# The Immunopathogenesis of Alopecia Areata

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**BSc Hons, MSc**

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

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September 2020

# Abstract

Alopecia areata (AA) is an organ specific polygenic autoimmune disease that causes patchy hair loss which can develop to affect the entire scalp and body. The inflammatory response driving AA is considered to be dominated by NKG2D$^+$ CD8 T cells, however the immune mechanisms driving hair loss are not sufficiently understood. Current treatments for AA, which include immunosuppressants, are often ineffective especially for severe AA. JAK inhibitors are useful for promoting hair regrowth, however they may be associated with side effects and disease relapse following treatment cessation.

Extensive immune profiling of peripheral blood and lesional AA skin was conducted to identify novel mechanisms of disease pathogenesis. Multiplex cytokine analysis indicated that AA is associated with a distinct type 17 and type 2 cytokine signature, characterised by increased circulating levels of IL-17A, IL-17F, IL-21, IL-23, IL-31, IL-33 and IL-17E/-25. The frequency of CCR6$^+$ CD4 T cells are increased in AA circulation, supporting the involvement of a Th17 response in AA pathogenesis. Stratification of the AA cohort revealed that changes in the frequency of CCR6$^+$ CD4 T cell populations are related to individuals with mild disease. Atopy is common in AA cohorts, however stratification indicated that the increase in circulating type 17 and type 2 cytokines are enriched in both atopic and non-atopic cohorts. We also observed an increase in the frequency of circulating transitional B cells, but this phenotype is specifically related to atopic AA individuals. Global transcriptomic analysis of AA skin from individuals with stable disease indicated enrichment of a macrophage signature characterised by expression of CD163, CD209 and CD206. Macrophages are known to be important for normal hair growth. Thus, it is proposed that the follicular inflammatory environment disrupts the homeostatic functions of macrophages, and that macrophages contribute to mechanisms maintaining hair loss.

This study provides novel observations indicating distinct systemic and tissue immune signatures, and indicates how features of the immune response are related to specific disease pathotypes. This study also implicates macrophages as pathogenic mediators of AA, and suggests that modulation of macrophage activity represents a novel therapeutic strategy for AA.

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# Acknowledgements

First, I would like to thank my supervisors Simon and Annika. Simon, thank you for literally keeping your door open, for enduring my lists and providing advice and guidance whenever I needed it. My confidence has massively grown from all of the support you have provided throughout my time in the lab. Thank you Annika for your encouragement throughout my PhD, for providing your expertise, infectious enthusiasm and hosting me when I visited Gothenburg; you even trusted me with your bike! Thank you to Iain, Georgia and Stefan for being so supportive and giving the best advice that helped me navigate through a PhD.

To the Millings, I am SO grateful to have been surrounded by the best bunch of people for the last four years. Thank you for never taking life too seriously and for always over-sharing. A special thank you has to go to my PhD partners. Anna, thank you for all of the glorious food, for the pigeon walks, and for being such a supportive friend. Thank you Hannah for providing the LOUDEST laughs, endless prosecco and for providing your bioinformatics skills over many Zooms. Thank you Verena (aka Virkon) for always providing an excuse to go the pub and for the spinning seal. Alberto, thank you for all of your help, and for our morning chats before the noisy ones arrived. Allan, thank you for all of the excellent bible classes, and for your scientific and life advice. Thank you to all of the other Mowlings for all of your help and all the laughs! I wouldn't have gotten through my admin' PhD without you all.

There are a whole bunch of people that made my PhD project possible. A huge thank you to Susan and Fiona for running the research clinic for the last four years. Fiona, I cannot thank you enough for the endless emails you have sent to make sure I had samples to collect every Monday. Thank you Diane for your time and advice; you are a flow guru. Thank you to Kostas, Rodanthi and Caroline for all of your time and help with my samples. Thank you Umer for being a mathematical wizard, and thank you John for being so patient when explaining informatics. Thank you Julia, Maryam and Lisa for all of your help when I visited Gothenburg for the RNA seq experiment. Thank you to Medical Research Scotland, AstraZeneca and Alopecia UK for providing support and funding my

project. Finally, a big thank you to ALL of the volunteers who have donated bits of their body; I literally wouldn't have gotten here without you.

Thank you to my beautiful friends, I am so lucky to have every single one of you. Caitlin, thank you for going through it all with me, I think it's finally time we get a real job! Thank you to my family who have been there from the beginning. An extra special thanks goes to my parents for always believing in me and making me take a break when I needed it most.

Last but not least, a special thank you to Bruce. Thank you for putting up with me whilst writing a thesis during lockdown. Thank you for always keeping me grounded, for being my biggest fan, but most of all for supporting me to pursue my passions.

# Author's Declaration

I declare that, except where reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature: Printed name: Kym Bain

# Abbreviations

| Abbreviation | Meaning |
|---|---|
| AA | Alopecia areata |
| ACTH | Adrenocorticotropic hormone |
| AD | Atopic dermatitis |
| AGA | Androgenetic alopecia |
| AIRE | Autoimmune regulator |
| aMSH | Alpha-melanocyte stimulating hormone |
| ANOVA | Analysis of variance |
| AS | Ankylosing spondylitis |
| AP1 | <50% scalp hair loss |
| AP2 | >50% scalp hair loss |
| AT | Alopecia totalis |
| AU | Alopecia universalis |
| BCR | B cell receptor |
| BM | Bone marrow |
| BMP | Bone morphogenetic protein |
| CD | Crohn's disease |
| cDC | Conventional dendritic cell |
| CeD | Coeliac disease |
| CGRP | Calcitonin gene-related peptide |
| CLA | Cutaneous lymphocyte antigen |
| CRH | Corticotrophin-releasing hormone |
| CTLA-4 | Cytotoxic T lymphocyte-associated antigen 4 |
| CVE | Cross validation error |
| DCP | Diphenylcyclopropenone |
| DE | Differential expression |
| DP | Double positive $CXCR3^+$ $CCR6^+$ CD4 T cells |
| DTH | Delayed-type hypersensitivity |
| eCRF | Electronic case report form |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | Epidermal growth factor receptor |
| ELISA | Enzyme-linked immunosorbent assay |
| FasL | Fas ligand |
| FGF-5 | Fibroblast growth factor 5 |
| FLG | Filaggrin |
| FMO | Fluorescent minus one |
| GO | Gene ontology |
| GWAS | Genome wide association study |
| H&E | Haematoxylin and eosin |

| | |
|---|---|
| HADS | Hospital anxiety and depression score |
| HC | Healthy control |
| HF | Hair follicle |
| HFSC | Hair follicle stem cells |
| HGSEA | Hypergeometric gene set enrichment analysis |
| HLA | Human leukocyte antigen |
| IBD | Inflammatory bowel disease |
| IDO | Indoleamine 2,3-dioxygenase |
| IFNγ | Interferon gamma |
| Ig | Immunoglobulin |
| IGF1 | Insulin like growth factor 1 |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| ILC | Innate lymphoid cell |
| IP | Immune privilege |
| IRAS | Integrated research application |
| KIRs | Killer cell immunoglobulin like receptor |
| LC | Langerhans cell |
| LH | Lithium heparin |
| MAIT | Mucosal associated invariant T cell |
| MHC | Major histocompatibility complex |
| MICA | MHC Class I polypeptide-related sequence A |
| MIF | Macrophage inhibitory factor |
| NK | Natural killer cells |
| NKG2D | Natural killer group 2D receptor |
| OD | Optical density |
| OSM | Oncostatin M |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate-buffered saline |
| PCA | Principal component analysis |
| pDC | Plasmacytoid dendritic cell |
| PRR | pathogen recognition receptor |
| PsA | Psoriatic arthritis |
| PsO | Psoriasis |
| RA | Rheumatoid arthritis |
| RCT | Randomised controlled trial |
| REC | Research ethics committee |
| RT | Room temperature |
| R&D | Research & development |
| SALT | Severity of alopecia tool |
| SCID | Severe combined immunodeficiency |
| SLE | Systemic lupus erythematosus |
| SNP | Single nucleotide polymorphism |
| T1D | Type 1 diabetes |

| | |
|---|---|
| Tbet | T-box transcription factor |
| TCM | Central memory T cell |
| TCR | T cell receptor |
| Teff | Effector T cell |
| TEM | Effector memory T cell |
| TGFB | Transforming growth factor beta |
| Th | T helper |
| TLR | Toll-like receptor |
| Treg | Regulatory T cell |
| TRM | Resident memory T cell |
| TTG | Tissue transglutaminase |
| UC | Ulcerative colitis |
| ULBP | UL16 binding protein |
| VDJ | Variable diversity joining |
| VIP | Vasoactive intestinal peptide |
| WIHG | Wound induced hair regrowth |

# 1 General introduction

## 1.1 Alopecia areata

Alopecia areata (AA) is an organ-specific autoimmune disease that causes hair loss. AA typically develops as circular patches of hair loss on the scalp that can spontaneously regrow, or can be associated with multiple episodes of relapsing-remitting hair loss. Between 14-25% of AA cases deteriorate to affect the entire scalp (alopecia totalis) and/or body (alopecia universalis), which often results in irreversible hair loss (Tosti et al., 2006; Gip et al., 1969).

AA is associated with a lifetime risk of 2% (Mirzoyev et al., 2014) and an incidence of 0.1-0.2% (Safavi et al., 1995; Benigno et al., 2020), making it one of the most common autoimmune diseases. Some studies indicate AA to be more common in females (Lundin et al., 2014), but this is believed to be an artefact of under-reporting of male cases. AA typically develops in adulthood, but can also affect children where the disease is more likely to become severe (Tosti et al., 2006). AA has been reported to be more common in African-Americans, however the disease is not typically associated with a particular ethnic background (Miteva & Villasante, 2015).

Despite being very common, AA has not received as much research interest as other autoimmune diseases. This lack of interest mainly stems from the ‘cosmetic’ label associated with AA, and an initial lack of robust evidence to indicate that AA is an autoimmune disease. However, in the last decades, AA research has gained momentum largely due to findings of genetic and clinical studies that strongly implicate the immune system (Petukhova et al., 2010; Xing et al., 2014; Jagielska et al., 2012). There is also an appreciation of comorbidities, both inflammatory and psychological (Lee et al., 2013), associated with AA that significantly impact quality of life, and therefore strongly support further research to understand the immune-mechanisms driving AA.

## 1.2 What causes alopecia areata?

***Environmental triggers***

A proportion of individuals with AA describe a 'trigger' that precedes onset of hair loss. Viral infection or vaccination have been associated with AA onset. Cytomegalovirus DNA was detected in AA skin (Skinner et al., 1995), but subsequent studies did not find a connection between this infection and occurrence of AA (Tosti et al., 1996). Epstein Barr virus infection has also been reported to precede hair loss in a small proportion of patients but a potential causative relationship hasn't been explored (Rodriguez & Duvic, 2008). AA episodes appear to be most common during winter months (Putterman & Castelo-Soccio, 2018), and it is suspected that seasonal fluctuations in hair loss patterns may be associated with viral emergence. Hair loss onset has also been associated with specific vaccines, including Japanese encephalitis (Chu et al., 2016) and hepatitis B vaccines (Wise et al., 1997). However, studies testing the effect of a recombinant hepatitis B vaccine in a mouse model of AA did not indicate an increased incidence of hair loss (Sundberg et al., 2009).

Psychological stress is one of the most common 'triggers' that affected individuals link to their onset of hair loss. A stress-related experience has been reported to precede AA in approximately 9.8% and 9.5% of adults and children, respectively (Kakourou et al., 2007; Tan et al., 2002). However, the link between AA onset and stress remains elusive because other studies have not identified a significant association (Brajac et al., 2003; van der Steen P et al., 1992). Nevertheless, a collection of studies provide evidence that suggest a connection from a biological perspective. In scalp skin from AA patients, the levels of corticotropin-releasing hormone (CRH) are elevated (Kim et al., 2006), and preliminary evidence indicates that CRH type 2 beta receptor expression is upregulated in AA skin (Katsarou-Katsari et al., 2001). CRH is upregulated in response to stress and drives adrenocorticotrophin hormone (ACTH) secretion which causes release of glucocorticoids (Brunson et al., 2001). CRH receptors are also upregulated in psoriasis lesions, and their expression correlates with disease severity (Cemil et al., 2012). It therefore seems plausible that stress-induced factors may contribute to induction of AA despite the current lack of definitive

evidence. The direct effects of stress and hormonal fluctuations on hair follicle physiology will be discussed in section 1.4.1.

AA patients anecdotally describe mild intestinal discomfort and occasionally report intestinal inflammation as a trigger for hair loss. At present, there are a selection of studies which indicate interesting observations, but no evidence indicates a direct link between the intestine and AA (Borde & Åstrand, 2018). Inflammatory bowel disease (IBD) was reported to occur at an incidence of 2% in a study examining comorbidities in 3568 AA patients (Huang & Qureshi, 2013a), however a systematic meta-analysis did not identify a significant association between AA and IBD (Lee et al., 2019). Interestingly, two AA patients with severe hair loss experienced hair regrowth following a feacal microbial transplant that was delivered to treat recurrent *C.difficile* infection (Rebello et al., 2017). This observation suggests a role for the microbiota in mechanisms driving hair loss. A recent study analysing the faecal microbiome of 25 AA patients did not identify a significant change in microbial diversity in comparison to the healthy samples, however the authors reported changes in the abundance of specific classes of bacteria (Juhasz et al., 2020). Further investigation is required to determine the potential relationship between AA, the intestine and the microbiome.

