMP-3 secretion was reduced in response to IL-17a (20ng/ml, p=0.06; 50ng/ml, p=0.06) (Figure 2.5(b)(ii)), however no significant changes were reported for MMP-1 and MMP-9 (Figure 2.5(a)(ii) and (c)(ii)). IFN-γ had no significant effect on MMP-1, MMP-3, or MMP-9 (Figures 2.5(a - c)(iii)).

Since RA FLS have been shown to undergo a metabolic shift to maintain their activated pathogenic phenotype (Fearon et al., 2022b), we next examined the effect of TNF-α, IL-17a and IFN-γ on key metabolic gene expression. A dose-dependent fold induction in the expression of the glucose receptor, GLUT-1, in response to TNF-α (0.1ng/ml, p=0.06; 1ng/ml, p<0.05), IL-17a (20ng/ml, p=0.06; 50ng/ml, p<0.01) and IFN-γ (50g/ml, p<0.05) was demonstrated (Figure 2.6(a)(i – iii)). Similarly, a dose-dependent increase in LDHA expression for TNF-α was demonstrated (0.1ng/ml, p=0.06; 1ng/ml, p<0.01) (Figure 2.6(b)(i)). IL-17a stimulation led to an increase in LDHA gene expression (50ng/ml, p<0.05) (Figure 2.6(b)(ii)). Additionally, an increase in LDHA in response to IFN-γ stimulation was also demonstrated (50ng/ml, p=0.06) (Figure 2.6(b)(iii)), however this did not reach
significance. A dose-dependent increase in the fold induction of the key metabolic sensor, HIF-1α, was demonstrated for DA FLS treated with TNF-α (0.1ng/ml, \( p<0.05 \); 1ng/ml, \( p<0.05 \)), IL-17a (50ng/ml, \( p=0.06 \)) and IFN-γ (50ng/ml, \( p<0.05 \)) (Figure 2.7(a)(i – iii)). Similarly, TNF-α stimulation also led to a significant increase in a dose-dependent manner of HK2 (0.1ng/ml, \( p=0.06 \); 1ng/ml, \( p<0.05 \)) (Figure 2.7(b)(i)) and PKM2 expression (1ng/ml, \( p<0.05 \)) (Figure 2.7(c)(i)). IL-17a significantly induced HK2 (1ng/ml, \( p<0.05 \)) (Figure 2.7(b)(ii)) with no significant effects observed for PKM2 (Figure 2.7(c)(ii)). Similarly, IFN-γ treated DA FLS showed a trending increase in HK2 (50ng/ml, \( p=0.06 \)) (Figure 2.7(b)(iii)) and PKM2 (10ng/ml, \( p=0.06 \)) (Figure 2.7(c)(iii)).
Figure 2. 1 TNF-α, IL-17a and IFN-γ induce IL-6 and MCP-1 secretion from DA FLS. DA FLS were cultured with (i) TNF-α (0.1ng/ml and 1ng/ml), (ii) IL-17a (20ng/ml and 50ng/ml), (iii) IFN-γ (10ng/ml and 50ng/ml) and (iv) GM-CSF (20ng/ml and 100ng/ml) for 24hr before supernatants harvested and cytokines measured. Bar charts show quantification of (a) IL-6 and (b) MCP-1 secretion from DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=4 or 7 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01, ####p<0.001 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. 2 TNF-α, IL-17a and IFN-γ induce RANTES and IL-8 secretion from DA FLS. DA FLS were incubated with (i) TNF-α (0.1ng/ml and 1ng/ml), (ii) IL-17a (20ng/ml and 50ng/ml), (iii) IFN-γ (10ng/ml and 50ng/ml) and (iv) GM-CSF (20ng/ml and 100ng/ml) for 24hr before supernatants harvested and cytokines measured. Bar charts show quantification of (a) RANTES and (b) IL-8 production from DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01, ###p<0.001 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. 3 TNF-α, IL-17a and IFN-γ induce IL-6 and MCP-1 gene expression in DA FLS. DA FLS were incubated with (i) TNF-α (0.1ng/ml and 1ng/ml), (ii) IL-17a (20ng/ml and 50ng/ml) and (iii) IFN-γ (10ng/ml and 50ng/ml) for 24hr before RNA was isolated and RT-PCR performed. Bar charts show quantification of (a) IL-6 and (b) MCP-1 gene expression in DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=5 expts, normalized to housekeeping control RPLPO. $2^{-\Delta\Delta Ct}$ indicates fold induction. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, #p<0.05, ##p<0.01 significantly different compared to other conditions.
Figure 2.4 TNF-α, IL-17a and IFN-γ induces RANTES and IL-8 gene expression in DA FLS. DA FLS were incubated with (i) TNF-α (0.1ng/ml and 1ng/ml), (ii) IL-17a (20ng/ml and 50ng/ml) and (iii) IFN-γ (10ng/ml and 50ng/ml) for 24hr before RNA was isolated and RT-PCR performed. Bar charts show quantification of (a) RANTES and (b) IL-8 gene expression in DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=4-5 expts, normalized to housekeeping control RPLPO. \(2^{-\Delta\Delta Ct}\) indicates fold induction. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively #\(p<0.05\), ###\(p<0.01\) significantly different compared to other conditions. *\(p<0.05\) significantly different compared to other conditions.
Figure 2. 5 TNF-α, IL-17a and IFN-γ induce MMP secretion in DA FLS. DA FLS were incubated with (i) TNF-α (0.1ng/ml and 1ng/ml), (ii) IL-17a (20ng/ml and 50ng/ml) and (iii) IFN-γ (10ng/ml and 50ng/ml) for 24hr before supernatants harvested and MMPs measured via MSD assay. Bar charts show quantification of (a) MMP-1, (b) MMP-3, and (c) MMP-9 secretion from DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=6 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. 6 TNF-α, IL-17a and IFN-γ induce GLUT-1 and LDHA gene expression in DA FLS. DA FLS were incubated with (i) TNF-α (0.1ng/ml and 1ng/ml), (ii) IL-17a (20ng/ml and 50ng/ml) and (iii) IFN-γ (10ng/ml and 50ng/ml) for 24hr before RNA was isolated and RT PCR performed. Bar charts show quantification of (a) GLUT-1 and (b) LDHA gene expression in DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=5 expts, normalized to housekeeping controls RPLPO. $2^{\Delta\Delta CT}$ indicates fold induction. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, #p<0.05, ##p<0.01 significantly different compared to other conditions.
Figure 2. 7 TNF-α, IL-17a and IFN-γ induce HIF-1α, HK2 and PKM2 gene expression in DA FLS. DA FLS were incubated with (i) TNF-α (0.1ng/ml and 1ng/ml), (ii) IL-17a (20ng/ml and 50ng/ml) and (iii) IFN-γ (10ng/ml and 50ng/ml) for 24hr before RNA was isolated and RT PCR performed. Bar charts show quantification of (a) HIF-1α, (b) HK2 and (c) PKM2 gene expression in DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=5-6 expts, normalized to housekeeping control RPLPO. 2^{ΔΔct} indicates fold induction. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
2.4.2 TNF-α, IL-17a and IFN-γ induce the adhesive ability of DA FLS

One of the main pathogenic functions of FLS is to retain infiltrating immune cells in the synovium, therefore we next examined the effect of TNF-α, IL-17a and IFN-γ on the adhesive capacity of DA FLS. DA FLS were cultured with increasing concentrations of TNF-α, IL-17a and IFN-γ for 24hr. Following culture, fresh media with healthy PBMC were added to the DA FLS for 1hr. Non-adherent cells were removed following PBS wash and the remaining adherent PBMC to the DA FLS monolayer were quantified using phase contrast microscopy. Figure 2.8(a) shows representative images of PBMC adhesion to DA FLS in response to cytokine stimulation. Quantification demonstrated an increase in the adhesive capacity of DA FLS, evident by the significant increase in the number of PBMC attached to DA FLS following stimulation with TNF-α (0.1ng/ml, p<0.05; 1ng/ml, p<0.01), IL-17a (20ng/ml, p=0.06; 50ng/ml, p<0.01), and IFN-γ (50ng/ml, p<0.01) (Figure 2.8(b – d)) compared to basal.

We next examined cell surface expression of key adhesion molecules, ICAM-1 and VCAM-1, on DA FLS by flow cytometry. Representative flow plots of the gating strategy are shown in Figure 2.9. Following exclusion of debris and doublets (Figure 2.9(a – c)), we gated on live cells (Figure 2.9(d)), finally, specific surface marker fluorescence was gated (Figure 2.9(e)). Figure 2.10(a) demonstrates representative dot plots for FMO control and basal unstimulated control for ICAM-1. Representative dot plots (Figure 2.10(b)(i)) and quantification (Figure 2.10(b)(ii)) demonstrate a significant increase in the frequency of ICAM-1 positive DA FLS in response to TNF-α (0.1ng/ml, p<0.01; 1ng/ml, p=0.05). In addition, we observed a stepwise increase in the MFI for ICAM-1 (Figure 2.10(b)(iii)) as reflected in the histogram plots (0.1ng/ml, p<0.01, 1ng/ml, p<0.0001) (Figure 2.10(b)(iv)). While there was no change in the frequency of ICAM-1 positive DA FLS in response to IL-17a (Figure 2.10(c)(i – ii)), a stepwise increase for ICAM-1 MFI compared to basal was observed (20ng/ml, p=0.07; 50ng/ml, p<0.01) (Figure 2.10(c)(iii – iv)). Representative dot plots (Figure 2.10(d)(i)) and quantification (Figure 2.10(d)(ii)) demonstrate a significant increase in frequency of ICAM-1 positive DA FLS in response to IFN-γ (10ng/ml, p<0.05; 50ng/ml, p<0.05). Quantification and representative histogram plots demonstrate a stepwise increase in ICAM-1 MFI (10ng/ml, p<0.01; 50ng/ml, p<0.001) (Figure 2.10(d) (iii – iv)).
Figure 2.11(a) shows representative flow plots for VCAM-1 FMO control and basal. There was a dramatic increase in the frequency of VCAM-1 positive DA FLS in response to TNF-α (0.1ng/ml, p<0.001; 1ng/ml, p<0.01) compared to basal (Figure 2.11(b)(i – ii)). Quantification and representative histograms plots demonstrate a dose-dependent increase in VCAM-1 MFI (0.1ng/ml, p<0.01; 1ng/ml, p<0.001) (Figure 2.11(b) (iii – iv)). No effect was observed in response to IL-17a for frequency of VCAM-1 positive DA FLS or VCAM-1 MFI (Figure 2.11(c)). A dose-dependent increase in the frequency of VCAM-1 positive DA FLS in response to IFN-γ stimulation was also observed (10ng/ml, p<0.01; 50ng/ml, p<0.01) (Figure 2.11(d)(i – ii)) paralleled by an increase in MFI for VCAM-1 (10ng/ml, p=0.07; 50ng/ml, p<0.05) (Figure 2.11(d)(iii – iv)).
Figure 2. TNF-α, IL-17a and IFN-γ induce the adhesive capacity of DA FLS. DA FLS were stimulated with TNF-α (0.1ng/ml and 1ng/ml), IL-17a (20ng/ml and 50ng/ml) or IFN-γ (10ng/ml and 50ng/ml) for 24hr before being washed then incubated with 5 x 10^5 PBMC for 1hr then washed. (a) Representative photomicrographs show the number of PBMC attached after stimulation with the various cytokines. (b) – (d). Bar charts show quantification of PBMC attached to DA FLS monolayers after stimulation with the various cytokines. Values expressed as mean ± SEM of n=6 expts. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. Gating strategy for flow cytometric analysis of DA FLS. DA FLS were stained with a panel of fluorochrome antibodies. Representative dot plots depicting the gating strategy used to identify and phenotype DA FLS. (a) – (d) the forward and side scatter parameters of cells were set before doublet exclusion and elimination of dead cells. (e) DA FLS were then gated for VCAM-1 (example marker).
Figure 2. 10 TNF-α, IL-17a and IFN-γ induce ICAM-1 expression in DA FLS. (a) Representative flow plots show FMO and basal. DA FLS were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots illustrate ICAM-1 positive DA FLS following treatment with the different cytokines. (ii) Bar chart displays the percentage frequency of ICAM-1 positive DA FLS in response to cytokines. (iii) Bar charts illustrate ICAM-1 MFI and (iv) representative histograms depict ICAM-1 MFI. Values expressed as mean ± SEM of n=9 expts, One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 significantly different compared to other conditions. *p<0.05, **p<0.01 significantly different compared to other conditions.
Figure 2. TNF-α or IFN-γ induce VCAM-1 expression in DA FLS. (a) Representative flow plots show FMO and unstimulated DA FLS. DA FLS were stimulated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots illustrate VCAM-1 positive DA FLS following treatment with the different cytokines. (ii) Bar chart displays the percentage frequency of VCAM-1 positive DA FLS in response to cytokines. (iii) Bar charts depict VCAM-1 MFI and (iv) representative histograms outline VCAM-1 MFI. Values expressed as mean ± SEM of n=9 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01, ###p<0.001 significantly different compared to other conditions. *p<0.05, **p<0.01 significantly different compared to other conditions.
2.4.3 TNF-α, IL-17a and IFN-γ induce expression of CXCR3, CXCR4 and CXCR5 on DA FLS

Next, we examined chemokine receptor expression – key molecules that facilitate the homing of leukocytes into the joint (Elemam et al., 2020). Representative dot plots for FMO and basal are shown for CXCR3 (Figure 2.12(a)). Representative flow dot plots and quantification demonstrate that both TNF-α (0.1ng/ml, \( p < 0.01 \); 1ng/ml, \( p < 0.001 \)) and IFN-γ (10ng/ml, \( p < 0.01 \); 50ng/ml, \( p < 0.001 \)) significantly increased the frequency of CXCR3 positive DA FLS (Figure 2.12(b)(i – ii) and Figure 2.12(d)(i – ii)). No effect was observed for the frequency of CXCR3 positive DA FLS in response to IL-17a stimulation (Figure 2.12(c)). Similar effects were observed for CXCR4. Figure 2.13(a) shows representative dot plots for FMO control and basal. Representative flow dot plots and quantification shows that both TNF-α (0.1ng/ml, \( p < 0.05 \); 1ng/ml, \( p < 0.01 \)) and IFN-γ (0.1ng/ml, \( p < 0.01 \); 1ng/ml, \( p < 0.01 \)) significantly increased the frequency of CXCR4 positive DA FLS compared to basal control (Figure 2.13(b)(i – ii) and Figure 2.13(d)(i – ii)). No effects were observed for the frequency of CXCR4 positive DA FLS in response to IL-17a stimulation (Figure 2.13(c)).

Figure 2.14(a) shows representative dot plots for CXCR5 FMO and basal control. A significant increase in the frequency of CXCR5 positive DA FLS was observed in response to TNF-α (1ng/ml, \( p < 0.05 \)) (Figure 2.14(b)(i – ii)). An increasing trend in the frequency of CXCR5 positive DA FLS in response to IFN-γ was also observed (50ng/ml, \( p = 0.07 \)) (Figure 2.14(d)), however this did not reach significance. No effect was observed for the frequency of CXCR5 positive DA FLS in response to IL-17a stimulation (Figure 2.14(c)). Finally, we assessed CCR6 expression. Representative dot plots for FMO and basal are shown for CCR6 in Figure 2.15(a). An increase in the frequency of CCR6 positive DA FLS in response to TNF-α (1ng/ml, \( p < 0.05 \)) and IFN-γ (50ng/ml, \( p < 0.05 \)) (Figure 2.15(b)(i – ii)) and Figure 2.15 (d)(i – ii)) was demonstrated. No effects were observed for the frequency of CCR6 positive DA FLS in response to IL-17a stimulation (Figure 2.15(c)).

Collectively, these data suggest that T cell-derived cytokines differentially induce the expression of adhesion molecules and chemokine receptors on DA FLS thus facilitating the migration and adhesion of immune cells into the joint therefore contributing to the pro-inflammatory response.
Figure 2. TNF-α or IFN-γ induces CXCR3 expression in DA FLS. (a) Representative flow dot plots show FMO and basal. DA FLS were incubated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show CXCR3 expression on DA FLS when treated with the different cytokines. (ii) Bar chart displays the percentage frequency of CXCR3 positive DA FLS following treatment with the different cytokines. Values expressed as mean ± SEM of n=9 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01, ###p<0.001 is significantly different compared to other conditions. *p<0.05, **p<0.01 significantly different compared to other conditions.
Figure 2. 13 TNF-α or IFN-γ induces CXCR4 expression in DA FLS. (a) Representative flow plots show FMO and basal. DA FLS were incubated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show CXCR4 expression on DA FLS when treated with the different cytokines. (ii) Bar chart displays the percentage frequency of CXCR4 positive DA FLS following treatment with the different cytokines. Values expressed as mean ± SEM of n=9 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively #p<0.05, ##p<0.01, is significantly different compared to other conditions. *p<0.05, **p<0.01 significantly different compared to other conditions.
Figure 2. 14 TNF-α or IFN-γ induces CXCR5 expression in DA FLS. (a) Representative flow plots show FMO and basal. DA FLS were cultured with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show CXCR5 expression on DA FLS following treatment with the different cytokines (ii) Bar chart displays the percentage frequency of CXCR5 positive DA FLS in response to cytokines. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, #p<0.05 is significantly different compared to other conditions.
Figure 2. 15 TNF-α or IFN-γ induces CCR6 expression in DA FLS. (a) Representative flow plots show FMO and representative flow plot for basal. DA FLS were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show CCR6 expression on DA FLS when treated with the different cytokines. (ii) Bar chart displays the percentage frequency of CCR6 positive DA FLS following treatment with the different cytokines. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA and Wilcoxon paired t-test were used for statistical analysis, respectively. #p<0.05 is significantly different compared to other conditions. *p<0.05 is significantly different compared to other conditions.
2.4.4 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces secretion and gene expression of proinflammatory mediators, and MMP production from DA FLS

We have shown that TNF-α, IL-17a and IFN-γ stimulation alone induce proinflammatory effects in DA FLS, however, within the joint microenvironment these cytokines are released from T cells simultaneously. Furthermore, based on our previous studies (Foley et al., 2020) there is a significant enrichment of polyfunctional T cells producing TNF-α, IL-17a and IFN-γ simultaneously in children with DA. Therefore, we next examined whether these cytokines could potentiate each other’s effects. DA FLS were stimulated with TNF-α (0.1ng/ml), IL-17a (50ng/ml), IFN-γ (50ng/ml) or GM-CSF (100ng/ml) alone and in combination for 24hr and supernatants were harvested for cytokine measurement.

IL-17a and IFN-γ potentiated/synergized the effect of TNF-α on IL-6 and MCP-1 secretion compared to basal or either cytokine alone (p<0.05) (Figure 2.16(a)(i – ii) and (b)(i – ii)). In contrast, no potentiation/synergistic effects were observed for GM-CSF (Figure 2.16(a)(iii) and (b)(iii)). IL-17a and IFN-γ had additive/potentiation effects on TNF-α induced RANTES (p<0.05) (Figure 2.17(a)(i – ii)) compared to basal or either cytokine alone. Interestingly, while IL-17a potentiated/synergized the effects of TNF-α on IL-8 secretion (p<0.01) (Figure 2.17(b)(i)), IFN-γ inhibited TNF-α induced IL-8 secretion (p<0.05) (Figure 2.17(b)(iii)). No effects were observed in response to GM-CSF with TNF-α (Figure 2.17(a)(iii) and (b)(iii)). Consistent with these results, both IL-17a and IFN-γ synergistically enhanced the effects of TNF-α on IL-6 gene expression (Figure 2.18(a)(i – ii)). IL-17a and IFN-γ potentiated the effects of TNF-α on MCP-1 gene expression (Figure 2.18(b)(i – ii)).

While IL-17a and TNF-α alone significantly stimulated RANTES gene expression compared to basal (p<0.01), no further effect was observed for the cytokine combination compared to either cytokine alone (Figure 2.19(a)(i)). IFN-γ potentiated the effect of TNF-α on RANTES gene expression compared to cytokines alone (p<0.05) (Figure 2.19(a)(iii)). For IL-8 gene expression, IL-17a potentiated the effect of TNF-α (Figure 2.19(b)(i)), like that observed at the protein level. In contrast, IFN-γ also potentiated the effect of TNF-α on IL-8 (p=0.06) (Figure 2.19(b)(iii)) which differs to that observed at the protein level where inhibition was demonstrated.
Next, we assessed the effect of cytokine combinations on MMP secretion from DA FLS. IL-17a potentiated the effect of TNF-α on secretion of MMP-1 ($p<0.01$), MMP-3 by four-fold ($p<0.01$) and MMP-9 by two-fold (Figure 2.20(a)(i), (b)(i) and (c)(i)). The combination of IFN-γ and TNF-α had no effect on MMP-1 or MMP3 compared to either cytokine alone (Figure 2.20(a)(ii) and Figure 2.20(b)(ii)). In contrast, IFN-γ and TNF-α inhibited MMP-9 gene expression by three-fold compared to cytokines alone ($p<0.05$) (Figure 2.20(c)(ii)).
Figure 2. Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces DA FLS IL-6 and MCP-1 secretion. DA FLS were stimulated with TNF-α (0.1ng/ml) in the presence or absence IL-17a (50ng/ml) or IFN-γ (50ng/ml) or GM-CSF (100ng/ml) for 24hr before analysing cytokine secretion via ELISA. Bar charts show quantification of (a) IL-6 and (b) MCP-1 secretion in DA FLS supernatants when treated with TNF-α alone and in combination with (i) IL-17a, (ii) IFN-γ or (iii) GM-CSF. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (†) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. †p<0.05, ††p<0.01, †††p<0.001, ††††p<0.0001 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. 17 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ differentially regulates DA FLS RANTES and IL-8 secretion. DA FLS were stimulated with TNF-α (0.1ng/ml) in the presence or absence IL-17a (50ng/ml) or IFN-γ (50ng/ml) or GM-CSF (100ng/ml) for 24hr before analysing cytokine secretion via ELISA. Bar charts show quantification of (a) RANTES and (b) IL-8 secretion in DA FLS supernatants when treated with TNF-α alone and in combination with (i) IL-17a, (ii) IFN-γ or (iii) GM-CSF. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01, ####p<0.001 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. 18 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces IL-6 and MCP-1 gene expression of DA FLS. DA FLS were cultured with TNF-α (0.1ng/ml) in the presence or absence of (i) IL-17a (50ng/ml) and (ii) IFN-γ (50ng/ml) of for 24hr before RNA was isolated and RT PCR performed. Bar charts show quantification of (a) IL-6 and (b) MCP-1 gene expression in DA FLS in response to cytokines. Values expressed as mean ± SEM of n=5-6 expts, normalized to housekeeping control RPLPO. 2^ΔΔct indicates fold induction. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, #p<0.05, ###p<0.01 significantly different from basal. *p<0.05 significantly different from basal.
Figure 2. Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces RANTES and IL-8 gene expression of DA FLS. DA FLS were incubated with TNF-α (0.1ng/ml) in the presence or absence of (i) IL-17a (50ng/ml) and (ii) IFN-γ (50ng/ml) for 24hr before RNA was isolated and RT PCR performed. Bar charts show quantification of (a) RANTES and (b) IL-8 gene expression in DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=5-6 expts, normalized to housekeeping control RPLPO. $2^{-\Delta\Delta ct}$ indicates fold induction. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. $p<0.05$, $##p<0.01$, $###p<0.001$ significantly different from basal. *$p<0.05$ significantly different from basal.
Figure 2. Synergy between TNF-α and IL-17a or TNF-α and IFN-γ differentially regulates MMP secretion by DA FLS. DA FLS were incubated with TNF-α (0.1ng/ml) in the presence or absence of (i) IL-17a (50ng/ml) and (ii) IFN-γ (50ng/ml) for 24hr before supernatants harvested and MMPs measured via MSD assay. Bar charts show quantification of (a) MMP-1, (b) MMP-3, and (c) MMP-9 secretion from DA FLS in response to cytokine combinations. Values expressed as mean ± SEM of n=6 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
2.4.5 TNF-α and IFN-γ synergistically induce cellular adhesion and ICAM-1 and VCAM-1 expression of DA FLS

To ascertain whether the synergistic relationship observed between cytokines had an effect on the adhesive capacity of DA FLS, we performed leukocyte-DA FLS adhesion assays along with quantification of adhesion molecules ICAM-1 and VCAM-1. Figure 2.21(a)(i) and Figure 2.21(b)(i) demonstrates the representative images for leukocyte adhesion on DA FLS following stimulation with TNF-α in the presence or absence of IL-17a or IFN-γ respectively. Quantification demonstrated that while both TNF-α (p<0.05) and IL-17a alone (p<0.05) stimulated leukocyte adhesion to DA FLS compared to basal, no further potentiation was observed for TNF-α and IL-17a combination (Figure 2.21(a)(ii)). While TNF-α (p<0.05) and IFN-γ alone (p<0.05) increased the number of adherent PBMC compared to basal alone, IFN-γ potentiated the effect of TNF-α (p<0.05) (Figure 2.21(b)(ii)).

Next, we investigated the effect of TNF-α in the presence or absence of IL-17a or IFN-γ on ICAM-1 and VCAM-1 cell surface expression. The gating strategy shown in Figure 2.9 was applied. Representative dot plots and quantification demonstrate the frequency of ICAM-1 positive DA FLS was increased in response to TNF-α alone (p<0.05) compared to basal, but no potentiation/synergy was observed in the presence of IL-17a (Figure 2.22(a)(i – ii)). Quantification of MFI and representative flow histograms demonstrates a significant increase in ICAM-1 MFI in response to TNF-α alone (p<0.0001) compared to basal (Figure 2.22(a)(iii – iv)). While TNF-α and IL-17a together significantly reduced the MFI for ICAM-1 (p<0.01) compared to TNF-α alone (Figure 2.22(a)(iii – iv)). Representative dot plots and quantification demonstrates a significant increase in the frequency of ICAM-1 positive DA FLS in response to IFN-γ (p<0.05) or TNF-α (p<0.05) compared to basal, but no potentiation/synergistic effects were observed (Figure 2.22(b)(i – ii)). IFN-γ (p<0.01) or TNF-α (p<0.01) alone significantly increased ICAM-1 MFI compared to basal, while IFN-γ significantly potentiated the effect of TNF-α on ICAM-1 MFI (p<0.05) compared to cytokines alone (Figure 2.22(b)(iii – iv)).

Representative dot plot and quantification demonstrates a significant increase in the frequency of VCAM-1 positive DA FLS in response to TNF-α alone (p<0.001) (Figure
2.23(a)(i – ii)) compared to basal, however no potentiation/synergistic effects were observed when DA FLS were stimulated with both TNF-α and IL-17a together for either frequency of VCAM-1 positive DA FLS or VCAM-1 MFI (Figure 2.23(a)(i – iv)). TNF-α (p<0.001) or IFN-γ (p<0.01) alone increased frequency of VCAM-1 positive DA FLS compared to basal but no potentiation/synergy was observed (Figure 2.23 (b)(i – ii)). IFN-γ (p<0.05) and TNF-α alone (p<0.01) increased VCAM-1 MFI, but IFN-γ potentiated the effect of TNF on VCAM-1 MFI compared to cytokines alone (p=0.06) (Figure 2.23 (b)(iii – iv)).
Figure 2. 21 TNF-α and IFN-γ synergy induces adhesion in DA FLS. DA FLS were stimulated with TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml) or IFN-γ (50ng/ml) of for 24hr before being washed then incubated with 5 x 10^5 PBMC for 1hr then washed again. (a)(i) and (b)(i) Representative photomicrographs show the number of PBMC attached after stimulation with the various cytokine combinations. (a)(ii) and (b)(ii) Bar charts show quantification of the number of PBMC attached to DA FLS after stimulation with the various cytokines. Values expressed as mean ± SEM of n=6 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01, ###p<0.001 significantly different compared to other conditions. *p<0.05 is significantly different compared to other conditions.
Figure 2. 22 TNF-α and IFN-γ synergy induces ICAM-1 expression in DA FLS. DA FLS were stimulated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow dots plots show the ICAM-1 positive DA FLS following treatment with cytokines. (ii) Bar chart displays the percentage frequency of ICAM-1 positive DA FLS in response to the various cytokines. (iii) Bar charts demonstrate ICAM-1 MFI and (iv) representative histograms for ICAM-1 MFI. Values expressed as mean ± SEM of n=9 expts, One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis respectively. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 significantly different compared to other conditions. *p<0.05, **p<0.01 significantly different compared to other conditions.
Figure 2. 23 TNF-α and IFN-γ synergy induces VCAM-1 expression in DA FLS. DA FLS were treated TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) of for 24hr before flow cytometry analysis. (i) Representative flow plots show the expression of VCAM-1 on DA FLS when stimulated with cytokines. (ii) Bar chart displays the percentage frequency of VCAM-1 positive DA FLS following treatment with cytokines. (iii) Bar charts demonstrate VCAM-1 MFI and (iv) representative histograms for VCAM-1 MFI. Values expressed as mean ± SEM of n=9 expts, One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis respectively. #p<0.05, ##p<0.01, ###p<0.001, significantly different compared to other conditions. *p<0.05, **p<0.01 significantly different compared to other conditions.
2.4.6 TNF-α and IFN-γ synergy drives the expression of chemokine receptors on DA FLS

Representative flow plots and quantification (Figure 2.24(a)(i – ii)) demonstrate that while there was an increase in the frequency of CXCR3 positive DA FLS in response to TNF-α (p<0.01) compared to basal, no effects were observed for IL-17a alone or in combination with TNF-α. Representative flow plots and quantification (Figure 2.24(b)(i – ii)) demonstrate that IFN-γ (p<0.05) or TNF-α (p<0.01) alone increased the frequency of CXCR3 positive DA FLS compared to basal. While TNF-α and IFN-γ together increased the frequency of CXCR3 positive DA FLS compared to cytokines alone (p<0.01), this was an additive effect.

Representative flow plots and quantification (Figure 2.25(a)(i – ii)) demonstrate the frequency of CXCR4 DA FLS in response to TNF-α alone compared to basal (p<0.05). No potentiation/synergy effect was observed for IL-17a and TNF-α combination. Representative flow plots and quantification (Figure 2.25(b)(i – ii)) show that the frequency of CXCR4 positive DA FLS was induced in response to IFN-γ (p<0.05) or TNF-α (p<0.05) compared to basal. IFN-γ potentiated the effects of TNF-α as observed by the significant increase in frequency of CXCR4 positive DA FLS (p<0.05).

Representative flow plots and quantification (Figure 2.26(a)(i – ii)) showed no change in the frequency of CXCR5 positive DA FLS in response to TNF-α or IL-17a alone compared to basal. No potentiation/synergistic effects were observed. Representative flow plots and quantification (Figure 2.26(b)(i – ii)) demonstrated no effect in the frequency of CXCR5 positive DA FLS in response to TNF-α or IFN-γ alone, however IFN-γ and TNF-α synergistically increased the frequency of CXCR5 positive DA FLS compared to cytokines alone (p<0.05).

Representative flow plots and quantification (Figure 2.27(a)(i – ii)) demonstrate that the frequency of CCR6 positive DA FLS did not change in response to TNF-α or IL-17a alone or in combination compared to basal. Representative flow plots and quantification (Figure 2.27(b)(i – ii)) demonstrated no changes in the frequency of CCR6 positive DA FLS in response to TNF-α or IFN-γ alone compared to basal. While there was a significant
increase in frequency of CCR6 positive DA FLS in response to TNF-α and IFN-γ together (p<0.05) compared to cytokines alone, this is an additive effect.
No synergistic effects were observed for CXCR3 expression in DA FLS. DA FLS were treated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow plots show the expression of CXCR3 on DA FLS when stimulated with cytokines. (ii) Bar chart displays the percentage frequency of CXCR3 positive DA FLS following treatment with cytokines. Values expressed as mean ± SEM of n=9 expts, One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis respectively. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 significantly different compared to other conditions. *p<0.05, **p<0.01 significantly different compared to other conditions.
Figure 2. 25 TNF-α and IFN-γ synergy induces CXCR4 expression in DA FLS. DA FLS were treated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow plots show the expression of CXCR4 on DA FLS when stimulated with cytokines. (ii) Bar chart displays the percentage frequency of CXCR4 positive DA FLS following treatment with cytokines. Values expressed as mean ± SEM of n=9 expts, One-way ANOVA (＃) and Wilcoxon paired t-test (*) were used for statistical analysis respectively. ＃p<0.05, ###p<0.01, ####p<0.001 significantly different compared to other conditions. *p<0.05, **p<0.01 significantly different compared to other conditions.
Figure 2. 26 TNF-α and IFN-γ synergy induces CXCR5 expression in DA FLS. DA FLS were treated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow plots show the expression of CXCR5 on DA FLS when stimulated with cytokines. (ii) Bar chart displays the percentage frequency of CXCR5 positive DA FLS following treatment with cytokines. Values expressed as mean ± SEM of n=9 expts, One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis respectively. #p<0.05, ##p<0.01, ###p<0.001 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. 27 No synergistic effects were observed for CCR6 expression in DA FLS. DA FLS were treated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow plots show the expression of CCR6 on DA FLS when stimulated with cytokines. (ii) Bar chart displays the percentage frequency of CCR6 positive DA FLS following treatment with different cytokines. Values expressed as mean ± SEM of n=9 expts, One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis #p<0.05, ##p<0.01 significantly different compared to other conditions.
2.4.7 Cytokine interactions favour a metabolic switch to glycolysis in DA FLS

To examine if the observed cytokine combinations alter the metabolic profile of DA FLS, OCR and ECAR were quantified by Seahorse analysis. The average ECAR and OCR bioenergetic profiles of DA FLS when stimulated with TNF-α and IL-17a are shown in Figure 2.28(a – b). TNF-α alone increased ECAR baseline compared to basal ($p=0.06$) (Figure 2.28(c)). IL-17a potentiated TNF-α and induced baseline ECAR levels compared to TNF-α ($p=0.06$) or IL-17a alone ($p<0.05$) (Figure 2.28(c)). In contrast, TNF-α and IL-17a alone and in combination inhibited baseline OCR levels ($p<0.05$) (Figure 2.28(d)). This cytokine combination also led to a significant reduction in ATP synthesis ($p<0.05$) (Figure 2.28(e)) and maximal respiratory capacity ($p<0.05$) (Figure 2.28(f)) compared to cytokines alone. This resulted in a shift in the metabolic profile of the DA FLS where a significant synergistic increase in the ECAR:OCR ratio was observed in comparison to cytokines alone ($p<0.05$) (Figure 2.28(g)). The metabolic map demonstrates that DA FLS treated with TNF-α and IL-17a shifted from a quiescent phenotype to a highly energetic profile (Figure 2.28(h)). No changes were reported for proton leak, respiratory reserve, non-mitochondrial respiration (Figure 2.29(a – c)). Pie charts describe the relative contribution of the OxPhos potential within each condition as a percentage of total OCR output for DA FLS in response to basal (Figure 2.29(d)(i)), TNF-α (Figure 2.29(d)(ii)), IL-17a (Figure 2.29(d)(iii)) and TNF-α and IL-17a (Figure 2.29(d)(iv)). ATP synthesis (black) demonstrated no differences in response to either TNF-α and IL-17a alone (Figure 2.29(d)(ii – iii)) and compared to basal (Figure 2.29(d)(ii)). However, ATP synthesis was reduced in response to TNF-α and IL-17a (Figure 2.29(d)(iv)) compared to either TNF-α or IL-17a alone (Figure 2.29(d)(ii – iii)) and basal (Figure 2.29(d)(i)). Proton leak (pink) was increased in response to either TNF-α or IL-17a alone (Figure 2.29(d)(ii – iii)) compared to basal (Figure 2.29(d)(i)). No changes in proton leak were observed in response to TNF-α and IL-17a (Figure 2.29(d)(iv)) compared to either TNF-α or IL-17a alone (Figure 2.29(d)(ii – iii)) but was increased when compared to basal (Figure 2.29(d)(i)). There were no changes in respiratory reserve (green) in response to TNF-α (Figure 2.29(d)(iii)) when compared to basal (Figure 2.29(d)(i)), but this decreased in response to IL-17a alone (Figure 2.29(d)(iii)). No changes in respiratory reserve in response to TNF-α and IL-17a (Figure 2.29(d)(iv)) was documented when compared to TNF-α (Figure 2.29(d)(ii)) or basal (Figure 2.29(d)(i)) but was increased when
compared to IL-17a alone (Figure 2.29(d)(iii)). No changes in non-mitochondrial respiration (purple) were observed.

Figure 2.30(a) and (b) demonstrates the average ECAR and OCR bioenergetic profiles of DA FLS when treated with TNF-α and IFN-γ. TNF-α ($p=0.06$) and IFN-γ ($p<0.05$) alone increased ECAR baseline compared to basal (Figure 2.30(c)). IFN-γ potentiated TNF-α to induce baseline ECAR levels compared to TNF-α alone ($p=0.06$) or IFN-γ alone ($p<0.05$) (Figure 2.30(c)). In contrast, TNF-α and IFN-γ inhibited baseline OCR levels ($p<0.01$) compared to cytokines alone (Figure 2.30(d)). This stimulation also resulted in a significant reduction in ATP synthesis ($p<0.05$) (Figure 2.30(e)) and max respiratory capacity ($p<0.05$) (Figure 2.30(f)) compared to basal or either cytokine alone. This resulted in a significant shift in the metabolic profile of DA FLS with a significant synergistic increase in the ECAR:OCR ratio in response cytokine combination in comparison to cytokines alone ($p<0.05$) (Figure 2.30(g)). The metabolic map demonstrates that DA FLS treated with TNF-α and IFN-γ shifted from a quiescent phenotype to a highly energetic profile (Figure 2.30(h)). We reported no changes for proton leak, respiratory reserve, non-mitochondrial respiration (Figure 2.31(a – c)). Pie charts describe the relative contribution of the OxPhos potential within each condition as a percentage of total OCR output for DA FLS in response to basal (Figure 2.31(d)(i)), TNF-α (Figure 2.31(d)(ii)), IFN-γ (Figure 2.31(d)(iii)) and TNF-α and IFN-γ (Figure 2.31(d)(iv)). No changes were observed for ATP synthesis (black) in response to TNF-α (Figure 2.31(d)(iii)) or IFN-γ (Figure 2.31(d)(iii)) compared to basal (Figure 2.31(d)(ii)). However, ATP synthesis was decreased in response to TNF-α and IFN-γ (Figure 2.31(d)(iv)) compared to TNF-α (Figure 2.31(d)(ii)), IFN-γ (Figure 2.31(d)(iii)) and basal (Figure 2.31(d)(i)). Proton leak (pink) was increased in response to TNF-α (Figure 2.31(d)(iii)) or IFN-γ (Figure 2.31(d)(iii)) when compared to basal (Figure 2.31(d)(i)). Similarly, proton leak was increased in response to TNF-α and IFN-γ (Figure 2.31(d)(iv)) compared to basal (Figure 2.31(d)(i)) with no changes when compared to TNF-α (Figure 2.31(d)(ii)) or IFN-γ (Figure 2.31(d)(iii)). No change in respiratory reserve (green) was observed in response to TNF-α (Figure 2.31(d)(ii)) compared to basal (Figure 2.31(d)(i)) but it was decreased in response to IFN-γ (Figure 2.31(d)(iii)) compared to basal (Figure 2.31(d)(i)). No changes in respiratory reserve were documented in response to TNF-α and IFN-γ (Figure 2.31(d)(iv)) compared to TNF-α (Figure 2.31(d)(ii)) or basal (Figure 2.31(d)(i))
but it was increased when compared to IFN-γ (Figure 2.31(d)(iii)). No changes in non-mitochondrial respiration (purple) were documented amongst the different conditions.

We next examined if the cytokines alone and in combination had an effect on metabolic gene expression. GLUT-1 gene expression increased in response to TNF-α ($p=0.06$) or IL-17a ($p=0.06$) compared to basal while IL-17a and TNF-α together induced a two-fold increase in GLUT-1 expression compared to either cytokine alone (Figure 2.32(a)(i)). TNF-α ($p=0.06$) or IFN-γ ($p=0.06$) alone increased GLUT-1 expression compared to basal while IFN-γ and TNF-α together induced a 30-fold increase in GLUT-1 expression compared to cytokines alone, however this did not reach significance (Figure 2.32(a)(iii)). LDHA expression was increased in response to TNF-α ($p=0.06$) or IL-17a ($p=0.06$) compared to basal while IL-17a and TNF-α together additively increased LDHA expression (Figure 2.32(b)(i)). TNF-α ($p=0.06$) or IFN-γ ($p=0.06$) alone increased LDHA compared to basal, while IFN-γ synergized with TNF-α to induce LDHA expression compared to either cytokine alone ($p=0.06$) (Figure 2.32(b)(ii)).

HIF-1α expression increased in response to TNF-α ($p<0.05$) or IL-17a ($p=0.06$) compared to basal while IL-17a and TNF-α together did not result in a potentiation/synergy effect but additive (Figure 2.33(a)(i)). HIF-1α was increased in response to TNF-α ($p<0.05$) or IFN-γ ($p<0.05$) compared to basal while IFN-γ potentiated the effect of TNF-α on HIF-1α expression, however this did not reach significance (Figure 2.33(a)(i)). HK2 expression was increased in response to TNF-α ($p<0.05$) or IL-17a ($p=0.06$) compared to basal, while IL-17a and TNF-α did not have a potentiation/synergy effect but additive (Figure 2.33(b)(i)). TNF-α ($p<0.05$) or IFN-γ ($p<0.05$) increased HK2 expression compared to basal, but IFN-γ potentiated the effect of TNF-α on HK2 expression ($p<0.001$) (Figure 2.33(b)(ii)). No changes in PKM2 were observed for TNF-α or IL-17a alone or in combination, although there was a trend for the cytokine combination (Figure 2.33(c)(i)). No changes in PKM2 were observed in response to TNF-α or IFN-γ or alone, but a synergistic increase for the combination of IFN-γ and TNF-α on PKM2 was demonstrated ($p<0.05$) (Figure 2.33(c)(ii)).
Figure 2. Synergy between TNF-α and IL-17a induces a glycolytic shift in DA FLS. (a) Average ECAR and (b) OCR profiles for DA FLS cultured with TNF-α (0.1 ng/ml) in the presence or absence of IL-17a (50ng/ml) before and after injections of oligomycin, FCCP, and antimycin A/rotenone. Bar charts show quantification of (c) ECAR baseline, (d) OCR baseline, (e) ATP synthesis, (f) max respiratory capacity, (g) ECAR:OCR ratio, and (h) metabolic map. Values expressed as mean ± SEM of n=6 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis respectively. #p<0.05, ##p<0.01 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. 29 Synergy between TNF-α and IL-17a induces a glycolytic shift in DA FLS. Bar charts show quantification of (a) proton leak, (b) respiratory reserve, (c) non-mitochondrial respiration in DA FLS in response TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml). (d) Pie charts reveal breakdown of OCR outputs within each condition (i) – (iv). Values expressed as mean ± SEM of n=6 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis respectively. #p<0.05 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. Synergy between TNF-α and IFN-γ induces a glycolytic shift in DA FLS. (a) Average ECAR and (b) OCR profiles for DA FLS cultured with TNF-α (0.1 ng/ml) in the presence or absence of IFN-γ (50ng/ml) in alone and in combination before and after injections of oligomycin, FCCP, and antimycin A/rotenone. Bar charts show quantification of (c) ECAR baseline, (d) OCR baseline, (e) ATP synthesis, (f) max respiratory capacity, (g) ECAR:OCR ratio, and (h) metabolic map. Values expressed as mean ± SEM of n=6 expts. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis respectively. #p<0.05, ##p<0.01 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 2. Synergy between TNF-α and IFN-γ induces a glycolytic shift in DA FLS. Bar charts show quantification of (a) proton leak, (b) respiratory reserve, (c) non-mitochondrial respiration in DA FLS in response to TNF-α (0.1ng/ml) in the presence or absence of IFN-γ (50ng/ml). Values expressed as mean ± SEM of n=6 expts. (d) Pie charts reveal breakdown of OCR outputs within each condition (i) – (iv). One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis #p<0.05 significantly different compared to other conditions.
Figure 2. Synergy between TNF-α and IL-17a or IFN-γ induces GLUT-1 and LDHA gene expression in DA FLS. DA FLS were incubated with TNF-α (0.1ng/ml) in the presence or absence of (i) IL-17a (50ng/ml) and (ii) IFN-γ (50ng/ml) for 24hr before RNA was isolated and RT-PCR performed. Bar charts show quantification of (a) GLUT-1 and (b) LDHA gene expression in DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=5 expts, normalized to housekeeping controls RPLPO. 2−ΔΔct indicates fold induction. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, #p<0.05, ###p<0.01 is significantly different from other conditions.
Figure 2. 33 TNF-α alone and in combination with IL-17a or IFN-γ induces HIF1a, HK2 and PKM2 gene expression in DA FLS. DA FLS were treated with TNF-α (0.1ng/ml) in the presence or absence of (i) IL-17a (50ng/ml) and (ii) IFN-γ (50ng/ml) of (0.1ng/ml) for 24hr before RNA was isolated and RT PCR performed. Bar charts show quantification of (a) HIF-1 α, (b) HK2 and (c) PKM2 gene expression in DA FLS in response to cytokine stimulation. Values expressed as mean ± SEM of n=5 expts, normalized to housekeeping controls RPLPO. 2^{ΔΔCt} indicates fold induction. One-way ANOVA (#) and Wilcoxon paired t-test (*) were used for statistical analysis, respectively. #p<0.05, ##p<0.01, ###p<0.001 is significantly different from other conditions. *p<0.05 significantly different compared to other conditions.
2.5 Discussion

TNF-α, IL-17a and IFN-γ are key mediators involved in the regulation of inflammatory responses. However, to date, no study has examined the contributions of these cytokines to the pathogenesis of synovial inflammation in DA. In this chapter, we demonstrate that TNF-α, IL-17a and IFN-γ regulate the expression of proinflammatory mediators, metabolic markers and matrix degrading enzymes in DA FLS. This is paralleled by a significant increase in leukocyte adhesive/migratory capacity, evident by the observed induced expression of key adhesion molecules and chemokine receptors. Next, we identified that IL-17a and IFN-γ either potentiated the effect of or synergized with TNF-α to differentially modulate pathogenic function of DA FLS, an effect that is more prominent for IFN-γ. The combination of cytokines was accompanied by a shift in the metabolic profile of DA FLS whereby a switch to a glycolytic profile, with concomitant inhibition of Ox-Phos in DA FLS was observed. This led to an increase in the ECAR:OCR ratio and a reduction in the ATP synthesis and max respiratory capacity, in addition to potentiated/synergistic induction of key metabolic markers including GLUT-1, LDHA, HK2 and PKM2. Together this data suggests that key cytokine interactions enhance the aggressive phenotype of DA FLS, and have implications for current therapeutic approaches, or potential targeting of metabolic pathways in DA.

In this chapter, we demonstrated that TNF-α, IL-17a and IFN-γ alone regulate the secretion of proinflammatory mediators IL-6, MCP-1, RANTES and IL-8 from DA FLS, in addition to increased expression of key matrix degrading enzymes MMP-1, MMP-3 and MMP-9, with no effect observed for GM-CSF. In the context of RA FLS, IL-6 has numerous functions including triggering bone resorption through RANKL-induction, skewing the balance between Th17 and Treg cells and inducing the production of MMPs (Hashizume et al., 2011). MCP-1 is a potent chemoattract that regulates monocyte migration into the RA joint while also promoting RA FLS proliferation and migration (Tong et al., 2021). Likewise, IL-8 and CCL8 induce neutrophil chemotaxis (Namba et al., 2017) while RANTES attracts lymphocytes and monocytes to the joint (Agere et al., 2017), in addition to promoting collagen degradation via MMP-1 and MMP-13 secretion from RA FLS. While this is the first study to focus on DA FLS, previous studies showed similar effects of these cytokines on FLS isolated from the RA joint and other diseases (Zrioual et al., 2009; McInnes et al., 2009).
Consistent with previous findings, TNF-α stimulation has been shown to regulate the production of IL-1β, IL-6 and IL-8, MMP-2, MMP-3, MMP-8, MMP-9 in RA FLS (Feldmann et al., 1996; Yoshida et al., 1999; Du et al., 2019), induce TGF-β expression in lung FLS (Sullivan et al., 2009) and reinforces nonapoptotic cell death mechanisms in collagen-induced arthritis models (CIA) (Wu et al., 2022). Previous studies have shown pleiotropic effects for IL-17 where it has been shown to coordinate interactions between stromal and immune cells while also determining pathophysiological processes, including stromal cell activation, angiogenesis and osteoclastogenesis (Taams, 2020; Lorè et al., 2021). IL-17a has been shown to increase IL-6, IL-8, VEGF, GROα, MMP-1, MMP-2, MMP-3, MMP-9 and MCP-1 production in RA FLS, induce migratory and invasive mechanisms via chemokine and cytoskeletal-dependent pathways in RA FLS and macrophages, in addition to promoting proteoglycan degradation in cartilage explants (Kehlen et al., 2002; Moran et al., 2009, 2011; Zrioual et al., 2009; Shahrara et al., 2010; Li et al., 2013; Kato et al., 2020). In the context of JIA, IL-17a induces IL-6, IL-8, MMP-1 and MMP-3 expression in JIA FLS (Agarwal et al., 2008), and promotes the generation of ECM proteins including collagen I and IV and fibronectin from lung FLS (Zhang et al., 2019). Supporting our data, previous studies have also shown IFN-γ induces IL-6 and IL-8 production from RA FLS (Möller et al., 2003; Karonitsch et al., 2018; Kato, 2020), induces CD4+ T cell-derived RANKL secretion (Kato, 2020), IL-18 binding protein isoforms (Möller et al., 2003) and soluble B cell activating factor (BAFF) (Lowin et al., 2020) and regulates migratory and invasive mechanisms in RA FLS (Möller et al., 2003; Karonitsch et al., 2018; Kato, 2020).

Consistent with previous RA FLS studies (Mullan et al., 2006; Fearon et al., 2007; Moran et al., 2009; Croft et al., 2016; Wu et al., 2022) our data demonstrates that TNF-α, IL-17a and IFN-γ alone increases leukocyte adhesive capacity of DA FLS, paralleled by increased expression of key adhesion molecules, ICAM-1 and VCAM-1 on DA FLS. While the regulation of these molecules has not been examined in DA FLS or in the DA synovium, other studies have shown increased expression of these molecules in the inflamed synovium of RA and JIA (Veale et al., 1993; Mullan et al., 2006; Maggi et al., 2016) and have shown regulation of both ICAM-1 and VCAM-1 in RA FLS or JIA FLS in response to TNF-α, OSM, IL-1β, IFN-γ, A-SAA (Mullan et al., 2006; Fearon et al., 2007; Mori et al., 2017;
Fromm et al., 2019). Both ICAM-1 and VCAM-1 are key molecules involved in facilitating the infiltration of immune cells into the synovium (Veale et al., 1996; Mullan et al., 2006; Maggi et al., 2016; Floudas et al., 2022). Furthermore, studies have shown that these adhesion molecules are associated with the invasive capacity of RA FLS (Seemayer et al., 2003).

Attracting and homing of infiltrating cells, such as leukocytes, neutrophils, macrophages, monocytes, eosinophils to the joint are crucial for pannus formation; a mechanism facilitated by chemokine receptors (Elemam et al., 2020). In this study, we show increased expression of CXCR3, CXCR4 and CXCR5 on DA FLS in response to the TNF-α, IL-17a and IFN-γ, which is supported by previous studies in RA FLS that show similar regulation of chemokines in response to either TNF-α or IL-1β (Trzybulska et al., 2018). Likewise, IL-17a stimulation has been demonstrated to induce CCL20 and CXCL1 secretion on CD4+ T cells (Seon et al., 2006). CXCR3 attracts neutrophils to the site of inflammation and has been shown to be elevated in the RA and JIA joint (Mohan et al., 2007; Julé et al., 2021) while CXCR5 attracts B cells and Tfh cells to the joint, which are known to be highly expressed in the RA synovium (Moser, 2015; Moschovakis et al., 2017). Interestingly, we previously demonstrated a depletion of B cells in the circulation of children with DA, suggesting they may be tracking to the joint (Foley et al., 2020). Furthermore, the same study demonstrated a significant increase in Tfh cells, that displayed markers of Th1/Th17 plasticity (CXCR3+ CCR6+) suggesting that depending on the microenvironment they could shift their pathogenic phenotype further contributing to synovial inflammation in DA (Foley et al., 2020). CXCR4 has also been shown to play an important role in FLS function with studies showing that inhibition of hypoxia induced RA FLS migration/invasion is mediated through transcriptional activation of CXCR4 (Li et al., 2013), with other studies demonstrating that CXCR4-positive chondrocytes lead to increased secretion of MMP-3 and subsequent cartilage breakdown (Kanbe et al., 2002). Studies also demonstrated increased expression of CXCR4 in RA tissues ectopic lymphoid structures (Timmer et al., 2007), suggesting a role for facilitating immune cell infiltration to the joint. Indeed, studies have shown increased expression of CXCR4 on CD4+ memory T cells in the RA synovium, in addition to other diseases including immunodeficiency virus (HIV), prostate cancer and breast cancer (Kircher et al., 2018). Thus, our data would suggest the increase in these key
chemokines not only alone can directly regulate FLS function but also contribute to the influx of neutrophils, T cells, B cells and Tfh cells to the joint, further potentiating the inflammatory response. The therapeutic potential of targeting chemokines was shown in mouse models of arthritis where anti-CXCR3 monoclonal antibodies significantly reduced the infiltration of leukocytes and decreased cartilage damage (Mohan et al., 2007). Furthermore, CXCR5-deficient mice do not develop CIA (Moschovakis et al., 2017), while blocking CXCR4 resulted in mitigating cell homing mechanisms resulting in ameliorating of inflammation in CIA (Vergunst et al., 2005). Moreover, other key cell types in the RA synovium (B cells, macrophages, neutrophils and DCs) secrete CCR1-7, CXCR1-6 and CX3CR1 (Haringman et al., 2004).

Changes in the pathogenic properties of DA FLS were mirrored by changes in their metabolic profile. Specifically, we demonstrated increased expression of the glucose receptor, GLUT-1, and key glycolytic enzymes, LDHA, HIF-1α, HK2 and PKM2, in DA FLS following stimulation with TNF-α, IL-17a and IFN-γ alone. While this is the first study to examine metabolic changes in DA FLS, previous studies have shown that the arthritic joint is profoundly hypoxic, and is paralleled by increased metabolic activity and increased levels of metabolites including succinate, lactate, citrate, pyruvate and α-Enolase, leading to a metabolic shift favouring glycolysis and a broken TCA cycle (Fearon et al., 2022). Studies have shown that TNF stimulation of JIA FLS leads to an increase in GLUT-1 expression (Bustamante et al., 2017). Furthermore, low pO2, oxidative stress or proinflammatory cytokines induce a shift in RA FLS towards glycolysis coupled with an increase in key glycolytic enzymes such as HK2 and PKM2 (Biniecka et al., 2016; McGarry et al., 2017; Bustamante et al., 2018; Hanlon et al., 2019). Indeed, blocking PFKFB3 and HK2 in RA FLS resulted in reduced migratory and invasive capacity of RA FLS, paralleled by inhibition of proinflammatory cytokines as well as HIF-1α and p-STAT3 transcriptional activation (Bae et al., 2012; Biniecka et al., 2016; Bustamante et al., 2017). There were also changes in glycolysis, OxPhos, lipid metabolism and amino acid metabolism reported in RA FLS compared to OA FLS (Fearon et al., 2019). Additionally, the accumulation of metabolites in the joint has a direct effect on the pathogenicity of RA FLS. In particular, both lactate and succinate have been shown to promote RA FLS invasive capacity (Biniecka
et al., 2016) while blockade of the monocarboxylate transporter 4 (MCT4), a lactate transporter, results in FLS apoptosis (Fujii et al., 2015).

In the joint microenvironment, there is a plethora of cytokines that interact with each other to drive RA pathogenesis (Ridgley et al., 2018; Zhang, 2020). As highlighted above we have previously demonstrated an enriched population of polyfunctional T cells that simultaneously produce TNF-α, IL-17a and IFN-γ in children with DA (Foley et al., 2020), thus we examined the effect of these cytokines alone and in combination on DA FLS function. Previous studies by our group and others have shown that polyfunctional T cells are more pathogenic, are resistant to Treg suppression, and are associated with disease activity and response (Basdeo et al., 2017; Wade et al., 2018; Taams, 2020; Floudas et al., 2022). Indeed, the polyfunctional T cell profile identified in children with DA (Foley et al., 2020) is consistent with the more erosive disease observed in these children (Foley et al., 2019), and supported by a study by Wade et al. (2018) which showed that only the polyfunctional T cells, and not the single cytokine producing T cells, were associated with disease activity and response (Wade et al., 2018). Both IL-17a and IFN-γ potentiated the effects of or synergised with TNF-α to induce the secretion of IL-6, MCP-1 and RANTES from DA FLS with no effect observed for GM-CSF. Interestingly, while IL-17a synergized with TNF-α to increase IL-8 production, IFN-γ inhibits TNF-α-induced IL-8 secretion. Similarly, IFN-γ inhibited TNF-α-induced MMP-9 while IL-17a synergized with TNF-α to induce MMP-1 and potentiated the effect of TNF-α on MMP-3, and MMP-9 secretion from DA FLS. Furthermore, IFN-γ and TNF-α led to a potentiation/synergistic increase in leukocyte-DA FLS adhesive capability, ICAM-1 and VCAM-1, in addition to potentiation/synergistic increases in the frequency of CXCR4 and CXCR5-positive DA FLS. Thus, these results suggest that cytokines interact with each other to further potentiate the inflammatory response, and in the context of DA FLS while these effects were observed for both IL-17a and IFN-γ with TNF-α, the effects were more pronounced for IFN-γ and TNF-α.

Our study demonstrated that IL-17a potentiated the effects of TNF-α by inducing the expression of IL-6, MCP-1, RANTES, IL-8, MMP-1, MMP-3, MMP-9 with no synergistic effects reported for adhesion molecules or chemokine receptors. This data is consistent with previous studies which have shown that IL-17a and TNF-α interaction synergistically
stimulates cartilage degradation through MMP secretion as well as IL-6 and IL-8 production in RA FLS (Moran et al., 2009; Mori et al., 2017). In contrast to our study, Mori et al. (2017) demonstrated IL-17a and TNF-α synergistically induce ICAM-1 and VCAM-1 expression thus highlighting that RA FLS are primed or influenced by their specific microenvironment. This is also consistent with studies that have shown RA FLS differ in their functional capacity depending on the FLS subtype or positional memory (Frank-Bertoncelj et al., 2017; Wei et al., 2020; Buckley et al., 2021). Indeed, FLS isolated from different joints demonstrate different functional capacity.

Numerous studies have demonstrated that IL-17a synergizes with TNF-α in different cell types including RA and corneal FLS, airway epithelial cells, hepatoma cells and adipocytes to secrete additional mediators such as VEGF, MMPs, neutrophil chemoattract proteins, e.g. CCL20, CXCL1, CXCL2 and CXCL5, and IL-6, IL-8 and GM-CSF across numerous autoimmune and inflammatory diseases like RA, nasal polyps, chronic liver and airway inflammation, herpetic stromal keratitis, inflammatory bowel disease, Type 2 diabetes and MS (Chabaud et al., 2001; Sung et al., 2001; Maertzdorf et al., 2002; Moran et al., 2009; Nonaka et al., 2009; Zrioual et al., 2009; Ermann et al., 2014; Honda et al., 2016; Shinjo et al., 2016; Beringer et al., 2018). Additionally, TNF-α and IL-17a work in concert to mediate IL-23 production in RA FLS thus creating a positive feedback loop in RA as IL-23 differentiates naïve T cells into Th17 cells (Goldberg et al., 2008). In JIA, IL-17 has been shown to induce the secretion of chemokine ligands such as CXCL1, CXCL6 and CCL16 from JIA FLS (Agarwal et al., 2008). However, synergy with IL-17a is not limited to TNF-α. TGF-β and IL-1β also potentiated the effects of IL-17 to enhance VEGF production thus regulating neovascularization (Sung et al., 2001) as well as promoting the transcriptional landscape of FAP-α+ Thy-1+ invasive RA FLS which are expanded in RA compared to PsA (Floudas, et al., 2022). Additionally, IL-17a or IL-17f enhance TNF-α or IL-1β-mediated MCP-1 and CXCL2 secretion from mouse mesangial cells (Iyoda et al., 2010). Furthermore, IL-17a has a similar synergistic relationship with OSM through the regulation of MMP-1 production from RA FLS and proteoglycan release from cartilage explants (Moran et al., 2009). Interestingly, soluble CD14 from monocytes, macrophages, DCs and neutrophils synergized with either TNF-α or IL-17a to increase IL-6 production from RA FLS (Ichise et al., 2020).
The pathways involved in these observed synergistic interactions between IL-17a and TNF-α are partially explained by Slowikowski et al. (2020). The synergy between IL-17a and TNF-α activates two relevant transcription factors (CUX1 and IkBζ) which form a molecular complex in the NFκB inflammatory pathway. This is responsible for chemokine transcription (CXCL1, CXCL2, CXCL3, CXCL6, CXCL8, IL-6, MCP-1 and CCL8), resulting in neutrophil and monocyte recruitment (Slowikowski et al., 2020). Interestingly, IkBζ is synergistically activated by IL-17a and TNF-α together and in turn activates the transcription factor, E74-like factor 3 (ELF3). Evidence for this was shown by the effective inhibition of IL-17a/TNF-α-mediated production of CCL8, CXCL5, CSF-3, MMP-3 and MMP-12 in OA FLS through ELF3-silencing (Kouri et al., 2022). In addition to the NFκB pathway, c-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) pathway is also implicated (Shinjo et al., 2016). As such, inhibition of either JNK or NFκB prevented IL-17a and TNF-α-induced IL-6 production from adipocytes (Shinjo et al., 2016). Furthermore, a recent study by Wei et al. (2020) demonstrated that the Notch signalling is involved in dictating the functional identity of RA FLS subtypes (Wei et al., 2020), and Wang et al. (2021) has demonstrated IL-17a increases Notch activity via the NFκB pathway in pancreatic ductal adenocarcinoma (Wang et al., 2021), thus suggesting that these two crucial signalling pathways in inflammation are connected.

In this study, we describe how IFN-γ synergized with TNF-α to induce IL-6, MCP-1, RANTES production with opposing synergy observed for IL-8, increased leukocyte-DA FLS capacity, paralleled by increased expression of ICAM-1, VCAM-1, CXCR4 and CXCR5. TNF-α and IFN-γ have been shown to synergistically induce proliferation, BAFF, IL-6, IL-8, CX3CL1 and CXCL9 expression in osteoblasts and RA FLS (Loos et al., 2006; Isozaki et al., 2008; Lowin et al., 2020). IFN-γ and TNF-α stimulation in systemic sclerosis FLS led to enhanced IL-6 and MCP-1 production (Antonelli et al., 2012) as well as increased IL-8 and CXCL10 production from FLS in temporomandibular joint (Ohta et al., 2017). Additionally the synergy between the two cytokines promoted a CXCL10+ CCL2+ macrophage phenotype and senescence-linked inflammation in coronavirus disease (COVID-19) (Zhang et al., 2021; Kandhaya-Pillai et al., 2022), as well as cytokine shock in COVID-19 mouse models resulting in tissue damage and further inflammation (Karki et al., 2021). The dynamic duo have also been shown to activate apoptosis of pancreatic cells in diabetic mouse models.
where they showed that IFN-γ potentiated the effect of TNF-α through the induction of caspase-dependent apoptosis pathways in pancreatic islet cells of diabetic mouse models (Suk et al., 2001). One beneficial effect of this pairing is that it induces apoptosis in neuroblastoma thus this could be used for therapeutic approaches (Shelke et al., 2018).

Not restricted to TNF-α, IFN-γ synergizes with TLR-4 to increase TNF-α, IL-6 and IL-12b production from macrophages (Qiao et al., 2013) which is important for rheumatic diseases. Pertaining to DS, a key study by Sullivan et al. (2016) hones in on how T21 activates the IFN transcriptional response in FLS, peripheral monocytes and T cells (Sullivan et al., 2016). Here, they show that T21 induces IFN-stimulated genes, with further studies of chromosome 21 showing it encodes for four of the six IFN receptors: Type 1 IFNR subunits (IFNAR1, IFNAR2), Type 2 IFNRs (IFNGR2) and Type 3 IFNR (IL-10RB), leading to high gene dosage of these receptors in DS (Powers et al., 2019). An elegant study by Ge et al. (2021) further highlighted the association of an IFN molecular signature in FLS. While over 100 susceptibility gene loci have been identified for RA, most loci were associated with immune cell function. In this study, they performed a genome-wide association study with FLS as the focal point and identified that TNF-α stimulation of RA FLS promoted IFN-responsive genes through the TNFA1P3 gene (Ge et al., 2021). This is consistent with the synergistic interactions observed in this study and the potential for IFN gene signature association with DA FLS.

NFκB and Jak-STAT have been implicated to play major roles in the mediation of the synergistic interactions between IFN-γ and TNF-α. NFκB-STAT elements control the ability of TNF-α and IFN-γ to induce gene transcription. Furthermore, NFκB and STAT-1 proteins directly modulate transcription by interacting with NFκB-STAT element/DNA binding in response to TNF-α and IFN-γ together (Ganster et al., 2005; Isozaki et al., 2008). Interestingly, IFN-γ via NFκB can activate promoters as well as enhancers for TNF-α, IL-6 and IL-12b loci (Qiao et al., 2013). While the primed chromatin did not induce transcriptional activation, it dramatically enhanced and lengthened the retention of TLR-4-mediated transcription factors. This priming led to reduced responsiveness of the cells to Jak inhibitors. Furthermore, Qiao et al. (2013) demonstrated unwanted priming of regulatory elements by IFN-γ suggesting that the observed priming of promoters and enhancers may be the potential mechanism for synergistic responses (Qiao et al., 2013).
This synergistic interaction is further highlighted in a study by Karonitsch et al. (2018) where TNF-α signalling adopts the mTOR pathway to induce IFN-γ activities via NFκB and STAT-1 signalling, a mechanisms that relies on amino acid availability (Karonitsch et al., 2018). NFκB-inducing kinase (NIK) is a constituent of the non-canonical part of the NFκB pathway and is crucial for NFκB-mediated lymphotxin B receptor activation in RA FLS which is associated with the aggressive function of RA FLS including accelerated proliferation, lower apoptosis, invasiveness and proinflammatory cytokine production (Nejatbakhsh-Samimi et al., 2020). The clinical impact of a role for IFN-γ signalling in DA, was highlighted by a study by Jones (2022) where they reported that tofacitinib (a small molecule Jak inhibitor) improved the clinical manifestations of disease in patients with DA (Jones, 2022).

Assessing the metabolism of DA FLS in response to cytokine combinations, we demonstrated that both IL-17a and IFN-γ potentiated/synergised with TNF-α to increase glycolytic activity in DA FLS which led to an increase in ECAR:OCR ratio. This was also associated with a decrease in ATP synthesis and max respiratory capacity. Thus, shifting the DA FLS from a quiescent metabolic state to a more energetic phenotype. The observed shift to a glycolytic profile was further supported by the observed potentiation/synergistic increases in key metabolic markers. IL-17a potentiated the effects of TNF-α on GLUT-1 and PKM2 expression while IFN-γ potentiated/synergised with TNF-α to induce the expression of GLUT-1, LDHA, HIF-1α, HK2 and PKM2. These changes to the metabolic profile of activated DA FLS is consistent with studies showing that the joint microenvironment is complex with an abundant array of proinflammatory cytokines capable of reprogramming the metabolic activity of synovial FLS in order to maintain their pathogenic state (Biniecka et al., 2016; Bustamante et al., 2017; McGarry et al., 2017; Hanlon et al., 2019; Petrasca et al., 2020; Fearon et al., 2022). Interestingly, previous studies have shown that TNF-inhibitors and tofacitinib switch the metabolic profile of cells within the RA inflamed joint, with numerous studies showing that this switch is associated with inducing resolution of inflammation in vivo, ex vivo and in vitro (Troughton et al., 1996; Nanki et al., 2005; Ng et al., 2010; Biniecka et al., 2011; Everts et al., 2014; Pantel et al., 2014; Biniecka et al., 2016; Ito et al., 2017) suggesting combination therapy of TNF-inhibitors and tofacitinib could mediate the IFN-γ/TNF-α synergistic effect. Metabolic
inhibitors including metformin, 3PO (PFKFB3 inhibitor), GSK2837808A (LDHA inhibitor), 3-BrPA (HK2 inhibitor) and AMPK-modifying compounds have all been shown to reduce the proinflammatory and invasive capacity of RA FLS and other synovial cells (Bae et al., 2012; Biniecka et al., 2016; Bustamante et al., 2018; Gallagher et al., 2020) in addition to inhibition of HIF-1α, pSTAT-3 and NFκB transcriptional pathways (Biniecka et al., 2016).