It is important to highlight that many individuals who develop hair loss caused by AA do not recall a specific trigger. Furthermore, whilst there are many studies describing associations between AA onset and 'potential' triggers, there are still no proven environmental factors that are directly linked to mechanisms driving hair loss.

***Genetic predisposition***

Multiple published studies have indicated a link between AA development and a positive family history. In adults, incidence rates associated with a family history of AA range between 0-8.6%, whereas the incidence relating to childhood onset is between 10-51.6% (Miteva & Villasante, 2015). Other studies have highlighted clear twin concordance (Scerri & Pace, 1992; Rodriguez et al., 2010), and more recently, it was reported that a positive family history is associated with more severe AA and earlier disease onset (Wang et al., 2018). Whilst these studies

indicate that increased susceptibility to AA is associated with a positive family history, the incidence is not predictable and therefore indicates involvement of environmental factors.

***Involvement of the immune system***

Initial genetic studies in AA cohorts clearly implicated the immune system, highlighting the association between AA and human leukocyte antigens (HLA), including HLA-DQB1, HLA-DRB1, HLA-A, HLA-B and MICA (Barahmani et al., 2006; Colombe et al., 1999; Andrade et al., 1999). In 2010, the first genome wide association study (GWAS) in AA was conducted. This supported the association between AA and HLA genes, but also highlighted novel associations with loci containing genes involved in the adaptive immune system (CTLA4, IL-2/-21, IL2RA, EOS) (Petukhova et al., 2010). Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is an inhibitory receptor important for tolerogenic responses, specifically related to regulatory T cell function (Wing et al., 2008). The transcription factor, Eos, is also vital for Treg function (Pan et al., 2009). Whereas IL-2/-21 and the IL-2 receptor (IL-2RA) are important for driving proliferation and survival of both effector T cells and Tregs (Liao et al., 2011; Chinen et al., 2016). Additionally, Petukhova et al (2010) identified novel associations between the loci containing the UL-16 binding protein (ULBP) gene cluster and AA. ULBP proteins are related to MHC class I molecules that bind to receptors expressed by natural killer cells and CD8 T cells to drive cytotoxic responses (Eagle et al., 2009).

In addition, GWAS studies also implicated genes related to the hair follicle. STX7, syntaxin 17, was significantly associated with AA (Petukhova et al., 2010), a protein which has been associated with greying hair in horses (Pielberg et al., 2008). The relationship with STX7 is compelling in the context of AA pathogenesis because grey hair does not seem to be affected (Jia et al., 2014). Another interesting genetic link with AA is PRDX5, which codes for an antioxidant enzyme involved in clearance of peroxides within cells (Perkins et al., 2015). Previous studies have reported dysregulation in levels of antioxidant enzymes in AA scalp (Akar et al., 2002), and its expression is increased in the context of other autoimmune diseases, including multiple sclerosis (Holley et al., 2007).

Importantly, many of these immunological genetic loci have also been associated with other autoimmune diseases, including rheumatoid arthritis (RA), type 1 diabetes (T1D) and systemic lupus erythematosus (SLE) (Petukhova et al., 2010; Betz et al., 2015). Collectively, these findings demonstrated that the genetic risk profile of AA is characteristic of a polygenic organ specific autoimmune disease. These findings provided the rationale for functional studies which indicated AA as a T-cell mediated autoimmune disease. Prior to sections describing the immune-mechanisms involved in AA pathogenesis, an introduction to the immune system and hair follicle biology is provided.

## 1.3 The immune system

The immune system is often described as being composed of two layers, the innate and adaptive systems. The innate immune system is the first line of defense. It responds quickly by recognising conserved sequences common to pathogens, and is responsible for activating the adaptive immune system. Once alerted, the adaptive immune system is able to specifically respond by differentiating and tailoring a response to eliminate a pathogen and confer lasting protection.

### 1.3.1 T cells

T cells are lymphocytes that develop in the thymus. They develop form bone-marrow progenitors and are a critical component of the adaptive immune system. Before leaving the thymus as naïve cells, T cells go through a number of developmental check points. First, T-cell receptor gene rearrangement takes place to produce unique $\gamma\delta$ or $\alpha\beta$ surface receptors with random specificities (Hedrick et al., 1984). Conventional $\alpha\beta$ T cells will then become double positive (DP, $CD4^+$ $CD8^+$) thymocytes. DP T cells are then tested for their ability to recognise MHC-self peptide complexes presented by specialised medullary thymic epithelial cells (Anderson & Takahama, 2012). This process of presenting tissue specific antigen is controlled by the expression of the autoimmune regulator (AIRE) gene (Yano et al., 2008). Thymocytes which weakly recognise MHC-self antigen complexes are positively selected, whereas cells with strong affinity for MHC-self-antigen complexes are deleted (negative selection). Thymocytes which survive subsequently differentiate into single positive T cells,

expressing CD4 or CD8. Some T cells with intermediate affinity for MHC-self peptide complexes will survive and become regulatory T cells (Tregs). Positive and negative selection of lymphocytes underpins central tolerance, and is fundamental for preventing autoreactive lymphocytes escaping the thymus. However, central tolerance mechanisms cannot remove all autoreactive lymphocytes, and therefore peripheral tolerance mechanisms are also essential. Peripheral tolerance mechanisms are described in section 1.3.3.

T cells exit the thymus as naïve cells and circulate between peripheral blood and secondary lymphoid organs. Human naïve T cells express CD45RA and the chemokine receptor, CCR7, which facilitates entry into lymph nodes (Sallusto et al., 1999; Michie et al., 1992). Activation of a naïve T cell requires a T cell to be presented with its cognate antigen, by major histocompatibility complex (MHC) on the surface of a dendritic cell in a lymph node (Langenkamp et al., 2000). Successful activation requires a second signal that involves engagement of costimulatory molecules that are adjacent to the TCR-MHC complex. Essential co-stimulation involves CD28 on the T cell interacting with CD80/86 on the APC (Mueller et al., 1989; Clark & Ledbetter, 1994), and may also involve CD40L and ICOSL binding to CD40 and ICOS, respectively.

Upon activation, T cells differentiate to become effector T cells, proliferate, and migrate to tissues by downregulating CCR7 (Sallusto et al., 1999). A proportion of differentiated T cells become $CD45RO^+$ central (TCM) or effector memory (TEM) cells. TCMs and TEMs recirculate to provide surveillance against future encounters against the same antigenic challenge. TCM cells retain expression of CCR7 and circulate between blood and lymph nodes, whereas TEM cells downregulate CCR7 expression to facilitate circulation between blood and tissues (Sallusto et al., 1999). Memory T cells can also permanently reside in tissue to provide rapid response to antigenic challenge; resident memory T cells (TRM) (Gebhardt et al., 2009).

***CD4 T cells***

$CD4^+$ T cells, referred to as T-helper (Th) cells, mediate their effector functions by secretion of cytokines to modulate the activity of other immune cells. CD4 T cells are activated when their T-cell receptor (TCR) recognises an MHCII-

exogenous antigen complex presented by dendritic cells (Grakoui et al., 1999). CD4 T cells are categorised into different subsets based on the type of response they drive, which is dictated by cytokines released by the presenting DC and surrounding environment (Mosmann & Sad, 1996). Th cell differentiation is summarized in Figure 1.1.

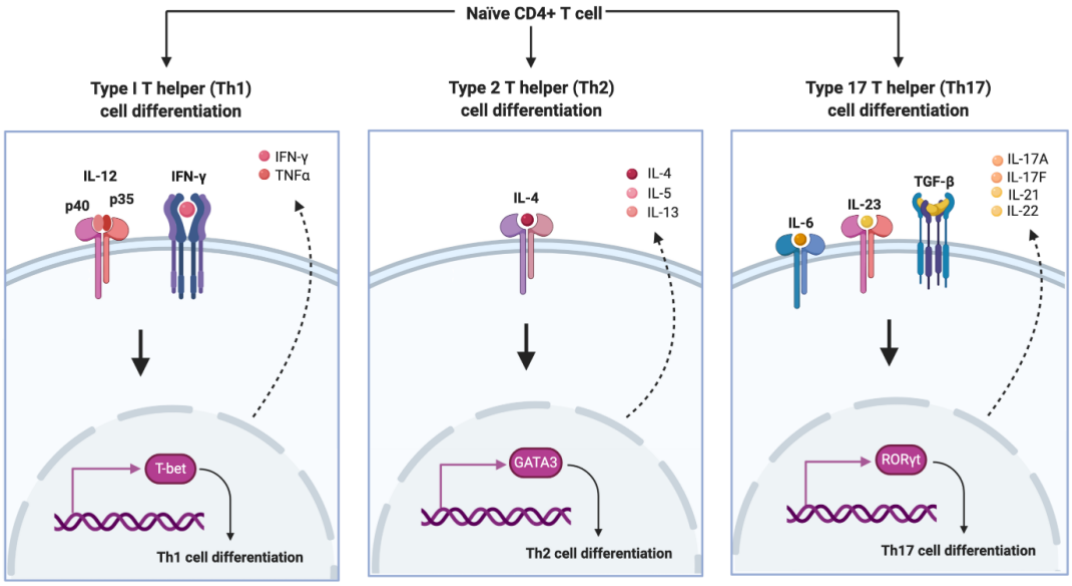


**Figure 1.1 CD4 T helper cell differentiation**
CD4 T helper cells differentiate from naïve T cells following recognition of antigen presented by dendritic cells. Th1 cells require IL-12 and IFNγ, which drives Tbet expression and production of type-1 cytokines. Th2 cells require IL-4, which drives GATA3 expression and production of type-2 cytokines. Th17 cells require IL-6 and TGF-ß, in addition to IL-23 for maintenance, which drives RORγt expression and type-17 cytokines. Created with Biorender.com.

Th1 cells are involved in driving responses against intracellular pathogens and successful priming requires IL-12 secretion from the presenting DC (Macatonia et al., 1995). Th1 cell differentiation requires expression of the T-box transcription factor (T-bet) which drives production of the signature Th1 effector cytokine, interferonγ (IFNγ) (Szabo et al., 2000). Th1 cells also express the chemokine receptor CXCR3 which is important for migration to effector sites (Yamamoto et al., 2000). IFNγ directly activates in excess of 200 genes involved in inflammatory immune responses (Bhat et al., 2018) but its most characterised effector functions are involved in the enhancement of macrophage phagocytic activity to kill bacteria, upregulation of molecules important for antigen processing and presentation, and for driving cytotoxic responses (Steimle et al., 1994; Decker et al., 2002; Schroder et al., 2004). Th1 cells are also an important

source of IL-2 which is important for development of robust CD8 T cell memory responses (Williams et al., 2006) and for suppressor function of regulatory T cells (Chinen et al., 2016).

Th2 cells are crucial for clearance of extracellular parasites, and are implicated in the pathogenesis of allergic responses (Robinson et al., 2010). IL-4 production from T cells and type-2 effector cells is reported to be involved in Th2 differentiation (Piehler et al., 2011; Ballesteros-Tato et al., 2016). However, it has been shown in mice that IL-4 is not required for Th2 differentiation (van Panhuys et al., 2008). Thus, the exact mechanisms required for Th2 differentiation require further investigation. Th2 cells express the transcription factor, GATA3, which drives expression of the signature Th2 cytokines, IL-13, IL-4 and IL-5 (Yamashita et al., 2004). Th2 cells can also produce IL-31, a cytokine which is upregulated in skin lesions from patients with eczema (Neis et al., 2006). Th2 cells are potentiated by the release of the alarmin, IL-33 (Komai-Koma et al., 2007). IL-33 is constitutively expressed by various cells, most notably epithelial and endothelial cells, and is only released in its activated form when cells die by necrosis (Lüthi et al., 2009). IL-33 is therefore a crucial signal which alerts the immune system of tissue damage. IL-25/17E is also a potent driver of type 2 responses and is involved in pathology related to dermatitis and asthma (Kim et al., 2002).

Th17 cells are important for defense against extracellular pathogens and fungi, and are commonly associated with aberrant autoimmune responses (Harrington et al., 2005). Th17 cells require IL-6 and TGFß signaling for their differentiation, which drives expression of the orphan nuclear receptor RORγt (Zhou et al., 2007; Harrington et al., 2005; Ivanov et al., 2006). This in turn drives expression of the chemokine receptor, CCR6, and the effector cytokines, IL-17A, IL-17F, IL-21 and IL-22 (Liang et al., 2006). Maintenance of Th17 cells requires IL-23 from innate cells (Stritesky et al., 2008). Th17 cells function to drive expression of pro-inflammatory cytokines such as TNF and IL-6, and induce migration of innate cells, such as neutrophils, to sites of inflammation by upregulating the expression of endothelial adhesion ligands (Roussel et al., 2010).