Studies have shown that various cytokine combinations can induce synergistic pro-inflammatory responses in synovial FLS including RA FLS (Moran et al., 2009; Nonaka et al., 2009; Hanlon et al., 2019; Kouri et al., 2022). While HC FLS have not been examined, the likelihood is that cytokine combinations would induce synergic inflammatory responses. This would be expected as stimulating HC FLS with pro-inflammatory cytokines would essentially be exposing the HC FLS to a pathogenic environment. However, while no studies have directly compared the effect of cytokines on pro-inflammatory responses in HC FLS compared to FLS from an inflamed joint (due to the difficulty of obtaining HC synovium), studies of circulatory immune cells have shown that immune responses are heightened in patients with IA compared to HC even in response to activation with inflammatory mediators (McGarry et al., 2021; Hanlon et al., 2023), suggesting that responses in pathogenic cells may be heightened. Finally, preliminary work in the group has shown that DA FLS display a more aggressive invasive and migratory phenotype, an effect associated with increased metabolic activity compared to JIA FLS.
Figure 2. 34 An overview of Chapter 2 focusing on TNF-α and IFN-γ. Synergy between TNF-α and IFN-γ uses the NFκB and JAK-STAT pathway to promote the glycolytic pathway where HIF-1α is induced along with PKM2, Glut-1, LDHA and HK2 in DA FLS. DA FLS favour a glycolytic switch to fuel their aggressive phenotypic activities such as enhanced production of IL-6, MCP-1, RANTES as well as increased expression of ICAM-1, VCAM-1 and CXCR4 and CXCR5 (made with BioRender).

In conclusion, we have demonstrated that IL-17a or IFN-γ on their own induce the destructive capacity of DA FLS while the combination of IL-17a/TNF-α or IFN-γ/TNF-α further potentiate the aggressive pathogenic phenotype of DA FLS. These data have major implications for combination therapy or manipulation of metabolic pathways for the treatment of DA.
CHAPTER 3

The effect of the DA joint microenvironment on Endothelial Cell function
3.1 Introduction

Synovial angiogenesis is a key event in the early pathogenesis of IA (Walsh et al., 2001; Fearon, et al., 2003; Fearon et al., 2019; Fearon et al., 2021) facilitating the influx of leukocytes to the joint through a process of leukocyte-EC adhesion and transendothelial migration. The first step of leukocyte adhesion and transendothelial migration includes early, weak adhesion termed “rolling”. This involves molecules called selectins and their associated ligands resulting in leukocyte movement. Secondly, strong leukocyte-EC adhesion is mediated by adhesion molecules, ICAM-1 and VCAM-1, as well as the secretion of numerous chemokines such as CXCR4 and CCR6 (Chou et al., 2010; Balogh et al., 2019). Thus, leukocyte adhesion and transendothelial cell migration are crucial processes involved in the initiation of synovial inflammation. Once activated, leukocytes enter the joint and secrete several proinflammatory mediators that activate resident synovial cells including resident macrophages and synovial FLS. Cellular function and activation are intrinsically linked to cellular metabolism. Consequently, there is a shift in the metabolic demand of ECs, in addition to both infiltrating and resident synovial cells, so that they can maintain their activated pathogenic phenotype. Thus, the metabolic demand of the inflamed synovium dictates the requirement of new blood vessel formation to provide adequate blood supply and nutrients to the expanding synovial pannus.

Under normal physiological conditions, angiogenesis is tightly controlled by both pro- and anti-angiogenic factors and is responsible for fundamental processes such as embryogenesis, menstrual cycle and tissue repair (Walsh et al., 2001; Balogh et al., 2019). Activation of ECs by pro-angiogenic stimuli leads to the secretion of degradative enzymes which dissolve ECM facilitating the migration of ECs away from the parent cell and the formation of new capillary sprouts. This is followed by EC proliferation, migration, tip-stalk cell selection, sprout elongation, synthesis of basement membrane, vessel fusion, pericyte stabilization, and lumen formation (Fearon et al., 2021). Once formed, anti-angiogenic inhibitors are released to ensure that uncontrolled angiogenesis does not occur. While many different forms of angiogenesis have been described including intussusceptive,
vessel co-option and vessel mimicry, the most well described form of angiogenesis in the inflamed joint is “sprouting” angiogenesis (Fearon et al., 2021).

The first sprout needs one EC that is more sensitive to the angiogenic stimuli in the surrounding microenvironment and becomes a guiding EC. As such, the Notch-Delta-like ligand 4 (DLL4) signalling pathway, through a process called lateral inhibition, dictates that one EC becomes highly polarized and will be defined as the “tip cell” (Fearon et al., 2021). VEGF receptor activation promotes tip cell formation and is dependent on a specific EC sensing the highest VEGF concentration. VEGF-VEGFR interactions on the tip cell induces the expression of DLL4. This leads to DLL4 binding to Notch on the neighbouring EC which inhibits VEGFR signalling, rendering this cell less responsive to stimulation by VEGF, thus instructing it to become a stalk cell (lateral inhibition). This results in the original selected tip cell maintaining its position as the leading migrating EC (Hellström et al., 2007). When migratory tip cells are chosen, they can sense their microenvironment through their filopodial extensions which grow towards the angiogenic stimulus. The neighbouring stalk cells then proliferate and support sprout elongation (Hoang et al., 2004). There are three EC subtypes: tip cell, stalk cell, and phalanx cell, each having a defined function that encompasses migration, proliferation, and quiescence respectively. Each EC subtype must understand the profile of their neighbouring EC to determine their functional role. For a proper functioning vessel, the communication between the different EC subtypes is vital and will dictate the function, i.e. tip cells will migrate, stalk cells will divide and form lagging cells, whereas the remaining ECs are phalanx cells and are quiescent (Fearon et al., 2021).

Angiogenesis and vascular abnormalities are well described in numerous previous studies in IA, including RA and PsA (Reece et al., 1999; Fearon et al., 2003; Kennedy et al., 2010; Elshabrawy et al., 2015; Fromm et al., 2019; He et al., 2020; Kim et al., 2020). As outlined in Chapter 1 (Figure 1.4), distinct macroscopic vascular morphology in the joint of early IA have been observed and reflect both differential pathogenesis and disease outcome (Veale et al., 1993; Reece et al., 1999; Ikeda et al., 2000; Fearon et al., 2003; Cañete et al., 2004; Izquierdo et al., 2009; Kennedy et al., 2010, 2011; Fisher et al., 2016; Pusztai et al., 2021). This is associated with differential synovial expression of angiogenic factors.
including VEGF, VEGF receptors, Ang1, Ang2, Tie 2, FGF-1/2, platelet-derived growth factor (PDGF), TGF-β1, (Koch et al., 1994; Hosaka et al., 1995; Shahrara et al., 2002; Fearon et al., 2003; Balogh et al., 2019), MMPs and the cytokines/chemokines (TNF-α, IL-10, OSM, IL-8, IL-17a, TGF-β, CCL2, and CX3CL1) (Fraser et al., 2001; Szekanecz et al., 2007; Marrelli et al., 2011; Moran et al., 2011; Tas et al., 2016).

VEGF is the ‘master’ regulator of pro-angiogenic mechanisms in the inflamed synovium; levels of which are significantly induced in both early and late stages of PsA synovial pathogenesis (Fearon et al., 2003; Fink et al., 2007). Furthermore, VEGF levels in early disease correlates with disease activity and is predictive of radiological progression within one year of diagnosis (Ballara et al., 2001; Clavel et al., 2007). VEGF modifies the endothelial barrier making it more responsive for activation of new vessel growth (Claesson-Welsh, 2016), and is produced by various cells including neutrophils, FLS and macrophages (Lapeyre-Prost et al., 2017). VEGF acts synergistically with Ang1 and Ang2 in controlling vessel stability and maturation (Asahara et al., 1996). Ang1 is responsible for pericyte recruitment and vessel stabilization. On the other hand, Ang2 opposes Ang1 and prevents the maturation of invading vascular sprouts in the presence of VEGF. The vessels are in a ‘plastic’ state and thus more responsive to VEGF stimulation (Asahara et al., 1996; Veale and Fearon, 2006). As the blood vessel matures, it becomes less dependent on VEGF. And so other factors, including Ang1, TGF-β, PDGF, and NCAM begin to regulate pericyte recruitment and EC-pericyte interactions wall (Tilton et al., 1979; Li et al., 2005; Kennedy et al., 2010). The formation of a pericyte layer stops the ‘plastic’ state thus stabilizing the vessel. In terms of the inflamed joint, studies have demonstrated that there are a mixture of immature and mature vessels that go through simultaneous angiogenesis, vessel maturation and regression depending on the stimuli (Izquierdo et al., 2009; Kennedy et al., 2010).

As highlighted in Chapter 2, numerous groups have observed increased expression of adhesion molecules including E-selectin, P-selectin, ICAM-1, VCAM-1, and chemokines in the inflamed synovium (Veale et al., 1993; García-López et al., 2001; Salcedo et al., 2003; Loos et al., 2006; Zhang et al., 2011; Veale et al., 2015; Agere et al., 2017; Balogh et al., 2019; Fromm et al., 2019). Interestingly, the synoviocyte-derived chemokine, CXCL12,
has been shown to bind to and accumulate on heparan sulfate molecules in RA ECs, where it colocalizes with the early angiogenic marker αvβ3 integrin inducing angiogenesis, an effect that is abrogated through CXCL12 immuno-depletion (Pablos et al., 2003). The CC branch includes chemoattractants such as CCL5 which recruits monocytes, macrophages and lymphocytes into the inflamed joint and can directly induce angiogenic mechanisms (Agere et al., 2017; Balogh et al., 2019). In addition, increased expression of CCR6 has been demonstrated to be localised to the vascular regions in RA synovial tissue (Matsui et al., 2001). Unsurprisingly, both the SF and synovial tissue from RA compared to HC have increased levels of numerous chemokines including CCL13, CCL18, CXCL9, CXCL12, CCL3, CXC3L1 (Iwamoto et al., 2008; Ridiandries et al., 2016).

In the context of JIA, studies have demonstrated increased synovial angiogenesis for all subgroups of JIA with no major differences reported; however, increased microvessel density has been observed in polyJIA compared to oligoJIA (Finnegan et al., 2011). The expression of the integrin, αVβ3, on ECs increases during angiogenesis. However, studies have demonstrated that its expression is significantly induced in the synovium of polyJIA. αVβ3 is a marker for immature, undifferentiated vessels, increased expression of which has also been observed in adult RA and PsA synovium (Brooks et al., 1995; Izquierdo et al., 2009; Kennedy et al., 2010). Furthermore, CXCR3, CXCL10, ICAM-1 and E-selectins are increased in the inflamed synovium of JIA patients, including vascular regions (Dolezalova et al., 2001; Martini et al., 2019). There is also a positive association between VEGF and diagnostic inflammatory markers, CRP and ESR (Maeno et al., 1999). To date, only one study has examined synovial vascularity in DA and reported an increase in the number of blood vessels in DA patients compared to HC and JIA (Foley et al., 2020).

Metabolic pathways are essential in controlling synovial inflammation. Although there is more vascularization in the inflamed joint, the uncontrolled newly formed vessels provide inadequate nutrient exchange. In combination with the expanding pannus, the new blood vessels cannot meet the elevated metabolic demands of infiltrating and tissue resident cells within the inflamed synovium (Kennedy et al., 2010; Ng et al., 2010; Biniecka et al., 2016; Fearon et al., 2016, 2022). This leads to a hypoxic microenvironment where the cells transform from a relatively quiescent state to a very metabolically active form; the
transformation of which is required to fuel their activated pathogenic phenotype (Schioppa et al., 2003; Fearon et al., 2016; Biniecka et al., 2016). As highlighted in Chapter 1 and 2, previous studies have shown a metabolic switch in the inflamed synovium, paralleled by the accumulation of metabolic intermediates (Haas et al., 2015; Biniecka et al., 2016; Michopoulos et al., 2016; Weyand et al., 2017; Fearon et al., 2019; Hanlon et al., 2019, 2022; Masoumi et al., 2020). Indeed, the metabolic intermediate, succinate, induces synovial neo-angiogenesis via VEGF-dependent HIF-1α pathways in CIA models (Li et al., 2018), lactate promotes the secretion of angiogenic growth factors from primary RA FLS (Biniecka et al., 2016), and G6PI induces synovial angiogenesis through hypoxic dependent mechanisms (Lu et al., 2017).

Although there is no data that examines the metabolic alterations in the DA joint, Bosco et al. (2009) demonstrated that the hypoxic microenvironment in JIA is modulated by VEGF and OPN, which are involved in promoting neoformation through EC survival, proliferation and migration (Bosco et al., 2009). Moreover, the lack of oxygen in the synovium along with the increased expression of CCL20, HIF-1α, and HIF-2α in JIA synovial monocytes contributes to the immune cell infiltration (Bosco et al., 2009). In addition, synovial macrophages in the JIA hypoxic joint express elevated levels of TREM-1 which has been shown to drive a proinflammatory macrophage phenotype (Raggi et al., 2017). In the context of DA, there are no studies which examine EC function.
3.2 Aims and objectives

Overall aims: To examine the effect of the DA joint microenvironment on endothelial cell function

- To determine the effect of primary DA FLS conditioned media (DA FLS-CM) on endothelial cell function
- To determine the effect of DA FLS-CM on adhesion molecule and chemokine receptor expression
- To determine the effect of DA FLS-CM on EC cellular metabolism
- To examine the effect of T cell-derived cytokines on endothelial cell function
- To examine the synergistic effect of T cell-derived cytokines on endothelial cell metabolism
3.3 Materials and methods

3.3.1 Patient recruitment and biopsies

Described in Section 2.3.1, Chapter 2. Each DA FLS-CM is from a different donor.

<table>
<thead>
<tr>
<th>DA n=7</th>
<th>Prior Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td><strong>NIL (inhibitor of inducible nitric oxide synthase)</strong></td>
</tr>
<tr>
<td>median (range) 10 (4-17)</td>
<td>2</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td><strong>NIL + NSAID</strong></td>
</tr>
<tr>
<td>F = 2</td>
<td>1</td>
</tr>
<tr>
<td>M = 5</td>
<td>MTX + adalimumab</td>
</tr>
<tr>
<td><strong>Active joint count</strong></td>
<td><strong>Adalimumab + Etanercept</strong></td>
</tr>
<tr>
<td>n (range) 17 (0-7)</td>
<td>2</td>
</tr>
<tr>
<td>unknown 1</td>
<td>Adalimumab</td>
</tr>
<tr>
<td><strong>ESR</strong></td>
<td><strong>Treatments at time of sample</strong></td>
</tr>
<tr>
<td>(14)</td>
<td>NIL</td>
</tr>
<tr>
<td>median 8</td>
<td>2</td>
</tr>
<tr>
<td>unknown 0</td>
<td>NIL + NSAID</td>
</tr>
<tr>
<td><strong>CRP</strong></td>
<td><strong>Etanercept</strong></td>
</tr>
<tr>
<td>(0)</td>
<td>1</td>
</tr>
<tr>
<td>median &lt;5</td>
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</tr>
<tr>
<td>unknown 0</td>
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<td>Golimumab</td>
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<tr>
<td>Unknown 0</td>
<td>Adalimumab</td>
</tr>
</tbody>
</table>

Table 3. 1 DA patient demographic, clinical, laboratory features and treatments prior sampling and at time of sampling.

3.3.2 Synovial biopsies-synovial fibroblast isolation

Isolation of primary DA FLS are described in Section 2.3.2, Chapter 2. To examine the effect of FLS-Conditioned Media (CM) on EC function, DA FLS were grown to confluence in a T25 flask (Sarstedt, Germany). Once confluent, media was replaced with 3.5ml cRPMI. Following 24hr, the supernatants were harvested and termed DA FLS-CM and stored at -20°C for EC functional experiments. Each DA FLS-CM was from a different donor.
3.3.3 Culture of HUVECs

Human umbilical vein endothelial cells (HUVECs) (ATCC, Manassas, USA) were grown to confluence in endothelial cell basal media (MCDB-131, Gibco) supplemented with L-glutamine (20ml of 100X solution), hydrocortisone (0.6 μg/ml), human epidermal growth factor (hEGF) (0.01 μg/ml), penicillin-streptomycin (100 units/ml and 100 μg/ml; Bioscience, UK), fungizone (0.25 μg/ml; Bioscience, UK) and 15% FCS (Gibco-BRL) (cMCDB-131).

3.3.4 HUVEC Tube formation Assay

Matrigel (50μl; BD Biosciences, San Jose, CA, USA) was plated in 96 well culture plates after thawing on ice and allowed to polymerise for 30 min at 37°C in humidified air with 5% CO₂. 2 x 10⁴ cells in cMCDB-131 were added to each well before being cultured with 33% v/v DA FLS-CM or cMCDB-131 (basal control media) for an additional 6hr. For cytokine stimulation, cells were cultured in cMCDB-131 for 24hr and subsequently stimulated with TNF-α (0.1 ng/ml and 1 ng/ml), IL-17a (20ng/ml and 50ng/ml) or IFN-γ (10ng/ml and 50ng/ml). For synergy experiments, cells were cultured in cMCDB-131 for 24hr before being treated with TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml) or IFN-γ (50ng/ml). EC tubule formation was then assessed using phase-contrast microscopy. Number of tubes were quantified by counting the number of connecting branches formed in a HPF (10x) and was performed for 3 random HPF by 2 assessors.

3.3.5 Protein analysis and Western Blotting

For protein analysis, HUVECs were seeded at a density of 1 x 10⁵/well in 6 well plates and cultured with DA FLS-CM or basal control media for 24hr. For cytokine stimulation, cells were cultured in 1% FBS MCDB-131 for 24hr and subsequently stimulated with TNF-α (0.1 ng/ml and 1 ng/ml), IL-17a (20ng/ml and 50ng/ml) or IFN-γ (10ng/ml and 50ng/ml). For synergy experiments, cells were cultured in 1% FBS MCDB-131 for 24hr before being treated with TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml) or IFN-γ (50ng/ml). Ice-cold RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma-Aldrich, USA) containing 10 μg/mL phosphatase inhibitor cocktail and 10 μg/mL protease inhibitor
cocktail (Sigma-Aldrich, Ireland) was used to lyse cell suspensions. Total protein concentration and normalisation was performed using a BCA assay (Pierce Chemical Co, USA). Protein (2 μg) was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (4% stacking, 8-10% resolving), gels were then transferred onto PVDF membranes (Amersham Biosciences, UK) prior to 1hr blocking in wash buffer (Tris-buffered saline (TBS) with 0.1% Tween-20) containing 3% BSA with gentle agitation at room temperature (RT). Membranes were incubated with rabbit polyclonal Notch1 intracellular domain (ICD) (Millipore, USA) diluted in 3% BSA in TBS/Tween-20 at 4°C overnight with gentle agitation. B-actin (Sigma -Aldrich, Ireland) was used as a loading control. The signal was detected using SuperSignal® West Pico Chemiluminescent Substrate (Amersham Biosciences, UK). Band densities were imaged using the ChemiDoc MP Imaging System (Bio-Rad, USA).

3.3.6 Wound Repair Assay
HUVECs (2 x 10^4 cells/well) were plated in 48 well plates and grown to confluency. Cells were scraped with a sterile pipette tip to create a linear wound across the centre of the well, debris was removed, and cells were cultured with 33% v/v DA FLS-CM and control media for 24hr. Migration was assessed through semi-quantitative analysis of cell repopulation of the wound margins by counting the number of cells migrating into the wound space and by calculating % wound closure.

3.3.7 Proliferation Assay
HUVECs (2 x 10^4 cells/well) were plated in 48 well plates before being treated with 33% v/v DA FLS-CM or control media for 24hr. Cells were then washed with 1 x PBS and fixed with 4% PFA for 30 min at RT. The 4% PFA was removed and 1ml/well of crystal violet (Cruinn, Ireland) was added for 30 min at RT. Plate was then washed x3 with distilled H₂O and left to dry overnight. The following day, 400μl/ well of 1% SDS was added for 30 min with gentle agitation. Proliferation results were read at 530nm using the Epoch reader (Agilent, USA) with 1% SDS as the blank control.
3.3.8 Adhesion assay

HUVECs (2 x 10^4 cells/well) were seeded in 48 well plates and incubated overnight. HUVECs were then cultured in 1% FBS MCDB-131 for 24hr and subsequently cells were stimulated with 33% v/v DA FLS-CM for another 24hr. For the dose response experiments, HUVECs were stimulated with TNF-α (0.1ng/ml and 1ng/ml), IL-17a (20ng/ml and 50ng/ml), and IFN-γ (10ng/ml and 50ng/ml) for 24hr. For synergy experiments, HUVECs were treated with IL-17a (50ng/ml) or IFN-γ (50ng/ml) in the presence or absence of TNF-α (0.1ng/ml) for 24hr. PBMC were isolated from healthy donors and the adhesion assays was performed as outlined in Section 2.3.7, Chapter 2.

3.3.9 Flow cytometry

HUVECs (1 x 10^5 cells/well) were grown to confluence in a 6 well plate and then cultured with 33% v/v DA FLS-CM or control media for another 24hr. For the cytokine experiments, HUVECs were cultured in 1% FBS MCDB-131 before being stimulated with TNF-α (0.1ng/ml and 1ng/ml), IL-17a (20ng/ml and 50ng/ml), and IFN-γ (10ng/ml and 50ng/ml) for 24hr. For synergy experiments, HUVECs were cultured in 1% FBS MCDB-131 before being stimulated with IL-17a (50ng/ml) or IFN-γ (50ng/ml) in the presence or absence of TNF-α (0.1ng/ml) for 24hr.

Following stimulation, Accutase (Sigma-Aldrich, Ireland) was utilised for cell detachment. Cells were washed in ice-cold PBS prior to incubation with Zombie NIR™ Fixable Viability Kit (BioSciences, UK) for 30 min at 4°C. To achieve blocking of Fc receptors, cells were incubated with TruStain FcX blocking solution (Biolegend, UK) for 10 min at 4°C, then stained with fluorochrome-conjugated antibodies against extracellular markers, vortexed, and then incubated in the dark at 4°C for 30 min. Table 3.1 outlines the antibodies assessed for EC treated with 33% DA FLS-CM while Table 3.2 outlines the antibodies assessed for EC stimulated with T cell-derived cytokines.

Samples were washed in FACS Buffer and pelleted at 400g. Supernatants were removed, and the cells were fixed with 1% PFA for 10 min before washing with FACS Buffer then resuspended in 200μl of FACS Buffer. Samples were acquired using the LSRFortessa Flow Cytometer (BD, UK) and analysed using FlowJo software (BD, UK).
<table>
<thead>
<tr>
<th>Marker</th>
<th>Other names</th>
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<th>Clone</th>
<th>Company</th>
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<tr>
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<td>BV605</td>
<td>HA58</td>
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Table 3. 2 Antibodies used for EC cultured with 33% DA FLS-CM

<table>
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<td>VCAM-1</td>
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<td>BV605</td>
<td>HA58</td>
<td>BD, UK</td>
</tr>
<tr>
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<td>-</td>
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<td>BV510</td>
<td>1A4</td>
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</tbody>
</table>

Table 3. 3 Antibodies used for ECs stimulated with T cell-derived cytokines

### 3.3.10 Cytokine and chemokine measurements

To assess the effects of DA FLS-CM and T cell cytokines (TNF-α, IL-17a and IFN-γ) in secretion of proinflammatory mediators, HUVECs were seeded in 48 well plates at a density of $2 \times 10^4$/well and allowed to adhere overnight. ECs were then cultured with 33% v/v DA FLS-CM for another 24hr and supernatants were harvested. For cytokine stimulation, cells were cultured in 1% FBS MCDB-131 for 24hr and subsequently stimulated with TNF-α (0.1 ng/ml and 1 ng/ml), IL-17a (20ng/ml and 50ng/ml) or IFN-γ (10ng/ml and 50ng/ml). For synergy experiments, cells were cultured in 1% FBS MCDB-131 for 24hr before stimulated with IL-17a (50ng/ml) or IFN-γ (50ng/ml) in the presence or absence of TNF-α (0.1ng/ml). Supernatants were harvested and proinflammatory mediators quantified by ELISA.
Quantification of IL-6, MCP-1, IL-8 and RANTES were measured by specific ELISAs as outlined in Section 2.3.5, Chapter 2.

### 3.3.11 Cellular bioenergetic analysis

Seahorse XFe96 analyzer (Seahorse Biosciences) was used to determine the real time levels of OCR and ECAR. HUVECs were seeded at 15 x 10^3 cells/well in a 96 well cell culture XFe microplate (Seahorse Biosciences) and allowed to adhere overnight. For the DA FLS-CM experiments, HUVECs were cultured with either 33% v/v DA FLS-CM or control media for another 24hr. For cytokine experiments, HUVECs were stimulated with either 33% v/v DA FLS-CM or control media for another 24hr. For cytokine experiments, HUVECs were cultured with either 33% v/v DA FLS-CM or control media for another 24hr. For cytokine experiments, HUVECs were cultured with either 33% v/v DA FLS-CM or control media for another 24hr. For cytokine experiments, HUVECs were cultured with either 33% v/v DA FLS-CM or control media for another 24hr. For cytokine experiments, HUVECs were cultured with either 33% v/v DA FLS-CM or control media for another 24hr. For cytokine experiments, HUVECs were cultured with either 33% v/v DA FLS-CM or control media for another 24hr. For cytokine experiments, HUVECs were cultured with either 33% v/v DA FLS-CM or control media for another 24hr. For cytokine experiments, HUVECs were cultured with either 33% v/v DA FLS-CM or control media for another 24hr. For cytokine experiments, HUVECs were cultured with either 33% v/v DA FLS-CM or control media for another 24hr.

Following 24hrs, cells were then washed with assay medium (unbuffered DMEM supplemented with 10mM glucose, pH 7.4) before incubation with assay medium for 30 min at 37°C in a non-CO2 incubator. Seahorse was performed and oxidative phosphorylation and glycolytic outputs calculated as outline in Section 2.3.10, Chapter 2.

For the glycolysis stress test, the assay was performed using glycolytic stress test assay protocol as per the manufacturer’s instructions (Agilent Technologies, USA). ECAR was measured under basal and treated with DA FLS-CM followed by the sequential addition of 10 mM glucose, 0.5 μM oligomycin, and 100 mM 2-DG. Glycolytic reserve was calculated by subtracting 2-DG from oligomycin, glycolysis was measured by subtracting glycolytic reserve from 2-DG while glycolytic capacity was measured by the addition of glycolysis and glycolytic reserve.

![Glycolytic Function Diagram](image.png)

**Figure 3.3.1** Representative Seahorse Glycolytic Stress test assay depicting measurable parameters.
3.3.12 Statistical analysis

Statistical analyses were performed on GraphPad Prism 9 software. Wilcoxon paired t-test (*) was used for nonparametric data (DA FLS-CM experiments), while RM One-way ANOVA (#) test or paired t-test (*) was used for analysis of parametric data (cytokine stimulations and cytokine synergy experiments). P-values of <0.05 (#p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001) were determined as statistically significant for One-way ANOVA while *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 were defined as statistically significant for paired t-test.
3.4 Results

3.4.1 DA FLS-CM induces tube formation and migration capacity in HUVECs

Previous studies have demonstrated distinct synovial vascular morphology, particularly, an increase in the number of blood vessels in the DA synovium compared to JIA. Therefore, in this chapter we investigate whether the DA joint microenvironment contributes to differential regulation of EC function. Representative images of HUVEC tube formation in response to DA FLS-CM compared to control media are shown in Figure 3.1(a). Quantification demonstrates that DA FLS-CM increased the number of connecting branches and formation of EC tubes compared to basal EC control media, however this did not reach significance (Figure 3.1(b)).

Given that one of the primary roles for ECs in RA is to promote angiogenesis, we next investigated the effect of DA joint microenvironment on the expression of the key angiogenic signalling protein, Notch 1 IC. Representative western blot image (Figure 3.1(c)) and quantification (Figure 3.1(d)) demonstrates that there was an increase in Notch1 IC in DA FLS-CM treated HUVEC compared to basal EC control media.

A key mechanism involved in new blood vessel formation is the ability of EC to migrate. Therefore, to assess the effect of DA FLS-CM on the migrative capacity of ECs, wound repair scratch assays were performed where the ability of the stimulated ECs to repopulate the wound margins was quantified. Representative images of HUVEC wound repair assays in response to DA FLS-CM are shown in Figure 3.2(a) where an increase in HUVEC repopulation of the wound margins is observed in response to DA FLS-CM (ii) compared to basal EC control media (i). Quantification demonstrated a significant increase in the number of cells repopulating the wound margins ($p=0.06$) (Figure 3.2(b)) and percentage wound closure in response to DA FLS-CM ($p<0.05$) (Figure 3.2(c)). In parallel, cell proliferation was assessed by a crystal violet assay (Figure 3.2(d)). While there was a minimal decrease in proliferation in response to DA FLS-CM, this was significant. This suggests that the effect of DA FLS-CM on EC migration was not due to increase in the number of cells, but actually the EC moving towards an angiogenic stimulus.
Figure 3. DA FLS-CM induces tube formation and NOTCH 1IC HUVECs. (a) Representative images of HUVEC tube formation under (i) basal conditions and in response to (ii) DA FLS-CM. (b) Graph represents semi-quantitative analysis of the number of EC connecting branches/high-powered field (of n=3 hpF). Original magnification 10x. Data are expressed as Mean ± SEM, of n=7 expts. Parametric t-test was used. (c) Representative western blot images demonstrating protein expression of Notch 1 ICD and (d) bar chart measuring the difference relative intensity of Notch 1 ICD in HUVECs (n=4) cultured with 33% DA FLS-CM or control media for 24 hr, β-actin was used as loading control. Wilcoxon signed rank paired t-test (*) was used for statistical analysis. Data expressed as mean ± SEM.
Figure 3. 2 DA FLS-CM induces migrative capacity of HUVECs. (a) Representative photomicrographs and accompanying dot plots demonstrating (b) average number of migrating cells and (c) % coverage of cells in basal controls (n=7) and EC stimulated DA FLS-CM (n=7). (d) Relative cell growth/ proliferation (n=7) under basal and DA FLS-CM conditions (n=7) for 24 hours. Values expressed as Mean +/- SEM of n=7 expts. Wilcoxon signed rank paired t-test (*) was used for statistical analysis., *p<0.05, significantly different from basal.
3.4.2 DA FLS-CM induces leukocyte adhesion through ICAM-1 expression on HUVECs

Activation of synovial ECs is critical for facilitating infiltration of immune cells to the synovium, a process that is mediated by adhesion molecule expression and homing chemokines which together promote transendothelial cell migration. Therefore, we next examined if DA FLS-CM promoted leukocyte-HUVEC adhesion. HC PBMC were incubated for 1hr on a confluent HUVEC monolayer that was pre-treated with DA FLS-CM for 24hr. Representative images show an increase in PBMC adhesion in response to stimulation with DA FLS-CM (Figure 3.3 (a)(i – ii)) with quantification demonstrating a significant increase in the adhesive capacity of EC compared to the basal EC control media ($p<0.05$) (Figure 3.3(b)).

To further examine the adhesive capacity of EC in response to DA FLS-CM we examined the expression of ICAM-1 and VCAM-1. As described above, HUVECs were cultured with 33% v/v DA FLS-CM or basal EC control media for 24hr before being stained with ICAM-1 and VCAM-1 (Table 3.1). The gating strategy for all EC flow experiments in this chapter are shown in Figure 3.4 where cells were gated based on (a) forward and side scatter and (b – c) doublets and (d) dead cells were removed and (e) FMO controls were used to determine gating boundaries. Zombie NIR™ Fixable Viability Kit (BioSciences, UK) were used to eliminate dead cells. To adjust for spectral overlap between detectors, compensation was applied using single stained compensation beads (BD, UK).

Representative dot plots (Figure 3.5(a)(i)) and quantification (Figure 3.5(a)(ii)) demonstrate a significant percentage increase in the frequency of ICAM-1 positive HUVEC in response to DA FLS-CM compared to basal EC control media ($p<0.05$). Additionally, a significant decrease ($p<0.05$) in ICAM-1 MFI was demonstrated in DA FLS-CM treated HUVECs compared to control (Figure 3.5(a)(iii – iv)). Representative dot plots (Figure 3.5 (b)(ii)) and quantification (Figure 3.5 (b)(iii)) demonstrate a significant decrease in frequency of VCAM-1 positive HUVEC in response to DA FLS-CM compared to basal EC control media ($p<0.05$). However, VCAM-1 expression on HUVEC was low and no significant changes were observed for VCAM-1 MFI (Figure 3.5 (b)(iii – iv)).
Figure 3. DA FLS-CM induces adhesive mechanisms in HUVEC. (a) Representative photomicrographs and (b) accompanying quantification graph demonstrating PBMC adhesion and average number of attached cells to HUVEC monolayers in (i) basal controls (n=7) and (ii) HUVEC treated with DA FLS-CM (n=7) for 24 hours. (b) Quantification of Leukocyte-EC adhesion. Values expressed as mean ± SEM of n=7 expts. Wilcoxon signed rank paired t-test (*) was used for statistical analysis. *p<0.05 significantly different compared to basal.
Figure 3. 4 HUVEC gating strategy. HUVECs were stained with a panel of fluorochrome antibodies (ICAM-1, VCAM-1, CXCR3, CXCR4, CXCR5, CCR6 and CD105). Representative dot plots depicting the gating strategy used to identify and phenotype HUVECs when cultured with DA FLS-CM. The forward and side scatter parameters of cells were set before doublet exclusion and elimination of dead cells (a – d). (e) Representative flow plot shows the gating for an example marker.
Figure 3. 5 DA FLS-CM induces ICAM-1 expression in HUVECs. HUVECs were cultured with 33% v/v DA FLS-CM or control media for 24hr before staining for flow cytometry. (i) Representative flow dot plots for FMO and basal control for (a) ICAM-1 and (b) VCAM-1. (ii) Bar charts display the percentage frequency of HUVECs that are positive for (a) ICAM-1 and (b) VCAM-1 following treatment with 33% DA FLS-CM. (iii) Bar charts demonstrate MFI values for (a) ICAM-1 and (b) VCAM-1. (iv) Representative histograms show MFI shift (a) ICAM-1 and (b) VCAM-1. Values expressed as mean ± SEM of n=7 expts, Wilcoxon signed rank paired t-test was used for statistical analysis. *p<0.05 significantly different compared to basal.
3.4.3 DA FLS-CM induces CXCR3, CXCR5 and CD105 expression on HUVECs

In addition to examining the adhesive mechanisms of HUVEC, we next examined the expression of key chemokine homing receptors which facilitate the movement of leukocytes into the joint. Specifically, we examined the chemokine receptors CXCR3, CXCR4, and CXCR5 which have been directly implicated in RA (MacDonald et al., 2018). Representative dot plots (Figure 3.6(a)(i)) and quantification (Figure 3.6(a)(ii)) demonstrate a trending increase in CXCR3 frequencies in DA FLS-CM treated HUVEC compared to basal with a significant increase in MFI ($p<0.05$) (Figure 3.6(a)(iii – iv)). Representative dot plots (Figure 3.6(b)(i)) and quantification (Figure 3.6(b)(ii)) show that the frequency of CXCR4 was lower in HUVECs cultured with DA FLS-CM compared to basal with no effect observed for MFI (Figure 3.6(b)(iii – iv)). Representative dot plots (Figure 3.6(c)(i)) and quantification (Figure 3.6(c)(ii)) demonstrate a trending increase in CXCR5 frequencies in DA FLS-CM treated cells compared to basal, however, this did not reach significance. A significant increase in CXCR5 MFI was observed (Figure 3.6(c)(iii – iv)) ($p<0.05$). No significant differences in CCR6 frequency or MFI were observed (Figure 3.7(a)(i – iv)).

We also examined the expression of CD105, as it has recently been shown to be expressed on activated ECs and involved in EC proliferation, differentiation, and migrational neovascularization. No increase in percentage frequency of CD105 positive HUVEC in response to DA FLS CM compared to basal was demonstrated (Figure 3.7(b)(i – (ii))), however, a trending increase in CD105 MFI was observed, although not significant (Figure 3.7(b)(iii – iv)).
Figure 3. The effect of DA FLS-CM on CXCR3, CXCR4 and CXCR5 expression in HUVECs. HUVEC were cultured with or without 33% v/v DA FLS-CM for 24hr before staining for flow cytometry. (i) Representative flow dot plots show FMO, basal and ECs following treatment with 33% v/v DA FLS-CM for (a) CXCR3, (b) CXCR4 and (c) CXCR5. (ii) Bar charts demonstrate the percentage frequency of HUVECs that are positive for (a) CXCR3, (b) CXCR4 and (c) CXCR5 in response to 33% DA FLS-CM. (iii) Bar charts demonstrate MFI values for (a) CXCR3, (b) CXCR4 and (c) CXCR5. (iv) Representative histograms show MFI shift. Values expressed as mean ± SEM of n=7 expts, Wilcoxon signed rank paired t-test was used for statistical analysis where *p<0.05 is significantly different compared to basal.
Figure 3. 7 The effect of DA FLS-CM on CCR6 and CD105 expression in HUVECs. HUVECs were cultured with or without 33% v/v DA FLS-CM before staining for flow cytometry. (i) Representative flow dot plots show FMO, basal and ECs following treatment with 33% v/v DA FLS-CM for (a) CCR6 and (b) CD105. (ii) Bar charts demonstrate the percentage frequency of HUVECs that are positive for (a) CCR6 and (b) CD105 following treatment with 33% DA FLS-CM. (iii) Bar charts demonstrate MFI values for (a) CCR6 and (b) CD105. (iv) Representative histograms show MFI shift. Values expressed as mean ± SEM of n=7 expts, Wilcoxon signed rank paired t-test was used for statistical analysis.
3.4.4 Effect of the DA joint microenvironment on the metabolic activity of HUVEC

We next investigated if the DA microenvironment can alter the metabolism of ECs. Average OCR bioenergetic profile is shown in Figure 3.8(a). No significant changes in baseline OxPhos (Figure 3.8(b)), ATP Synthesis (Figure 3.8(c)), max respiration (Figure 3.8(d)), proton leak (Figure 3.8(e)), respiratory reserve (Figure 3.8(f)), and non-mitochondrial respiration (Figure 3.8(g)) in response to DA FLS-CM compared to basal were observed. Pie charts showing the relative contribution of the OxPhos potential within each condition demonstrated no differences in ATP synthesis (black) and proton leak (pink) outputs as a percentage of total OxPhos potential between basal (Figure 3.8(h)(i)) and DA FLS-CM treated HUVECs (Figure 3.8(h)(ii)). However, respiratory reserve (green) was increased by 10% in DA FLS-treated HUVECs (Figure 3.8(h)(ii)) compared to basal (Figure 3.8(h)(i)). Non-mitochondrial respiration (purple) was decreased by 11% in DA FLS-treated HUVECs (Figure 3.8(h)(ii)) compared to basal (Figure 3.8(h)(i)).

In addition to oxidative phosphorylation, we also examined the glycolytic potential of DA FLS-CM treated HUVECs. Average ECAR bioenergetic profile is shown in Figure 3.9(a). No changes were observed for ECAR baseline (Figure 3.9(b)), non-glycolytic capacity (Figure 3.9(c)), glycolytic reserve (Figure 3.9(d)), glycolytic capacity (Figure 4.9(e)), ECAR:OCR ratio (Figure 3.9(f)), and metabolic map (Figure 3.9(g)). The average of the ECAR outputs within each condition are shown in the pie charts (Figure 3.9(h)). Glycolytic reserve (black) was increased by 7% in DA FLS-CM-treated HUVECs (Figure 3.9(h)(ii)) compared to basal (Figure 3.9(h)(i)). Non-glycolytic acidification (green) was the same as a percentage of total ECAR potential between basal (Figure 3.9(h)(i)) and DA FLS-CM treated HUVECs (Figure 3.9(h)(ii)). Furthermore, glycolysis (pink) as an ECAR output, was lost in DA FLS-CM treated HUVECs (Figure 3.9(h)(ii)) compared to basal (Figure 3.9(h)(i)).
Figure 3. 8 DA joint microenvironment does not alter metabolic activity of HUVECs. (a) Average OCR profile for basal and ECs cultured with 33% DA FLS-CM before and after injections of oligomycin, FCCP, and antimycin A/rotenone. Bar charts show quantification of (b) OCR baseline, (c) ATP synthesis, (d) max respiratory capacity, (e) proton leak, (f) respiratory reserve and (g) non-mitochondrial respiration for both basal and ECs following treatment with 33% DA FLS-CM. Pie charts shown in (h) outline the proportion of the average of each OCR output from both (i) basal and (ii) ECs treated with DA FLS-CM. Values expressed as mean ± SEM of n=7 expts. Wilcoxon signed rank paired t-test was performed for statistical analysis.
Figure 3. 9 DA joint microenvironment does not alter the glycolytic capacity of HUVECs. 
(a) Average ECAR profile for basal and ECs cultured with 33% DA FLS-CM before and after injections of glucose, oligomycin and 2-DG. Bar charts show quantification of (b) ECAR baseline (c) non-glycolytic capacity (d) glycolytic reserve (e) glycolytic capacity (f) ECAR: OCR ratio (g) metabolic map of both basal and ECs treated with 33% DA FLS-CM. Pie charts shown in (h) outline the proportion of the total ECAR outputs from both (i) basal and (ii) ECs following treatment with DA FLS-CM. Values expressed as mean ± SEM of n=7 expts. Wilcoxon signed rank paired t-test was performed for statistical analysis.
3.4.5 To examine the effect of TNFα, IL-17a and IFN-γ on HUVEC tube formation.

We next investigated if T cell-derived cytokines contribute to tube formation. Representative images of HUVEC tube formation in response to increasing concentrations of TNF-α, IL-17a, and IFN-γ are shown in Figure 3.10(a). Quantification demonstrates that no significant changes in the number of tube formations were observed in response to TNF-α when compared to basal (Figure 3.10(b)(i)). While not significant, there was a stepwise increase in tube formations when stimulated with IL-17a (Figure 3.10(b)(ii)). Similarly, while an increase in the number of tube formations were observed in response to IFN-γ, this was not statistically significant (Figure 3.10(b)(iii)).

Figure 3. 10 The effect of TNFα, IL-17a or IFN-γ on tube formation in HUVECs. (a) Representative images of HUVEC tube formation in response to basal, TNF-α (0.1ng/ml and 1ng/ml), IL-17a (20ng/ml and 50ng/ml) and IFN-γ (10ng/ml and 50ng/ml). (b) Graph represents semi-quantitative analysis of the number of EC connecting branches/high-powered field (of n=3 hpF) in response to (i) TNF-α, (ii) IL-17a and (iii) IFN-γ. Original magnification 10x. Data are expressed as Mean ± SEM, of n=3 expts. Paired t-test was performed for statistical analysis. **p<0.01 significantly different from other conditions.
3.4.6 TNF-α, IL-17a and IFN-γ induce HUVEC cytokine secretion.

We next examined the effect of TNF-α, IL-17a and IFN-γ on secretion of proinflammatory mediators. We demonstrated a significant increase in the secretion of IL-6 in response to TNF-α (0.1ng/ml, \( p<0.01 \); 1ng/ml, \( p<0.01 \)) (Figure 3.11(a)(i)) and IFN-γ (10ng/ml, \( p<0.05 \); 50ng/ml, \( p<0.05 \)) (Figure 3.11(a)(iii)), with a trending increase observed for IL-17a (20ng/ml, \( p=0.06 \); 50ng/ml, \( p=0.07 \)) (Figure 3.11(a)(ii)). Furthermore, we also demonstrated a significant increase in MCP-1 secretion in response to TNF-α (0.1ng/ml, \( p<0.01 \); 1ng/ml, \( p<0.0001 \)) (Figure 3.11(b)(i)) and IFN-γ (10ng/ml, \( p<0.05 \); 50ng/ml, \( p<0.01 \)) (Figure 3.11(b)(iii)). No differences were observed for IL-17a stimulation (Figure 3.11(b)(ii)).

We also reported an increase in RANTES secretion from HUVECs in response to TNF-α (0.1ng/ml, \( p=0.05 \); 1ng/ml, \( p<0.05 \)) (Figure 3.12(a)(i)). While both IL-17a (20ng/ml) and IFN-γ (10ng/ml) increased RANTES in comparison to basal (Figure 3.12(a)(ii - iii)), this was not significant nor was it dose-dependent. While it is not clear why a dose dependent response was not observed for RANTES following stimulation with increasing concentrations of IL-17a and IFN-γ, we speculate that it may be due to saturation of cytokine activation through intracellular pathways. Indeed, some studies have shown that if cells are over stimulated, the saturation effect can result in an inverse response and lead to inhibition outputs (Mimeault et al., 2010; McFarlane et al., 2023). Studies have also shown pleiotropic responses to cytokines depending on the cell type, microenvironment and concentration of the stimulant (Mimeault et al., 2010; McFarlane et al., 2023). IL-8 secretion was increased in response to TNF-α (0.1ng/ml, \( p<0.05 \); 1ng/ml, \( p<0.01 \)) (Figure 3.12(b)(i)). No effects on IL-8 secretion were observed for IL-17a and IFN-γ stimulation (Figure 3.12(b)(ii – iii)).
Figure 3. 11 TNF-α, IL-17α and IFN-γ induce IL-6 and/or MCP-1 secretion from HUVECs. HUVECs were cultured with (i) TNF-α (0.1ng/ml and 1ng/ml), (ii) IL-17α (20ng/ml and 50ng/ml) and (iii) IFN-γ (10ng/ml and 50ng/ml) for 24hr, supernatants were harvested, and cytokines measured by ELISA. Bar charts show quantification of (a) IL-6 and (b) MCP-1 secretion in cultured supernatants in response to cytokines. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA was used for statistical analysis. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 significantly different compared to other conditions.
Figure 3. 12 Effect of TNF-α, IL-17a and IFN-γ on RANTES and IL-8 secretion from HUVECs. HUVECs were cultured with (i) TNF-α (0.1ng/ml and 1ng/ml), (ii) IL-17a (20ng/ml and 50ng/ml) and (iii) IFN-γ (10ng/ml and 50ng/ml) for 24hr, supernatants were harvested, and cytokine measured by ELISA. Bar charts show quantification of (a) RANTES and (b) IL-8 secretion in cultured supernatants in response to cytokine stimulation. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis, respectively. #p<0.05, ##p<0.01 significantly different compared to other conditions.
3.4.7 The effect of TNF-α, IL-17a, and IFN-γ on adhesion molecule expression on HUVECs

We next treated HUVECs with TNF-α, IL-17a, and IFN-γ for 24 hr and flow cytometric staining was performed for ICAM-1 and VCAM-1 with extracellular antibodies according to Table 3.2 and applied gating strategy as per Figure 3.4. Representative dot plots (Figure 3.13(a) and (b)(i)) and quantification (Figure 3.13(b)(ii)) demonstrated an increase in the percentage frequency of ICAM-1 positive HUVEC following TNF-α stimulation (0.1ng/ml, \( p < 0.001 \); 1ng/ml, \( p < 0.01 \)) compared to basal. There was a significant increase in ICAM-1 MFI in response to TNF-α (0.1ng/ml, \( p = 0.06 \); 1ng/ml, \( p < 0.0001 \)) compared to basal (Figure 3.13(b)(iii – iv)). No significant changes were observed for percentage frequency of ICAM-1 positive HUVECs (Figure 3.13(c)(i – ii)) or ICAM-1 MFI (Figure 3.13(c)(iii – iv)) in response to IL-17a stimulation. We also demonstrated a significant increase in the percentage frequency of ICAM-1 positive HUVECs in response to IFN-γ stimulation (50ng/ml, \( p < 0.01 \); 50ng/ml, \( p < 0.01 \)) (Figure 3.13(a) and (d)(i – ii)) compared to basal with no change in ICAM-1 MFI observed (Figure 3.13(d)(iii – iv)).

Representative dot plots (Figure 3.14(a) and (b)(i)) and quantification (Figure 3.14(b)(ii)) demonstrate a dramatic increase in the percentage frequency of VCAM-1 positive HUVECs treated with TNF-α (1ng/ml, \( p < 0.0001 \)) (Figure 3.14 (b)(i – ii)), paralleled by a significant increase in VCAM-1 MFI compared to basal (1ng/ml, \( p < 0.05 \)) (Figure 3.14(b)(iii – iv)). Representative dot plots (Figure 3.14(c)(i) and (d)(i)) and quantification demonstrate no significant differences in percentage frequency of VCAM-1 positive HUVEC (Figure 3.14(c)(ii) and (d)(ii)) or VCAM-1 MFI (Figure 3.14(c)(iii – iv) and (d)(iii -iv)) in response to IL-17a or IFN-γ.
Figure 3. 13 TNF-α and IFN-γ induce ICAM-1 expression in HUVECs. (a) Representative flow dot plots show FMO and basal controls. HUVECs were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots demonstrate percentage frequency of ICAM-1 positive HUVECs when treated with the different cytokines. (ii) Bar chart displays the percentage frequency of ICAM-1 positive HUVECs in response to the different cytokines. (iii) Bar charts demonstrate ICAM-1 MFI and (iv) representative histograms show ICAM-1 MFI. Values expressed as mean ± SEM of n=7 expts, paired t-test was used for statistical analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 significantly different compared to other conditions.
Figure 3. TNF-α induces VCAM-1 expression in HUVECs. (a) Representative flow dot plots show FMO and basal control. HUVECs were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show VCAM-1 expression on HUVECs following stimulation with the different cytokines. (ii) Bar charts demonstrate the percentage frequency of VCAM-1 positive HUVECs in response to the different cytokines. (iii) Bar charts demonstrate VCAM-1 MFI and (iv) representative histograms show VCAM-1 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) or paired t tests (*) were used for statistical analysis, respectively #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
3.4.8 Effect of TNF-α, IL-17a, and IFN-γ on expression of CXCR3, CXCR4, CXCR5, CCR6, VEGFR, and decreases Claudin 5 on HUVECs

Representative dot plots (Figure 3.15(a) and (b)(i)) and quantification (Figure 3.15(b)(ii)) demonstrate percentage frequency of CXCR3 positive HUVEC is significantly increased in response to TNF-α (0.1ng/ml, \( p<0.05 \); 1ng/ml, \( p<0.0001 \)). Quantification (Figure 3.15(b)(iii)) and representative histogram plots (Figure 3.15(b)(iv)) demonstrate there was a significant increase in the MFI for CXCR3 compared to basal (0.1ng/ml, \( p<0.05 \); 1ng/ml, \( p<0.05 \)). Representative dot plots (Figure 3.15(c)(i)) and quantification (Figure 3.15(c)(ii)) demonstrate no difference in the frequency of CXCR3 positive HUVECs in response to IL-17a, however, CXCR3 MFI was significantly increased in response to IL-17a (20ng/ml, \( p<0.05 \)) (Figure 3.15(c)(iii) and (Figure 3.15(c)(iv)). No differences in CXCR3 frequency or MFI in response to IFN-γ was observed (Figure 3.15(d)). Representative dot plots (Figure 3.16(a) and (b)(i)) and quantification (Figure 3.16(b)(ii)) demonstrate a significant increase in the percentage frequency of CXCR4 positive HUVEC in response to TNF-α (0.1ng/ml, \( p<0.01 \); 1ng/ml, \( p<0.001 \)). CXCR4 MFI was also increased in response to TNF-α (1ng/ml, \( p<0.05 \)) (Figure 3.16(b)(iii – iv)). No differences in the frequency of CXCR4 positive HUVEC or MFI in response to IL-17a or IFN-γ was observed (Figure 3.16(c) and (d)). Representative dot plots (Figure 3.17(a) and (b)(i)) and quantification (Figure 3.17(b)(ii)) demonstrated an increase in the percentage frequency of CXCR5 positive HUVEC in response to TNF-α (1ng/ml, \( p<0.05 \)). No change in CXCR5 MFI in response to TNF-α was observed (Figure 3.17(b)(iii) and (Figure 3.17(b)(iv)). No changes in frequency of CXCR5 positive HUVEC or in CXCR5 MFI in response to IL-17a or IFN-γ was observed (Figure 3.17(c) and (d)). Representative dot plots (Figure 3.18(a) and (b)(i)) and quantification (Figure 3.18(b)(ii)) demonstrated a significant increase in the percentage frequency of CCR6 positive HUVEC in response to TNF-α (1ng/ml, \( p<0.01 \)). A significant increase in CCR6 MFI in response to TNF-α compared to basal was observed (1ng/ml, \( p<0.05 \)) (Figure 3.18(b)(iii)) (Figure 3.18(b)(iv)). No differences in the frequency of CCR6 positive HUVEC or CCR6 MFI in response to IL-17a or IFN-γ was demonstrated (Figure 3.18(c) and (d)).

Next, we examined the surface expression of Claudin 5, which regulates tight junctions in the transmembrane, and thus may be implicated in the regulation of the dysfunctional blood vessels observed in the inflamed joint (Reece et al., 1999). Representative dot plots
(Figure 3.19(a) and (b)(i)) and quantification (Figure 3.19(b)(ii)) demonstrates a decrease in the percentage frequency of Claudin 5 positive HUVEC in response to TNF-α (0.1ng/ml, \( p=0.07 \)) compared to basal while quantification (Figure 3.19(b)(iii) and representative histogram (Figure 3.19(b)(iv)) demonstrated no differences in Claudin 5 MFI. No differences in the frequency of Claudin 5 positive HUVEC or Claudin 5 MFI in response to IL-17a or IFN-γ was observed (Figure 3.19(c) and (d)).

We next assessed CD141, a marker of EC activation in inflammation (Goncharov et al., 2017). No significant differences in percentage frequency of CD141 positive HUVEC or CD141 MFI in response to TNF-α, IL-17a or IFN-γ were observed (Figure 3.20(b - d)).

Representative dot plots (Figure 3.21(a) and (b)(i)) and quantification (Figure 3.21(b)(ii)) demonstrate there was an increasing trend in the frequency of VEGFR positive HUVEC in response to TNF-α compared to basal. Quantification (Figure 3.21(b)(iii) and histogram plots (Figure 3.21(b)(iv)) show no difference for VEGFR MFI in response to TNF-α compared to basal. No effects for either the frequency of VEGFR positive HUVEC or VEGFR MFI in response to IL-17a or IFN-γ was demonstrated (Figure 3.21(c)) and Figure 3.21(d)).
Figure 3. 15 TNF-α and IL-17a induce CXCR3 expression in HUVECs. (a) Representative flow dot plots show FMO and basal controls. HUVECs were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained by flow cytometry. (i) Representative flow dot plots show CXCR3 positive HUVECs when treated with the different cytokines. (ii) Bar charts demonstrate the percentage frequency of HUVECs positive CXCR3 in response to the various cytokines. (iii) Bar charts demonstrate CXCR3 MFI and (iv) representative histograms show CXCR3 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) was used for statistical analysis, respectively. ####p<0.0001, ######p<0.0001 is significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 3. 16 TNF-α induces CXCR4 expression in HUVECs. (a) Representative flow dot plots show FMO and basal controls. HUVECs were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show CXCR4 positive HUVECs when treated with the different cytokines. (ii) Bar chart displays the percentage frequency of CXCR4 positive HUVEC in response to the different cytokines. (iii) Bar charts demonstrate CXCR4 MFI and (iv) representative histograms show CXCR4 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) was used for statistical analysis, *p<0.05, **p<0.01, ***p<0.001 significantly different compared to basal.
Figure 3. 17 TNF-α induces CXCR5 expression in HUVECs. (a) Representative flow dot plots show FMO and basal control. HUVECs were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show CXCR5 positive HUVECs when treated with the different cytokines. (ii) Bar chart demonstrates the percentage frequency of CXCR5 positive HUVEC in response to the various proinflammatory cytokines. (iii) Quantification demonstrates CXCR5 MFI and (iv) representative histograms show CXCR5 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis, respectively. #p<0.05 significantly different compared to basal, *p<0.05 significantly different compared to basal.
Figure 3. 18 TNF-α induces CCR6 expression in HUVECs. (a) Representative flow dot plots show FMO and basal control. HUVECs were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show CCR6 positive HUVECs when treated with the different cytokines. (ii) Bar charts demonstrate the percentage frequency of CCR6 positive HUVECs in response to the different cytokines. (iii) Quantification demonstrates CCR6 MFI and (iv) representative histograms show CCR6 MFI Shift., Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) was used for statistical analysis, respectively. #p<0.05, ##p<0.01 significantly different compared to basal. *p<0.05, **p<0.01 significantly different compared to basal.
Figure 3. 19 TNF-α decreases Claudin 5 expression in HUVECs. (a) Representative flow dot plots for Claudin 5 FMO and basal controls. HUVECs were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show Claudin 5 positive HUVECs when treated with the different cytokines. (ii) Bar charts demonstrate the percentage frequency of Claudin 5 positive HUVECs in response to the different cytokines. (iii) Bar charts demonstrate Claudin 5 MFI and (iv) representative histograms show Claudin 5 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis.
Figure 3. 20 TNF-α induces CD141 expression in HUVECs. (a) Representative flow dot plots for CD141 show FMO and basal controls. HUVECs were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17a (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show CD141 positive HUVECs when treated with the different cytokines. (ii) Bar charts demonstrate the percentage frequency of CD141 positive HUVECs when stimulated with different cytokines. (iii) Bar charts demonstrate CD141 MFI and (iv) representative histograms show CD141 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis, #p<0.05 is significantly different compared to basal for One-way ANOVA.
Figure 3. 21 TNF-α, IL-17α or IFN-γ had no effect on VEGFR expression in HUVECs. (a) Representative flow dot plots for VEGFR show FMO and basal controls. HUVECs were treated with (b) TNF-α (0.1ng/ml and 1ng/ml), (c) IL-17α (20ng/ml and 50ng/ml) and (d) IFN-γ (10ng/ml and 50ng/ml) for 24hr before being stained for flow cytometry. (i) Representative flow dot plots show VEGFR positive HUVECs when treated with the different cytokines. (ii) Bar charts demonstrate the percentage frequency of VEGFR positive HUVECs when stimulated with different cytokines. (iii) Bar charts demonstrate VEGFR MFI and (iv) representative histograms show VEGFR MFI Shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis.
3.4.9 No synergistic effects on HUVEC tube formation

Next, we examined the potential effect of cytokine synergy on HUVEC tube formation. Representative images (Figure 3.22(a)(i)) and quantification (Figure 3.22(b)(i)) demonstrate that while not significant, the number of tube formations increased in response to TNF-α and IL-17a alone compared to basal. However, no synergistic effect was observed for TNF-α and IL-17a compared to cytokines alone. Similarly, representative images (Figure 3.22(a)(ii)) and quantification (Figure 3.22(b)(ii)) demonstrate no significant effect in the number of tube formations in response to IFN-γ alone or in combination with TNF-α.

Figure 3. 22 No synergistic effects on HUVEC tube formation in response to cytokine stimulation. (a) Representative images of HUVEC tube formation under basal conditions and in response to TNF-α (0.1ng/ml) IL-17a (50ng/ml) or IFN-γ (50ng/ml) alone or in combination. (b) Bar graphs represent semi-quantitative analysis of the number of EC connecting branches/high-powered field (of n=3 hpF) in response to (i) TNF-α (0.1ng/ml) and IL-17a (50ng/ml) and (ii) TNF-α (0.1ng/ml) and IFN-γ (50ng/ml). Original magnification 10x. Data are expressed as Mean ± SEM, of n=3 expts. One-way ANOVA was or paired t test were performed for statistical analysis.
3.4.10 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces proinflammatory mediator secretion from HUVECs

Next, HUVECs were treated with TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml) or IFN-γ (50ng/ml) for 24hr. Following culture, supernatants were harvested and secretion of proinflammatory mediators quantified. IL-6 secretion was significantly increased in response to TNF-α compared to basal \( (p<0.01) \) (Figure 3.23(a)(i)). IL-17a potentiated the effect of TNF-α on IL-6 secretion compared to basal \( (p<0.05) \) or cytokine alone \( (p<0.05) \) (Figure 3.23(a)(ii)), IFN-γ alone increased IL-6 secretion \( (p<0.01) \) (Figure 3.23(a)(ii)), and in combination with TNF-α, IFN-γ synergistically induced IL-6 secretion \( (p<0.0001) \) (Figure 3.23(a)(ii)). MCP-1 secretion was increased in response to TNF-α alone \( (p<0.01) \) (Figure 3.23(b)(i)), with no further effects observed for the combination of IL-17a and TNF-α (Figure 3.23(b)(ii)). In contrast, IFN-γ and TNF-α synergistically induced MCP-1 secretion compared to basal \( (p<0.0001) \) or either cytokine alone \( (p<0.001) \) (Figure 3.23(b)(ii)).

No effect on RANTES secretion was observed in response to TNF-α or IL-17a alone or in combination (Figure 3.24(a)(i)). No effect on RANTES secretion was demonstrated in response to IFN-γ alone (Figure 3.24(a)(ii)). RANTES secretion was synergistically increased in response to the combination of IFN-γ and TNF-α compared to basal \( (p<0.05) \) or either cytokine alone \( (p<0.05) \) (Figure 3.24(a)(ii)). IL-8 secretion was significantly increased in response to TNF-α alone compared to basal \( (p<0.05) \) (Figure 3.24(b)(i)), with no effect observed for IL-17a alone. IL-17a and TNF-α synergistically increased IL-8 secretion compared to basal \( (p<0.05) \) or cytokines alone \( (p<0.05) \) (Figure 3.24(b)(ii)). No change in IL-8 secretion was observed in response to IFN-γ alone (Figure 3.24(b)(ii)), however, IFN-γ and TNF-α synergistically increased IL-8 secretion from HUVECs compared to basal \( (p<0.001) \) and either cytokine alone \( (p<0.001) \) (Figure 3.24(b)(ii)).
Figure 3. 23 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induced IL-6 and MCP-1 secretion from HUVECs. HUVECs were stimulated with TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml) or IFN-γ (50ng/ml) for 24hr before analysing cytokine secretion via ELISA. Bar charts show quantification of (a) IL-6 and (b) MCP-1 secretion in cultured HUVECs supernatants when treated with TNF-α alone and in combination with (i) IL-17a and (ii) IFN-γ. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 significantly different compared to other conditions.
Figure 3. Synergy between TNF-α and IL-17a or TNF-α and IFN-γ on RANTES and IL-8 secretion from HUVECs. HUVECs were stimulated with TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml) or IFN-γ (50ng/ml) for 24hr before analysing cytokine secretion via ELISA. Bar charts show quantification of (a) RANTES and (b) IL-8 secretion in cultured HUVECs supernatants when treated with TNF-α alone and in combination with (i) IL-17a and (ii) IFN-γ. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis, #p<0.05, ##p<0.01, ###p<0.001 significantly different compared to other conditions.
3.4.11 Synergy between TNF-α and IL-17a or TNF-α and IFN-γ induces ICAM-1 and VCAM-1 expressions on HUVECs

HUVECs were cultured with TNF-α (0.1ng/ml) in the presence or absence of IL-17a (50ng/ml) or IFN-γ (50ng/ml) for 24hr. Following culture, HUVECs were stained for extracellular markers as per Table 3.2. Figure 3.4 outlines the gating strategy used, while FMOs, representative flow plots for basal and individual cytokine stimulations are outlined in Figures 3.13 – 3.20. Representative dot plots (Figure 3.25(a)(i)) and quantification (Figure 3.25(a)(ii)) demonstrate that TNF-α alone increases the percentage frequency of ICAM-1 positive HUVECs compared to basal (p<0.01) with no effect observed for IL-17a alone. While IL-17a and TNF-α combination increased the frequency of ICAM-1 positive HUVECs compared to IL-17a alone (p<0.05) and basal (p<0.05). No synergy between IL-17a and TNF-α was observed (Figure 3.25(a)(i – ii)). ICAM-1 MFI was significantly increased in response to TNF-α alone (p=0.06) (Figure 3.25(a)(iii – iv)) compared to basal, with no effect observed for IL-17a alone. While IL-17a and TNF-α in combination increased ICAM-1 MFI compared to IL-17a alone (p<0.01) and basal (p<0.05), no synergy was observed compared to cytokines alone (Figure 3.25(a)(iii – iv)). Representative dot plots Figure 3.25(b)(i)) and quantification (Figure 3.25(b)(ii)) show that IFN-γ alone increased the percentage frequency of ICAM-1 positive HUVECs (p<0.05) compared to basal, however, it did not further potentiate the effect of TNF-α (Figure 3.25(b)(ii)). No differences in ICAM-1 MFI were demonstrated in response to IFN-γ alone compared to basal (Figure 3.25(b)(iii – iv)), however the combination of IFN-γ and TNF-α synergistically increased ICAM-1 MFI compared to cytokines alone (p<0.05) and basal (p<0.05) (Figure 3.25(b)(iii – iv)). Representative dot plots Figure 3.26(a)(i)) and quantification (Figure 3.26(a)(ii)) demonstrate that IL-17a or TNF-α alone had no effect on the frequency of VCAM-1 positive HUVECs compared to basal. While the combination of IL-17a and TNF-α increases frequency of VCAM-1 positive HUVECs compared to basal (p<0.05) and cytokines alone (p=0.05), no synergy was observed. No differences in VCAM-1 MFI were observed (Figure 2.26(a)(iii – iv)). Representative dot plots Figure 3.27(b)(i)) and quantification (Figure 3.26(b)(ii)) demonstrate that while IFN-γ or TNF-α alone had no effect of frequency of VCAM-1 positive HUVECs compared to basal, the combination of IFN-γ and TNF-α together synergistically increased the frequency of VCAM-1 positive
HUVEC compared to cytokines alone (p<0.05 and p=0.05) and basal (p<0.05). No changes in VCAM-1 MFI were observed (Figure 3.26(b)(iii – iv)).
Figure 3. Synergy between TNF-α and IFN-γ induced ICAM-1 expression in HUVECs. HUVECs were stimulated with TNF-α (0.1ng/ml) in the absence or presence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative dot plots show the expression of ICAM-1 positive HUVECs following treatment with cytokines. (ii) Bar charts demonstrate the percentage frequency of ICAM-1 positive HUVECs when stimulated with the different cytokines. (iii) Bar charts demonstrate ICAM-1 MFI and (iv) representative histograms show ICAM-1 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
Figure 3. Synergy between TNF-α and IFN-γ induced VCAM-1 expression in HUVECs. HUVECs were treated with TNF-α (0.1ng/ml) in the absence or presence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow dot plots show the expression of VCAM-1 positive HUVECs following treatment with cytokines. (ii) Bar charts demonstrate the percentage frequency of VCAM-1 positive HUVECs when stimulated with different cytokines. (iii) Bar charts demonstrate VCAM-1 MFI and (iv) representative histograms show VCAM-1 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis respectively. #p<0.05 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
3.4.12 Cytokine synergistic effects on chemokine and angiogenic markers on HUVECs

We next examined the expression of chemokine receptors involved in EC migration specifically CXCR3, CXCR4, CXCR5, and CCR6 via flow cytometry. Representative dot plots Figure 3.27(a)(i) and quantification (Figure 3.27(a)(iii)) demonstrate that frequency of CXCR3 positive HUVEC increased in response to TNF-α alone ($p<0.05$), with no effect observed for IL-17a alone or in combination with TNF-α. Quantification (Figure 3.27(a)(iii) and representative histogram (Figure 3.27(a)(iv)) demonstrate that CXCR3 MFI was significantly increased in response to TNF-α alone ($p<0.05$), with no effects observed for IL-17a. IL-17a inhibited TNF-α CXCR3 MFI by two-fold compared to cytokines alone ($p=0.06$) (Figure 3.27(a)(iii – iv)). Representative dot plots (Figure 3.27(b)(i)) and quantification (Figure 3.27(b)(iii)) demonstrate that IFN-γ alone or in combination with TNF-α had no further effect on frequency of CXCR3 positive HUVECs compared to basal or cytokines alone. Similarly, no changes in CXCR3 MFI were observed in response to IFN-γ alone or in combination with TNF-α (Figure 3.27(b)(iii – iv)). Representative dot plots (Figure 3.28(a)(i)) and quantification (Figure 3.28(a)(iii)) demonstrate a significant increase in the frequency of CXCR4 positive HUVEC in response to TNF-α alone ($p<0.05$), with no effect observed for IL-17a alone. While IL-17a and TNF-α increased the frequency of CXCR4 positive HUVEC compared to IL-17a alone ($p<0.05$) and basal ($p<0.01$), no synergy/potentiation was observed compared to TNF-α alone (Figure 3.28(a)(ii)). No significant differences in CXCR4 MFI were observed (Figure 3.28(a)(iii – iv)). Representative dot plots Figure 3.28(b)(i)) and quantification (Figure 3.28(b)(ii)) demonstrated that frequency of CXCR4 positive HUVEC increased in response to TNF-α alone ($p<0.05$), with no effect observed for IFN-γ alone, however IFN-γ potentiated the effect of TNF-α compared to cytokines alone ($p<0.01$) and basal ($p<0.05$). CXCR4 MFI did not change in response to IFN-γ or TNF-α, while a trending synergistic increase in CXCR4 MFI by three-fold in response to the combination of IFN-γ and TNF-α was observed, this was not significant (Figure 3.28(b)(iii – iv)).

Representative dot plots (Figure 3.29(a)(i)) and quantification (Figure 3.29(a)(iii)) demonstrated a trending increase in the frequency of CXCR5 positive HUVEC in response to TNF-α. No effects were observed for IL-17a alone or in combination with TNF-α. No differences in CXCR5 MFI were observed (Figure 3.29(a)(iii – iv)). Representative dot plots
Representative dot plots (Figure 3.29(b)(i)) and quantification (Figure 3.29(b)(iii)) demonstrate a trending increase in the frequency of CXCR5 positive HUVECs in response to TNF-α, with no difference in response to IFN-γ. IFN-γ potentiated the effect of TNF-α on frequency of CXCR5 positive HUVECs. No differences were observed in CXCR5 MFI (Figure 3.29(b)(iii – iv)).