Expression of subset-specific chemokine receptors, including CXCR3 and CCR6, are important for T cells to traffic to sites of inflammation. The ligands for CXCR3 and CCR6, include CXCL9, CXCL10 and CCL20, and are expressed by immune cells, endothelial and epithelial cells (Taub et al., 1993; Hirota et al., 2007; Le Borgne et al., 2006). During T cell priming, T cells will also upregulate homing receptors that facilitate migration to specific tissues. Activation of T cells in a skin-draining lymph node causes upregulation of cutaneous lymphocyte antigen (CLA) which binds to E-selectin expressed in the skin (Picker et al., 1993). In contrast, mucosal-activated T cells are imprinted to express the gut-homing receptor, CCR9 (Iwata et al., 2004).

Tregs also belong to the CD4 T cell family and are vital for driving tolerogenic response towards self or non-self antigen, and during the resolution phase of immune responses. Tregs can be thymically derived (natural Tregs) or can develop from naïve T cells (inducible Tregs). All Tregs express the transcription factor, Foxp3, and IL2RA which is essential for their differentiation and function (Fontenot et al., 2003; Malek et al., 2002). The mechanism Tregs use to induce tolerance include directly inhibiting T cell activation by binding to CD80/86 on the surface of DCs using the inhibitory receptor, CTLA-4, and by secreting anti-inflammatory cytokines including IL-10 (Rubtsov et al., 2008; Read et al., 2000).

***CD8 T cells***

CD8 T cells, also known as cytotoxic T cells, mediate direct cell killing of infected cells and 'stressed' cells, including cancer cells, by recognising MHCI-endogenous antigen complexes. CD8 T cells are activated in a lymph node by cross-presenting dendritic cells (Haan et al., 2000). CD4 T cells are vital for CD8 T cell activation, by licensing of DCs, which involves simultaneous interaction of CD40 and CD70 on the DC with CD40L on the CD4 T cell, and CD27 on the CD8 T cell, respectively (Taraban et al., 2004). Similar to Th1 cells, CD8 T cell differentiation requires IL-12 which drives expression of Tbet (Takemoto et al., 2006). CD8 T cells kill target cells by releasing granules containing perforin and granzymes or by direct FAS-FASL interaction leading to apoptosis (Henkart & Sitkovsky, 1994). CD8 T cells also express classical type-1 cytokines, including IFN$\gamma$ and TNF to drive anti-microbial activity in innate cells.

In tissues, CD8 T cell can also respond to ‘stressed’ cells by recognising ligands including major histocompatibility complex (MHC) class 1 related chain (MICA/B) and ULBP binding proteins (Eagle et al., 2009; Bauer et al., 1999). These ligands bind to the natural killer group 2 member D (NKG2D) receptor that is constitutively expressed by human CD8 T cells and NK cells. During inflammatory responses, NKG2D can function as co-stimulation for TCR signaling, and is thought to lower the TCR-activation threshold (Jamieson et al., 2002). NKG2D is also reported to be involved in formation of CD8 T cell memory cells (Wensveen et al., 2013). Furthermore, NKG2D appears to be important for rescuing CD4-unhelped CD8 T cells (Zloza et al., 2012). It has also been shown that NKG2D ligation in addition to IL-15 signalling can activate intestinal intraepithelial CD8 T cells from active coeliac (CeD) biopsies in a TCR independent manner (Meresse et al., 2004). Importantly, this is the only setting where TCR-independent NKG2D activation of CD8 T cells has been observed. In the context of autoimmunity, NKG2D is implicated strongly in AA pathogenesis, and in other diseases including vitiligo and T1D (Ogasawara et al., 2004). In these diseases, NKG2D ligands are upregulated by tissue cells, including epithelial cells, which are reported to play a role in the activation of autoreactive T cells.

### 1.3.2 B cells

B cells are lymphocytes that initially develop in the foetal liver and bone marrow (BM), and then migrate to secondary lymphoid organs for functional maturation. First, V-D-J recombination takes place in multiple steps to produce heavy and light chains that together produce a unique immunoglobulin (Ig) that forms a pre-B cell receptor (BCR). V-D-J recombination is the process mediated by RAG genes which involves combining variable (V), diversity (D) and joining (J) gene segments to form an Ig. This process of randomly selecting and joining V, D and J gene segments produces a repertoire of antibodies which enables the immune system to recognise a huge volume of antigen. Similar to developing T cells, the random process of receptor generation results in a large proportion of BCRs which recognise self. These B cells will then go through receptor-editing which aims to generate a BCR that does not recognise self-antigen. B cells which are still self-reactive are then deleted via apoptosis in response to repetitive self-antigen signaling (Hartley et al., 1993).

Immature IgM$^+$ CD19$^+$ B cells emerge from the BM and migrate to the spleen. Here the maturation process continues. Immature B cells progress through transitional B cell stages, where they express both IgM and IgD, before differentiating into mature marginal zone and follicular B cells. Upon antigen encounter in a LN, mature follicular B cells move to the T cell zone to search for 'help' from an activated T cell (Garside et al., 1998). With T cell help, via CD40-CD40L ligation, the germinal center forms and somatic hypermutation takes place to generate high affinity antibodies. This process is coupled with class switch recombination, determined by the cytokine milieu generated by the CD4 T cell, to generate antibodies with different constant regions that determine their functional capability (IgG, IgE, IgA) (Stavnezer et al., 2008). B cells with high affinity antibodies then become memory or plasma B cells (Phan et al., 2006). Memory B cells can be identified by expression of CD27. In human peripheral blood, a large population of IgM$^+$ memory cell exist, alongside circulating transitional, naïve and memory subsets. The exact developmental trajectory of IgM$^+$ memory cells is not fully understood, but it is reported that these cells represent circulating marginal zone B cells which respond to antigen in a T-cell independent manner (Weller, 2004).

The predominant role of B cells is to produce antigen specific antibodies which are particularly important during recall responses, but B cells are also known to function as antigen presenting cells. In the context of CeD, B cells are reported to play an important role in the generation of autoantibodies against the enzyme, tissue transglutaminase (TTG), which modifies gliadin peptides to generate immunogenic epitopes. The current model suggests that TTG acts as a carrier protein for the gliadin peptide. The BCR binds to a TTG-gliadin complex and presents it to gliadin-specific CD4 T cell resulting in the generation of TTG-specific autoantibodies (Dieterich et al., 1997; Sollid et al., 1997). Thus, B cells can contribute to the activation of autoreactive T cells.

### 1.3.3 Peripheral tolerance

Central tolerance mechanisms which take place in the bone marrow and thymus during B and T cell development will remove the majority of self-reactive lymphocytes. However, a proportion of self-antigens are not expressed during

development, in addition to other non-harmful antigens, including dietary peptides. Peripheral tolerance mechanisms are therefore essential for preventing activation of potentially harmful lymphocytes in the periphery.

Peripheral tolerance mechanisms include anergy induction and deletion of lymphocytes upon encounter of cognate antigen. Deletion of peripheral autoreactive T cells is mediated by apoptosis induced by extrinsic pathways, as opposed to clonal deletion which occurs in the thymus as a result of strong affinity for self-antigen. This deletion is caused by excessive exposure to cognate antigen, leading to expression of the FAS ligand (FasL) and subsequent cell-death upon engagement with the Fas receptor (Bonfoco et al., 1998). In contrast, anergy is used to describe a state where immune cells become unresponsive to cognate antigen. T cell anergy can be induced by tolerogenic dendritic cells which do not provide co-stimulatory signals in steady-state conditions. Instead, inhibitory signals, including CTLA-4 and PDL1, are provided (Probst et al., 2005). The lack of co-stimulation prevents proliferation and differentiation of the anergic T cell. Similarly, anergy can be induced in B cells which have bound to antigen but fail to receive signals from T cells (Gauld et al., 2005).

The failure of peripheral tolerance mechanisms substantially contribute to multiple autoimmune diseases. Anergy is reversible and therefore a lack of specific signals can release lymphocytes from this unresponsive state. For example, B cells require chronic antigen binding to maintain anergy (Gauld et al., 2005) and it has been shown that mechanisms maintaining anergy are defective in SLE patients (Malkiel et al., 2016). During chronic inflammation, autoreactive B cells can lead to epitope spreading whereby multiple epitopes of the same antigen or different antigens become pathogenic targets of the immune system. This can occur via different mechanisms including processing of antigen by autoreactive B cells to generate new epitopes which can subsequently drive activation of autoreactive naïve T cells (Cornaby et al., 2015). Defects in apoptosis mechanisms are also linked with development of autoimmunity. Polymorphisms in the PTPN22 gene, which codes for a tyrosine phosphatase protein, is associated with apoptosis resistance in naïve and transitional B cell subsets. Importantly, polymorphisms in PTPN22 are associated with multiple

autoimmune diseases (Habib et al., 2012). Thus, defects in anergy induction and mechanisms involved in deletion of autoreactive lymphocytes underpin pathogenic mechanisms driving autoimmunity.

## 1.3.4 Innate immune cells

***Dendritic cells***

Conventional dendritic cells (DCs) are professional antigen presenting cells that are fundamental for connecting the innate to the adaptive immune system. By presenting antigen via MHC, DCs are able to prime naïve T cells and dictate the flavour of the ensuing T cell response by secretion of specific cytokines. DCs are identified as lineage negative ($CD3^-CD19^-CD56^-CD15^-$, $Lin^-$), $MHCII^+$ $CD11c^+$ cells. Two broad DC subsets exist, namely cDC1 and cDC2, which in humans are identified by CD141 and CD1c expression, respectively (Villani et al., 2017). cDC1 are responsible for cross-presenting antigen to activate CD8 T cells (Haan et al., 2000). Whereas, cDC2 would appear to be most important for driving CD4 T cell responses (Gao et al., 2013). Notably, transcriptomic analysis has enabled elucidation of how DCs can adapt during inflammation, where cDC2s have been shown to activate both CD4 and CD8 T cell responses (Bosteels et al., 2020). Thus, emerging evidence indicates that DCs adapt in relation to the inflammatory environment.

Plasmacytoid dendritic cells (pDC) come from the same lineage precursor as cDCs (common DC precursor) but cannot prime naïve T cells; their main function is to produce type 1 interferon. In humans, pDCs are identified as $MHCII^+$ $CD11c^-$ $Lin^-$ $CD123^+$ cells. However, this identification also appears to capture a proportion of $AXL^+$ DCs (Villani et al., 2017) that were subsequently shown to represent a circulating pre-DC population (See et al., 2017). pDCs sense danger using toll-like receptors 7 and 9, which specifically detect RNA viruses and DNA viruses (Swiecki & Colonna, 2015). pDCs are implicated in autoimmunity, particularly in the pathogenesis of SLE. Here, immune complexes composed of endogenous DNA and antinuclear antibodies can active pDCs (Means et al., 2005). This mechanism is of clinical importance because pDC activity contributes to refractory responses to glucocorticoids in SLE patients (Guiducci et al., 2010).

***Macrophages***

Macrophages are tissue-resident innate immune cells that are important for homeostatic and repair processes, but can also play a pathogenic role in the context of inflammation. Macrophages are derived from embryonic precursors arising from the yolk sac and foetal liver, and have the capacity to self-renew (Ginhoux & Guilliams, 2016). Macrophages can also develop from infiltrating monocytes where they adopt a tissue-specific signature based on the surrounding niche (van de Laar et al., 2016).

Activation of macrophages is broadly defined as classical activation or alternative activation. First, macrophages recognise conserved pathogenic signatures using their surface receptors which include toll-like receptors (TLRs). Classical activation occurs when type-1 cytokines, typically IFN$\gamma$, are released by other immune cells including NK and T cells. Macrophages are then polarised for microbicidal activity against pathogens and express inflammatory mediators including reactive oxygen species (Ding et al., 1988; Mosser & Edwards, 2008). In contrast, type-2 associated cytokines, IL-4, IL-13 and IL-10, induce alternative macrophage activation. These macrophages are associated with wound healing responses, and express markers such as arginase 1 (Wager & Wormley, 2014). Whilst these terms may be used to characterise macrophages, similar to the 'M1' and 'M2' designation, it is now widely recognised that macrophages are plastic, and that their phenotype and function can vary greatly depending our their surrounding environment (Guilliams & Scott, 2017).

Under homeostatic conditions, the main functions of macrophages include removal of dead cells and debris, and aiding tissue remodelling. Importantly, macrophage tissue residence is associated with the ability to become highly specialised to deal with homeostatic functions and threats associated with specific tissues. For example, alveolar macrophages regulate surfactant levels in the lung (Gautier et al., 2012). Whereas, macrophages in the intestine possess anti-inflammatory properties to maintain a homeostatic environment, and are constantly replaced by infiltrating monocytes (Bain et al., 2014).

In the context of inflammation, macrophages can be harmful because they secrete potent pro-inflammatory cytokines including TNF, IL-1ß and IL-6. During

chronic inflammation, problems with pro-inflammatory macrophages are coupled with the effect of an inflammatory environment on homeostatic functions of resident macrophages. Furthermore, macrophages contribute to fibrosis which can also negatively impact organ function (Misharin et al., 2017).

***Natural killer cells***

Natural killer (NK) cells are innate lymphocytes which function as the innate cytotoxic counterpart to CD8 T cells. Similar to CD8 T cells, they are involved in killing virally infected or transformed cells and are poised to release granules containing perforin and granzymes. NK cells recognise target cells in a non-antigen specific manner using activation or inhibitory receptors (Lanier, 2008). As discussed, activation is mediated by the NKG2D receptor which recognises 'stress' ligands. Whereas, killer cell immunoglobulin-like receptors (KIRs) prevent NK cell activation by recognising MHCI or other 'self' ligands expressed by healthy cells. During type 1 responses, NK cells are an early provider of IFN$\gamma$ which contributes to the generation of adaptive CD8 T cell and Th1 responses (Biron & Brossay, 2001).