Representative dot plots Figure 3.30(a)(i)) and quantification (Figure 3.30(a)(iii)) show that TNF-α alone increased the frequency of CCR6 positive HUVEC compared to basal (p=0.07) with no effects observed for IL-17a alone or in combination with TNF-α. No changes were observed for CCR6 MFI (Figure 3.30(a)(iii – iv)). Representative dot plots Figure 3.30(b)(i)) and quantification (Figure 3.30(b)(iii)) demonstrated that IFN-γ alone had no effect on the frequency of CCR6 positive HUVEC, however a synergistic increase in the frequency of CCR6 positive HUVECs in response to the combination of IFN-γ and TNF-α compared to cytokines alone (p<0.05) and basal (p<0.05) was observed. No changes for CCR6 MFI were demonstrated (Figure 3.30(b)(iii – iv)).

Representative dot plots (Figure 3.31(a)(i)) and quantification (Figure 3.31(a)(ii)) demonstrated a trending decrease in the frequency of Claudin 5 positive HUVEC in response to IL-17a or TNF-α alone or in combination, no changes in Claudin 5 MFI were observed (Figure 3.31(a)(iii – iv)). Representative dot plots (Figure 3.31(b)(i)) and quantification (Figure 3.31(b)(iii)) demonstrated a trending decrease in frequency of Claudin 5 positive HUVEC in response to TNF-α and IFN-γ alone and in combination (p<0.05). No differences in Claudin 5 MFI were observed (Figure 3.31(b)(iii – iv)).

No changes were observed for frequency of CD141 positive HUVECs or CD141 MFI in response to cytokine alone or in combination (Figure 3.20(b – d) and Figure 3.32(a) and (b)).

Representative dot plots Figure 3.33(a)(i)) and quantification (Figure 3.33(a)(ii)) demonstrate no changes in the frequency of VEGFR positive HUVEC in response to TNF-α or IL-17a alone, however, the combination of TNF-α and IL-17a reduced VEGFR expression compared to basal (p=0.06). No changes in VEGFR MFI were observed in response to TNF-α or IL-17a alone or in combination compared to basal or cytokine alone (Figure 3.33(a)(iii – iv)). No changes were observed for frequency of VEGFR positive HUVEC or VEGFR MFI
in response to TNF-α or IFN-γ alone or in combination (Figure 3.21(b) and (d) and Figure 3.33(b)).
Figure 3. Synergy between TNF-α and IL-17a and TNF-α and IFN-γ had differential effects on expression in HUVECs. HUVECs were treated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow dot plots show the expression of CXCR3 positive HUVECs when stimulated with cytokines. (ii) Bar charts demonstrate the percentage frequency CXCR3 positive HUVECs when stimulated with different cytokines. (iii) Bar charts demonstrate CXCR3 MFI and (iv) representative histograms show CXCR3 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis *p<0.05 is significantly different compared to other conditions.
Figure 3. Synergy between TNF-α and IFN-γ induced CXCR4 expression in HUVECs. HUVECs were stimulated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow dot plots show the expression of CXCR4 positive HUVECs when stimulated with cytokines. (ii) Bar charts demonstrate the percentage frequency CXCR4 positive HUVECs following treatment with different cytokines. (iii) Bar charts demonstrate CXCR4 MFI and (iv) representative histograms show CXCR4 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis. #p<0.05, ##p<0.01 significantly different compared to other conditions.
Figure 3. Synergy between TNF-α and IFN-γ induced CXCR5 expression in HUVECs. HUVECs were stimulated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow dot plots show the expression of CXCR5 positive HUVECs following treatment with the different cytokines. (ii) Bar charts demonstrate the percentage frequency of CXCR5 positive HUVECs when stimulated with the different cytokines. (iii) Bar charts demonstrate CXCR5 MFI and (iv) representative histograms show CXCR5 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis.
Figure 3. Synergy between TNF-α and IFN-γ induced CCR6 expression in HUVECs. HUVECs were incubated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow dot plots show the expression of CCR6 positive HUVECs when stimulated with cytokines. (ii) Bar charts demonstrate the percentage frequency of CCR6 positive HUVECs following treatment with the different cytokines (iii) Bar charts demonstrate CCR6 MFI and (iv) representative histograms show CCR6 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis. *p<0.05 significantly different compared to other conditions.
Figure 3. 31 TNF-α and IL-17a or IFN-γ did not affect Claudin 5 expression in HUVECs. HUVECs were treated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow dot plots demonstrate the expression of Claudin 5 positive HUVECs following treatment with cytokines. (ii) Bar charts demonstrate the percentage frequency of Claudin 5 positive HUVECs when stimulated with different cytokines. (iii) Bar charts demonstrate Claudin 5 MFI and (iv) representative histograms show Claudin 5 MFI shift. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis. *p<0.05 significantly different compared to other conditions.
Figure 3. 32 TNF-α and IL-17a or IFN-γ did not affect CD141 expression in HUVECs. HUVECs were treated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow plots demonstrate the expression of Claudin 5 positive HUVECs following treatment with cytokines. (ii) Bar charts demonstrate the percentage frequency of CD141 positive HUVECs when stimulated with the different cytokines. (iii) Bar charts demonstrate CD141 MFI and (iv) representative histograms show Claudin 5 MFI shift. Values expressed as mean ± SEM of n=7 expts, One-way ANOVA (#) and paired t-test (*) were used for statistical analysis.
Figure 3. 33 TNF-α and IL-17a or IFN-γ did not affect VEGFR expression in HUVECs. HUVECs were stimulated with TNF-α (0.1ng/ml) in the presence or absence of (a) IL-17a (50ng/ml) or (b) IFN-γ (50ng/ml) for 24hr before flow cytometry analysis. (i) Representative flow dot plots show the expression of VEGFR positive HUVECs following treatment with cytokines. (ii) Bar charts demonstrate the percentage frequency of VEGFR positive HUVECs when stimulated with the different cytokines. (iii) Bar charts demonstrate VEGFR MFI and (iv) representative histograms show VEGFR MFI shift. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis.
3.4.13 TNF-α and IL-17a or TNF-α and IFN-γ did not affect HUVEC cell metabolism

Representative OxPhos bioenergetic profile for TNF-α and IL-17a stimulations are shown in Figure 3.34(a). No changes in OCR baseline (Figure 3.34(b)), ATP synthesis (Figure 3.34(c)), max respiratory capacity (Figure 3.34(d)), proton leak (Figure 3.34(e)) were observed. Respiratory reserve was reduced in response to TNF-α (\(p<0.05\)) compared to basal with no changes observed for IL-17a, however, in combination with TNF-α it was reduced compared to cytokine alone although this did not reach significance (Figure 3.34(f)). There were no changes in mitochondrial respiration (Figure 3.34(g)). Pie charts represent the relative contribution of the OxPhos potential within each condition (Figure 3.34(h)(i) – (iv)). No changes in ATP synthesis (black) as a percentage of total OxPhos potential was observed for any conditions. Proton leak (pink) was increased in TNF-α alone (Figure 3.34(h)(ii)) and in combination with IL-17a (Figure 3.34(h)(iv)) compared to basal (Figure 3.34(h)(i)) and IL-17a alone (Figure 3.34(h)(iii)). Respiratory reserve (green) output was decreased in response to TNF-α (Figure 3.34(h)(ii)) compared to basal (Figure 3.34(h)(i)) and increased in response to the combination of TNF-α and IL-17a (Figure 3.34(h)(iv)) compared to TNF-α alone (Figure 3.34(h)(ii)) or IL-17a alone (Figure 3.34(h)(iii)). Non-mitochondrial respiration (purple) output was reduced in response to TNF-α and IL-17a alone (Figure 3.34(h)(ii)) compared to cytokine alone (Figure 3.34(h)(ii – iii)) but decreased when compared to basal (Figure 3.34(h)(i)).

Representative glycolytic bioenergetic profiles are shown in Figure 3.35(a). No changes in ECAR baseline, ECAR:OCR ratio, or metabolic map were observed in response to TNF-α alone or IL-17a alone compared to basal, and TNF-α and IL-17a did not change ECAR baseline when compared to cytokines alone or basal (Figure 3.35(b – d)).

Representative OxPhos bioenergetic profiles are shown for TNF-α and IFN-γ alone and in combination in Figure 3.36(a). No differences were observed for OCR baseline (Figure 3.36(b)), ATP synthesis (Figure 3.36(c)), max respiratory capacity (Figure 3.36(d)), proton leak (Figure 3.36(e)) in response to TNF-α or IFN-γ alone or in combination. Respiratory reserve was decreased in response to TNF-α alone (\(p<0.05\)), with no effect observed for IFN-γ alone or in combination with TNF-α (Figure 3.36(f)). No changes were demonstrated
for non-mitochondrial respiration (Figure 3.36(g)). Pie charts showing the relative contribution of the OxPhos potential as a percentage of total OCR outputs within each condition were demonstrated (Figure 3.36(h)(i – iv)). No changes in ATP synthesis (black) output were observed across all the conditions. Proton leak (pink) output was increased in response to TNF-α alone (Figure 3.36(h)(ii)) compared to basal (Figure 3.36(h)(i)) while no changes were reported in response to IFN-γ alone (Figure 3.36(h)(iii)). Proton leak output was decreased in response to the TNF-α and IFN-γ combination (Figure 3.36(h)(iv)) when compared to TNF-α alone (Figure 3.36(h)(ii)) with no changes compared to IFN-γ alone (Figure 3.36(h)(iii)) or basal (Figure 3.36(h)(i)). Respiratory reserve (green) output was decreased in response to TNF-α alone (Figure 3.36(h)(ii)) compared to basal (Figure 3.36(h)(i)) with no changes observed in response to IFN-γ alone (Figure 3.36(h)(iii)). Respiratory reserve output was increased in response to the combination of TNF-α and IFN-γ (Figure 3.36(h)(iv)) when compared to TNF-α alone (Figure 3.36(h)(ii)).

Representative glycolytic bioenergetic profile is demonstrated in Figure 3.37(a). No changes in ECAR baseline, ECAR:OCR ratio or metabolic map were observed in response to TNF-α alone or IFN-γ alone when compared to basal nor in combination compared to cytokine alone or basal (Figure 3.37(b – d)).
Figure 3. TNF-α and IL-17a did not affect the metabolic capacity of HUVECs. (a) Average OCR profile for HUVECs cultured with TNF-α (0.1 ng/ml) in the presence or absence of IL-17a (50ng/ml) before and after injections of oligomycin, FCCP, and antimycin A/ rotenone. Bar charts show quantification of (b) OCR baseline, (c) ATP synthesis, (d) max respiratory capacity, (e) proton leak, (f) respiratory reserve and (g) non-mitochondrial respiration. Pie charts shown in (h) outline the relative contribution of the total OCR outputs as a percentage of OCR output in (i) basal, HUVECs treated with (ii) TNF-α (0.1 ng/ml), (iii) IL-17a (50 ng/ml) and (iv) TNF-α (0.1 ng/ml) and IL-17a (50 ng/ml). Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis where *p<0.05 significantly different compared to other conditions.
Figure 3. 35 TNF-α and IL-17a did not affect the glycolytic capacity of HUVECs (a) Average ECAR profile for HUVECs cultured with TNF-α (0.1 ng/ml) in the presence or absence of IL-17a (50ng/ml) before and after injections of oligomycin, FCCP, and antimycin A/rotenone. Bar charts show quantification of (b) ECAR baseline, (c) ECAR:OCR ratio, (d) metabolic map. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis.
Figure 3. TNF-α and IFN-γ did not affect the metabolic capacity of HUVECs. (a) Average OCR profile for HUVECs cultured with TNF-α (0.1 ng/ml) in the presence or absence of IFN-γ (50 ng/ml) before and after injections of oligomycin, FCCP, and antimycin A/rotenone. Bar charts show quantification of (b) OCR baseline, (c) ATP synthesis, (d) max respiratory capacity, (e) proton leak, (f) respiratory reserve and (g) non-mitochondrial respiration. Pie charts shown in (h) outline the relative contribution of the total OCR outputs as a percentage of OCR output in (i) basal, HUVECs treated with (ii) TNF-α (0.1 ng/ml), (iii) IFN-γ (50 ng/ml) and (iv) TNF-α (0.1 ng/ml) and IFN-γ (50 ng/ml). Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis *p<0.05 significantly different compared to other conditions.
Figure 3. The effects of TNF-α and IFN-γ cytokine synergy on the glycolytic capacity of HUVECs (a) Average ECAR profile for HUVECs cultured with TNF-α (0.1 ng/ml) in the presence or absence of IFN-γ (50ng/ml) before and after injections of oligomycin, FCCP, and antimycin A/rotenone. Bar charts show quantification of (b) ECAR baseline, (c) ECAR:OCR ratio, (d) metabolic map. Values expressed as mean ± SEM of n=7 expts. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis.
3.5 Discussion

The inflamed joint microenvironment is complex due to cell-cell and soluble mediator interactions (Ikeda et al., 2000; Vignola et al., 2002; Świdrowska et al., 2015; Pavlova et al., 2022). While we don’t know the primary trigger of joint inflammation, one of the primary events across all forms of IA, including DA, is dysfunctional neo-angiogenesis. To further examine the mechanisms underlying the pathogenesis of synovial inflammation in DA, in this chapter we examined the effect of conditioned media from primary DA FLS (reflecting the joint microenvironment) on EC function, to assess whether soluble mediators that are secreted from DA FLS induce a pathogenic phenotype in EC. We demonstrated that the DA joint microenvironment promotes EC tube formation, migratory capabilities, and leukocyte-EC adhesion, effects that were paralleled by increases in adhesion molecules and chemokine receptor expression. However, no changes to the metabolic capacity of DA FLS-CM treated ECs were demonstrated. Furthermore, we examined the effect of TNF-α, IL-17a, and IFN-γ alone and in combination on EC function. IFN-γ and/or IL-17a potentiated/synergistically induced the effect of TNF-α on the secretion of IL-6, MCP-1, RANTES, and IL-8 from ECs. In parallel, IFN-γ potentiated/synergistically induced the effect of TNF-α on ICAM-1 and VCAM-1, and on chemokine receptor expression of CXCR3, CXCR4, CXCR5, and CCR6, paralleled by a decrease in the tight junction marker Claudin 5. Finally, while TNF-α induced a shift towards glycolysis, no significant changes were observed for IL-17a or IFN-γ alone, while IFN-γ reduced TNF-α induced-baseline glycolysis. Collectively, this data suggests that the DA joint microenvironment induces a pathogenic EC phenotype further contributing to joint inflammation.

In this chapter, we demonstrated that DA FLS-CM promotes EC pathogenic function evident by the observed increase in tube formation, migrative capacity, and expression of Notch 1IC signalling. This increase in EC activation and function is consistent with the observed increased synovial neovascularisation previously demonstrated in DA (Foley et al., 2020), an observation also demonstrated in other forms of IA including RA and PsA (Fearon et al., 2003; Elshabrawy et al., 2015; Fromm et al., 2019; He et al., 2020; Kim et al., 2020). As highlighted in the previous chapter, FLS significantly contribute to synovial inflammation, and while they have not been examined before in the context of DA, studies in RA and PsA have demonstrated that they spontaneously release proinflammatory mediators,
proteases, and proangiogenic factors (Fromm et al., 2019; Floudas et al., 2022). Furthermore, numerous studies have shown that FLS secrete key proangiogenic mediators including VEGF, Ang1, Ang2, FGF, Vascular Endothelial Growth Factor Receptor-1 (FLT-1), Tie2, thymic stromal lymphopoitin (TSLP), and macrophage inaglarflammatory protein (MIP-1) (Scott et al., 2002; Honorati et al., 2006; Cho et al., 2007; Bartok et al., 2010; Szekanecz et al., 2010; Balogh et al., 2018; Fromm et al., 2019; Wade et al., 2019). Of particular note, VEGF and Ang2 are highly expressed in the inflamed joint (Fearon et al., 2003; Van De Sande et al., 2013; Kelly et al., 2015), and synergistically induce angiogenesis via the Notch-DLL4 signalling pathway (Gao et al., 2013; Gao et al., 2015) which determines tip-stalk cell interactions, that in turn dictates blood vessel directed migration away from its parent vessel (Fearon et al., 2021). Furthermore, hypoxia can induce both EC and FLS function via Notch-DLL4 interaction (Gao et al., 2013; Gao et al., 2015), with previous studies demonstrating that the RA, PsA and JIA joint microenvironments are profoundly hypoxic (Bosco et al., 2009; Ng et al., 2010). Increased expression of Notch1 IC and its ligand, DLL4, is colocalised to both the vasculature and lining layer regions in the inflamed RA pannus (Gao et al., 2012; Gao et al., 2013; Choe et al., 2016). In fact, silencing DLL4-Notch signalling inhibits cellular invasion, angiogenesis and migration (Gao et al., 2013; Akil et al., 2021), in addition to significantly decreasing VEGF/Ang2-induced IL-6, IL-8, MMP-2, and 9 expression in RA synovial explant ex vivo (Gao et al., 2013). Our data is also consistent with previous studies examining FLS in other forms of IA, where FLS-CM induces EC tube formation and migratory capacity (Fromm et al., 2019). Furthermore, ECM degradation is required for EC-directed migration, a process mediated by MMPs, in particular, the pro-angiogenic proteases MMP-2 and MMP-9 (Fearon et al., 2021). Numerous studies have shown that RA FLS secrete these molecules (Fearon, et al., 2003; Hellström et al., 2007; Li et al., 2013; Zhou et al., 2014; Petrasca et al., 2020), in addition, we also showed in Chapter 2 that MMP-9 is secreted from DA FLS, thus most likely contributes to the altered EC migratory function observed in this chapter. Currently, there are no effective anti-angiogenic therapies known to treat IA. One such reason could be due to the multitude of factors involved in regulating angiogenesis which could make it difficult to be target-specific. Ramucirumab is a monoclonal antibody against VEGFR2 and when used in combination with MTX has been proven to be effective in experimental models (Abdel-Maged et al., 2019). However, Ramucirumab is ineffective
in RA and one reason could be that there are other VEGF receptors that VEGF or TNF-α interact with (Elshabrawy et al., 2015). Vitaxin, an anti-αvβ3 integrin antibody, was another anti-angiogenic therapy tested in RA but Phase I/II clinical trials were cancelled due to inefficacy of the antibody (Semerano et al., 2011). Since αvβ3 is a fibronectin receptor there are other fibronectin receptors such as α5β1 which fibronectin, an RA autoantigen, could bind to (Nagae et al., 2012). Both αvβ3 and α5β1 are highly expressed in the inflamed joints (Morshed et al., 2019). Paclitaxel (Angiotech Pharmaceuticals) is an anti-HIF-1α antibody and microtubule destabiliser (Lainer-Carr et al., 2007). This antibody did not pass Phase II of the clinical trials. Thus while angiogenesis plays a key role to driving synovial inflammation, effective targeting of angiogenesis is difficult due to redundancy of pathways, in addition regression of blood vessels also depends on blood vessel stability and maturity (Kennedy et al., 2011).

One limitation of this study is that macrovascular endothelial cells were used as opposed to synovial microvascular cells, which are difficult to culture, particularly after the first passage. Macro-vessel compared to micro-vessel function can vary, in addition to variation between different endothelial cell beds, with pericyte-endothelial cell-contact and vessel maturity/stability constantly changing during the course of the disease. However, studies have shown similar increased expression of growth factors, chemokines, adhesion molecules and MMPs, in other more accessible microvascular beds (dermal) to that of macrovascular beds (Müller et al., 2002; Gao et al., 2013; Zhou et al., 2014) suggesting a similar response and thus can inform us on potential pathogenic mechanisms.

We also observed DA FLS-CM increased leukocyte-EC adhesion, an effect possibly mediated through the observed increase in the frequency of ICAM-1 positive EC. In contrast VCAM-1 frequency was decreased, however, percentage expression was extremely low. While this is the first study to show increased ICAM-1 expression on ECs in response to the DA microenvironment, ICAM-1 is highly expressed on ECs in the inflamed synovium, particularly in RA and OA (Mulherin et al., 1996; Szekanecz et al., 2000; Elshabrawy et al., 2015). It is also consistent with studies in RA and PsA, where FLS-CM was shown to induce leukocyte-EC adhesion, in addition to ICAM-1 and VCAM-1 expression (Fromm et al., 2019). Furthermore, ICAM-1 and VCAM-1 are associated with the invasive capacity of FLS and are key
molecules involved in facilitating leukocytes trans-endothelial cell migration to the joint. Another adhesion molecule of interest is platelet-derived cell adhesion molecule-1 (PECAM-1). It plays an integral role in normal angiogenesis (Feng et al., 2016), however, increased EC expression of PECAM-1 has been shown to facilitate leukocyte migration to the RA joint (Szekanecz et al., 2000), neutrophils adhesion to blood vessels in atherosclerosis (Xie et al., 2020), and to mediate angiogenesis in brain gliomas (Feng et al., 2016). Other members of the cell adhesion molecule family worth noting are the selectins. Mouse ECs expressing P-selectins have been reported to drive homing of mouse eosinophils in murine models of asthma (Xiong et al., 2021), while coronary artery ECs expressing E-selectins induce monocyte adhesion in Kawasaki disease (Zhang et al., 2021), and serum soluble P- and E-selectins were increased in PsA patients compared to HC (Ataseven et al., 2014).

In this study, we demonstrated divergent effects of DA FLS-CM on HUVEC ICAM-1 and VCAM-1 which contrasts previous studies in RA and PsA (Fromm et al., 2019). While it is not clear why the DA FLS-CM decreased VCAM-1 expression, a study in JIA examining the effect of T cell-CM, demonstrated Th1, but not of Th17-CM, induces VCAM-1 expression in JIA FLS (Agarwal et al., 2008), thus this effect may be dependent on the cellular composition within the inflamed joint. Different cellular regulatory function is also consistent with recent studies demonstrating numerous FLS subtypes existing in the inflamed synovium that display differential functions defined by their invasive capacity, immune effector function, and positional memory (Frank-Bertoncelj et al., 2017; Wei et al., 2020). In this study, we utilised expanded primary FLS, therefore a mixed population of FLS and thus may lead to divergent functional effects depending on the percentage composition of different population from individual DA patients. Indeed, scRNAseq analysis of RA FLS clusters has demonstrated differential pathways are associated with different FLS clusters including ECM breakdown, focal adhesion, and invasive capacity (Floudas et al., 2022).

The DA microenvironment had minimal effect on chemokine receptor expression, albeit we did demonstrate a trending increase for both CXCR3 and CCR6. Previous studies have shown CXCR3 expression on ECs are increased in the joints of patients with JIA (Loos et al., 2006), in the synovium of patients with chronic RA (García-López et al., 2001), and in thyroid gland EC from patients with Grave’s disease and Hashimoto’s thyroiditis (García-López et al.,
CXCR3/CXCL10 interactions in JIA have been shown to regulate activation of T cells and their recruitment into the inflamed synovium (Martini et al., 2005), with CXCL10 also known to be highly expressed on ECs and synovial macrophages in JIA (Lee et al., 2013). CCR6 expression is upregulated on ECs in RA compared to HC, an effect that promotes neutrophil recruitment into the joint (Chou et al., 2010). CCR6 is also expressed on ECs in chronic renal inflammation, and thought to be involved in the recruitment of T and B cells to organized nodular infiltrates (Welsh-Bacic et al., 2011). A trending increase was also observed for CD105, which is supported by a study by Scola et al. (2001), which demonstrated increased CD105 gene expression in JIA synovium compared to OA (Scola et al., 2001). A useful marker for activated ECs, as well as angiogenesis, CD105 is a receptor for TGF-β and regulates the TGF-β pathway in ECs (Ollauri-Ibáñez et al., 2020). Furthermore, CD105 expression on ECs is induced under hypoxic conditions and prevents EC apoptosis thus contributing to the pannus formation (Li et al., 2003). In cervical cancer, the frequency of CD105+ vessels are positively associated with number of lymph node metastasis (Zijlmans et al., 2009) while in small cell lung cancer, CD105-mediated angiogenesis potentiates the formation of new blood vessels (Takase et al., 2010) and is associated with tip-cell formation (Virgintino et al., 2012).

Interestingly, overall, we observed minimal change in the metabolic profile of EC in response to the DA microenvironment. However, we did demonstrate differences in respiratory reserve and non-mitochondrial respiration as a percentage of the overall OCR output (Figure 3.8(h)), and in glycolytic reserve (Figure 3.9(h)) as a percentage of overall glycolytic output following treatment with 33% DA FLS-CM, suggesting there are subtle shifts in the metabolic energy being utilised by the EC. We also demonstrated an increase in Notch1 IC in response to DA FLS CM, which is required for tip-stalk selection, a mechanism that also requires a shift in the metabolic profile. Indeed it is known that activated ECs require a metabolic shift to glycolysis, and that this profile is associated with phenotypic differentiation of EC to tip and stalk cells (Fearon et al., 2021). Previous studies have also shown that hypoxia, oxidative stress, and proinflammatory mediators induce EC tube formation, migrative, invasive and adhesive capacity, paralleled by a glycolytic metabolic shift (Gao et al., 2015; Biniecka et al., 2016; Hanlon et al., 2019). Furthermore, PFKFB3 blockade in microvascular ECs (a key glycolytic enzyme) inhibits angiogenic function and Notch1 IC (Schoors et al., 2014; Biniecka et al., 2016), and
silencing of G6PI (another glycolytic enzyme) inhibits hypoxia-mediated angiogenesis in RA (Lu et al., 2017). Based on these studies it is unclear why a similar shift wasn’t observed in this study. It may be due to divergent effects of pro/anti-inflammatory mediators in the DA FLS-CM that leads to different metabolic reprogramming as highlighted above (Figure 3.8(h) and Figure 3.9(h)), in additional to potential utilisation of alternative pathways including the PPP, fatty acid synthesis/oxidation and amino acid metabolism. Future experiments plan to examine these alternative pathways, through gene/protein expression and carbon tracing, in addition to examining the effect in primary microvascular synovial EC. One of the limitations of the Seahorse Technology is that it can only measure glycolysis and OxPhos thus there are other metabolic pathways including fatty acid synthesis/oxidation, PPP (oxidative/non-oxidative), and amino acid metabolism which could also supply energy to support pathogenic phenotypic changes observed in HUVEC. Thus, future studies examining the other metabolic pathways may give insight to the metabolic reliance of HUVEC in response to the joint microenvironment.

Next, we examined the effect of TNF-α, IL-17a, and IFN-γ alone and in combination on EC function based on our previous observations of increased polyfunctional T cells in children with DA (Foley et al., 2020). We demonstrated that TNF-α, IL-17a, and IFN-γ alone differentially regulate the secretion of IL-6, MCP-1, RANTES, and IL-8 from ECs, similar to what was observed for DA FLS (Section 2.5). We also showed that IL-17a and/or IFN-γ potentiated the effect of or synergised with TNF-α to induce the secretion of IL-6, MCP-1, RANTES, and IL-8 from ECs, effects that were more pronounced for IFN-γ. While IL-17a had a minimal effect on TNF-α-induced ICAM-1/VCAM-1 and chemokine receptor expression, IFN-γ potentiated the effect of TNF-α on both ICAM-1 and VCAM-1, in addition to CXCR4, CXCR5 and CCR6, paralleled by a decrease in the tight junction marker Claudin 5.

Secretion of IL-6 from ECs in RA have been shown to stimulate VEGF production and thus pannus formation (Srirangan et al., 2010), an effect that induces a positive feedback loop that further regulates EC activation and MCP-1 production (Jarlborg et al., 2022). The impact of IL-6 secretion from ECs are also highlighted in studies showing that IL-6R inhibition resulted in the restoration of normal EC function paralleled by reduced joint
pain in RA patients (Kume et al., 2011; Bacchiega et al., 2017), while MCP-1 secretion facilitates monocyte and macrophage recruitment to the inflamed joint. In other types of autoimmunity/inflammation, MCP-1 secretion from retinal ECs contributes to inflammatory responses in diabetic retinopathy patients (Taghavi et al., 2019), while inhibiting MCP-1 in mouse endothelial precursor cells in breast cancer prevents tumour neovascularization in murine mammary carcinoma (Chen et al., 2016). RANTES, much like other chemokines, modulates cell migration, cell proliferation, and lymphocyte/monocyte trafficking in IA (Agere et al., 2017). Pharoah et al. (2006), demonstrated high expression of RANTES localised to the vascular and lining layer regions of the synovium in JIA (Pharoah et al., 2006). Furthermore, IL-8 is a potent chemoattractant that recruits neutrophils to the joint (Namba et al., 2017), expression of which is increased on RA synovial endothelium (Middleton et al., 2004).

Previous studies have shown that TNF-α-treated ECs lead to an increase in IL-6, MCP-1, RANTES, IL-8, and GROα secretion (Hanlon et al., 2019). TNF-α stimulation of human aortic smooth muscle cells and ECs mediate the secretion of MCP-1 in atherosclerosis (Kim et al., 2007) and induces VEGF from ECs through the NFκB pathway (Certo et al., 2021). TNF-α stimulation of dermal microvascular ECs resulted in increased tube formation compared to resting ECs (Shu et al., 2012). Furthermore, IL-17a stimulation of ECs, FLS, keratinocytes, and neurons in autoimmune models led to increased IL-6 production (Ogura et al., 2008; Ma et al., 2018), in addition to IL-8, GM-CSF and GROα secretion and neutrophil recruitment in ECs, effects which were mediated through STAT3 phosphorylation (Yuan et al., 2015). Moreover, IL-17 stimulation of lung microvascular ECs, induced tube formation and production of VEGF and bFGF via IL-17RC (Pickens et al., 2010).

We demonstrate that TNF-α and IFN-γ induced ICAM-1 and VCAM-1 expression with no effects observed for IL-17a. Supporting our findings, previous studies have shown that TNF-α induces ICAM-1, VCAM-1, and E-selectin expression on dermal microvascular ECs and subsequent neutrophil recruitment (Pober, 2002; Shu et al., 2012). TNF-α also increases VE-cadherin on primary lung ECs, leading to increased vessel permeability and enhanced leukocyte infiltration (Reglero-Real et al., 2016). While in this study IL-17a did not affect adhesion molecule expression, previous studies have shown that IL-17a
indirectly recruits leukocytes through the expression of ICAM-1, VCAM-1 and E-selectin on microvascular ECs and lung microvascular ECs (Roussel et al., 2010; Nguyen et al., 2013; Wang et al., 2022). However, consistent with our study, others have shown that IFN-γ stimulation results in increased ICAM-1 and prolonged E-selectin expression on activated ECs compared to resting ECs (Pober, 2002). In addition IFN-γ stimulation increases ICAM-1, VCAM-1, and IFN-inducible T cell-α chemoattract expression paralleled by increased induction of leukocyte infiltration in concanavalin A-induced T cell hepatitis (Jaruga et al., 2004).

In this chapter, we demonstrate that TNF-α or IL-17a induced the expression of CXCR3 while TNF-α increased expression of CXCR4, CXCR5, CCR6, CD141, and VEGFR as well as decreased expression of Claudin 5 from ECs with minimum effects reported for IFN-γ. Consistent with previous studies, TNF-α stimulation has been shown to mediate the expression of CXCL1 and CCL2 in vascular regions in cardiovascular disease and in colon, breast, and ovarian cancer (Lo et al., 2014; Nallasamy et al., 2021). Additionally, TNF-α or IL-1β stimulation of mouse ECs, FLS, and macrophages induce CCL5, CXCL1, CXCL2, and CXCL5 compared to resting ECs in serum transfer arthritis (Chou et al., 2010). Consistent with our findings, Sartain et al. (2016) described that TNF-α increased the surface expression of CD141 in HUVECs and glomerular microvascular ECs (Sartain et al., 2016) suggesting that TNF-α is required for EC activation. Not limited to ECs, CD141 is expressed on a particular monocyte subset. Specifically, monocytes cultured with CM from human bronchial epithelial cells containing TNF-α, IL-1β, IL-6, IL-8, IL-15, and GM-CSF mediated a CD1s41+ CD123+ DC-SIGN+ monocyte subtype in patients with adenocarcinoma and sarcoidosis (Gazdhar et al., 2017). Similar to our findings, other groups have shown that TNF-α stimulation reduced Claudin 5 expression in dermal microvascular ECs leading to a disrupted tight junction barrier via the NFkB pathway allowing more immune cells to infiltrate (Clark et al., 2015). Not only is this important in the context of DA and RA, but studies examining other autoimmune diseases such as Alzheimer’s disease, demonstrated that TNF-α, IL-6 and IL-17 stimulation reduced Claudin 5 expression on ECs lining the cerebral vasculature, thus facilitating immune cells to enter through the blood brain barrier (Greene et al., 2019; Voirin et al., 2020).
Similarly, previous studies have also shown IL-17a stimulation leads to CXCR2, CXCL1, CXCL8, CXCL10, CCL2, and CCL20 production from dysfunctional dermal microvascular ECs and mouse aortic ECs in atherosclerosis and RA (Bosteen et al., 2014; Yuan et al., 2015; Zhang et al., 2021; Wang et al., 2022). While we observed no effects on chemokine expression in ECs in response to IFN-γ, other studies have shown that IFN-γ stimulation of ECs regulates CXCL10 and CXCL11—chemokine ligands known for inhibiting apoptosis (Cazzaniga et al., 2022). Endothelial progenitor cells (EPC) have a higher proliferation potential compared to ECs and act as an extra source of ECs. IFN-α activation in systemic lupus erythematosus induces EPCs to express CXCR3, where CXCR3 has the primary role of recruiting Th1 cells to the site of inflammation (Ding et al., 2020).

We also demonstrated that both IL-17a and/or IFN-γ potentiated the effect of or synergised with TNF-α to induce secretion of proinflammatory mediators, adhesion molecules, or chemokines. IL-17a potentiated the effect of TNF-α on IL-6 and IL-8 secretion from ECs while TNF-α and IFN-γ synergistically induced IL-6, MCP-1, RANTES, and IL-8 secretion from ECs. For adhesion molecules and chemokine receptors, IL-17a did not potentiate the effects of TNF-α, however, IFN-γ synergistically induced the effects of TNF-α on ICAM-1, VCAM-1, CXCR4, CXCR5, and CCR6 expression on ECs.

Previous studies have shown synergistic interactions between TNF-α and IL-17a in the stimulation of IL-6, IL-8, ICAM-1, and VCAM-1 in RA FLS, in addition to inducing cartilage degradation (Moran et al., 2011; Mori et al., 2017). TNF-α and IL-17a synergistically induced IL-6 and IL-8 production from RA FLS, psoriasis FLS, myoblasts and hepatocytes (Noack et al., 2019). Additionally, Bosteen et al. (2014) demonstrated TNF-α and IL-17a synergistically induced the production of GM-CSF from dermal microvascular ECs (Bosteen et al., 2014) and induced RANTES production from HUVECs (Hot et al., 2012). Previous studies have also demonstrated that IL-17a upregulates the expression of TNFR2 in synovial FLS (Miossec et al., 2012). However, we found no further potentiation effect on adhesion molecule or chemokine receptors in response to the combination of IL-17a and TNF-α, which contrasts with previous studies that showed IL-17a synergizes with TNF-α, IL-1β and IL-6 to increase ICAM-1, E-selectin, CXCL1, CXCL8, and CXCL10 secretion from human dermal microvascular ECs, resulting in increased neutrophil adhesion in these ECs (Bosteen et al., 2014) and synergistically increased neutrophil chemotaxis via CXCL1,
CXCL2, CXCL5, CXCR2, and CXCR4 expression in mouse aortic ECs and HUVECs (Hot et al., 2012; Griffin et al., 2018).

Consistent with the TNF-α and IFN-γ interactions observed in this study, previous studies in other cell types have demonstrated TNF-α and IFN-γ synergistically induce proliferation, BAFF, IL-6, IL-8, CX3CL1, and CXCL9 expression in osteoblasts and RA FLS, and induce IL-6 and MCP-1 secretion from systemic sclerosis FLS (Loos et al., 2006; Zaba et al., 2007; Isozaki et al., 2008; Lowin et al., 2020). Studies have also shown synergistic regulation of apoptotic pathways an effect mediated through caspase-dependent pathways in pancreatic cells and in neuroblastoma (Suk et al., 2001; Shelke et al., 2018). In the context of DS, enrichment of an IFN-γ molecular signature has been identified (Frank-Bertoncelj et al., 2017; Powers et al., 2019). This synergistic interaction is thought to be mediated through NFκB-STAT signalling elements (Ganster et al., 2005; Isozaki et al., 2008). IFN-γ via NFκB can activate promoters as well as enhancers for TNF-α, IL-6, and IL-12b (Qiao et al., 2013). Furthermore, Karonitsch et al. (2018), demonstrated that TNF-α signalling adopts the mTOR pathway to induce IFN-γ activities via NFκB and STAT-1 signalling (Karonitsch et al., 2018). IFN-γ synergises with TNF-α to increase IL-17 and RANTES expression compared to TNF-α alone in human and mouse aortic ECs (Mehta et al., 2017; Griffin et al., 2018). IFN-γ and TNF-α has also been shown to inhibit the expression of connective tissue growth factor, an angiogenic and profibrotic factor (Laug et al., 2012). Additionally, IFN-γ synergizes with TNF-α to induce PECAM-1 expression in HUVECs (Reglero-Real et al., 2016). This relationship is not restricted to TNF-α as IFN-γ also synergizes with TGF-β and IL-1α to enhance cadherin and VCAM-1 expression in human microvascular ECs (De Caterina et al., 2001; Chrobak et al., 2013).

While this is the first study to demonstrate that IFN-γ and TNF-α synergy leads to CXCR3, CXCR4, CXCR5, and CCR6 expression on ECs, in human microvascular ECs and mouse aortic ECs, IFN-γ synergises with TNF-α to increase CXCL9 and CXCL10 expression (Loos et al., 2006; Griffin et al., 2018). Another group demonstrated that ligands to TLR2, TLR3, and TLR4 in combination with IFN-γ dramatically enhanced CXCL9, CXCL10, and CXCL11 expression on human microvascular ECs (Loos et al., 2006). Loos et al. (2006) also described how IFN-γ synergized with IL-1β to induce CXCL9 and CXCL11 expression on human microvascular ECs (Loos et al., 2006). Interestingly, CXCL8 can synergize with MCP-
1, CCL7, CCL8, and CXCL12 to greatly increase neutrophil chemotaxis compared to chemokines alone (Cambier et al.). Interestingly, IFN-γ further reduced the effect of TNF-α on expression of Claudin 5, which supports previous studies showing TNF-α decreases CD105 expression on vascular ECs under hypoxic conditions (Li et al., 2003) highlighting the pathogenic activity between TNF-α and IFN-γ.

Similar to what we observed for the DA FLS-CM, minimal effect on metabolism was demonstrated for the cytokines alone or in combination, however, changes in the percentage of overall OCR and glycolytic outputs were observed. Interestingly, we also demonstrated minimal effects on EC tube formation in response to cytokine stimulation, thus suggesting that ECs were not differentiating into tip-stalk cells. Indeed, the effect of the cytokines alone and in combination were more pronounced for the secretion of proinflammatory mediators, adhesion molecules, and chemokine receptors. However, previous studies have shown that TNF-α and IFN-γ synergy resulted in an induced nitric oxide by lung ECs and vascular ECs (Yamaoka et al., 2002; Certo et al., 2021). Furthermore, studies have shown increased expression of the glucose transporter, GLUT-1, and key glycolytic enzymes, GAPDH and PKM2, localised in the vascular region of the RA synovium, expression of which are decreased in response to TNF-inhibitors (Biniecka et al., 2016). Blockade of key glycolytic enzymes inhibit both EC and synovial FLS function (Schoors et al., 2014; Biniecka et al., 2016; Wade et al., 2019). Interestingly, Wade et al. (2019) demonstrated that activated ECs display increased expression of G6PD, a rate-limiting enzyme of the PPP, suggesting that glucose-derived carbons may be diverted away from the glycolytic pathway to the PPP pathway to sustain rapid proliferation of the tip-stalk cell phenotype. Finally, the effect of cytokines on EC tip-stalk cell signalling is most likely indirect through mediators secreted from EC in response to the cytokines, therefore future studies aim to examine these mechanisms at longer time points, in addition to examining alternative pathways, through gene/protein expression and carbon tracing.
Figure 3. Summary of Chapter 3. ECs in the DA joint microenvironment that would have interacted with TNF-α and IFN-γ synergy as well as TNF-α and IL-17a induce high expressions of ICAM-1, CXCR3, CXCR4, CXCR5, and CCR6 in addition to increased production of IL-6, MCP-1, RANTES, and IL-8 (Made with Biorender).

In conclusion, the DA joint microenvironment and key cytokine interactions induce endothelial dysfunction, including tube formation, migration, adhesion, and chemokine receptor expression. The DA joint is shaped by complex interactions between immune-stromal cells and the secretion of proinflammatory mediators. Similar to that observed in Chapter 2, this also has implications for therapeutic strategies, particularly the consistently observed potentiation/synergistic interactions between IFN-γ and TNF-α, where NFκB and Jak-STAT signalling have been implicated (Ganster et al., 2005; Isozaki et al., 2008; Qiao et al., 2013).
CHAPTER FOUR

Defective immune cell landscape with a classical monocyte phenotype in DA
4.1 Introduction

The most potent function of T cells is to respond to a diverse array of pathogens. Therefore the immune system needs to create an extremely diverse set of T cell receptors (TCRs) (Kambayashi, 2018) capable of recognizing the vast array of disease causing pathogens. VDJ recombination in the thymus leads to potentially $10^9$ different TCRs (Kamradt et al., 2001). While T cells, and other cell of the adaptive immune system have enhanced specificity to antigens, compared to innate immune cells, the emergence of TCR recognizing self-antigen can emerge. It is therefore important that mechanisms of tolerance are initiated to prevent the development of autoimmunity. The immune system has developed mechanisms of both central and peripheral tolerance to ensure that autoreactive T cells do not become self-reactive to endogenous harmless antigens (Kamradt et al., 2001; Kambayashi, 2018).

Central tolerance occurs during T cell selection in the thymus where positive selection through survival signals results in CD4 or CD8 expressing T cells with a functional TCR (Sha et al., 1988). These cells then move to the corticomedullary junction of the thymus and bind with self-peptide loaded MHC. T cells with high affinity to self-antigens go through negative selection and are eliminated via apoptosis preventing autoreactive T cells exiting the thymus (Klein et al., 2014). However, we know that some self-reactive T cells can escape the thymus into the periphery. When this happens, peripheral tolerance can use one of three mechanisms (Mueller, 2010). Anergy is where T cells become inactivated and are unable to respond to antigens (Macián et al., 2004); autoreactive T cell apoptosis (Gronski, 2006); and induced Treg development where Tregs are produced in the periphery compared to the thymus (Sakaguchi et al., 2009).

If self-reactive T cells do escape both central and peripheral tolerogenic mechanisms, an additional process that has the potential to limit autoreactive T cells, is the expression of checkpoint inhibitors, which act as negative regulators to T cell signalling. One such checkpoint inhibitor, known as programmed cell death protein 1 (PD-1) – upon binding to either of its two ligands, PD-L1 and PD-L2, raises the TCR signalling threshold required for T cell activation and cytokine production, through dephosphorylation of signalling intermediates (Khan et al., 2018). This downregulates T cell signalling since TCRs will be internalized (Arasanz et al., 2017).
However, despite these combined tolerogenic mechanism of T cell activation, self-reactive T cells can escape these processes and thus T cells remain key drivers of several autoimmune diseases.

T1D is dominated by CD4+ T cell infiltration into the pancreatic islets resulting in the damage of insulin-secreting β cells (Sharma et al., 2019). In addition, CD4+ T cells amplify the B cell production of autoantibodies – an important trait of T1D (Soeldner et al., 1985). Not only that but activated CD4+ T cells drive neutrophil and macrophage infiltration into the islets leading to further destruction of β cells (Thayer et al., 2011; Padgett et al., 2015; Huang et al., 2016). Multiple sclerosis (MS) is another autoimmune disorder influenced by dysfunctional T cell regulation in several ways (van Langelaar et al., 2020). IFN-γ-secreting Th17 cells increases the permeability of the blood brain barrier. T cells also secrete GM-CSF and IFN-γ and may encounter T-bet+ memory B cells leading to inflammation and demyelination – a trademark of MS (van Langelaar et al., 2020).

Multiple studies have demonstrated major T cell dysfunction in the RA joint. This includes increased effector T cell populations, impaired Tregs, enhanced expressions of chemokine receptors such as CCR4, CCR5, CXCR3 and CXCR5 and activate resident macrophages and FLS that proliferate and degrade the cartilage and encompasses T cells expressing higher levels of proinflammatory cytokines including TNF-α and IFN-γ (Cope, 2008; Rao et al., 2017; Fonseka et al., 2018; Ponchel et al., 2020; Floudas et al., 2022; Yamada, 2022).

Cytokine production from T cells remain at the epicentres of RA pathogenesis. Including the ones mentioned in Section 1.26 (Chapter 1), IL-21 also plays a role in RA. The primary source of IL-21 is Tfh cells (Gong et al., 2019). Additionally, IL-2 promotes T cell proliferation and Treg growth and survival thus IL-2 deficiency is observed throughout RA pathogenesis (Graßhoff et al., 2021). Inadvertently, Tregs suppress T cell responses by depriving effector T cells of IL-2 since they consume more IL-2 from the RA microenvironment (Pandiyan et al., 2007).

Another cytokine of interest is IL-4. It has the dual role of being both anti and proinflammatory and thus is a pleotropic mediator and it controls T cell activation, survival, differentiation, and proliferation (Magyari et al., 2014; Dong et al., 2018). In RA, the IL-4 gene is increased in T cells compared to HC (Rivas et al., 1995). Additionally, high
levels of IL-22, produced primarily by Th17 cells, was reported in RA (Fakher et al., 2022) however, the first clinical trial for the use of fezukinumab (anti-IL-22 blocking antibody) has failed to meet the efficacy criteria (Pfizer, 2022).

In addition to T cell derived cytokines being implicated in the pathogenesis of RA - the migration of T cells directly into the joint also facilitates pannus formation in IA. Without the aid of chemokines and their receptors, this would be impossible. As such, CCR1 levels are amplified on T cells in the RA synovium (Elemam et al., 2020). CXCR3 is also expressed on T cells, specifically IFN-γ secreting Th1 cells, and has multiple ligands (CXCL9, CXCL10 and CXCL11) that facilitate Th1 migration into the synovial tissue (Di Domenicantonio, 2014). In addition to Th cells, cytotoxic T cells have also been implicated in RA. The expression of granzyme B from CD8+ cytotoxic T cells in RA is increased compared to HC in the periphery (Carvalheiro et al., 2015). This is important since granzyme B is linked to joint erosions in RA (Goldbach-Mansky et al., 2005).

As mentioned previously, polyfunctional T cells are defined by their capacity to produce multiple cytokines simultaneously (Basdeo et al., 2015). Further examination proved that polyfunctional CD161+ Th17 lineage cells are highly enriched in the RA synovium. Their pathogenic traits include production of proinflammatory cytokines, thriving in a hypoxic environment and activating FLS (Basdeo et al., 2015). A comprehensive study by Floudas et al. (2022) highlighted heightened plasticity in Tfh cells and CD4+ T cell polyfunctionality in the RA synovium, as well as enriched memory Treg cell activities. Importantly, RNAseq analysis of RA synovial tissue outlines that T cell activation and differentiation signalling pathways precede the onset of RA (Floudas, et al., 2022). This emphasizes the early emergence of T cell polyfunctionality during inflammation.

What is even more striking for polyfunctional T cells is that they are resistant to suppression by Tregs (Basdeo et al., 2015, Floudas et al., 2022). ExTh17 cells from RA patients are resistant to Treg-mediated suppression of proliferation compared to autologous Th1 and Th17 T cells (Basdeo et al., 2017). It is worth noting that synovial tissue Tregs from patients RA primarily belong to the memory Treg compartment (Floudas et al., 2022). This may indicate that impaired memory FoxP3 Tregs are unable to regulate synovial immune responses (Hoffmann et al., 2006).
Tregs are powerful immunosuppressors capable of downregulating inflammation from Th1 and Th17 cells through various mechanisms (Joller et al., 2014; Levine et al., 2017) including IL-10 production, CTLA-4 and PD-1 expression. Specifically, PD-1 binds to PD-L1 on DCs to inhibit effector T cells (Jiang et al., 2021) thus preventing antigen presentation from the DC to the effector T cell. In RA, Treg function is impaired as they cannot inhibit B cell activity (Basdeo et al., 2015; Rapetti et al., 2015). While changes in the numbers of RA Tregs remain in question (Jiang et al., 2021), our group reports that Treg frequency in DA is reduced compared to T21 and JIA (Foley et al., 2020).

A restricted Treg TCR repertoire exists at sites of inflammation in JIA (Rossetti et al., 2017) and in circulation (Henderson et al., 2016) thereby limiting the interaction between Tregs and APCs. Furthermore, Tregs in JIA are dysfunctional (Hoepli et al., 2019) - these cells display fluctuating FoxP3 and CD25 expression providing unreliable IL-2R expression leading to a low affinity to IL-2, changed cytokine production and insufficient chemokine secretion (Pesenacker et al., 2013; Bending et al., 2014; Patterson et al., 2016).

In the context of DS, smaller thymic structure, impaired naïve T cell development and higher frequency of memory T cells are reported (Marcovecchio et al., 2019). Additionally, Table 1.3 (Chapter 1) outlines the abnormal frequencies of other cell types found in DS. The triplication of chromosome 21 in DS leads to an enhanced dosage of IFN receptors resulting in increased IFN stimulation in T cells (Sullivan et al., 2016; Powers et al., 2019). T21 encodes for four of the six interferon receptors (Sullivan et al., 2016) making this a plausible cause of autoimmunity seen in DS. Interestingly, Ge et al. documented that FLS stimulated with TNF led to changes in their essential genes such as TNFAIP3 and IFNAR1 (Ge et al., 2021) thus indicating an IFN gene signature in DS.

The role of T cells in children with DA is less understood. Currently, our group have conducted the only published study to date which has examined this. Foley et al. (2020) demonstrated increased T cell polyfunctionality and high levels of Th17 cell plasticity in children with DA along with augmented production of TNF-α, IFN-γ and IL-17a from CD8-, CD8+ and CD161+ T cells in comparison to children JIA or T21 (Foley, et al., 2020). In addition, children with DA had increased frequencies of CXCR3+ and CCR6+ Tfh cells compared to JIA and T21 (Foley et al., 2020). At a histological level an increase in CD3+ T cell infiltrates were also demonstrated in DA synovium compared to JIA (Foley et al., 2020).
206). Together, this study highlights the need for further elucidation of T cell plasticity and activation that may contribute to the pathogenesis of DA.

Myeloid cells encompass granulocytes, monocytes, DCs and macrophages that derive from haematopoietic stem cells (De Kleer et al., 2014). Monocytes are bone marrow-derived cells, essential in the immune system as their main functions are to link the innate and adaptive immune system acting as an APC, phagocytosing foreign particles (Uribe-Querol et al., 2020) and producing TNF-α, IL-1β, IL-6 and CXCL10 as well as being involved in producing reactive oxygen species (Cros et al., 2010; Yoon et al., 2014; Rana et al., 2018). They express chemokine receptors including CCR2 and CX3CR1 which interact with MCP-1 and CX3CL1 that are produced mainly by FLS (Rana et al., 2018). Peripheral blood monocytes can serve as biomarkers of RA severity, with increased frequencies of peripheral monocytes in treatment-naïve RA patients predictive of low therapeutic response to MTX (Chara et al., 2015; Tsukamoto et al., 2017). As described in Section 1.24, Chapter 1, monocytes are categorised into three subtypes based on their expression of CD14 and CD16: CD14- CD16+ are nonclassical monocytes, CD14+ CD16+ are intermediate monocytes, and CD14+ CD16- classical monocytes (Luo et al., 2018), with the latter thought to differentiate into bone-resorbing osteoclasts in RA (Xue et al., 2020). Several studies demonstrate that monocyte subtypes in RA differ compared to HC (Rossol et al., 2012; Tsukamoto et al., 2017). Our group have shown that circulating CD14+ monocytes from individuals ‘at-risk’ (IAR) of developing RA are metabolically reprogrammed and are hyper-inflammatory highlighting that monocyte activation occurs before clinical manifestations of RA diseases (McGarry et al., 2021). Subsequently we have also demonstrated that monocyte-derived macrophages maintain the inflammatory phenotype of their precursor monocyte parent cell (Hanlon et al., 2023). In the context of JIA, studies have shown that both synovial and peripheral intermediate and nonclassical monocytes in JIA were higher compared to septic arthritis (Cren et al., 2020). Similar to RA (Mc Garry et al., 2021), peripheral blood monocytes in JIA have also been shown to be primed and display a heightened response to stimulation (Wang et al., 2021). Differences have also been observed across JIA subtypes where oligoJIA have synovial monocytes with an M1/M2-like pattern (Schmidt et al., 2020) while sJIA patients have a IFN-γ-producing plastic macrophage phenotype in the periphery (Schulert et al., 2021). Consistent with
this, studies have shown that the increased monocyte activation in the JIA synovium is regulated at an epigenetic level, and is associated with an IFN molecular signature, an effect that is reversed by JAK-STAT inhibition with ruxolitinib (Peeters et al., 2023). As highlighted in Chapter 2, children with DA had a strong IFN-γ signature that led to enhanced production of proinflammatory mediators coupled with increased chemokine receptor and adhesion molecule expressions brought about through the Jak-STAT and NFκB pathway. Children with DS were found to have lower total monocytes in the periphery compared to HC but increased nonclassical monocytes compared to HC (Bloemers et al., 2010).

It is likely that DA is an autoinflammatory disease as there is no known autoantigen. However, there are seven subsets of JIA, some of which are associated with autoantigens such as ANA, and there are reports of ANA positivity in a number of DA subjects. So, the distinction between autoinflammatory and autoimmune disease remains somewhat blurred depending on the presence or absence of a specific autoantigen.

To date, there are no studies that examine monocytes in DA.
4.2 Aims and Objectives

Overall aim: to investigate the immune cell landscape in DA

1. To examine T cell chemokine receptor expression profile differences in children with DA compared to JIA, T21 and HC
2. To examine the potential correlation between immune cell IFN receptor expression and T cell plasticity in children with DA compared to JIA, T21 and HC
3. To assess the distribution of monocyte subsets in children with DA compared to JIA, T21 and HC
4.3 Materials and methods

4.3.1 Patient recruitment

This project was approved by the Ethics Committee of CHI at Crumlin and SVUH. All families and participants received verbal and documented information on the study and written consent was obtained prior to recruitment. Children with DA and JIA were enrolled from the NCPR in CHI at Crumlin. Children with T21 were recruited from Tallaght University Hospital (TUH). This group of children were diagnosed with T21 but showed no evidence of past or present arthritis. HC were recruited from the orthopaedic department at CHI at Crumlin, and defined as children without T21, had no medical history of inflammatory diseases or autoimmune diseases. Fully informed parental consent and participant acceptance were obtained for all individuals who provided blood donations.

| HC  
<table>
<thead>
<tr>
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</tr>
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<tbody>
<tr>
<td>Age</td>
</tr>
<tr>
<td>median (range)</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>F = 4</td>
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</tbody>
</table>

Table 4. 1 HC demographics that includes age and gender.
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</thead>
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</tr>
<tr>
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<tr>
<td>11 (3-15)</td>
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</tr>
<tr>
<td>Gender</td>
<td>Etanercept 2</td>
</tr>
<tr>
<td>F = 9</td>
<td>Etanercept + Intraarticular corticosteroids (IAI) 1</td>
</tr>
<tr>
<td>M = 2</td>
<td>MTX 1</td>
</tr>
<tr>
<td>Active joint count</td>
<td>MTX + prednisolone 1</td>
</tr>
<tr>
<td>n (range)</td>
<td>Adalimumab + MTX + IAI 1</td>
</tr>
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</tr>
<tr>
<td>median</td>
<td>MTX</td>
</tr>
<tr>
<td>9</td>
<td>MTX + prednisolone</td>
</tr>
<tr>
<td>unknown</td>
<td>Adalimumab + MTX + IAI</td>
</tr>
<tr>
<td>high CRP (%) (0)</td>
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<td>median</td>
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<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
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<tr>
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<td>Adalimumab + MTX 1</td>
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<td>11</td>
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**Table 4.** JIA patients’ demographic, clinical, laboratory features and treatments prior sampling and at time of sampling.

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<td>Age</td>
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</tr>
<tr>
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<td>Etanercept 2</td>
</tr>
<tr>
<td>10m (5m-2y 11m)</td>
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</tr>
<tr>
<td>Gender</td>
<td>Adalimumab + MTX 1</td>
</tr>
<tr>
<td>F = 7</td>
<td>Tocilizumab + MTX 3</td>
</tr>
<tr>
<td>M = 4</td>
<td>MTX 1</td>
</tr>
<tr>
<td>unknown</td>
<td>0</td>
</tr>
<tr>
<td>high CRP (%) (0)</td>
<td>unknown</td>
</tr>
<tr>
<td>median</td>
<td>&lt;5</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RF/ ACPA</td>
<td>NIL 1</td>
</tr>
<tr>
<td>Positive</td>
<td>Etanercept 2</td>
</tr>
<tr>
<td>0</td>
<td>Etanercept + MTX 3</td>
</tr>
<tr>
<td>Negative</td>
<td>Adalimumab + MTX 1</td>
</tr>
<tr>
<td>11</td>
<td>Tocilizumab + MTX 3</td>
</tr>
<tr>
<td>Unknown</td>
<td>MTX 1</td>
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**Table 4.** T21 patients’ demographics that includes age and gender.
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<td>10</td>
</tr>
<tr>
<td>(range)</td>
<td>(4-17)</td>
</tr>
<tr>
<td>Gender</td>
<td>NIL + NSAID</td>
</tr>
<tr>
<td>F = 7</td>
<td>MTX + Etanercept</td>
</tr>
<tr>
<td>M = 9</td>
<td>MTX + Adalimumab + Etanercept</td>
</tr>
<tr>
<td>Active joint count</td>
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</tr>
<tr>
<td>n</td>
<td>Etanercept</td>
</tr>
<tr>
<td>(range)</td>
<td>(0-6)</td>
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<tr>
<td>unknown</td>
<td>2</td>
</tr>
<tr>
<td>ESR</td>
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<tr>
<td>high ESR (%)</td>
<td>Tocilizumab</td>
</tr>
<tr>
<td>median</td>
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<tr>
<td>CRP</td>
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<td>&lt;5</td>
</tr>
<tr>
<td>unknown</td>
<td>0</td>
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<tr>
<td>RF/ ACPA</td>
<td>Unknown</td>
</tr>
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<td>Negative</td>
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<td>0</td>
</tr>
<tr>
<td>Treatments at time of sample</td>
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</tr>
<tr>
<td>NIL</td>
<td>NSAID</td>
</tr>
<tr>
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<td>Adalimumab</td>
</tr>
<tr>
<td>Tocilizumab</td>
<td>Tofacitinib</td>
</tr>
<tr>
<td>Ustekinumab (anti-IL12/IL-23)</td>
<td>Golimumab</td>
</tr>
</tbody>
</table>

Table 4. DA patients’ demographic, clinical, laboratory features and treatments prior sampling and at time of sampling.

4.3.2 PBMC isolation

PBMC were isolated according to 2.3.7, Chapter 2. PBMC were resuspended in cRPMI before cryogenically stored at -80°C until required for analysis.

4.3.3 Cell stimulation

For PBMC stimulation and cytokine detection via flow, 5 x 10^5 cells were placed per well in a flat bottom 96 well plate in 200ul of cRPMI. Cells were incubated with cell stimulation cocktail (PMA/ ionomycin) (ThermoFisher, UK) for 1hr at 37°C prior to addition of 0.05µg/ml Brefeldin A and monensin (ThermoFisher, UK) for another 4hr incubation at 37°C.
4.3.4 Flow cytometric analysis

$5 \times 10^5$ cells were washed in PBS prior to incubation with Zombie NIR™ Fixable Viability Kit (BioSciences, UK) for 30mins at 4°C. An Fc receptor blocking step was performed by incubating the cells with TruStain FcX blocking solution (Biolegend, UK) for 10m at 4°C. Cells were then stained with fluorochrome-conjugated antibodies against specific extracellular markers (described in Table 4.1 – 4.4), vortexed then incubated in the dark at 4°C for 30mins. For intracellular cytokine staining, cells were permeabilised according to the manufacturer’s instructions and using the FOXP3 staining buffer set (eBiosciences, UK) before the addition of intracellular cytokine antibodies then fixed with the staining buffer set. Samples were then washed in 1x Perm/Wash and centrifuged at 400g. Supernatants were removed before being washed and resuspended in 200μl of FACS Buffer. Samples were acquired using the LSRFortessa Flow Cytometer (Beckman Coulter, UK) and analysed using FlowJo software (BD, UK). To adjust for spectral overlap between detectors, compensation was applied using single stained compensation beads (BD, UK). Specific surface marker fluorescence gating was performed by comparison with an FMO. Gating strategy as per Figure 4.1 was applied.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Other names</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
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<td>BV785</td>
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</tr>
<tr>
<td>*IFN-γ</td>
<td>Type II interferon</td>
<td>APC</td>
<td>4S.B3</td>
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<tr>
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<td>TCGF</td>
<td>BV605</td>
<td>MQ1-17H12</td>
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<td></td>
<td>BV711</td>
<td>MP4-25D2</td>
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<td>BV650</td>
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<td></td>
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<td>*IL-22</td>
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<td>PE</td>
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<td>*GM-CSF</td>
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<td>BVD2-21C11</td>
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<td>*TNF-α</td>
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Table 4.5 Th cell antibody panel * indicates intracellular stains
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Table 4. 6 Chemokine receptor antibody panel

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<td>T4</td>
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Table 4. 7 IFN receptor antibody panel

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Table 4. 8 Monocyte antibody panel
4.3.5 Statistical analysis

Statistical analyses were performed on GraphPad Prism 9 software. Kruskal-Wallis One-way ANOVA (#) and Mann-Whitney unpaired t-test (*) was used for analysis of non-parametric data. P-values of <0.05 (#p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001) were determined as statistically significant for One-way ANOVA while *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 were defined as statistically significant for paired t-test.
4.4 Results

4.4.1 Increased cytokine production and higher polyfunctionality in DA compared to HC, JIA and T21

To determine whether there are differences in the T cell subpopulation distribution in patients with DA compared to children with T21, JIA or HC controls, we examined cell surface expression of peripheral blood T cells by flow cytometric analysis. Representative flow plots of the gating strategy used are shown in Figure 4.1. Following exclusion of debris and doublets (Figure 4.1(a – c)), we gated on live cells (Figure 4.1(d)). CD3+ cells were gated (Figure 4.1(e)) before further phenotyping of T cells based on expression of CD4 and CD8 (Figure 4.1(f)). CD4+ CD8+ T cells were excluded from the analysis since they represent less than 10% of the population thus would provide inaccurate data. Representative dot plots for an example marker and FMO is observed in Figure 4.1(g - h). Firstly, we examined the frequency of CD4+ T helper, CD8+ and the double negative CD4-CD8-. The DA cohort had a significantly reduced CD4+ T cell frequency compared to JIA (p<0.01) and T21 (p<0.05) with no significant changes observed compared to HC (Figure 4.2(a)). Additionally, DA had a significantly increased CD8+ T cell frequency compared to JIA (p<0.01) and T21 (p<0.001) (Figure 4.2(b)). No significant changes in CD4- CD8- T cell frequency was reported in the DA group compared to the three other cohorts (Figure 4.2(c)).

Next, the expression of key cytokines that were previously associated with IA were examined (McInnes et al., 2007; Foley et al., 2020). First, we assessed the percentage of cytokines expressed by CD4+ T cells. Specifically, IL-2 is needed for T cell survival and is consumed by Tregs while TNF-α is one of the primary mediators of inflammation. IL-2 expression was significantly increased in DA compared to HC (p<0.05) (Figure 4.3(a)), however IL-2 expression in DA was similar to T21. TNF-α expression was increased in DA compared to HC (p<0.01), JIA (p=0.05), and T21 (p<0.05) (Figure 4.3(b)). Next, we examined Th2 cytokines i.e. IL-4 which downregulates Th1 responses and GM-CSF which helps differentiate Th17 cells. IL-4 expression was significantly increased in DA compared to T21 (p<0.05) (Figure 4.3(c)). GM-CSF was highly expressed in patients with DA compared to HC (p<0.05) (Figure 4.3(d)), however was similar to its control T21. We then analyzed Th17 cytokines, namely IL-17a and IL-22, since IL-17a drives synovial cell chemotaxis and IL-22 provides autoantibodies with inflammatory properties (Pfeifle et al., 2017). Furthermore, the two main subtypes in DA are poly-RF neg and psoriatic arthritis,
with numerous studies demonstrating efficacy for anti-IL17 therapies in adult PsA. IL-17a expression was significantly increased in DA compared to T21 ($p<0.01$) (Figure 4.3(e)), while IL-22 expression was significantly increased in DA compared to HC ($p<0.01$), with a trending increase when compared to T21 (Figure 4.3(f)). Next, we examined IL-21 as this Tfh cytokine aids B cells to generate autoantibodies (Jandl et al., 2017), and we have previously shown increased Tfh plasticity in DA (Foley et al., 2020). IL-21 expression was increased in DA compared to T21 ($p<0.05$) (Figure 4.3(g)). Finally, we examined IFN-γ expression as this Th1 cytokine activates macrophages (Chemin et al., 2019). IFN-γ expression was significantly increased in DA compared to HC ($p<0.01$) and T21 ($p<0.01$) with no changes reported compared to JIA (Figure 4.3(h)). We then assessed the cytokine expression of CD8+ T cells across all four cohorts. IL-2 expression was increased in DA compared to HC ($p<0.05$) with no changes observed when compared to T21 (Figure 4.4(a)). TNF-α expression was increased in DA compared to HC ($p<0.01$) (Figure 4.4(b)) with no changes observed when compared to T21. No differences were observed for IL-4 in DA compared to the three groups (Figure 4.4(c)), although similar to IL4+ CD4+ cells, a trending increase was observed compared to its T21 control group. While not significant, GM-CSF expression was higher in DA, JIA and T21 compared to HC (Figure 4.4(d)). IL-17a expression was increased in DA compared to T21 ($p=0.05$) (Figure 4.4(e)) with no changes observed for IL-22 expression (Figure 4.4(f)). IL-21 was reduced in DA compared to HC ($p=0.05$) (Figure 4.4(g)), with no difference compared to T21. IFN-γ expression was significantly increased in DA compared to HC ($p<0.01$), JIA ($p<0.01$) and T21 ($p<0.05$) (Figure 4.4(h)). We also analysed the cytokine expression of CD4- CD8- T cells in the four groups. IL-2 expression was decreased in DA compared to T21 ($p<0.05$) (Figure 4.5(a)). TNF-α expression was increased in DA compared to HC ($p<0.001$) (Figure 4.5(b)), with no differences observed when compared to T21. No significant differences were observed for IL-4 across all groups (Figure 4.5(c)), although similar to CD4+ and CD8+ T cells, a trending increase was demonstrated in DA compared to its T21 control group. GM-CSF was higher in DA compared to HC ($p=0.05$) with no differences observed when compared to T21 (Figure 4.5(d)). IL-17a expression was increased in DA compared to T21 ($p<0.01$) (Figure 4.5(e)). While not significant, IL-22 expression was higher in DA compared to HC ($p=0.06$) (Figure 4.5(f)). IL-21 expression was reduced in DA compared to HC ($p<0.05$) (Figure 4.5(g)).
4.5(g)). IFN-γ expression was significantly increased in DA compared to HC ($p<0.01$) with a trending increase compared to T21 ($p=0.07$)) (Figure 4.5(h)).
Figure 4. Gating strategy for Th panel. PBMC were stained with a panel of fluorochrome antibodies according to Table 4.1. Representative dot plots depicting the gating strategy used to identify and phenotype PBMC. (a)-(d) the forward and side scatter parameters of cells were set before elimination of dead cells and doublet exclusion. (e) CD3+ T cells were selected against CD45+ cells, (f) further phenotyping into the different T cell subpopulations, (g) example marker applied against (h) FMO.
Figure 4.2 Increased CD4+ and CD8+ T cell subpopulations in DA peripheral blood. Bar charts depict the frequency of (a) CD4+, (b) CD8+ and (c) CD4-CD8- T cells as part of total CD3+ T cells for HC (n=5), JIA (n=11), T21 (n=12) and DA (n=16). Values expressed as mean ± SEM symbols represent individual samples, n=5-16 donors/group. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis, respectively #p<0.05, ##p<0.01, ###p<0.001 significantly different compared to other groups.
Figure 4. Increased proinflammatory cytokine expression from patients with DA peripheral blood CD4+ T cells. Bar charts depict the frequency percentage of CD4+ T cells positive for (a) IL-2, (b) TNF-α, (c) IL-4, (d) GM-CSF, (e) IL-17a, (f) IL-22, (g) IL-21 and (h) IFN-γ amongst HC (n=5), JIA (n=7 or 11), T21 (n=7 or 12) and DA (n=11 or 16). Values expressed as mean ± SEM of n=5-16 donors/group. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other groups. *p<0.05 significantly different compared to other groups.
Figure 4. Increased proinflammatory cytokine expression from patients with DA peripheral blood CD8+ T cells. Bar charts depict the expression of (a) IL-2, (b) TNF-α, (c) IL-4, (d) GM-CSF, (e) IL-17a, (f) IL-22, (g) IL-21 and (h) IFN-γ amongst HC (n=5), JIA (n=7 or 11), T21 (n=7 or 12) and DA (n=11 or 16) in the CD8+ T cell subpopulation. Values expressed as mean ± SEM of n=5-16 donors. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis #p<0.05, ##p<0.01 significantly different compared to other groups.
Figure 4. Increased proinflammatory cytokine expression from patients with DA peripheral blood CD4-CD8- T cells. Bar charts depict the expression of (a) IL-2, (b) TNF-α, (c) IL-4, (d) GM-CSF, (e) IL-17a, (f) IL-22, (g) IL-21 and (h) IFN-γ amongst HC (n=5), JIA (n=7 or 11), T21 (n=7 or 12) and DA (n=11 or 16) in the CD4-CD8- T cell subpopulation. Values expressed as mean ± SEM of n=5-16 donors. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis respectively #p<0.05, ##p<0.01, ###p<0.001 significantly different compared to other groups. *p<0.05 significantly different compared to other groups.
4.4.2 Increased CD4+, CD8+ and CD4-CD8- polyfunctionality in DA

As we have previously demonstrated, T cell polyfunctionality is enhanced in DA (Foley et al., 2020). Therefore, we next expanded these studies to include a larger panel of cytokines. Analysis was performed by SPICE which allows the visualisation of polydimensional data therefore we can look at multiple parameters simultaneously. In Figure 4.6, we describe a fraction of the 256 possible combinations of cytokines. In Figure 4.7, each arc represents a specific cytokine where the overlapping of arcs above a pie segment equates to more than one cytokine (see arc legend) produced in combination. We demonstrate that about 10% of the CD4+ T cell population in the HC group produced one or more cytokines together, (Figure 4.7(a)(i)), in contrast to CD4+ T cells in JIA (40%) (Figure 4.7(a)(ii)), T21 (46%) (Figure 4.7(a)(iii)) and DA (70%) (Figure 4.7(a)(iv)), thus highlighting an increase in T cell polyfunctionality in all groups with respect to HC. This is also evident from the increase in overlapping arcs on the pies, determining an increase of cytokines produce alone or in different combination in disease compared to HC (Figure 4.7(a)). The DA group had an enhanced increase in CD4+ T cells expressing IFN-γ in combination with TNF-α and IL-2 (purple arc overlapping with yellow and red arcs) (Figure 4.7(a)(iv)) in respect to the T21 control (Figure 4.7(a)(iii)). When analysing the CD8+ T cells population, although we observed a higher frequency of cells expressing one or more cytokine together in HC (22%) (Figure 4.7(b)(i)), the frequency of cells expression cytokines was still higher in JIA (60%) (Figure 4.7(b)(ii)), T21 (67%) (Figure 4.7(b)(iii)) and DA (68%) (Figure 4.7(b)(iv)), thus confirming an increase in polyfunctionality in all the groups in respect to HC. Similar to the CD4+ T cells, this was reflected by the increase in overlapping arcs, highlighting the increase in cells expressing one or more cytokines together in disease compared to HC (Figure 4.7(b)). The DA group had an enhanced increase in CD8+ T cells expressing IFN-γ in combination with TNF-α and GM-CSF (purple arc overlapping with yellow and green arcs) (Figure 4.7(b)(iv)), in respect to the T21 control (Figure 4.7(b)(iii)). Within the CD4-CD8- T cell population, approximately 21% of cells in HC are producing one cytokine or more (Figure 4.7(c)(i)), which is in stark contrast to JIA (48%) (Figure 4.7(c)(ii)), T21 (46%) (Figure 4.7(c)(iii)) and DA (60%) (Figure 4.7(c)(iv)). The increased polyfunctionality observed in all groups in contrast to HC, is highlighted by the increase of cells expressing one or more cytokines together (overlapping of arcs), specifically an increase in cells expressing IFN-γ, in combination with TNF-α can be observed in DA vs T21.
groups (purple and yellow arcs) (Figure 4.7(c) (iii – iv)). Together this data suggest that DA has overall the highest degree of polyfunctionality in the three T cell subpopulations.