## 1.4 The hair follicle

The skin is a multifunctional barrier tissue and its primary function is to provide physical protection from external stressors and challenges. The skin is also vital for physiological processes including thermal regulation, water retention and sensory detection of touch and pain. To carry out these functions the skin is home to a collection of mini-organs including sebaceous and eccrine glands, sensory fibers and hair follicles (HFs).

The human skin is home to approximately 5 million HFs. The scalp contains the highest density of HFs where hair primarily acts as a heat insulator. Humans have two different types of hair: scalp HFs grow terminal pigmented hairs, whereas the face and limbs usually grow short vellus hairs. Aside from warmth, hair is also important for protective purposes. Eye lashes and hair in the nose and ears prevent entry of irritants such as dust and potentially harmful microbes.

Each HF is a self-sufficient mini-organ that is situated alongside a sebaceous gland and arrector pili muscle in the skin, as depicted in Figure 1.2. The HF is composed of many specialist structures; the epithelial components include the infundibulum, isthmus, suprabulbar, bulb and bulge and are ectoderm-derived, whereas the dermal papilla is mesoderm-derived (Schneider et al., 2009). The dermal papilla (DP) is composed of fibroblasts which control the hair cycle, and the bulge region is home to hair follicle stem cells (HFSCs) (Yang et al., 2017). The growing hair develops from proliferating keratinocyte cells that migrate from the bulge region into the matrix to form the hair shaft. Melanocytes are also present in the matrix and the outer root sheath, and are responsible for the pigmentation of keratinocytes during anagen (Slominski et al., 2005). Initiation of anagen, the active growth phase, requires contact between the dermal papilla and HFSCs in the bulge region. Once growth is initiated, the dermal papilla controls the duration of anagen, thus dictating hair length, and the diameter of the hair shaft (Stenn & Paus, 2001).

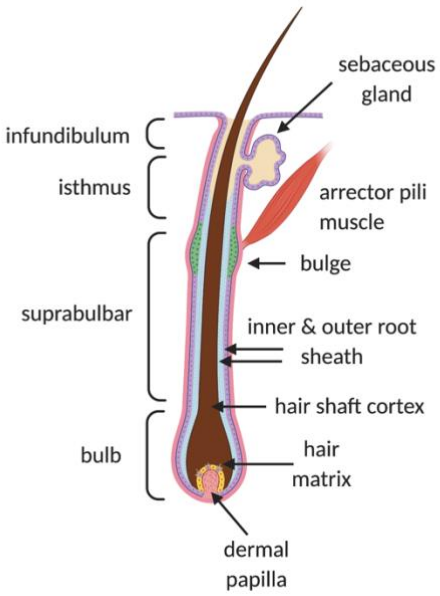


**Figure 1.2 The hair follicle**
Schematic diagram representing an anagen hair follicle. The four epithelial regions of the HF are highlighted: infundibulum, isthmus, suprabulbar and bulb, alongside important structures involved in hair cycling: the bulge containing hair follicle stem cells and the dermal papilla. Adapted from Schneider et al (2009) and created with BioRender.com

## 1.4.1 The hair follicle cycle

Hair growth occurs in cycles and each cycle is composed of three main phases: anagen, catagen and telogen (Figure 1.3). During each cycle, the distal region of

the HF is effectively regrown by a series of growth and apoptosis driven stages. As mentioned previously, anagen is the active growth phase of the HF cycle. On the human scalp, 80-90% of HFs are in anagen and the rate of hair growth can be up to 20uM per hour (Yang et al., 2017). Anagen is followed by catagen which is an apoptosis driven stage involving regression of the HF, and then a dormant phase called telogen (Stenn & Paus, 2001). Complex molecular pathways are involved in the intricate control of the HF cycle, and a slight disruption in the ratio of anagen/catagen/telogen hairs on the scalp is associated with conditions characterised by hair thinning, known as telogen effluvium or diffuse alopecia (Jain et al., 2000).

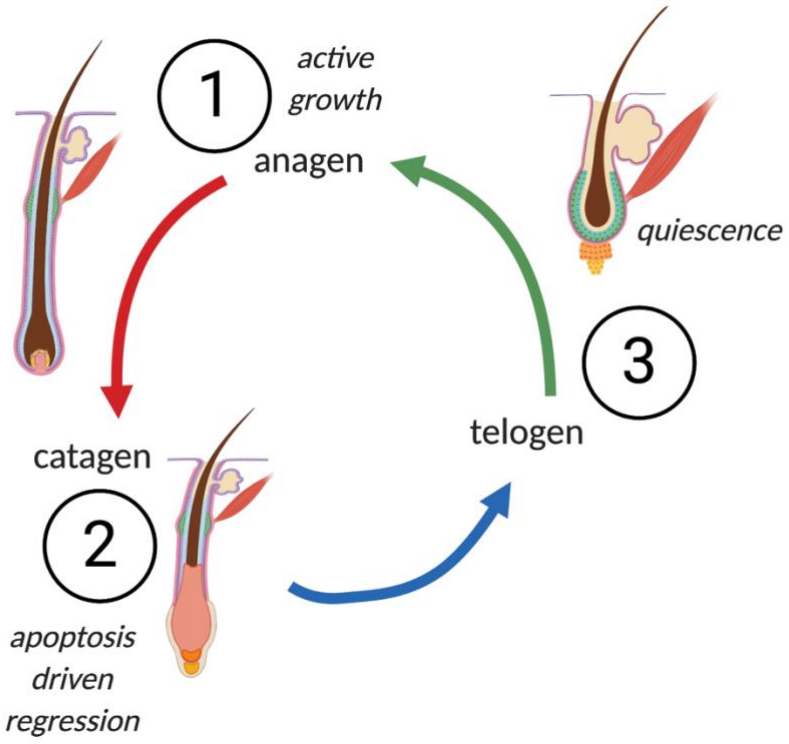


**Figure 1.3 The hair follicle growth cycle**
The HF cycle involved three key phases: anagen, catagen and telogen. Anagen represents the active growth phase and is followed by an apoptotic-driven phase where the distal region of the follicle is removed. The quiescent telogen phase follows before moving back into anagen. Created with BioRender.com

The molecular pathways and signals involved in HFSC activation are well studied due to the accessibility of HFs, and their cyclic regenerative capacity which is of interest in the field of organ regeneration (Toyoshima et al., 2012). Anagen initiation is dependent on interaction between the DP and HFSCs within the bulge region, which causes HFSCs to form a hair matrix. Once the matrix is

formed, the matrix cells grow inwards to form the hair shaft which is composed of terminally differentiated keratinocytes. It has been shown that activation of quiescent HFSCs requires inhibition of bone morphogenetic proteins (BMP) and activation of ß-catenin via Wnt signalling (Plikus et al., 2008; Celso, 2004; Van Mater et al., 2003). Variation in the expression of BMPs and WNT/ß-catenin molecules throughout micro-niches of the DP facilitates the cyclic differentiation and self-renewal capacity of epithelial cells forming the different parts of the HF matrix (Yang et al., 2017). The relationship between Wnt, BMP and ß-catenin signalling in relation to HFSC activation is depicted in Figure 1.4. The transcriptional repressor, Blimp-1, is an important regulator of Wnt/ß-catenin signalling in the DP which can be induced by TGFß (Telerman et al., 2017). Other appendages in the surrounding skin, including adipocytes (Plikus et al., 2008) and nerve fibres (Brownell et al., 2011) also play a role in maintaining the HFSC niche. Interestingly, adipocytes situated below HFs have the capacity to express BMP molecules, highlighting potential communication between two tissues important for thermal regulation (Plikus et al., 2008).

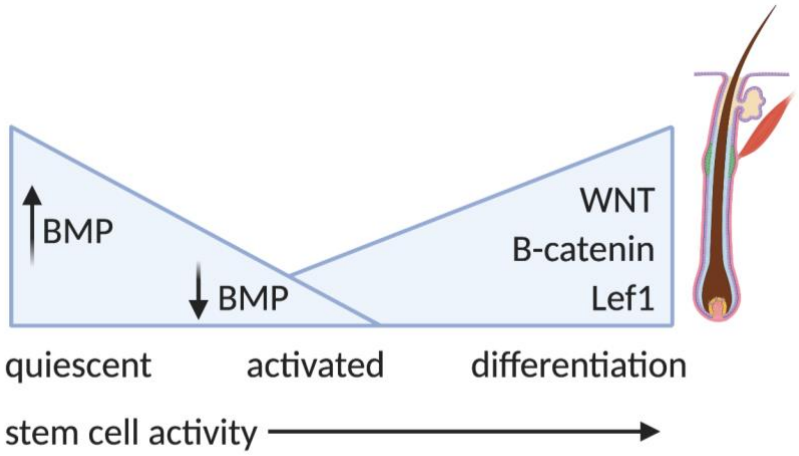


**Figure 1.4 Molecules regulating stem cell activation**
Hair follicle stem cells activity is regulated by a complex interplay of molecules. Stem cell quiescence is associated with expression of BMP molecules, whereas WNT/ß-catenin signalling induces stem cell activation. Diagram was adapted from Schneider et al (2009) and created with BioRender.com.

The HF has also been shown to be sensitive to changes in the skin's macroenvironment, particularly in relation to hormonal fluctuations or responses to stress. Oestrogen negatively impacts hair growth by promoting anagen to catagen transition and this mechanism is thought to play a role in hair loss and thinning in pregnant and menopausal women (Hu et al., 2012). In animal models,

stress-inducing stimuli leading to release of substance P causes premature catagen induction indicated by enhanced epithelial apoptosis (Arck et al., 2003). It has also been shown *in vitro* that human HFs can produce cortisol, and that corticotropin releasing hormone (CRH) directly modulates hair growth by influencing keratinocyte activity and catagen onset (Ito et al., 2005).

### 1.4.2 Hair follicle immune privilege

The HF is regarded as a relative site of immune privilege (IP). HFs were first identified as an IP zone by the observation that HFs survive following allograft transplantation (Billingham & Silvers, 1971). Follicular IP is described as relative because the protection varies throughout the hair cycle, and requires active regulatory mechanisms to be maintained (Paus et al., 2003).

IP is classically associated with organs in the body that can protect grafted tissues (Niederkorn, 2006). Immunological concepts related to IP are reported to involve both passive and active mechanisms. Clonal ignorance describes the model where autoreactive T cells exist in the periphery but do not react to their cognate self-antigen because the tissue is inaccessible to the immune system (Forrester et al., 2008). Whilst immunological ignorance appears to play a role, it is now appreciated that active regulatory mechanisms are in place to maintain tolerance in specific sites. The testis are a well-characterised IP zone, and it appears that both the physiological blood-testis barrier, and expression of anti-inflammatory mediators, including cytokines contribute to mechanisms preventing immune-attack (Fijak & Meinhardt, 2006). The eye is also protected by immune-inhibitory mechanisms including expression of ocular FasL which causes apoptosis of immune cells, and the presence of secretory molecules within the anterior chamber including TGFß and α-melanocyte stimulating hormone (α-MSH) (Griffith et al., 1995; Taylor, 1999). It appears that mechanisms protecting HFs are predominantly associated with active regulation.

Hair follicle IP is primarily associated with the anagen bulb and bulge region. IP is reported to exist in these regions to prevent aberrant responses directed against potentially immune-reactive antigen, including melanin-associated peptides that are expressed during anagen (Trautman et al., 2009). Multiple mechanisms maintaining HF-associated IP have been described that involve

direct cell contact or secretory mediators. Direct mechanisms include downregulation of MHCI expression by the follicular epithelium (Christoph et al., 2000; Paus et al., 2005). The follicular epithelium also expresses CD200, which is reported to inhibit myeloid function and therefore contribute to immune regulation (Meyer et al., 2008; Hoek et al., 2000). Secretory immuno-inhibitory molecules associated with HF immune privilege include TGFß, a-MSH, IL-10, idoleamine-2,3-dioxygenase (IDO) and macrophage migratory inhibitory factor (MIF) (Meyer et al., 2008). Notably, administration of IDO, TGFß or the growth factor, IGF-1, to HFs *in vitro* can reduce expression of MHCI by hair bulbs (Ito et al., 2004). Usually, downregulation of MHCI would drive NK activation, but it is reported that NK cell attack is prevented by low expression of NK cell activating ligands, including MICA, in combination with MIF expression (Ito et al., 2008).

Hormones and neuropeptides have also been shown to contribute to HF-associated IP mechanisms. *In vitro*, calcitonin gene-related peptide (CRGP) causes downregulation of MHCI expression on human hair follicle (Pi et al., 2013). Furthermore, vasoactive intestinal peptide (VIP) receptor in expressed by the epithelium of normal human HFs and *in vitro*, VIP expression prevents IP collapse (Bertolini et al., 2016). These findings are interesting in the context of the previously discussed influence that stress has of HF growth, and highlight the important balance that must be maintained for normal hair growth.

In the context of AA pathogenesis, HF-associated IP collapse is regarded to be essential for initiation of mechanisms driving hair loss. This relationship will be discussed in section 1.6.