Next, we examined in more detail the cytokine coexpression within CD4+, CD8+ and CD4-CD8- T cells across the four groups. Interestingly, the frequency of CD4+ T cells coexpressing IFN-γ, IL-2 and TNF-α is increased in DA compared to HC ($p<0.01$), JIA ($p=0.05$) and T21 ($p<0.01$) (Figure 4.8(a)(i)). The frequency of CD4+ T cells co-expressing IFN-γ, IL-2, TNF-α and IL-22 in DA is increased compared to HC ($p<0.01$) with a trending increase compared to T21 (Figure 4.8(a)(ii)). The frequency of CD4+ T cells that do not express any cytokines in DA are decreased compared to HC ($p<0.01$) (Figure 4.8(a)(iii)), thus confirming an increase in polyfunctionality.

The frequency of CD8+ T cells that coexpress IFN-γ and TNF-α are increased in DA compared to HC ($p<0.05$), JIA ($p<0.05$) and T21 ($p=0.05$) (Figure 4.8(b)(i)). The frequency of CD8+ T cells that coexpress IFN-γ, IL-2 and TNF-α are increased in DA compared to HC ($p<0.01$) with a trending decrease compared to T21 (Figure 4.8(b)(ii)). The frequency of CD8+ T cells that co-express IFN-γ, TNF-α and GM-CSF are increased in DA compared to HC ($p<0.05$), JIA ($p=0.07$) and T21 ($p<0.05$) (Figure 4.8(b)(iii)). The frequency of CD8+ T cells that do not express any cytokines are decreased in DA compared to HC ($p<0.01$) and JIA ($p<0.05$) (Figure 4.8(b)(iv)). The frequency of CD4-CD8- T cells that coexpress IFN-γ and TNF-α are increased in DA compared to T21 ($p<0.05$) (Figure 4.8(c)(i)). No changes were observed in the frequency of CD4-CD8- T cells that coexpress IFN-γ and IL-22 across all groups (Figure 4.8(c)(ii)). As observed for the other T cell populations, the frequency of CD4-CD8- T cells that do not express these cytokines are decreased in all group in respect to HC, with a significant decrease observed for DA compared to HC ($p<0.01$) while no differences were observed between DA and T21 (Figure 4.8(c)(iii)).
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Figure 4. 6 Snapshot of all possible 256 combinations of cytokines using Boolean gating. Due to visual limitations of the SPICE software, we can only show 64 possible combinations.
Figure 4. 7 Children with DA have increased polyfunctional CD4+, CD8+ and CD4-CD8- T cells. Pie charts describe polyfunctionality of (a) CD4+, (b) CD8+ and (c) CD4-CD8- T cells from (i) HC (n=5), (ii) JIA (n=7), (iii) T21 (n=7) and (iv) DA (n=11) patients stained with fluorochrome-conjugated antibodies specific for IL-2, TNF-α, GM-CSF, IL-17a, IL-21 and IFN-γ then analysed via flow cytometry. Results were collated using SPICE. Each pie chart represents the average frequencies of cytokine-producing T cells generating every possible combination of the six cytokines. The arc above each circumference describes the specific cytokine produced by the proportion of cells under the arc. Multiple arcs above a pie segment represent polyfunctional cells. The size of the pie segment or arc correlates to the frequency of the particular population.
Figure 4. 8 Children with DA have increased coexpression of proinflammatory cytokines. Bar charts demonstrate CD4+ T cells that coexpress (a)(i) IFN-γ+ IL-2+ TNF-α, (a)(ii) IFN-γ+ IL-2+ TNF-α IL-22+ and (a)(iii) none of these cytokines; CD8+ T cells that coexpress (b)(i) IFN-γ+ TNF-α, (b)(ii) IFN-γ+ IL-2+ TNF-α, (b)(iii) IFN-γ+ TNF-α+ GM-CSF+ (b)(iv) none of these cytokines; CD4- CD8- T cells that coexpress (c)(i) IFN-γ+ TNF-α, (c)(ii) IFN-γ+ IL-22+ and (c)(iii) none of these cytokines from HC (n=5), JIA (n=7), T21 (n=7) and DA (n=11) patients stained with fluorochrome-conjugated antibodies specific for IL-2, TNF-α, GM-CSF, IL-17a, IL-21 and IFN-γ then analysed via flow cytometry. Values expressed as mean ± SEM of n=5-11 donors/group. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other groups. *p<0.05 significantly different compared to other groups.
4.4.3 Increased frequency of chemokine receptor expression on T cell subpopulations in DA

We next analysed chemokine receptor expression on the CD4+, CD8+ and CD4-CD8- T cell subpopulations using a specific gating strategy (Figure 4.9). Firstly, we examined CCR1 frequencies since CCR1 binds to CCL3 and CCL5 (RANTES) and is known for neutrophil chemotaxis into the inflamed joint (Chou et al., 2010). CCR1 was reduced in DA compared to HC within both CD4+ ($p=0.05$) (Figure 4.10(a)) and CD8+ T cells ($p=0.05$) (Figure 4.10(b)) with no significant changes observed in CD4-CD8- T cell subpopulation (Figure 4.10(c)).

We then examined CCR2 frequencies since CCR2 is known to recruit Th1 cells into the synovium (Moadab et al., 2021). CCR2 was increased in DA compared to T21 ($p<0.001$) on CD4+ T cells (Figure 4.10(d)), significantly increased compared to HC ($p<0.05$) and T21 ($p<0.05$) on CD8+ T cells (Figure 4.10(e)) and significantly increased compared to T21 ($p<0.01$) on CD4- CD8- T cells (Figure 4.10(f)). We also analyzed CCR4 frequencies since CCR4 recruits Th17 cells into the synovium (Honzawa et al., 2022). CCR4 frequencies in DA was increased compared to T21 ($p=0.05$) on CD4+ T cells (Figure 4.11(a)) with no differences observed within the CD8+ T cell compartment (Figure 4.11(b)). CCR4 frequency was decreased in DA compared to T21 ($p<0.01$) (Figure 4.11(c)). We assessed CCR5 frequencies also as it recruits monocytes and macrophages into the RA synovium (Prahalad, 2006). CCR5 frequency was increased in DA compared to T21 within CD4+ T cells ($p<0.05$) (Figure 4.11(d)), CD8+ T cells ($p=0.07$) (Figure 4.11(e)) and CD4- CD8- T cells ($p<0.05$) (Figure 4.11(f)). We examined CXCR3 frequencies since CXCR3 positive T cells are found in RA synovial fluid (Mohan and Issekutz, 2007). CXCR3 frequency was increased in DA compared to JIA ($p<0.05$) and T21 ($p<0.05$) CD4+ T cells (Figure 4.12(a)), with no significant changes seen across all groups within CD8+ and CD4- CD8- T cells (Figure 4.12(b-c)). We also analysed CXCR6 since this was found on T cells in the RA synovium (Tu et al., 2021). CXCR6 frequency in DA was increased compared to JIA ($p<0.05$) and T21 ($p<0.05$) CD4+ T cells (Figure 4.12(d)). CXCR6 frequency was increased in DA compared to JIA ($p<0.01$) CD8+ T cells (Figure 4.12(e)), with no changes seen across all groups on CD4-CD8-T cells (Figure 4.12(f)).
**Figure 4. 9 Gating strategy for chemokine panel.** PBMC were stained with a panel of fluorochrome antibodies according to Table 4.2. Representative dot plots depicting the gating strategy used to identify and phenotype PBMC with the gating strategy from Figure 4.1(a) - (d) used. (a) CD4+ T cells were selected against CD3- cells (b) CD4+ T cells were gated against CD8+ T cells (c) example marker gated according to (d) example FMO, (e) further phenotyping into the different memory T cell subpopulations, (f) example marker gated according to (g) example FMO.
Figure 4. 10 CCR1 and CCR2 expressions on CD4+, CD8+ and CD4-CD8- T cells in DA differ. Bar charts depict the expression of CCR1 on (a) CD4+ T cells (b) CD8+ T cells, (c) CD4-CD8- T cells; CCR2 on (d) CD4+ T cells, (e) CD8+ T cells and (f) CD4-CD8- T cells from HC (n=5), JIA (n=10), T21 (n=11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors/group. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis respectively #p<0.05, ###p<0.01, ####p<0.001 significantly different compared to other groups. *p<0.05 significantly different compared to other groups.
Figure 4. 11 CCR4 and CCR5 expressions on CD4+, CD8+ and CD4-CD8- T cells in DA differ. Bar charts depict the expression of CCR4 on (a) CD4+ T cells, (b) CD8+ T cells, (c) CD4-CD8-T cells; CCR5 on (d) CD4+ T cells, (e) CD8+ T cells (f) CD4-CD8- T cells from HC (n=5), JIA (n= 10), T21 (n= 11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors/group. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other groups. *p<0.05 significantly different compared to other groups.
Figure 4. 12 CXCR3 and CXCR6 expressions on T cells in DA differ. Bar charts depict the expression of CXCR3 on (a) CD4+ T cells (b) CD8+ T cells, (c) CD4- CD8- T cells; CXCR6 on (d) CD4+ T cells, (e) CD8+ T cells (f) CD4- CD8- T cells from HC (n=5), JIA (n= 10), T21 (n= 11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other groups. *p<0.05 significantly different compared to other groups.
4.4.4 IFN-γ R1 expression on CD8 T cells higher in DA compared to HC, JIA and T21

Since it is understood that T21 results in increased expression of IFN receptors which constantly activate IFN pathways (Sullivan et al., 2016; Araya et al., 2019; Powers et al., 2019), we next examined the IFN-γ R1 (CD119) expression on T cells. CD4-CD8- T cell subpopulations were excluded from this analysis since their total cell percentages were less than 10% thus it would provide inaccurate data. Gating strategy as per Figure 4.13 was applied. Representative dot plots (Figure 4.14(a)(i)) and quantification (Figure 4.14(a)(ii)) demonstrate no changes were observed in the IFN-γ R1 expression in the CD4+ T cell population across all groups. IFN-γ R1 expression in the CD8+ T cell population was significantly increased in DA compared to HC ($p<0.05$) and JIA ($p<0.01$) with a trending increase compared to T21 (Figure 4.14(b)).
Figure 4. 13 Gating strategy for IFN-γ R1 panel. PBMC were stained with a panel of fluorochrome antibodies according to Table 4.3. Representative dot plots depicting the gating strategy used to identify and phenotype PBMC with the gating strategy from Figure 4.1(a)-(d) used. (a) CD3+, monocytes and CD3- cells were selected (b) CD4+ T cells were gated against CD8+ T cells and from these T cell subpopulations (c) IFN-γ R1 was gated against (d) FMO (e) further phenotyping into the different memory T cell subpopulations and from these subpopulations (f) IFN-γ R1 was gated against (g) FMO.
Figure 4. 14 IFN-γ R1 expressions on CD8+ T cells populations in DA are increased. (i) Representative flow plots describe frequency percentage of (a) CD4+ T cells and (b) CD8+ T cells positive for IFN-γ R1 across all four groups. (ii) Quantification describes IFN-γ R1 positive T cells of HC (n=5), JIA (n=10), T21 (n=11) and DA (n=15). Values expressed as mean ± SEM of n=5-15 donors. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis, #p<0.05, ##p<0.01 significantly different compared to other groups.
4.4.5 Increased frequency of memory T cell subpopulations in DA

Memory T cells are antigen-specific T cells that remain long-term after antigen-exposure (Chang et al., 2021). Specifically, CD4+ memory T cells and CD8+ memory T cells can stimulate site-specific disease recurrence and joint injury as well as recruit other immune cells to the site of inflammation (Brennan et al., 2008; Cho et al., 2012; Khanniche et al., 2019; Takeshita et al., 2019; Chang et al., 2021). We assessed these populations via flow cytometric analysis according to Table 4.2 and applied the gating strategy according to Figure 4.9. We examine effector memory T cells (CD45RO+ CCR7-) which are memory T cells that are in the periphery and can navigate through peripheral organs to the inflamed tissue after cytokine or chemokine stimulation and exacerbate the immune response; central memory T cells (CD45RO+ CCR7+) are stimulated by APCs before recirculating to the inflamed tissue; naïve T cells (CD45RO+ CCR7-) are T cells which have not encountered an APC or antigen before; and terminally differentiated T cells (TEMRA) (CD45RO- CCR7-) display potent effector function after activation (Golubovskaya et al., 2016; Gray et al., 2018; Sani et al., 2019; Chang et al., 2021).

First, we assessed the different memory T cell subpopulations. Effector memory T cell frequency was higher in DA compared to T21 (p<0.01) CD4+ T cells (Figure 4.15(a)) and CD8+ population (p<0.05) (Figure 4.15(e)), in addition, they were increased in DA compared to JIA (p<0.05) within CD8+ T cells (Figure 4.15(e)). Central memory T cell frequency was increased in DA compared to T21 (p<0.01) in CD4+ T cells (Figure 4.15(b)), and CD8+ T cells (p<0.05) (Figure 4.15(f)). CD4+ naïve T cells were decreased in DA compared to T21 (p<0.05) on CD4+ T cells (Figure 4.15(c)) while CD8+ naïve T cells were decreased in DA compared to JIA (p<0.05) and T21 (p<0.01) (Figure 4.15(g)). No changes were observed in TEMRA for all groups within both CD4+ and CD8+ T cell compartments (Figure 4.15(d) and (h)).
Figure 4. Memory T cell subpopulations differ in DA compared to HC, JIA and T21. Bar charts depict the expression of (a) effector memory, (b) central memory, (c) naive, (d) \text{T}_{EMRA}\ T cells in the CD4+ T cell populations and (e) effector memory, (f) central memory, (g) naive (h) \text{T}_{EMRA}\ T cells in the CD8+ T cell populations of HC (n=5), JIA (n= 10), T21 (n= 11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other groups. *p<0.05, **p<0.01 significantly different compared to other groups.
4.4.6 Chemokine Receptor Expression on Memory T cells

Comparing the CCR1 expression within memory T cell subpopulations, we demonstrated that CCR1 frequency was decreased in DA compared to HC ($p=0.05$) (Figure 4.16(a)) in CD4+ effector memory T cells, with similar frequencies also observed for T21. CCR1 was decreased in DA compared to T21 ($p=0.05$) CD8+ effector memory T cells (Figure 4.16(e)). CCR1 frequency was decreased in DA compared to HC ($p<0.01$) in CD4+ central memory T cells with similar frequencies observed for T21 (Figure 4.16(b)). No changes in CCR1 frequency in CD8+ central memory T cells were observed across all groups (Figure 4.16(f)).

CCR1 frequency was reduced in DA compared to HC ($p<0.05$) CD4+ naïve T cells (Figure 4.16(c)), again similar frequencies were also observed for T21. No changes were demonstrated across all groups in CD8+ naïve T cells (Figure 4.16(g)) and for both CD4+ and CD8+ TEMRA cells (Figure 4.16(d) and (h)).

CCR2 frequency was increased in DA compared to T21 ($p<0.01$) in CD4+ effector memory T cells (Figure 4.17(a)), while no changes were documented across all groups in CD8+ effector memory T cells (Figure 4.17(e)). CCR2 frequency was increased in DA compared to JIA ($p<0.05$) and T21 ($p<0.0001$) CD4+ central memory T cells (Figure 4.17(b)). No changes were reported across all groups in CD8+ central memory T cells (Figure 4.17(f)).

CCR2 frequency was increased in DA compared to T21 in CD4+ naïve T cells ($p<0.05$) (Figure 4.17(c)) and CD8+ naïve T cells ($p=0.07$) (Figure 4.17(g)). No changes were observed for CD4+ and CD8+ TEMRA cells (Figure 4.17(d) and (h)).

CCR4 frequency was decreased in DA compared to T21 ($p<0.01$) within the CD4+ effector memory T cell compartment (Figure 4.18(a)) while no changes were observed across any groups within CD8+ effector memory T cells (Figure 4.18(e)). CCR4 frequency was decreased in DA compared to T21 ($p<0.05$) CD4+ central memory T cells (Figure 4.18(b)) while no changes were observed in CD8+ central memory T cells in any group (Figure 4.18(f)). No changes in CD4+ naïve T cells were observed in any group (Figure 4.18(c)) while CCR4 frequency increased in DA compared to HC ($p<0.05$) in CD8+ naïve T cells with a trending increase compared to T21 (Figure 4.18(g)). CCR4 frequency was decreased in DA compared to T21 on both CD4+ TEMRA cells ($p<0.01$) (Figure 4.18(d)) and CD8+ TEMRA cells ($p<0.05$) (Figure 4.18(h)).
There were no changes in CCR5 frequency reported across all groups for both CD4+ and CD8+ effector memory T cells (Figure 4.19(a) and (e)), CD4+ and CD8+ central memory T cells (Figure 4.19(b) and (f)), CD4+ and CD8+ naïve T cells (Figure 4.19(c) and (g)) and CD4+ TEMRA cells (Figure 4.19(d)). CCR5 frequency was increased in DA compared to HC (p<0.05) in CD8+ TEMRA cells (Figure 4.19(h)).

No changes were observed for CXCR3 frequency across all groups for both CD4+ and CD8+ effector memory T cells (Figure 4.20(a) and (e)), CD4+ and CD8+ central memory T cells (Figure 4.20(b) and (f)). CXCR3 frequency in DA was increased compared to T21 (p=0.06) in CD4+ naïve T cells (Figure 4.20(c)). No changes were reported in CD8+ naïve T cells (Figure 4.20(g)) and CD4+ and CD8+ TEMRA cells (Figure 4.20(d) and (h)).

There were no changes documented for CXCR6 frequency across all groups in CD4+ and CD8+ effector memory T cells (Figure 4.21(a) and (e)) and CD4+ central memory T cells (Figure 4.21(b)). CXCR6 frequency was increased in DA compared to HC (p<0.05) (Figure 4.21(f)) within the CD8+ central memory T cells, however similar frequencies were observed for T21. No changes were reported across all groups in CD4+ and CD8+ naïve T cells (Figure 4.21(c) and (g)) and CD4+ and CD8+ TEMRA cells (Figure 4.21(d) and (h)).
Figure 4. 16 CCR1 expression on different memory T cells populations in DA differ. Bar charts depict the expression of CCR1 on (a) CD4+ effector memory (b) CD4+ central memory (c) CD4+ naïve T cells (d) CD4+ T<sub>EMRA</sub> (e) CD8+ effector memory (f) CD8+ central memory (g) CD8+ naïve T cells (h) CD8+ T<sub>EMRA</sub> T cells of HC (n=5), JIA (n=10), T21 (n=11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors/group. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis, #p<0.05, ##p<0.01 significantly different compared to other groups.
Figure 4. 17 CCR2 expression on different memory T cells populations in DA differ. Bar charts depict the expression of CCR2 on (a) CD4+ effector memory (b) CD4+ central memory (c) CD4+ naïve T cells (d) CD4+ TEMRA (e) CD8+ effector memory (f) CD8+ central memory (g) CD8+ naïve T cells (h) CD8+ TEMRA T cells of HC (n=5), JIA (n=10), T21 (n=11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis respectively #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 significantly different compared to other groups. *p<0.05, **p<0.01 significantly different compared to other groups.
Figure 4. 18 CCR4 expression on different memory T cells populations in DA differ. Bar charts depict the expression of CCR4 on (a) CD4+ effector memory (b) CD4+ central memory (c) CD4+ naïve T cells (d) CD4+ TEMRA (e) CD8+ effector memory (f) CD8+ central memory (g) CD8+ naïve T cells (h) CD8+ TEMRA T cells of HC (n=5), JIA (n=10), T21 (n=11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis #p<0.05, ##p<0.01 significantly different compared to other groups.
Figure 4. 19 CCR5 expression on different memory T cells populations in DA differ. Bar charts depict the expression of CCR5 on (a) CD4+ effector memory (b) CD4+ central memory (c) CD4+ naive T cells (d) CD4+ TEMRA (e) CD8+ effector memory (f) CD8+ central memory (g) CD8+ naive T cells (h) CD8+ TEMRA T cells of HC (n=5), JIA (n=10), T21 (n=11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors. One-way ANOVA (#) and paired t-test (*) were used for statistical analysis #p<0.05, ##p<0.01 significantly different compared to other groups.
Figure 4. 20 CXCR3 expression on different memory T cells populations in DA differ. Bar charts depict the expression of CXCR3 on (a) CD4+ effector memory (b) CD4+ central memory (c) CD4+ naïve T cells (d) CD4+ T<sub>EMRA</sub> (e) CD8+ effector memory (f) CD8+ central memory (g) CD8+ naïve T cells (h) CD8+ T<sub>EMRA</sub> T cells of HC (n=5), JIA (n=10), T21 (n=11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other groups.
Figure 4. 21 CXCR6 expressions on different memory T cells populations in DA differ. Bar charts depict the expression of CXCR6 on (a) CD4+ effector memory (b) CD4+ central memory (c) CD4+ naïve T cells (d) CD4+ TEMRA (e) CD8+ effector memory (f) CD8+ central memory (g) CD8+ naïve T cells (h) CD8+ TEMRA T cells of HC (n=5), JIA (n=10), T21 (n=11) and DA (n=15) groups. Values expressed as mean ± SEM of n=5-15 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis #p<0.05 significantly different compared to other groups.
4.4.7 IFN-γ R1 expression across the different memory T cell subpopulations were similar

We then examined the IFN-γ R1 expression across the different memory T cell subpopulations. Due to technical issues, HCs were not included. No changes in IFN-γ R1 expression were observed across all groups for the CD4+ and CD8+ effector memory T cells (Figure 4.22(a) and (d)), CD4+ and CD8+ central memory T cells (Figure 4.22(b) and (f)), CD4+ and CD8+ naive T cells (Figure 4.22(c) and (g)), CD4+ TEMRA (Figure 4.22(d) and (h)).

Figure 4. 22 IFN-γ R1 expression on different memory T cell subpopulations are similar in JIA, T21 and DA. Bar charts depict the expression of IFN-γ R1 on (a) CD4+ effector memory (b) CD4+ central memory (c) CD4+ naive T cells (d) CD4+ TEMRA (e) CD8+ effector memory (f) CD8+ central memory (g) CD8+ naive T cells and (h) CD8+ TEMRA T cells of JIA (n=8), T21 (n=10) and DA (n=11) groups. Values expressed as mean ± SEM of n=8-11 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis.
4.4.8 Nonclassical monocytes express reduced CD86 in DA compared to T21

Next we examined monocyte phenotype and activation as they are known to be primed in the circulation in adult forms of IA (Marzaioli et al., 2020; McGarry et al., 2021; Hanlon et al., 2023). We used fluorochrome-conjugated antibodies outlined in Table 4.4 and applied the gating strategy as per Figure 4.23. First, we examined the frequencies of different monocyte subsets across the three groups. In Figure 4.24, we demonstrate that while the frequency of nonclassical monocytes is increased in T21 compared to JIA ($p=0.05$), no significant changes were observed for DA compared to T21 (Figure 4.24(a)). While no significant changes were observed in intermediate monocytes frequencies across all groups, there was a trending decrease in DA compared to T21 (Figure 4.24(b)). While the frequency of classical monocytes was decreased in T21 compared to JIA ($p=0.06$), no significant changes were documented in DA compared to T21 (Figure 4.24(c)).

Next, we investigated different markers involved in autoimmunity and inflammation within the nonclassical monocyte subset. We assessed the expression of CD15 as this is a marker of activated monocytes (Nakayama et al., 2001). No changes in CD15 expression were observed across all groups (Figure 4.25(a)). Next, we analysed CD33 surface expression on monocyte subsets since high frequencies of CD33+ monocytes are found in the periphery of RA patients (Cravens et al., 2007). CD33 was expressed at very low levels in the nonclassical monocytes subsets, and no changes were observed across the 3 groups (Figure 4.25(b)). We then assessed the expression of CD38 since CD38+ monocytes are induced in inflammation (Amici et al., 2018) and involved in bone resorption (Ma et al., 2022). No changes in CD38 expressions were observed across all groups (Figure 4.25(c)). We also analysed the expression of PD-1 as this is a known inhibitor of T cell activation (Latchman et al., 2001), and no changes were observed across the groups (Figure 4.25(d)). We also assessed the expressions of CD80 and CD86 since synovial monocytes are known to have increased expression of costimulatory CD80 and CD86 in RA compared to HC and these molecules also activate T cells in RA (Yoon et al., 2014; Roszkowski et al., 2021). No changes were observed for the expression of CD80 across all groups (Figure 4.25(e)). The frequency of CD86 was increased in T21 compared to JIA ($p<0.05$) and reduced in DA compared to T21 ($p<0.01$) (Figure 4.25(f)). We then examined the expression of a checkpoint inhibitor, V-domain Ig suppressor of T cell activation (VISTA), as VISTA knock-
out reduced CIA in mouse models (Ceeraz et al., 2017; Broughton et al., 2019). No changes were observed for the frequency of VISTA across all groups (Figure 4.25(g)).

Within the intermediate monocyte subpopulation, we observed no changes in the expression of CD15 (Figure 4.26(a)). The expression of CD33 was increased in DA compared to HC ($p<0.01$) and compared to T21 ($p<0.05$) (Figure 4.26(b)). The expression of CD38 was increased in DA compared to T21 ($p=0.07$) (Figure 4.26(c)). No changes in the expression of PD-1 and CD80 were reported across all groups (Figure 4.26(d–e)). The expression of CD86 was increased in T21 compared to JIA ($p=0.05$) and reduced in DA compared to T21 ($p<0.05$) (Figure 4.26(f)). No changes in the expression of VISTA were reported across all groups (Figure 4.26(g)).

Within the classical monocyte subpopulation, the expression of CD15 was increased in T21 compared to JIA ($p<0.05$) and decreased in DA compared to T21 ($p<0.05$) (Figure 4.27(a)). The expression of CD33 was increased in DA compared to T21, however this did not reach significance (Figure 4.27(b)). No changes were reported in the expression of CD38, PD-1, CD80 and CD86 across all groups (Figure 4.27(c–f)). The expression of VISTA was decreased in DA compared to T21; however, this did not reach significance (Figure 4.27(g)).
Figure 4. 23 Gating strategy for monocyte panel. PBMC were stained with a panel of fluorochrome antibodies according to Table 4.4. Gating strategy used to exclude debris, doublets and dead cells from gating strategy 4.1 (a)-(d) were applied. (a) CD3+ cells were removed from analysis (b) CD11c+ cells were selected before (c) CD14- CD16- cells were removed and (d) further phenotyping into the different monocyte subsets with (e) example marker gated according to (f) example FMO.
Figure 4. Frequencies of monocyte subsets are similar in JIA, T21 and DA. Bar charts demonstrate the percentage frequency of (a) nonclassical monocytes, (b) intermediate monocytes and (c) classical monocytes in JIA (n=8), T21 (n=10) and DA (n=11). Values expressed as mean ± SEM of n=8-11 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis.
Figure 4. Frequency of nonclassical monocytes expressing CD86 is reduced in DA compared to T21. Bar charts demonstrating the frequency percentage of nonclassical monocytes that express (a) CD15, (b) CD33, (c) CD38, (d) PD-1, (e) CD80, (f) CD86 and (g) VISTA in JIA (n=8), T21 (n=10) and DA (n=11). Values expressed as mean ± SEM of n=8-11 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis respectively #p<0.05, ###p<0.01 significantly different compared to other groups. *p<0.05 significantly different compared to other groups.
Figure 4. Frequency of intermediate monocytes expressing CD86 is reduced in DA compared to T21. Bar charts demonstrating the frequency percentage of intermediate monocytes that express (a) CD15, (b) CD33, (c) CD38, (d) PD-1, (e) CD80, (f) CD86 and (g) VISTA in JIA (n=8), T21 (n=10) and DA (n=11). Values expressed as mean ± SEM of n=8-11 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other groups. *p<0.05 significantly different compared to other groups.
Figure 4. Frequency of classical monocytes expressing CD15 is reduced in DA compared to T21. Bar charts demonstrating the frequency percentage of classical monocytes that express (a) CD15, (b) CD33, (c) CD38, (d) PD-1, (e) CD80, (f) CD86 and (g) VISTA in JIA (n=8), T21 (n=10) and DA (n=11). Values expressed as mean ± SEM of n=8-11 donors. One-way ANOVA (#) and paired t-test (*) was used for statistical analysis respectively #p<0.05 significantly different compared to other groups.
4.4.9 Classical monocytes are dominant in DA

We then analysed peripheral monocytes by unbiased, unsupervised, multidimensional analysis of flow cytometric data results in cell clustering which was performed using the tSNE algorithm (Kobak et al., 2019). Additionally, tSNE performs dimensionality reduction of complex data by clustering cells that have similar expression profiles. HC were excluded from this analysis due to the smaller sample size across all groups for that particular experiment run which included HC.

Total peripheral monocytes were analysed using unsupervised clustering by tSNE algorithm after data concatenation. Figure 4.28(a)(i) demonstrates that 12 clusters of different monocyte phenotypes were identified through concatenated data for JIA, T21 and DA. Following this, the clusters were further categorised according to their frequency percentage of each marker included in the flow cytometric staining (Table 4.4) for all donors (Figure 4.28(a)(ii)). The line graph describes the intensity of which each marker was expressed in all 12 clusters (Figure 4.28(a)(iii)). Quantification revealed that the most dominant cluster in JIA was cluster 4 (Figure 4.28(b)(i)), cluster 2 was the most dominant cluster in T21 (Figure 4.28(b)(ii)) while cluster 1 and 3 were the most dominant clusters in DA (Figure 4.28(b)(iii)).