### 1.4.3 Hair follicle interactions with the immune system

In recent years, research has indicated that the HF interacts with immune cells and that this interaction is fundamental for normal HF function and growth (Rahmani et al., 2020). Understanding of the relationships that exist between HFs and immune cells has stemmed from studies reporting the importance of stem cell and immune interactions for organ morphogenesis and regeneration. Functions of immune cells at regenerative sites may involve the removal of dead cells and debris from the environment, and those involved in angiogenesis. Recent studies have eloquently shown that immune cells, particularly T cells and

macrophages, interact with HFs and are critical for controlling hair growth in healthy skin.

In murine skin, T cells have been shown to use the HF to migrate to the epidermis in response to stressful stimuli (Nagao et al., 2012). It has also been shown that resident CD8 and CD4 T cell populations are specifically located at the HF epithelium, and that this tropism is maintained by follicular expression of IL-15 and IL-7 (Adachi et al., 2015). These studies highlight the role that HFs play in skin homeostasis.

In terms of hair follicle cycling, regulatory Tregs are indicated to be crucial. Human skin is home to many resident Tregs that are important for maintaining tolerance against the skin microbiota and for tissue repair processes following injury. In human skin, Tregs are found in close proximity to HFs (Sanchez Rodriguez et al., 2014). In mice, it has been shown that administration of a WNT inhibitor, leading to inhibition of HF morphogenesis, is associated with a significant decrease in Tregs in postnatal skin (Scharschmidt et al., 2017). The direct requirement of Tregs for HF cycling was then eloquently demonstrated by a study where Treg ablation prevented anagen induction (Ali et al., 2017). This study demonstrated that Treg-HF interaction via Jagged-1 is required to drive HFSC differentiation (Ali et al., 2017). Of note, alopecia universalis has been reported to develop in people with the rare genetic condition caused by a FOXP3 mutation (IPEX, immune dysregulation polyendocrinopathy enteropathy X-linked) that causes lethal autoimmunity (Nieves et al., 2004).

It has been known for many years that macrophages interact with the HF at specific stages of the growth cycle (Parakkal, 1969). However, the cruciality of macrophages for hair growth has not been fully appreciated until fairly recently (summarised in Figure 1.5). At present, there do not appear to be any observations that phagocytosis mechanisms play a role in HF-macrophage interaction. However, factors secreted by macrophages do appear to be important. $TREM2^{+}$ macrophages have been shown to directly contribute to HFSC quiescence by secretion of oncostatin M (OSM) (Wang et al., 2019). OSM signals via the JAK-STAT pathway, and JAK-STAT signalling has been shown to be crucial for telogen-associated HFSC quiescence (Harel et al., 2015). Furthermore,

anagen onset is associated with apoptosis-driven reduction in macrophages (Castellana et al., 2014; Wang et al., 2019). Castellana et al (2014) demonstrated that clodronate-induced ablation of macrophages caused release on Wnt ligands, including Wnt7b and Wnt10a, leading to anagen induction. Macrophages have also been shown to partake in the catagen phase via secretion of the fibroblast growth factor, FGF-5 (Suzuki et al., 1998; 2000).

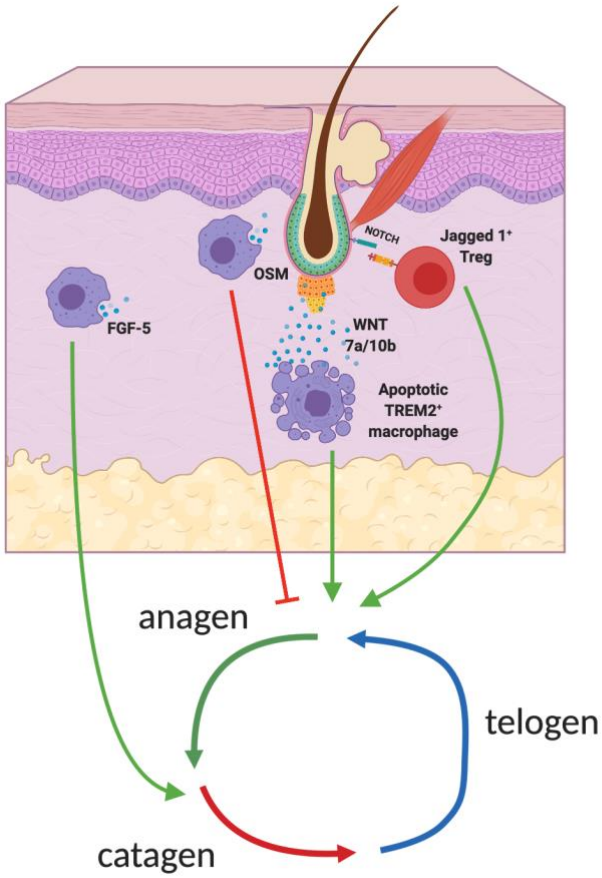


**Figure 1.5 Immune interactions with the HF**
Schematic diagram highlighting macrophage and Treg mediated functions in hair cycling. Apoptotic macrophage release of Wnt ligands and Jagged 1 Treg interaction contribute to anagen onset. Oncostatin M (OSM) release from TREM2$^+$ macrophages maintains hair follicle stem cell quiescence via JAK-STAT signalling. Fibroblast growth factor 5 (FGF-5) macrophage production contributes to catagen associated HF regression. Diagram was adapted from Rahmani et al (2020) and created with BioRender.com

The involvement of immune cells in hair growth has also been demonstrated in injury models. It is well known that full-thickness injury to the skin leads to wound induced hair regrowth (WIHG) which is caused by activation of telogen follicles. In a mouse model, it was shown that ASK1-/- wounds were associated

with delayed WIHG which was accompanied with a reduction in macrophage infiltration (Osaka et al., 2007). Subsequently, it was shown that pro-inflammatory macrophages are essential for hair regeneration following hair plucking. Plucked follicles recruited $TNF^+$ macrophages by expression of CCL2 (Chen et al., 2015) and this activated HFSC ß-catenin signalling (Wang et al., 2017). It was then shown that $CCR2^+$ CX3CR1hi Ly6Clo $TNF^+$ macrophages are involved in WIHG, which was dependent on TGFß and CX3CR1 expression (Rahmani at al., 2018a). Interestingly, applying imiquimod (TLR agonist) to mouse skin led to anagen induction which was associated with infiltration of T cells and an increase in pro-inflammatory macrophages (Amberg et al., 2016).

Taken together, the data from numerous studies highlight that macrophages are essential for homeostatic regulation of HF growth. The current evidence indicates that macrophages contribute to telogen and catagen in normal skin, whereas pro-inflammatory macrophages promote HFSC activation in the context of skin injury.

## 1.5 Immunopathogenesis of AA

AA is recognised as a T-cell mediated polygenic autoimmune condition. However, the acceptance of AA as a *bona fide* autoimmune disease was somewhat delayed despite early observations indicating dense lymphocytic infiltrate surrounding anagen HFs (Todes-Taylor et al., 1984). Genetic studies, particularly the first GWAS in 2010 (Petukhova et al., 2010), have been fundamental in generating interest in the field of AA immune pathology which have led to critical findings of the immune mechanisms driving hair loss.

### 1.5.1 Presentation and histopathology of AA

AA primarily presents as circular patches of hair loss. Although the presentation of hair loss dramatically varies between affected individuals, where it can involve one patch, multiple patches or develop to affect the entire scalp (totalis, AT) or body (universalis, AU) (Alkhalifah et al., 2010). Hair lost associated with AA may spontaneously regrow, follow multiple episodes of relapsing-remitting disease, or hair loss may last for a lifetime. AT and AU usually develop quickly and are unlikely to be spontaneously resolved. In

comparison to other hair loss conditions, AA is a form of non-scarring hair loss meaning that HFs are not destroyed. In the clinic, AA is usually diagnosed by the characteristic appearance of circular patches, the existence of abnormal hairs in lesional margins (referred to as exclamation point hairs) and an increased catagen-telogen to anagen follicle ratio (Pratt et al., 2017).

Histopathological analysis of AA skin reveals the characteristic lymphocyte infiltration surrounding the bulb region of anagen HFs. Multiple studies have indicated that the follicular infiltrate is composed CD8 T cells, CD4 T cells, mast cells, antigen presenting cells, eosinophils and NK cells (Perret et al., 1984; Fuentes-Duculan et al., 2016; Bertolini et al., 2014; Yoon et al., 2014). Epithelial cells that form the matrix of the HF appear to be the main target of infiltrating immune cells, which leads to hair shaft breakage (Messenger & Bleehan, 1984). The damaging inflammatory response causes the follicle to abandon anagen and prematurely move into catagen and then telogen.

### 1.5.2 Model of immune privilege collapse

Hair follicle IP collapse is considered to be essential for development of AA (Bertolini et al., 2020; Pratt et al., 2017). Evidence to support this model indicates that hair loss is associated with upregulation of MHCI by the bulbar HF epithelium and MHCII by HF keratinocytes (Paus et al., 1993). Whilst this model is widely accepted to drive initiation of AA, there is still no direct evidence for a role of IP collapse in mechanisms driving hair loss. Nevertheless, it is reported that specific stimuli in the skin, that may be related to stress or infection, can lead to release of inflammatory mediators which drives IP collapse and activation of autoreactive T cells. To date, the self-antigen responsible for driving T cell activation is unknown. However, it is anticipated that the auto-antigen is melanin-associated because remission is often associated with grey hair growth prior to formation of normal pigmented hair shafts (Trautman et al., 2009).

### 1.5.3 NKG2D$^+$ CD8 T cells are essential for AA pathogenesis

CD8 T cells and their production of IFN$\gamma$ are known to be vital for driving hair loss. Early observations in AA skin indicated a role for CD8 T cells due to their

abundance in follicular infiltrates and the upregulation of MHC I by the follicular epithelium. However, only recently have details of the mechanisms been elucidated.

A significant association between AA and 'stress' ligands including the ULBP locus was observed in the AA GWAS (Petukhova et al., 2010). These ligands are usually regarded as danger signals for NK cells, however in humans, the NKG2D receptor which recognises ULBP ligands is constitutively expressed by CD8 T cells (Prajapati et al., 2018; Eagle et al., 2009). Petukhova et al (2010) observed that expression of the ULBP ligand is upregulated by the HF DP and dermal sheath in early human AA lesions, and that these regions are associated with T cell infiltrates. Furthermore, they observed that the majority of infiltrating $NKG2D^+$ cells were T cells, rather than NK cells. These findings were supported by others who observed $NKG2D^+$ cells in close proximity with affected HF in lesional human skin (Ito et al., 2008). These findings led to mechanistic T cell studies involving the C3H/Hej mouse models of AA.

The majority of functional studies indicating a direct role for CD8 T cells in AA pathogenesis have been obtained from experiments using mouse models. The most commonly used AA mouse models include the C3H/Hej mice which spontaneously develop hair loss, and the humanised model which involves transplanting normal human scalp onto SCID (severe combined immunodeficient) mice and then injecting stimulated human PBMCs into the skin to drive hair loss (Sundberg et al., 1994; Gilhar et al., 2013).

The C3H/Hej is a mutant strain characterised by a TLR4 mutation. Approximately 20% of ageing C3H/Hej mice spontaneously develop AA (Sundberg et al., 1994). Spontaneous hair loss development takes time and is not guaranteed, thus grafting of lesional skin from mice who spontaneously developed hair loss to 'healthy' 10-week old C3H/Hej mice is used to initiate disease onset (McElwee et al., 1998). This model causes hair loss to develop within 6-10 weeks. Here, we refer to these mice as 'grafted recipients'. The C3H/Hej mouse has been used to demonstrate that subcutaneous injection of CD8 T cells from lymph nodes of affected mice into normal C3H/Hej mice induces localised hair loss (McElwee et al., 2005). In line with observations in

human AA lesions, affected HFs in C3H/Hej skin are associated with infiltration of NKG2D$^+$ CD8 T cells and upregulation of Rae-1 and H60 by the HF epithelium, ligands which are homologous to human ULBP proteins (Xing at al., 2014b). Importantly, the authors show that transfer of NKG2D$^+$ CD8 T cells from cutaneous lymph nodes of grafted recipients into skin of normal C3H/Hej mice caused hair loss onset (Xing et al., 2014). The MICA protein, another ligand of the NKG2D receptor, is genetically associated with AA and in addition to ULBP ligands, is upregulated in human AA lesions (Petukhova et al., 2010; Ito at al., 2008a). However, mechanistic experiments involving MICA-NKG2D engagement cannot be conducted because mice do not express the MICA protein. Nevertheless, these data indicate key milestones which implicated NKG2D$^+$ CD8 T cells as pathogenic mediators driving hair loss. CD8 T cell interaction with anagen HFs is illustrated in Figure 1.6.

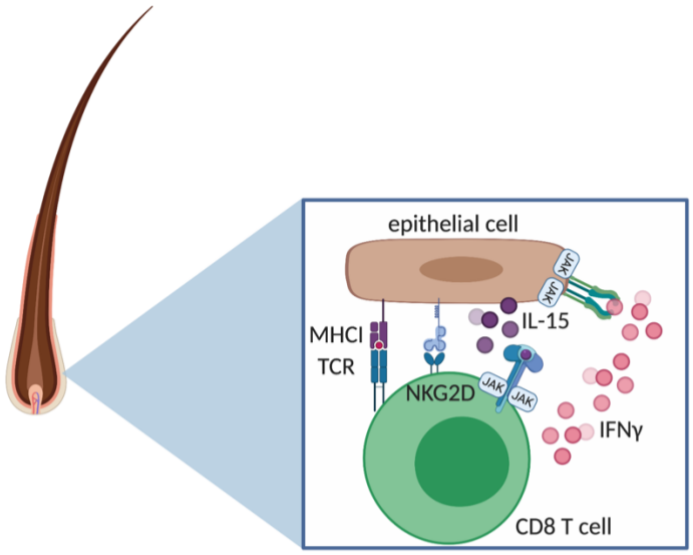


**Figure 1.6 CD8 T cell mechanisms driving hair loss**
Schematic diagram highlighting molecules involved in CD8 T cell mediated inflammation against anagen HFs. MHCI and ULBP/MICA ligands expressed by the HF epithelium bind to the CD8 T cell TCR and NKG2D receptors, respectively. IL-15 and IFNγ signal via JAK molecules to drive hair loss. Diagram was adapted from Pratt et al (2017) and created with BioRender.com

## 1.5.4 CD4 T cells in AA

CD4 T cells are known to be vital for activation of CD8 T cell responses by licensing cDC1s (Smith et al., 2004). Thus, in the context of AA, CD4 T cells must play a role in the mechanisms initiating activation of autoreactive CD8 T cells. Furthermore, a strong genetic linkage exists between HLA-DR loci and AA,

implicating APC-CD4 T cell interactions (Betz et al., 2015). Studies in the C3H/Hej AA mouse model indicate that CD4 T cells are particularly important for development of severe AA. Subcutaneous transfer of magnetic bead-purified $CD4^+CD25^-$ T cells and $CD8^+$ T cells from lymph nodes of mice with hair loss into normal C3H/Hej mice caused extensive hair loss (McElwee et al., 2005). In contrast, subcutaneous delivery of CD8 T cells alone was only capable of inducing localised hair loss. Collectively, these observations indicate that CD4 T cells are important for the development of AA. It is very likely that CD4 T cells are also important for initial activation of autoreactive CD8 T cells. However, it is difficult to study mechanisms initiating AA because the mouse model involves transfer of previously activated CD8 T cells.

In human AA lesions, CD4 T cells are found in follicular infiltrates, and some reports indicate that they are more abundant than CD8 T cells (Perret et al., 1984). Additionally, histological analysis indicates that IL-$17^+$ CD4 T cells are located in the dermis surrounding HFs in human AA skin, but they do not appear to be infiltrating HFs (Tanemura et al., 2013). Microarray transcriptome profiling in AA skin indicated upregulation in genes associated with both Th1 and Th2 responses (Suarez-Farinas et al., 2015). In peripheral blood, the proportion of IL-$17^+$ CD4 T cells are elevated in AA compared to HCs (Han et al., 2015). In contrast, the proportions of Tregs (FoxP$3^+$ CD4) were found to be significantly reduced in comparison to HCs (Han et al., 2015). Another study profiling circulating T cells did not observe any changes in the frequencies of IL-$17^+$ CD4 T cells (Czarnowicki et al., 2017). However, the authors observed an increase in the frequency of IL-$13^+$ CD4 T cells (Th2) and central memory CD4 T cells ($CD45RO^+CCR7^+$), alongside a decrease in the frequency of effector memory CD4 T cells ($CD45RO^+CCR7^-$) in AA patients compared to HCs. Collectively, these studies describe an association between AA and changes in Th1, Th2 and Th17 cells. Whilst the importance of CD4 T cells in AA pathogenesis remains to be thoroughly investigated, it is clear that multiple cytokines associated with the generation and activity of specific CD4 T cell populations are dysregulated in AA.

### 1.5.5 Cytokines in AA

The cytokines which are most commonly associated with AA pathogenesis are IFNγ and IL-15 (illustrated in Figure 1.6). The importance of IFNγ in mechanisms driving hair loss has been demonstrated using the C3H/Hej model. Intravenous administration of IFNγ to C3H/Hej mice causes transient hair loss (Gilhar et al., 2005). In contrast to wild-type grafted recipient C3H/Hej mice, IFNγ knock-out grafted recipient mice do not develop hair loss (Freyschmidt-Paul et al., 2006). Furthermore, intraperitoneal administration of anti-IFNγ antibodies at time of grafting prevents hair loss onset in grafted recipient mice (Xing et al., 2014). This study did not test the effect of IFNγ neutralisation on reversing hair loss. Interestingly, IFNγ potently induces upregulation of follicular MHCI and MHCII expression, and therefore is thought to play an important role in IP collapse (Rückert et al., 1998). Collectively, these observations led to experiments testing the ability of blocking JAK-STAT signalling to prevent hair loss development in the C3H/Hej model, and then in humans.

Oral administration of a JAK 1/2 inhibitor successfully prevented hair loss and associated infiltration of NKG2D$^{+}$ CD8 T cells in grafted recipients (Xing et al., 2014). The authors also demonstrated that topical application of JAK1/2 inhibitors to grafted recipients who had already developed hair loss was able to induce regrowth (Xing et al., 2014). This seminal study led to clinical studies testing the efficacy of Ruxolitinib and Tofacitinib in promoting hair regrowth in AA patients. Various open-label studies have indicated that JAK inhibitors cause hair regrowth (>50%) in between 60-75% of patients (Mackay-Wiggan et al., 2016; Jabbari et al., 2018; Liu et al., 2017). A phase 3 JAK-inhibitor open label trial conducted by Pfizer is now in progress (https://www.pfizer.com/science/find-a-trial/nct04006457).

The clinical studies generated in response to findings by Xing et al (2014) have been fundamental in generating recent pharmaceutical interest in AA. Whilst these data indicate a fundamental role for JAK-STAT signalling in human AA pathogenesis, they do not precisely confirm the importance of IFNγ because of the broad targeting of these small molecule inhibitors. For example, Ruxolitinib in targeting JAK 1 and 2, prevents IFNγ receptor signalling, in addition to the

signalling of the common γ chain cytokines, including IL-2, IL-15 and IL-21. Furthermore, the success of JAK-inhibitors is also attributed to promoting factors that directly activate HFSCs (Harel et al., 2015). The broad targeting of JAK inhibitor therapy is related to the occurrence of side-effects in treated individuals, which most commonly include bacterial, fungal and viral infection, thrombocytopenia and neutropenia (Wang et al., 2018).

Assessment of C3H/Hej mice led to the observation that IL-15 and IL-15RB are upregulated by the follicular epithelium (Xing et al., 2014). Thus, it was demonstrated that blocking IL-15 signalling using IL-15RB antibodies prevented development of hair loss in grafted recipients (Xing et al., 2014). Importantly, immunofluorescent staining in affected human skin indicated that IL-15 and IL-15RA are expressed by the HF epithelium (Xing et al., 2014). IL-15 is a cytokine known to play a critical role in survival and generation of CD4 and CD8 memory T cells (van Leeuwen et al., 2009; Richer et al., 2015). As mentioned previously, IL-15 is one of the cytokines that signals through a receptor complex which utilises the common γ receptor chain (Willem W Overwijk, 2009). In the context of CD8 T cell effector function, IL-15 is reported to play a role in NKG2D-mediated activation of CD8 T cells. Liver damage in patients with acute hepatitis A has been reported to be a result of IL-15 mediated bystander activation of non-specific NKG2D$^{+}$ CD8 T cells (Kim et al., 2018). Furthermore, IL-15 has been shown to modulate NKG2D signalling to cause TCR-independent activation of CD8 T cells (Meresse et al., 2004). Interestingly, IL-15 induces upregulation of MICA in biopsies from CeD and HCs (Hüe et al., 2004). As previously noted, it is unclear whether NKG2D and IL-15 can facilitate T cell activation in the absence of TCR stimulation, however these studies indicate that they are important for re-activation of memory T cells and appear to drive an innate-like cytotoxic state in effector T cells. Notably, follicular IL-15 expression has been shown to support CD8 T cell residency, and it's expression is important for T cell-mediated responses in the skin (Adachi et al., 2015). Thus, it appears that follicular IL-15 expression is important for dermal immunity, but it's expression in the context of AA promotes autoreactive T cell activity.

Cytokine profiling in human serum has indicated dysregulation in various cytokines associated with specific T-helper subsets. The levels of circulating IL-

17A, IL-21, IL-22 and IL-23 are significantly increased in AA compared to controls (Tembhre & Sharma, 2013; Bilgic et al., 2015; Atwa et al., 2015). In addition, the levels of TNF and IL-6 are elevated (Bilgic et al., 2015; Atwa et al., 2015). IFNγ and IL-15 levels are also elevated in serum of AA cohorts (Ebrahim et al., 2019; Tembhre & Sharma, 2013; Kasumagic-Halilovic et al., 2010). At a tissue level, micro array transcriptome analysis indicates upregulation of IFNγ, in addition to IL-23 and IL-13 in human AA skin (Suarez-Farinas et al., 2015). Thus, published studies indicate broad dysregulation in multiple cytokines that indicate involvement of type 1, type 2 and type 17 responses.

Biologic therapies targeting cytokines are successful in the treatment of multiple inflammatory disorders, including IBD, RA and PsA. Studies highlighting dysregulation of type-17 related cytokines in AA patients prompted studies testing the ability of biologics targeting IL-17A and IL-23 pathways for promoting hair regrowth. Blockade of IL-17A using subcutaneous administration of Secukinumab was not proven to be effective for promoting hair regrowth in a randomised double blinded pilot study (Guttman-Yassky et al., 2018). Eleven AA patients were enrolled, but only one patient experienced partial regrowth out of the 3 patients who remained in the study until the primary end point. Clinical studies testing the efficacy of Ustekinumab, an antibody which targets the p40 subunit which is part of both IL-12 and IL-23, have also been conducted in small AA cohorts. An initial study administered subcutaneous Ustekinumab to three AA patients with varying levels of hair loss (Guttman-Yassky et al., 2016). Impressive hair regrowth was observed in a patient with AT, whereas hair regrowth was marginal in the other patients who had patchy hair loss. Notably, the patient with AT who responded to treatment, had the shortest disease duration. Subsequently, four AA patients with AT who received Ustekinumab did not experience any hair regrowth (Ortolan et al., 2019). At present, the current evidence from human studies is not satisfactory to draw conclusions of the involvement of IL-12 and IL-23 in AA pathogenesis.

### 1.5.6 The involvement of other immune cells

Few studies have focused on the role of innate immune cells in AA pathogenesis. NK cells are an obvious contender because they are activated by NKG2D ligation

(Eagle et al., 2009). In healthy skin, it is peculiar that NK cells do not attack normal HFs because of the absence of MHCI expression. Interestingly, NKG2D$^+$ NK cells are not observed in close proximity to HFs in normal skin, but are abundant within follicular infiltrate in AA skin (Ito et al., 2008). IP collapse, with associated downregulation of MIF and upregulation of MICA ligands, are thought to contribute to this aggregation of NK cells (Ito et al., 2008). This study postulates that NK cell activity is suppressed in normal skin and that NK cell contribute to pathology in AA. In contrast, another study using the grafted recipient C3H/Hej model indicated that depletion of NK cells using anti-asialo GM1 caused exacerbation of hair loss, indicated by accelerated hair loss occurrence in comparison to untreated grafted recipients (Kaufman et al., 2010).

Autoantibodies are commonly generated in patients with autoimmune diseases, but their presence does not necessarily indicate their involvement in disease pathology. In the context of lupus and RA, autoantibodies are pathogenic because they activate complement and target citrullinated self-proteins, respectively (Walport, 2002; Dahlqvist et al., 2003). Autoantibodies against follicular antigens have been detected in AA circulation (Tobin et al., 1994). Notably, autoantibodies against follicular antigen were also observed in control sera, but were lower titer compared to AA sera (Tobin et al., 1994).

To our knowledge, conventional DCs have not been profiled in AA patients. However, a study analysing AA skin biopsies indicated that the number of CD1a$^+$ cells are increased following application of a contact sensitizing treatment that is commonly used to treat patchy AA (details of this treatment are provided in section 1.6) (Heffler et al., 2002). CD1a is receptor which binds lipids, and is a characteristic marker of Langerhans cells (LCs) (Porcelli et al., 1989). LCs are cells found in the epidermis and have a mixed DC/macrophage transcriptome (Mowat et al., 2017). They are able to migrate to lymph nodes, and in humans are able to cross-present antigen to CD8 T cells, thus functioning similar to cDC1 cells (Artyomov et al., 2015). Whilst the study conducted by Heffler et al (2002) is interesting in the context of response to a contact sensitiser, it does not indicate that LCs are involved in AA pathogenesis. Another study indicated that pDCs are present in perifollicular infiltrates in AA skin (Abou Rahal et al., 2014),

but investigation of their role in AA pathology has not been reported. Additionally, despite the important relationship between macrophages and HFs for normal hair growth, macrophages have not been profiled in the context of AA.