Quantification (Figure 4.29(a)(i)) demonstrated that 32.5% of the total clusters in JIA are cluster 4 with data concatenation showing the cluster visualization (Figure 4.29(a)(iii)). Line graphs describe the intensity of which each marker is expressed in that specific cluster where the most prominent markers within cluster 4 are CD14, CD38 and CD11c (Figure 4.29(a)(iii)). This demonstrates that the most dominant cells within JIA are classical monocytes. Quantification (Figure 4.29(b)(i)) demonstrated that 18% of the total clusters in T21 are cluster 2 with data concatenation showing the cluster visualization (Figure 4.29(b)(iii)). The most prominent markers within this cluster are CD16, CD38 and CD11c (Figure 4.29(b)(iii)). This describes a nonclassical monocyte phenotype. Quantification (Figure 4.29(c)(i) and (iv)) demonstrated that 21% of the total clusters are cluster 1 and 3 with data concatenation showing the cluster visualization (Figure 4.29(c)(ii) and (v)). The most prominent markers within these clusters are CD11c, CD14, CD38 and CD86 (Figure 4.29(b)(iii)). Together, this reveals that DA has a classical monocyte phenotype that is more activated and has a high potential to activate effector T cells.
Figure 4.30(a)(i) and (ii) demonstrates the concatenated data and visualization according to the four main clusters observed altogether across JIA, T21 and DA. The heatmap (Figure 4.30(a)(iii)) demonstrates that cluster 1 and 3 (found in DA), in addition to the high expression of markers described in Figure 4.30(b)(iii), have low CD16, CD80, VISTA, PD-1, BTLA and CD15. Cluster 2 (found in T21) demonstrates that the lowly expressed markers are CD11b, CD33, CD14, CD80, VISTA, PD-1, BTLA, CD86 and CD15 (Figure 4.30(a)(iii)). Cluster 4 (found in JIA) demonstrated low expressions of CD11b, CD33, CD16, CD80, VISTA, PD-1, BTLA and CD15.
Figure 4. 28 Cluster 1 and 3 prominent in DA and is different compared to JIA and T21. Multiparametric flow cytometric data of (a) total peripheral monocytes subjected to unsupervised clustering by tSNE algorithm following (i) visualization of monocyte clusters based on CD14 and CD16 populations. (ii) Bar chart describing the number of events related to each cluster (iii) Line graphs describe the intensity of which each marker is expressed within the cluster. (b) Bar charts describe the most dominant cluster within (i) JIA (n=4), (ii) T21 (n=5) and (iii) DA (n=5).
Figure 4. 29 Cluster 1 and 3 prominent in DA and is different compared to JIA and T21. Multiparametric flow cytometric data of total peripheral monocytes subjected to unsupervised clustering by tSNE algorithm within (a) JIA (n=4), (b) T21 (n=5) and (c) DA (n=5). (i) and (iv) Bar charts describe the most dominant clusters within each group, (ii) visualization of cluster based on CD14 and CD16 expression (v) data concatenation of monocytes based on CD14 and CD16 populations and (iii) and (vi) Line graphs describe intensity of each marker expressed within the cluster.
Figure 4. 30 Cluster 1, 2, 3 and 4 prominent in DA, JIA and T21. Multiparametric flow cytometric data of (a) total peripheral monocytes subjected to unsupervised clustering by tSNE algorithm with (i) visualization of clusters based on CD14 and CD16 expression for JIA (n=4), T21 (n=5) and DA (n=5) combined. (ii) Line graphs describe intensity of which each marker is expressed within the four clusters, (iii) heatmap or phonograph describes the intensity of how each marker is expressed in each cluster.
4.5 Discussion

In this chapter, we performed in depth phenotypical and functional analysis of both T cell and monocyte subsets, thus exploring innate and adaptive immune dysregulation in children with DA. We demonstrate that children with DA have increased frequency of peripheral CD8+ T cells concomitant with a reduction in circulating CD4+ T cells. This dysregulation in T cell frequencies is coupled with increased polyfunctional and proinflammatory functions compared to children with DS alone. Furthermore, we highlight differential expression of tissue homing chemokine receptors in both CD4+ and CD8+ T cells in DA which may be responsible for T cell infiltration to the joint. We demonstrate increased expression of both effector and central memory T cell subpopulations in DA, coupled with differential expression of their respective chemokine receptors. Finally, we highlight for the first time, a previously undescribed dysregulation in innate immune regulation whereby children with DA display a monocyte signature that is predominantly that of a classical profile coupled with differential expression of costimulatory and coinhibitory receptors CD86, BTLA and VISTA.

In this study, we demonstrate an enrichment of proinflammatory T cells in DA, characterised by increased TNF-α, IL-17a and IFN-γ expression within the CD4+ T cell compartment in addition to a marked increase in IFN-γ expression within the CD8+ T cell subpopulation. Furthermore, in addition to an enrichment in these aforementioned cytokines we report increased polyfunctionality and plasticity in the cytokine response from CD4+, CD8+ and CD4-CD8- T cells. Polyfunctionality, or the ability of cells to produce multiple cytokines simultaneously within the same cell has previously been described in the pathogenesis of adult rheumatological diseases such as RA and PsA (Basdeo et al., 2015; Wade et al., 2018). Polyfunctional T cells are immunologically distinct from monofunctional T cells whereby they display enhanced chemotaxis, activation and indeed metabolism (Basdeo et al., 2015; Wade et al., 2018; Floudas et al., 2022) and are resistant to Treg suppression (Basdeo et al., 2015). Furthermore, previous studies demonstrated that specific targeting of pathogenic polyfunctional T cells may offer greater therapeutic benefit than that of monofunctional cells (Wade et al., 2018). Collectively the emergence of a polyfunctional T cell phenotype across all T cell lineages (CD4+, CD8+ and CD4-CD8-) strongly suggests that they represent important contributors to DA disease pathogenesis.
While PMA and ionomycin were utilized in this to examine cytokine levels *ex vivo*, additional experiments utilising anti-CD3 and anti-CD28 would allow T cell proliferation and more robust cytokine responses.

In addition to increased polyfunctionality, T cells from DA patients also display an enhanced chemotactic profile as demonstrated by increased expression of chemokine receptor expression across all T cell lineages examined (CD4+, CD8+ and CD4- CD8-). Specifically, we report a significant increase in CCR2, CCR5 and CXCR3 expression in peripheral CD4+ T cells in DA compared to T21 in addition to increased CCR2 expression in CD8+ and CD4- CD8- cells. Increased CCR5 expression on CD4+ T cells can be a hallmark of expanded Tfh cells, and while not exclusively examined within this study, previous work by Foley *et al.* (2020) reported increased Tfh and Tph in children with DA. (Foley *et al.*, 2020). Additional studies examining immune cell dysregulation in DA are rare, however, chemokines such as CCL5 and CCL3 are enriched in the synovium of JIA patients (Pharoah *et al.*, 2006) and thus may facilitate the recruitment of these CCR5+ or CXCR3+ immune cells into the inflamed joint. Furthermore, we also report a significant increase in the expression of CCR1 in CD4+ and CD8+ T cells DA compared to children in the general population – thus suggesting that dysregulated chemokine expression may be an attributed combined feature of both DS and autoimmunity. At present there are conflicting studies which show that peripheral immune cells may or may not correlate with joint inflammation in RA and PsA. Van Amelsfort *et al.*, (2004) demonstrated that there is a higher frequency of immunosuppressive CD4+ CD25+ Tregs in the RA joint compared to the periphery (Van Amelsfort *et al.*, 2004). Interestingly, another study showed that there is a positive relationship between synovial IL-17a+ CD8+ T cells and PsA disease activity (Menon *et al.*, 2014). Additionally, Su *et al.*, (2016) demonstrated how RA patients treated with $^{99}$Tc-methylene diphosphonate had increased peripheral γδ T cells and Tregs which was associated with reduced disease activity (Su *et al.*, 2016). However, in general there are no defined routine systemic biomarkers (soluble mediators or cellular phenotype) that are associated with joint inflammation in inflammatory forms of arthritis, other than ACPA for the definition of RA. While CRP and ESR are utilised as a measure of inflammation across all patients with rheumatic diseases, studies have highlighted the limitations of CRP and ESR in assessing Disease Activity at the
site of inflammation (Orr et al., 2018). According to the patients’ clinical data and the polyfunctionality data in Chapter 4, there is a positive correlation between the donors who have exhibited increased polyfunctionality and active joint count. However, as highlighted by Foley et al., (2019), systemic markers of inflammation including CRP and ESR do not reflect the level of joint inflammation in children with DA (Foley et al., 2019). Indeed, this is possibly one of the reasons for delayed diagnosis of these vulnerable children.

Next, we examined the frequency and phenotype of memory and naive T cells within the circulation of children with DA. Interestingly we report that there is a significant increase in both central memory and effector memory T cells (CD4+ and CD8+) in DA compared to T21 paralleled by a decrease in circulating naive T cells. Thus, this suggests that there is a significant increase in antigen experienced T cells coupled with increased T cell differentiation in DA compared to T21, further highlighting the additional autoimmune dysregulation which occurs in these patients. Interestingly, previous studies by Patrick et al. (2022) reported no significant difference between circulating naive and memory CD4+ and CD8+ T cells in HC and JIA patients, in agreement with the data presented in this chapter (Patrick et al., 2022). However, the increased dysregulation in memory and naive T cells reported in this study between DA and T21 may provide further evidence of the increased aggressive and erosive clinical phenotype associated with DA compared to children with JIA, whereby increased memory cell infiltration to the joint may potentiate synovial inflammation even in the presence of limited self-antigen. Of note we also report subtle differences in chemokine receptor expression on central and effector memory T cells which may also affect recruitment to the inflamed joint. Specifically, we observed higher CCR2 expression in the effector and central memory T cells coupled with a decrease in both CCR4 and CCR1 effector memory T cells. While the functional implications of these differential chemotactic phenotypes have yet to be explored, this study is the first to demonstrate alterations in both memory T cell frequency and chemokine expression in DA.

Interestingly we report no significant alterations in the expression of IFN-γ R1 on CD4+/CD8+/CD-CD8- and all T memory cell subsets in DA compared to T21. Given that chromosome 21 encodes for four of the six IFN receptors (Sullivan et al., 2016; Araya et
al., 2019) this was an unexpected result. However, given the complexity of immune
dysregulation reported in T21 and indeed DA, additional immune subsets not examined
within this study may be the source of enhanced IFN signalling. Furthermore, an additional
plausible explanation for unaltered IFN-γ R1 on circulating T cells may be due to the
increased IFN-γ levels found in the DA periphery, binding of which to the IFN-γ R1 may
cause internalization of the receptor.

One limitation of this chapter was that the absolute numbers of cells should be included
in addition to percentage frequencies. Future flow experiments will include absolute
numbers.

Uniquely within this study we also examined key innate immune cells, i.e. monocytes
which have previously been shown to harbour increased inflammatory potential in the
circulation of RA patients (Marzaioli et al., 2020; McGarry et al., 2021). While this is the
first study to investigate monocyte phenotypes in DA, we observed no significant changes
in the monocyte subset frequencies. Furthermore, we highlighted decreased expression
of CD86 in both nonclassical and intermediate monocytes in DA compared to T21.
Moreover, we also report increased expression of CD38 and CD33 on intermediate
monocytes coupled with increased frequency of CD15+ classical monocytes. While
decreased in CD86 expression may suggest a potential reduction in T cell stimulatory
capacity in DA, increased CD38 expression is known to boost antigen presentation to T
cells (Zilber et al., 2000) thus highlighting the complexity of monocyte maturation in DA.
Indeed, it is likely that a sensitive balance of costimulatory/coinhibitory molecules is
needed, respective to the particular self-antigen, which in the case of DA, is still unknown.
In order to assess the downstream functional effects of our reported dysregulation in
monocyte maturation markers, co-culturing with T cells and assessment of T cell
proliferation is likely to yield more definitive answers.

Additionally unsupervised clustering demonstrates differential clustering, showing that
DA monocytes predominately belong to clusters which display a classical monocyte
phenotype. Further analysis reveals that DA cluster 1 and 3 both have a classical
phenotype expressing high CD14 and low CD16. Additionally, both cluster 1 and 3 express
high levels of CD38 – a marker that is linked to osteoclast formation and bone resorption
(Ma et al., 2022) therefore contributing to bone degradation as well as T cell costimulatory
molecule, CD86 (Sansom et al., 2003; Qureshi et al., 2011) resulting in higher levels of T cell activation in DA. Both clusters also have low BTLA expression thus potentially reinforcing their inability to inhibit T cell and B cell responses leading to inflammation (Higashioka et al., 2021; Ning et al., 2021). The major difference between cluster 1 and 3 is that cluster 3 has low expressions of CD80, another T cell costimulatory molecule (Yoon et al., 2014). T21 monocytes are mainly cluster 2 and are phenotypically intermediate monocytes. Interestingly, cluster 2 has low expressions of CD80 and CD86 making cluster 1 and cluster 3 in DA more phenotypically proinflammatory.

**Figure 4. 31 Summary of Chapter 4.** Peripheral immune cells in DA with increased expression of chemokine receptors, production and expression of cytokines from T cells and a classical monocyte phenotype (made with BioRender).

In conclusion, we report for that first time that children with DA have dysregulated frequencies of CD4+ and CD8+ T cells with increased polyfunctional, proinflammatory functions and chemotactic phenotypes. We demonstrate a dominance in effector and central memory T cell subsets in DA coupled with differential expression of their respective chemokine receptors. Moreover, we highlight for the first time a previously
undescribed innate dysregulation in monocytes whereby children with DA display a signature that is predominantly that of a classical phenotype coupled with differential expression of costimulatory and coinhibitory receptors. Collectively, these perturbations may be responsible for the increased erosive disease observed at the clinical level reported in DA, either via increased production of T cell derived cytokines, increased migration of T cells to the joint, enhanced T cell responses or via increased T cell activation via monocytes. Interestingly, the predominant cytokines associated with polyfunctional T cells in DA, were TNF-α and IFN-γ, which we demonstrated had a significant synergistic effect on both DA FLS and EC pathogenic function in Chapter 2 and 3, therefore it has implications for combination/adjuvant therapy. Finally, results in this chapter examined T cell and monocyte dysregulation within the peripheral blood of DA patients. Previous studies by Spreafico et al. (2016) successfully identified a subset of peripheral blood CD4+ T cells which accurately reflected the phenotypical signature of T cell infiltrating the inflamed synovium of JIA patients (Spreafico et al., 2016). Thus, further emphasising that pathogenic immune cells contributing to synovial inflammation may be identifiable within the circulation of patients thus increasing the potential impact of this work.
CHAPTER 5

General Discussion and Future Direction
5.1 General Discussion

Until recently, studies on Down syndrome-associated arthritis were limited (Yancey et al., 1984; Olson et al., 1990; Padmakumar et al., 2002; Juj et al., 2009). Over the last seven years, our collaborators in CHI at Crumlin performed a nationwide screening service for children with DS and demonstrated an increased risk of IA in these vulnerable children. Indeed, the data demonstrated that the prevalence was 2-3 times greater (1/50) than previously reported, and was 20 times greater than that of JIA (1/1000) (Foley et al., 2019). This study also demonstrated a significant delay in diagnosis and a more aggressive erosive disease in children with DA compared to JIA, in addition to identifying distinct clinical phenotypes. Expanding these studies, our group obtained matched PBMC and tissue, and demonstrated enrichment of polyfunctional T cells in DA compared to JIA, in addition to an increase in immune cell infiltrates in the synovium, paralleled by a thickened lining layer (Foley et al., 2020). Thus, the cellular and molecular analysis reflected the clinical aggressive erosive phenotype. First line treatment for DA was MTX, however it was exceptionally poorly tolerated. Intra-articular steroid joint injections and TNF-inhibitors have shown better responses with less side effects, however a significant proportion of children still fail to adequately respond or have sub-optimal responses, indicating that additional treatment options are required. As highlighted in Figure 1.3 (Chapter 1), DA has three clinical phenotypes compared to JIA which has seven clinical phenotypes, this observation along with the differences demonstrated in the cellular and molecular studies outlined above, strongly suggests that DA is a distinct disease to JIA.

The joint microenvironment involves complex cell-cell interactions and the secretion of copious pro-inflammatory mediators, which together shape the inflammatory responses across phenotypes, and even within phenotypes making the treatment strategies for individuals difficult. In adult IA including RA and PsA, complex ‘omic’ molecular analysis is being performed to help stratify treatment approaches with the ultimate goal of precision medicine. The range of targeted biotherapeutics currently available have significantly improved outcomes for RA and PsA. However, as highlighted above a significant proportion of patients still don’t respond or have sub-optimal responses, with only 1 in 3 achieving remission. Despite having a range of biotherapeutics, it is currently difficult to predict who will develop severe, erosive disease or who will respond to treatment. These
drugs are very expensive; thus ‘trial and error’ is not a cost-effective strategy and reduces the opportunity of treatment during the therapeutic window of opportunity to avoid irreversible joint destruction. This is largely due to lack of biomarkers that could identify the presence of disease, but also the lack of biomarkers that predict the right treatment from the onset of disease. Indeed, Foley et al. (2019), described lower systemic levels of inflammatory markers CRP and ESR in children with DA compared to JIA, despite the former displaying significantly more erosive disease (Foley et al., 2019). Therefore, current routine systemic markers of inflammation are not useful for identifying IA in children with DS, and diagnosis is more difficult due to the multifaceted co-morbidities associated with DS.

This thesis examined the effect of key cytokines and the joint microenvironment on cellular function of DA FLS and ECs, key cell types that play a critical role in synovial inflammation, through facilitating immune cell extravasation into the joint and in the invasion/degradation of adjacent cartilage and bone. Furthermore, we examined the immune cell profile in these children, specifically T cell activation, polyfunctionality, homing capacity, in addition to monocyte profiles. In this thesis, we examined the effect of T cell-derived cytokines on stromal cell activation in DA, thus providing better understanding of complex interactions between various cytokines that together potentiate the inflammatory response in the joint. Furthermore, we show that in addition to cytokine stimulation, the joint microenvironment (DA FLS-CM) can also induce a pathogenic phenotype in healthy ECs. Finally, we demonstrated that patients with DA have increased T cell polyfunctionality paralleled with increased chemokine receptors in T cells, enhanced memory T cell frequencies along with a nonclassical monocyte phenotype.

Thus, this thesis provides the first in-depth analysis of DA FLS function and regulation and provides significantly better knowledge of the immune cell dysregulation that orchestrates the inflammatory response. Better understanding of the cellular and molecular mechanisms involved in the pathogenesis of DA, will allow the development of biomarkers for identification of disease onset, progression and response to treatment, in addition to better selection of targeted therapies from the outset, thus improving patient outcomes long-term.
In Chapter 2, we examined the effect of cytokine synergy on the pro-inflammatory and invasive capacity of primary DA FLS. Specifically, we examined the effect of TNF-α, IL-17a and IFN-γ alone and in combination on DA FLS function. TNF-α, IL-17a and IFN-γ alone differentially regulated the expression of proinflammatory mediators, metabolic markers and matrix degrading enzymes in DA FLS. This was paralleled by a significant increase in leukocyte adhesive/migratory capacity, evident by the observed increased expression of key adhesion molecules and chemokine receptors. IL-17a and IFN-γ potentiated the effects of TNF-α on IL-6, MCP-1 and RANTES secretion compared to stimulation alone. Additionally, IFN-γ potentiated the effects of TNF-α on CXCR3, CXCR4 and ICAM-1, with no synergistic effect observed for IL-17a. The combination of cytokines was accompanied by a shift in the metabolic profile of DA FLS to glycolysis, in addition to potentiated/synergistic induction of key metabolic markers. Together these data suggests that key cytokine interactions enhance the aggressive phenotype of DA FLS, thus this has potential implications for combination therapy in the treatment of DA. Synergistic interactions between IL-17a and TNF-α are possibly mediated through the CUX1/IκBζ transcriptional factors that can form a molecular complex with NFκB, an effect that potentiates it’s transcriptional activation (Slowikowski et al., 2020; Kouri et al., 2022). Other studies have shown interactions between the NFκB pathway and MAPK pathways (Shinjo et al., 2016), in addition to Notch/STAT/NFκB/HIF-1α in RA FLS (Wei et al., 2020; Wang et al. 2021). Interestingly the dominant subtype of DA is poly-RF-neg, with data now emerging that these patients also develop psoriasis long-term, suggesting that targeting the IL-17a pathway could be a potential strategy considering it is efficacious in PsA (which is a seronegative arthritis) and not in RA. However, in our study the potentiation/synergistic effects were more pronounced for IFN-γ and TNF-α. Sullivan et al. (2016) previously demonstrated that T21 is associated with enhanced IFN transcriptional responses in FLS, peripheral monocytes and T cells (Sullivan et al., 2016), with studies also showing an enrichment of IFN receptors on chromosome 21, resulting in enhanced gene dosage of these receptors in DS (Powers et al., 2019). In the context of the current study in DA FLS, a genome-wide association study identified that TNF-α stimulated RA FLS promoted IFN-responsive genes, an effect mediated through epigenetic chromatin alterations (Ge et al., 2021). Thus there is an enrichment of a IFN molecular signature in DS and DA, and in TNF-α-stimulated RA FLS suggesting potential interactions.
between NFκB and Jak-STAT signalling (Ganster et al., 2005; Isozaki et al., 2008). A study by Qiao et al. (2013) demonstrated that IFN-γ activated promoters for the TNF-α gene loci via NFκB (Qiao et al., 2013), an effect that was potentiated by IFN-γ priming. Thus, this suggests that the enriched IFN molecular signature on chromosome 21, may influence or prime cellular responses, an in the context of this study, cellular responses to TNF-α.

As stated in both chapter 1 and 2, we now know FLS are not a homogenous population of cells, and at least 11 FLS populations exist within the inflamed joint (Croft et al., 2019; Wei et al., 2020; Floudas et al., 2022). These FLS clusters have different functional effects depending on their spatial organisational niches within the synovium, with some clusters displaying invasive function, while others regulate effector immune responses. Therefore, while the study by Ge et al. (2021) identified an enhanced IFN molecular signature on TNF-α stimulated FLS (i.e. expanded homogenous population), it would be interesting to identify which specific FLS clusters in the synovium display this enhanced signature and thus the functional implications. From a clinical perspective these finding suggest tofacitinib, a Jak-STAT pathway inhibitor (approved for adult IA) could be a potential treatment strategy for children with DA. Indeed, clinical follow-up of our current cohort has shown that even though TNF-inhibitors are better tolerated than MTX, still a significant proportion do not respond.

In Chapter 3, we demonstrated that the DA joint microenvironment and cytokine interactions could drive a pathogenic phenotype in healthy ECs. DA FLS-CM induced EC tube formation, migratory capabilities and leukocyte-EC adhesion, effects that were paralleled by increases in adhesion molecule and chemokine receptor expression. Furthermore, we examined the effect of TNF-α, IL-17a and IFN-γ, alone and in combination on EC function. IFN-γ and/or IL-17a potentiated/synergistically induced the effect of TNF-α on the secretion of IL-6, MCP-1, RANTES and IL-8, in addition to adhesion molecule and chemokine receptor expression. Thus, the DA joint is shaped by complex interactions between immune-stromal cells and their secreted pro-inflammatory mediators. Interestingly, while we demonstrated induction of a pathogenic EC phenotype, minimal changes in metabolism were observed, which was not expected. This could be due to limitations of the study which include time points and analysis of alternative pathways, in addition to the use of a cell line as opposed to primary synovial EC which are difficult to...
isolated and cultured. To address these further additional experiments will be performed to examine alternative energy pathways in addition to carbon tracing. Furthermore, while we demonstrated that DA FLS-CM induced EC function, it is unclear what mediators are present in the CM that drive this effect. We know from Chapter 2 that numerous pro-inflammatory mediators are secreted including IL-6, MCP-1, RANTES and IL-8, in addition to the pro-angiogenic protease MMP-9. Therefore, future studies will include performing a protein array, specifically focused on angiogenic mediators. In addition, we have obtained healthy control FLS, therefore the spontaneous secretion of angiogenic mediators will be compared between the DA and HC FLS. Similar to chapter 2, interactions between cytokines led to potentiation/synergistic effects on EC function. As outlined above and in chapter 3, several studies have shown potential transcriptional interactions between NFκB/STAT/Notch/MAPK/HIF-1α (Qiao et al., 2013; Gao et al., 2013; Gao et al., 2015). Interestingly, an eloquent study by Wei et al. (2020) in the RA synovium, demonstrated that Notch signalling drives both transcriptional and spatial gradients of FLS from the vascular ECs. Thus, FLS display spatial positional identity that is regulated by endothelium-derived Notch activation (Wei et al., 2020), further emphasising the complex intricate pathways that drive the synovial pathology in IA.

In Chapter 4, we demonstrated that patients with DA have impaired immune cell frequencies and function. Patients with DA have higher CD8+ T cell frequency with a reduced number of CD4+ T cells, increased proinflammatory cytokine production from both CD4+ and CD8+ T cells mirrored with an enhanced CD4+, CD8+ and CD4-CD8- T cell polyfunctionality with a particularly increased simultaneous production of both TNF-α and IFN-γ. This not only supports but it also expands on our previous work (Foley et al., 2020) as we are including CD4-CD8- T cell subpopulations and IL-2, IL-4, IL-21 and IL-22 in this analysis. The coexpressions of multiple cytokines were also increased in DA across all three T cell subpopulations. Furthermore, we demonstrated increased frequencies of effector memory and central memory T cells in DA, in contrast to decreased frequencies of naïve T cells. This thesis also adds new insights into the homing mechanisms that facilitate T cell migration to the joint where differential expression of CCR1, CCR2, CCR4 and CXCR3 across the T cell subsets were observed. Using tSNE analysis, we demonstrated that DA monocytes are phenotypically classical displaying CD11c, CD14, CD38 and CD86 with low
CD16, CD80, VISTA, PD-1, BTLA and CD15. JIA clusters also displayed a classical monocyte phenotype. In contrast, T21 displayed a non-classical monocyte phenotype. The profiles in both DA and JIA, are consistent with previous studies in monocytes in RA and PsA, where they display a more activated phenotype (typically classical and intermediate subtypes) (Marzaioli et al., 2020; McGarry et al., 2021), had increased homing markers (Marzaioli et al., 2020) and were shown to be more responsive to stimuli compared to HC monocytes (McGarry et al., 2021) suggesting monocytes in the systemic circulation of patients with IA (adults and children) are primed for heightened pro-inflammatory responses.

In this study we have demonstrated T cell polyfunctionality, DA FLS pathogenic responses to T cell-derived cytokines and angiogenic responses to the joint microenvironment. While we do not know the primary trigger of DA, it is most likely a combination of a genetic predisposition and an environmental insult (mouth, lungs or gut). The combination of genetics and an environmental insult leads to an abnormal systemic immune response, which would align with the data in this thesis where we observed an increase in circulatory pathogenic polyfunctional T cells in DA. For these T cells to migrate to the joint, a second hit is required. While we do not fully understand the sequence of events, the likelihood is that the T cells in the circulation are already primed to migrate to the joint, and that the second hit may simultaneously induce adhesion molecules and chemokine gradients on synovial EC and DA FLS that induce the migration of the pathogenic polyfunctional T cells from the systemic circulation to the joint. Once the T cells infiltrate the joint, the secretion of pro-inflammatory cytokines will induce FLS invasive mechanisms, in addition to activation of angiogenesis which will allow further infiltration of immune cells. Pathogenic DA FLS can in turn then activate T cells and ECs in joint, thus creating a vicious pro-inflammatory response leading to synovial hyperplasia and joint damage.

5.2 In Summary
In summary, the results outlined in chapters 2, 3 and 4 of this thesis clearly demonstrate a role for cytokine interactions in promoting mechanisms of synovial inflammation, ultimately leading to increased structural damage and disease progression. Our data shows that the joint microenvironment and cytokine interactions, particularly IFN-γ/TNF-α, can drive a number of complex mechanisms including secretion of proinflammatory
mediators, expression of adhesion molecule and chemokine receptors, in addition to altered cellular bioenergetics leading to a chronic cycle of pro-inflammatory responses. Our data also demonstrates pathogenic immune cell mechanisms that are distinct to DA, this along with the stromal cell responses gives further insight into the potential therapeutic agents that could be effectively targeted for the treatment of DA. Compared to research performed in JIA, the clinical, cellular and molecular research is very much in its infancy for DA. Thus, further in-depth analysis of immune-stromal cell function, histological and transcriptomic dysregulation in children with DA, across phenotypes, should provide the insight needed to make biologically and therapeutically meaningful changes.

Figure 5. 1 Summary of the aggressive stromal cell phenotypes in the DA microenvironment and aggressive immune cell phenotypes in the periphery (made with BioRender).
5.3 Future Direction

In this thesis we provide better understanding of the pathogenic mechanisms that are involved in driving inflammation in DA. Indeed, this is the only study to date to examine primary FLS from the joint of children with DA. In addition, we have significantly expanded our knowledge on the pathogenic profile of immune cells that define the DA phenotype.

Data from both Chapter 2 and 3 demonstrate synergistic interactions between IFN-γ and TNF-α, and data from Qiao et al. (2013), showed that IFN-γ can prime the TNF-α response in monocytes (Qiao et al., 2013). Thus, we are currently expanding these observations and performing experiments where DA FLS and primary synovial ECs are primed for 24 hrs with IFN-γ before stimulation with TNF-α. Preliminary data has shown that priming of DA FLS further potentiates the response of DA FLS to TNF-α stimulation compared to non-primed cells. Figure 5.2 below demonstrates that priming of DA FLS further potentiates the secretion of both IL-6 and RANTES from DA FLS in response to TNF-α, compared to non-primed cells. Current experiments are examining the downstream signalling pathways particularly NFκB, STAT and Notch. Furthermore, to examine if the enhanced inflammatory responses in primed cells is due to epigenetic alteration, DNA methylation and chromatin immunoprecipitation analysis will be performed. Thus, this will allow us to identify if IFN-γ alters promoter/enhancer at specific transcriptional sites.

Figure 5.2 Primary DA FLS were primed with IFN-γ for 24 hrs, before stimulation with TNF-α alone and in combination with IFN-γ for a further 24 hrs. Data was compared to non-primed DA FLS. IL-6 and RANTES were quantified in cultured supernatants by ELISA. Values expressed as mean ± SEM of n=4 expts.
However, the potential synergy between these cytokines also has implications for treatment of disease. Therefore, we plan to perform ‘proof of concept’ studies creating models that more closely reflect the in vivo joint microenvironment. We are establishing collagen-based ECM scaffolds in the lab for the study of FLS, EC and immune cells. FLS and CellTrace Far Red labelled EC will be used to populate ECM scaffolds prior to the addition of CFSE-labelled peripheral blood immune cells. In combination with second harmonic generation, multiphoton microscopy system will be used to track both stromal and immune cell motility, interaction, and metabolism. Thus, once scaffolds are established, they will be utilised to test various inhibitors, including TNF-inhibitors, anti-IL-17 and tofacitinib, in addition to novel targets. Following culture, scaffolds can be digested, and cells will be examined using flow cytometric analysis. Thus, this 3D model, will more closely reflect the in vivo microenvironment and along with conventional 2D culture models will give better insights for development of stratified therapeutic strategies.

As highlighted in Chapter 1, there are 3 main subtypes of DA (Poly RF-, Oligo and Psoriatic) therefore studies will be expanded to compare the primary FLS and immune profile across the different subtypes. Furthermore, PBMC have been obtained from patients following treatment, and stratified to responders and non-responders. Therefore, bio-samples will be examined to identify if a cellular or molecular signature can be identified that define the 3 phenotypes but also can differentiate between responders vs non-responders.

Finally, another exciting area in the field is the role of metabolism in the regulation of both stromal and immune cells, and in the context of the inflamed joint, their competition for nutrients. In Chapter 2, we provided data in support of altered metabolism in DA FLS in response to cytokine stimulation, with a shift towards a glycolytic metabolic profile. I performed preliminary data to examine the effect of metabolic blockade on TNF-α-induced DA FLS pathogenic function. Initial data demonstrated that the metabolic inhibitor, 2-DG, decreases TNF-α-induced secretion of IL-6, MCP-1 and RANTES (Figure 5.3(a) – (c)), in addition to decreased ICAM-1 MFI and the percentage frequency of CCR6, CXCR3, CXCR4 and CXCR5 positive DA FLS in response to TNF-α and 2-DG compared to 2-DG alone (Figure 5.3(d) – (f) and Figure 5.4(a) – (d)). Furthermore, this change in DA FLS function, was paralleled by a shift in their metabolic profile from a highly energetic phenotype under TNF-α stimulated conditions to a quiescent phenotype in the presence
of 2-DG (Figure 5.5(e) –(h)). These preliminary data along with the outputs from Chapter 2, suggest targeting metabolic pathways as a potential strategic approach. This is consistent with previous studies focused on RA stromal and immune cells where metabolic reprogramming of FLS, T cells, macrophages and monocytes isolated from RA patients led to resolution of inflammation (Chan et al., 2002; McInnes et al., 2007; Gao et al., 2013; Biniecka et al., 2016; Canavan et al., 2020; Gallagher et al., 2020; Petrasca et al., 2020; Wang et al., 2020; Falconer et al., 2021). Furthermore, metabolic inhibition in whole tissue synovial explants ex vivo (which maintain the architecture cell-cell contact of the synovium) and in animal models of arthritis also demonstrated an inhibition of inflammatory pathways (Son et al., 2014; Biniecka et al., 2016; Garcia-Carbonell et al., 2016; Okano et al., 2017; Xia et al., 2017; McGarry et al., 2018).
Figure 5. 3 DG reduces the production of IL-6, MCP-1 and RANTES and reduces the expression of ICAM-1 from DA FLS. DA FLS were cultured with 2-DG (50mM) for 2hr before the addition of TNF-α (1ng/ml) for 24hr. Following this, supernatants were harvested and quantified for cytokine by ELISA. DA FLS were also stained for ICAM-1 by flow cytometry. Bar graphs demonstrate the (a) IL-6, (b) MCP-1 and (c) RANTES secretion from DA FLS. Bar graphs show (d) frequency of ICAM-1 positive DA FLS, (e) ICAM-1 MFI and (f) ICAM-1 MFI shift. Values expressed as mean ± SEM of n=4-7 expts. One way ANOVA (#) and Wilcoxon paired t-test (*) was used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.

As highlighted previously, we plan to expand these studies to measure metabolic intermediates, alternative metabolic pathways and carbon tracing. Analysis of metabolism and its role in cellular function will be particularly interesting when we compare 2D models to 3D model, as the latter will display nutrient competition by stromal and immune cells, so the cellular metabolic pathways utilised may diverge to adapt to this competitive microenvironment. Finally, analysis of the metabolic profile of immune and stromal cells in responder vs non responder patients, would give insight into the association between the inflammatory responses and metabolic reprogramming.
Figure 5. 4 2-DG reduces the expression of CCR6, CXCR3, CXCR4, CXCR5 and switches the metabolic profile of DA FLS to glycolysis. DA FLS were cultured with 2-DG (50mM) for 2hr before the addition of TNF-α (1ng/ml) for 24hr and stained for flow cytometry or used for seahorse analysis. Bar charts illustrate the percentage frequency of (a) CCR6, (b) CXCR3, (c) CXCR4 and (d) CXCR5 positive DA FLS. (e) Average ECAR and (f) OCR profiles for DA FLS cultured with 2-DG (50mM) for 2hr before the addition of TNF-α (1ng/ml) for 24hr before and after injections of oligomycin, FCCP, and antimycin A/ rotenone. Bar charts (g) show quantification of ECAR:OCR ratio and (h) metabolic map. Values expressed as mean ± SEM of n=4-6 expts. One way ANOVA (#) and Wilcoxon paired t-test (*) was used for statistical analysis respectively #p<0.05, ##p<0.01 significantly different compared to other conditions. *p<0.05 significantly different compared to other conditions.
In summary, the lack of information and awareness regarding the clinical, epidemiological and cellular/molecular characteristics of this form of IA, suggests that children with ‘Down syndrome-associated Arthritis’ are potentially at greater risk of long-term complications. The research outlined in this thesis represents a significant advancement in our understanding of the disease and identifies distinct immune-stromal cell dysregulation in the pathogenesis of DA. Further studies and support for this condition, will lead to improved diagnostic and prognostic outcomes for these vulnerable children. It also demonstrates the importance of translational research, and the involvement of patients with research, thus we would like to thank the children and their parents as this research could not have been performed without them.
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