### 1.5.7 Comorbidities

AA is associated with several comorbidities. They most commonly include atopic conditions (asthma, rhinitis, eczema), thyroid problems (under and overactive) and other autoimmune diseases, such as vitiligo, psoriasis, RA and inflammatory bowel disease (IBD). Atopy is very common in AA cohorts with an incidence of approximately 38% (Huang et al., 2013). Studies have shown that comorbid atopy is associated with more severe and earlier onset AA suggesting atopic mechanisms may contribute to hair loss (Mohan & Silverberg, 2015; Lee et al., 2014). Furthermore, elevated serum IgE is associated with AA patients who have no history of atopy (Attia et al., 2010; Bakry, 2014). Notably, Ustekinumab was reported to promote hair regrowth in one AA patient, and this patient also had atopic dermatitis (AD), whereas the two non-responders had no history of atopy (Guttman-Yassky et al., 2016). In another study, Ustekinumab did not promote hair regrowth in any of the four patients who were treated, but the authors highlighted that one patient subsequently received JAK inhibitor treatment and experienced hair regrowth. In addition to hair regrowth, the patient's dermatitis improved (Ortolan et al., 2019). Taken together, this evidence indicates cross-over between immunological mechanisms driving atopy and AA.

Vitiligo has been significantly associated with AA occurrence in some studies (Chu et al., 2011), but a large-scale meta-analysis did not identify a significant odds ratio connecting the two diseases (Lee et al., 2019). Thyroid conditions are also significantly associated with AA, with a collective incidence of approximately 8-14% in AA cohorts (Huang et al., 2013; Lee et al., 2019). Thyroid conditions related to AA include both Hashimoto's and Grave's disease, with associated prevalence of 2.9% and 1.4%, respectively (Lee et al., 2019). Presence of thyroid disease comorbidity has been associated with increased severity of hair loss, but in contrast to atopy, thyroid conditions appear to be more common in AA patients who develop the disease later in life (Lee et al.,

2014). Interestingly, thyroid dysfunction is also reported to be common in cohorts of vitiligo patients (Díaz-Angulo et al., 2015).

Other autoimmune diseases including IBD and RA have also been linked with AA, with prevalence rates of 2% and 3.9%, respectively (Huang et al., 2013). These estimates are higher that the incidence rates of IBD and RA in the general population, reported to be approximately 1% (Symmons, 2002; Alatab et al., 2020). However, as mentioned previously, a meta-analysis did not identify a significant association between AA and IBD (Lee at al., 2019). The connection between T1D and AA in less clear. Huang et al (2013) report a high prevalence of 11%, whereas the meta-analysis conducted by Lee at al (2019) indicated that T1D was associated with a lower odds ratio. In contrast, AA is associated with higher risk of comorbid hyperinsulinemia and metabolic syndrome (Lee et al., 2019). Lupus erythematosus (SLE) is also a comorbidity associated with AA, however identifying the true incidence rates of a comorbid association is difficult as SLE is known to cause hair loss (Concha & Werth, 2018). Nevertheless, a recent study assessing over 50,000 AA patients indicated that the prevalence of SLE in AA populations is 0.3%, which is higher than the 0.1% prevalence in the general population (Kridin et al., 2020).

Psychological morbidity is also extremely common in AA cohorts and poses a huge burden for many people affected by AA. Strikingly meta-analysis of AA comorbidity studies reported that psychiatric disease in AA patients has a prevalence of 49% (Lee et al., 2019). These data are predominantly associated with anxiety and depression which are reported here to be at a prevalence of 27% and 19%, respectively. At an individual study level, psychiatric morbidity is reported to affect between 60-70% of AA individuals at least once during their lifetime (Ruiz-Doblado et al., 2003; Colón et al., 1991).

## 1.6 Current treatment strategies for AA

The current therapeutic landscape for AA is bleak. The most common treatments involve steroids (topical or intralesional, IL), diphenylcyclopropenone (DCP) and immunosuppressants. The use of the treatments varies according to hair loss severity. Whilst some of the treatments may provoke hair regrowth in patients with patchy hair loss, they are usually ineffective for those with severe AA (Pratt

et al., 2017). In the clinic, hair loss severity is determined using the Severity of Alopecia Tool (SALT) (Olsen et al., 2004). The SALT score is obtained by combining the percentage of hair loss from the four regions of the scalp: back, front, left side and right side.

Corticosteroids may be administered topically or injected into AA lesions. IL steroids are first line therapy for adults with <50% patchy hair loss. For this treatment, triamcinolone acetonide is injected subcutaneously into lesional scalp. This treatment may also be used to treat eyebrow hair loss. Injections are administered every 4-6 weeks and treatment is stopped at 6 months if no regrowth is observed. IL therapy was shown to be effective in 82% of patients with limited AA (>50% improvement from baseline) (Tan, Tay, Goh, & Giam, 2002b). Skin atrophy is a common side effect of IL steroids, but the skin will recover within months following treatment (Pratt et al., 2017). To date, there have been no randomised controlled trials (RCTs) for the treatment of AA with IL corticosteroids (Spano & Donovan, 2015). Whilst multiple IL steroid injections may promote hair regrowth in those with mild AA, they are ineffective for severe AA (Hull et al., 2003). Topical corticosteroid administration may be used in children with AA. In an RCT, application of topical betamethasone valerate promoted hair regrowth in 60% of patients with mild AA (<26% scalp involvement) (Mancuso et al., 2003).

Diphenylcyclopropenone (DCP) is a topical contact sensitiser used for the treatment of moderate to severe AA (>50% scalp involvement). The treatment involves applying a small amount of DCP to the scalp, and applying more two weeks later with the goal of inducing a dermatitis reaction. No RCTs have been conducted for the use of DCP in AA treatment. However, it is reported that approximately 58% of AA patients respond (Rokhsar et al., 1998). Efficacy is mostly related to those with patchy hair loss, as response rates in AT/AU patients is low (Rokhsar et al., 1998). Furthermore, DCP therapy can be particularly unpleasant because the treatment requires induction of a hypersensitivity reaction.

The mechanism of action involved in DCP induced hair regrowth in not known. Possible mechanisms have been postulated which involve promoting ‘antigen

competition' (Happle, 1980; Spano & Donovan, 2015). Analysis of inflammatory transcripts following DCP treatment indicate increased expression of IL-10 which may be involved in controlling autoreactive T cell responses (Hoffmann et al., 1994). As previously mentioned, DCP treatment caused an increase in $CD1a^{+}$ cells in AA skin but it is not known how this contributes to hair regrowth (Heffler et al., 2002). Whilst DCP may appear to be successful, a response often takes months, and AA relapse is very common when treatment is stopped (Rokhsar et al., 1998). Unfortunately, relapse has been reported to occur in 62% of patients who previously responded (Wiseman et al., 2001).

A proportion of individuals may be offered systemic treatment with methotrexate or other systemic immunosuppressant but these options are usually a last option for severe refractory AA. Methotrexate is a folic acid antagonist that functions as a broad immunosuppressant which is used for the treatment of various inflammatory conditions, including psoriasis and RA (Haustein & Rytter, 2000; Bedoui et al., 2019). A meta-analysis of studies assessing methotrexate response indicated a complete response (100% regrowth) rate of 35.8% (Phan et al., 2019). Whereas, a mean response rate of 63% was associated with 50-100% regrowth (Phan et al., 2019). The drawbacks of methotrexate treatment include the side effect profile and the relapse rates. The pooled AA relapse rate associated with methotrexate treatment was identified as 47.7% (Phan et al., 2019). Side effects do appear to be rare but can be dangerous, therefore patients require regular monitoring.

## 1.7 Hypothesis and aims

The current treatment options for AA are severely limited. AA has endured significant neglect in comparison to other autoimmune diseases which can now be treated with a repertoire of sophisticated therapeutics. Thankfully, the emergence of data indicating the immunological mechanisms involved in AA pathogenesis have caused a step-wise change in terms of scientific and pharmaceutical interest, which has led to wide-scale testing of JAK-inhibitor therapy. Whilst small-molecule inhibitors offer promise, they are costly and are associated with side-effects which may impact the uptake of these drugs. It is

also apparent that some individuals with AA do not respond to JAK inhibitor therapy.

CD8 T cells are clearly important for driving hair loss. However, autoimmune diseases often involve dysregulation of many components of the immune system, and at present it is not well understood how other immune cells and inflammatory mediators contribute to AA pathogenesis. It is also not well understood how the acute response driving AA may differ from the response involved in chronic AA. We hypothesise that better characterisation of other immune cells in addition to CD8 T cells will identify novel therapeutic targets.

Thus, the experiments described in this thesis set out to address four key aims:

1. To extensively profile the systemic cytokine signature associated with AA

2. To characterise the peripheral immunophenotype associated with AA by assessing circulating frequencies of CD8 T cells, CD4 T cells, B cells, dendritic cells and natural killer cell populations

3. To identify relationships between circulating immune parameters and clinical features

4. To identify key pathways associated with AA at a tissue level using an un-biased transcriptomic approach

# 2 Materials and methods

## 2.1 NHS ethical approval process

Research involving human specimens requires ethical approval to safeguard study volunteers by ensuring appropriate measures are in place regarding consent and for the collection, storage and use of samples. The various steps involved in obtaining approval are summarised in Figure 2.1. We first prepared the ethical documents for submission to the West of Scotland Research Ethics Committee (REC). Documents including the study protocol detailing the scope and aims of the project, participant information sheet, participant questionnaires and consent forms were drafted and submitted via the integrated research application system (IRAS). We were then invited to attend a meeting to discuss our study rationale and protocol with the REC committee members and were issued with comments to improve and amend our study documents. Following completion of requested alterations, ethical approval was obtained on the 20th March 2017. Research and development approval was granted shortly after, which allowed us to initiate participant recruitment.


**Figure 2.1 Ethical approval process**
Timeline representing the various steps involved in obtaining ethical and R&D approval for the AA research clinic. Research ethics committee, REC; research and development, R & D; integrated research application system, IRAS.

## 2.2 Participant recruitment

Participants with a diagnosis of AA were recruited via the Glasgow hair clinic or through advertisement at the Queen Elizabeth University Hospital, Glasgow

Royal Infirmary or on the Alopecia UK website. Recruitment to the clinic was facilitated by Dr Susan Holmes and Fiona Moffat. Recruited participants were >18 years old and had a confirmed diagnosis of alopecia areata. Participants had no diagnosis of another major inflammatory disease (rheumatoid arthritis, inflammatory bowel disease, psoriasis, psoriatic arthritis, ankylosing spondylitis) but may have had a history of atopy (asthma, eczema or hayfever) or thyroid condition. All participants provided written informed consent in line with research ethics committee approval (West of Scotland REC 1, 17/WS/0029). On providing consent, participants could opt to donating peripheral blood and additional samples including a stool sample and skin biopsy.

Age and sex-matched healthy control (HC) participants were recruited to the AA research clinic to donate blood, stool samples and skin biopsies under the same ethical approval as AA participants (West of Scotland REC 1, 17/WS/0029). HCs were also recruited to donate blood at the clinical facility at the British Heart Foundation building at the University of Glasgow. Ethical approval for volunteers recruited at the University of Glasgow was provided by the College of Medical, Veterinary, & Life Sciences Ethics Committee for Non-Clinical Research involving Human Subjects (project no. 200180145).

Participants with psoriatic arthritis were recruited as part of another cross-sectional study under separate ethics at the Universities of Glasgow and Manchester. Data obtained from samples collected from these participants were used for comparison purposes.

The cohorts used in each part of this study are described in detail in the relevant chapters. Here we provide an overview of how the samples contributed to each part of the analyses. The same cohort was used for the cytokine and phenotyping studies described in chapters 3, 4 and 5. A small proportion of samples were not used for the B cell phenotyping analysis due to low number of cells obtained during extraction of PBMCs. In chapter 7, five of the AA skin samples were collected from participants also used in the peripheral blood experiments, whereas the remaining skin samples were collected from participants recruited later in the study. The majority of samples used to measure markers of intestinal inflammation, including calprotectin, zonulin and

TTG IgA, were measured using samples which overlapped with the peripheral blood studies, in addition to a proportion of samples collected later in the study.

### 2.2.1 Collection of clinical data

Clinical information was recorded on an electronic case report form (eCRF) stored in the Castor EDC database (https://www.castoredc.com). The eCRF was specifically designed for this study according to the REC approved questionnaires. All clinical data was anonymised and associated with participant ID numbers. Clinical information was collected from each AA volunteer as described in Table 2.1. Detailed information regarding the participants' disease status was recorded including disease duration, current treatment, comorbidities and severity of current scalp and body hair loss.

Severity of scalp hair loss was determined using the severity of alopecia tool (SALT) score (Olsen et al., 2004). The SALT score represents the percentage of hair loss which is calculated by estimating the amount of hair loss on the top, back, left side and right side of the scalp. In chapters 3 and 5, AA participants were stratified into four groups based on their SALT score: inactive, <50% hair loss, >50% hair loss and total hair loss (>95%). The inactive group represented individuals who had previously lost hair but were experiencing regrowth when attending the clinic to donate samples. Body hair loss was recorded as no hair loss, partial or total involvement. On attending the clinic, consented participants were also asked to complete the hospital anxiety and depression scale (HADS) questionnaire (Zigmond & Snaith, 1983). This questionnaire was used to generate a score to indicate the participant's current depression and anxiety status (no depression/anxiety, 0-7; mild, 8-10; moderate, 11-14; severe, 15-20).

**Table 2.1 Patient information questionnaire**
Anonymised clinical information was recorded for each participant and recorded electronically in CASTOR EDC.

| | |
|---|---|
| **Participant information** | Age |

| | | |
|---|---|---|
| | Gender | |
| **Alopecia areata information** | Disease duration | Years since onset of first episode of hair loss |
| | Current treatment | Topical steroid, intralesional steroid, diphenylcyclopropenone, methotrexate |
| | Comorbidities | Hayfever, asthma, eczema, anaemia, vitiligo, thyroid condition, type 2 diabetes, coeliac disease |
| | Severity of alopecia areata tool (SALT) score | 0-100% |
| | Disease severity category | no loss, <50%, >50%, >95% scalp loss |
| | Facial and body hair loss | no loss, partial loss, total loss |
| | Current and previous episodes of hair loss | regrowth, partial regrowth, no regrowth |
| **Sample collection** | Peripheral blood | 27 mL |
| | Skin biopsy | Location of biopsy- temporal, parietal, occipital scalp |
| | Stool sample | Collection pack provided to participant |
| **Quality of life questionnaire** | Hospital anxiety and depression (HADS) score | no depression/anxiety (0-7)<br>mild (8-10)<br>moderate (11-14)<br>severe (15-20) |

## 2.3 Sample collection and processing

### Peripheral blood

Whole blood was collected in two 9mL lithium heparin (LH) vacutainers, one 6mL

EDTA vacutainer and one 3 mL SST vacutainer (BD Biosciences, US). Phlebotomy procedures were carried out by medical practitioners or trained phlebotomists. All blood was processed within two hours of collection. Plasma and serum were isolated from EDTA and SST vacutainers, respectively. Vacutainers were centrifuged at 1200 G for 10 minutes at room temperature (RT). Plasma and serum were aliquoted and stored at -80°C prior to analysis of inflammatory proteins.

**Skin biopsies**

4mm punch biopsies were excised from the scalp and immediately immersed in 4% formalin for fixation or in RNAlater (Qiagen, Germany) to preserve RNA. Formalin fixed tissue was stored at RT prior to processing for histology as described in section 2.7. Tissue stored in RNAlater was incubated at 4°C for 4 hours and then stored at -80°C prior to extraction of RNA as described in section 2.5.1.

**Stool samples**

Stool sampling kits were provided to participants to self-collect samples. Collected samples were posted by participants to the laboratory at the New Lister Building, University of Glasgow for processing. On collection, samples were weighed and aliquoted. Aliquots were frozen at -80°C prior to extraction of calprotectin as described in section 2.6.2.

## 2.4 Flow cytometry

### 2.4.1 Peripheral blood mononuclear cell (PBMC) isolation

Whole blood collected in LH vacutainers was diluted (1:1) in phosphate-buffered saline (PBS, Gibco, Life Technologies, UK) and layered onto 4 mL histopaque-1077 (Sigma-Aldrich, UK) in 10 mL aliquots. Optimal separation required blood and histopaque to be at RT. Samples were centrifuged at 2100 rpm for 25 minutes with no brake. Following centrifugation, four layers are formed: plasma, PBMCs, histopaque and red blood cells. The PBMC layer was carefully removed and washed in 30 mL of PBS at 400 G for 10 minutes. The supernatant was removed and PBMCs were washed again with 10 mL of PBS at 200 G for 10 minutes. Cells were resuspended in 1 mL PBS and counted using a

haemocytometer. The number of cells per mL of blood was calculated and recorded.

### 2.4.2 Surface staining and acquiring of cells

Cells were stained for viability (1:1000, fixable viability dye, eBioscience) and FC receptor blocked (1:200, Fc receptor binding inhibitor, eBioscience, US) for 30 minutes at 4°C in the dark. Cells were washed in 1 mL FACS buffer (PBS, 2% foetal calf serum, 2mM EDTA) at 400 G for 10 minutes. The supernatant was removed and cells were incubated with fluorescently labelled antibody panels suspended in 200 µL of FACS buffer. Antibodies used are depicted in Table 2.2. 30 µL of brilliant stain buffer (BD Biosciences, US) was added to panels containing both brilliant violet and ultraviolet fluorophores. Cells were incubated with antibodies for 30 minutes at RT in the dark. Cells were washed in 2 mL of FACS buffer at 400 G for 10 minutes. Where appropriate, secondary antibodies were added to cells and incubated for a further 20 minutes at RT in the dark. Cells washed again in 2 mL of FACS buffer at 400 G for 10 minutes. The supernatant was removed and cells were suspended in 500 µL of fixation buffer (Biolegend, US). Cells in fixation buffer were stored at 4°C overnight.

On the following morning, cells were washed twice in 1 mL of FACS buffer at 400 G for 10 minutes. Samples were filtered and acquired on the LSR Fortessa instrument (BD Biosciences, US) at the Flow Core Facility at the Institute of Infection, Immunity and Inflammation, University of Glasgow. Flow cytometry data were analysed using FlowJo Software (version 10.2). Frequency and cell counts were exported and graphed using GraphPad Prism7 (US).

**Table 2.2 Flow cytometry antibodies**
PBMCs were isolated from peripheral blood and stained with antibody panels to identify populations of CD8 T cells, CD4 T cells, B cells, natural killer cells and dendritic cells.

| Marker | Fluorochrome | Concentration | Clone | Manufacturer |
|---|---|---|---|---|
| **CD1c** | PECy7 | 1/100 | L161 | Biolegend |
| **CD10** | BV605 | 1/40 | HI10a | Biolegend |
| **CD11c** | AF700 | 1/100 | Bu15 | Biolegend |

| | | | | |
|---|---|---|---|---|
| **CD123** | BV421 | 1/66 | 6H6 | Biolegend |
| **CD127** | BV421 | 1/40 | A019D5 | Biolegend |
| **CD14** | Biotin | 1/200 | HCD14 | Biolegend |
| **CD141** | PE | 1/100 | IT2.2 | Biolegend |
| **CD15** | Biotin | 1/200 | W6D3 | Biolegend |
| **CD16** | FITC<br>BUV395 | 1/200<br>1/200 | 3G8<br>3G8 | Biolegend<br>BD Biosciences |
| **CD19** | BV510<br>Biotin | 1/100<br>1/400 | HIB19<br>HIB19 | Biolegend<br>Biolegend |
| **CD27** | PECy7 | 1/50 | O323 | Biolegend |
| **CD3** | AF700<br>Biotin | 1/100<br>1/100 | UCHT1<br>UCHT1 | Biolegend<br>Biolegend |
| **CD38** | AF700 | 1/40 | HB-7 | Biolegend |
| **CD4** | FITC<br>BUV395 | 1/100<br>1/100 | OKT4<br>RPA-T4 | Biolegend<br>BD Biosciences |
| **CD43** | FITC | 1/40 | MEM-59 | Biolegend |
| **CD45** | BV510 | 1/100 | HI30 | Biolegend |
| **CD45RA** | PerCP Cy5.5 | 1/100 | HI100 | Biolegend |
| **CD45RO** | BV605 | 1/100 | UCHL1 | Biolegend |
| **CD56** | PECy7<br>Biotin | 1/40<br>1/200 | HCD56<br>M-DC8 | Biolegend<br>Miltenyi |
| **CD69** | BV605 | 1/40 | FN50 | Biolegend |
| **CD8** | BUV395 | 1/100 | RPA-T8 | BD Biosciences |
| **CCR6** | BV605 | 1/50 | G034E3 | Biolegend |
| **CCR7** | BV421 | 1/100 | G043H7 | Biolegend |
| **CCR9** | APC | 1/40 | L053E8 | Biolegend |
| **CXCR3** | PerCPCy5.5 | 1/50 | G025H7 | Biolegend |

| | | | | |
|---|---|---|---|---|
| **CX3CR1** | APC | 1/50 | 2A9-1 | Biolegend |
| **HLA-DR** | BV510 | 1/100 | L243 | Biolegend |
| **NKG2D** | PE | 1/50 | 1D11 | Biolegend |
| **CLA** | FITC | 1/50 | HECA-452 | Biolegend |
| **IgD** | BV421 | 1/100 | IA6-2 | Biolegend |
| **IgM** | PerCP Cy5.5 | 1/40 | MHM-88 | Biolegend |
| **Viability** | eFluor 780 | 1/1000 | NA | eBioscience |

## 2.5 RNA sequencing

### 2.5.1 RNA extraction and library preparation

Whole skin biopsies that had been frozen in RNAlater (ThermoFisher, UK) were disrupted and homogenised using stainless steel beads and QIAzol Lysis Reagent (Qiagen, Germany) in a TissueLyser at 25Hz (Qiagen, Germany) for 5 minutes. RNA was isolated according to manufacturer's protocol (RNeasy Lipid Tissue Mini Kit, Qiagen, Germany) with inclusion of the genomic DNA removal step (RNase-Free DNase set, Qiagen, Germany). RNA quality was assessed using a Fragment Analyser (Agilent Technologies, USA) using an RNA kit (Agilent Technologies, Netherlands).

1000ng of high quality RNA (RIN 8.7 ± 0.5) from each sample was used for library preparation. Fragmentation (5 minutes at 94°C), conversion to cDNA, adapter ligation (KAPA Dual-Indexed Adapter Kit, KAPA Biosystems, USA) and amplification (9 cycles) were performed according to manufacturer's protocol (mRNA HyperPrep Kit, KAPA Biosystems, USA). Appropriate library fragment size was confirmed on the Fragment Analyser (Agilent Technologies, Netherlands) using the standard sense NGS kit (Agilent Technologies, Netherlands). Sample libraries were pooled at 15 nM. The sample pool was loaded (final loading concentration 1.9pM) onto NextSeq500 instrument (Illumina, USA) according to manufacturer's guide (NextSeq System: Denature and Dilute Libraries, Illumina,

USA). Paired end (2x75 bp), dual index sequencing was performed using an Illumina using a mid-output flow cell.

### 2.5.2 Data processing, alignment and quality control

High quality run was confirmed using Illumina sequencing analysis viewer. A cluster density of 268.5 gave in total 189 million (M) pass filter reads. Data was demultiplexed, resulting in 29.1 – 42.1M (mean 35.1M) paired reads per sample. A standard processing pipeline was used to align and process the RNA-seq data set. The fastQ files were quality controlled using FastQC (v0.11.7, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then aligned to the reference genome and transcriptome GRCh38 (release 91) using STAR (v2.6) with -- quantMode GeneCounts --outFilterMultimapNmax 1 and --outFilterMatchNmin 35-- (Dobin et al., 2012). We used a Star index with a --sjdbOverhang of 74. Read counts were then merged and genes with an average of <1 read per sample were excluded from the analysis.

### 2.5.3 Differential expression analysis

Expression and differential expression (DE) values between AA and HC samples were generated using DESeq2 (v1.24) (Love et al., 2014). Differential expression data were visualised using Searchlight (v2.0.0, https://github.com/Searchlight2/Searchlight2) conducted by John Cole (Cole et al., 2018), using the GRCh38 (release 91) genome file with an absolute fold cut-off of 1 and an adjusted p value cut-off of 0.05. Benjamini-Hochberg correction was applied to control the false discovery rate. Principal component analysis (PCA) was performed indicating % variation attributed to PC1 and PC2. The heatmap represents differentially expressed genes using Spearman distances, UPMA agglomeration and mean reordering, on pre-scaled (Z-scores) gene expression values. A volcano plot was also generated indicating the relationship between fold change and adjusted p values for each differentially expressed gene.

### 2.5.4 Gene set enrichment and Cibersort analysis

Hyper-geometric gene-set enrichment analysis (HGSEA) was used to identify gene sets significantly up or down regulated in AA RNA-seq data compared to HCs. We specified the gene ontology (GO) biological process database for over-representation analysis with adjusted p value cut-off of 0.05. The most enriched gene sets identified in the upregulated and downregulated DE gene lists were represented in bar charts indicating the -log10 p-value on the x axis and the gene set name on the y axis. For upstream regulator analysis, the TRRUST database (Han et al., 2017) with a z-score cut-off of 2 was used (https://www.grnpedia.org/trrust/). HGSEA and TRRUST interpretation were also visualised using Searchlight (v2.0.0).

Distribution of immune cell types within skin RNAseq datasets were calculated using the de-convolution package Cibersort (https://cibersort.stanford.edu/) (Newman et al., 2015). The LM22 (22 whole blood immune cell types) signature gene file was selected for Cibersort analysis.

### 2.5.5 Comparison of patchy and severe data sets

Differential expression values comparing patchy (AP), severe (AT) and HC RNAseq datasets were generated using bcbioRNAseq Differential Expression MarkDown (https://github.com/hbc/bcbioRNASeq) using the GRCh38 (release 91) genome file with an absolute fold cut-off of 0.5 and an adjusted p value cut-off of 0.05. The heatmap represents differentially expressed genes between AP and HCs. Data were scaled by row using the ward.d2 method for clustering (Ward, 1963). The Venn diagram was generated using the online Venny (v2.1.0) tool (https://bioinfogp.cnb.csic.es/tools/venny/).

## 2.6 Measurement of inflammatory proteins

### 2.6.1 Multiplex U-plex assay

Plasma aliquots from AA patients, PsA patients and HCs were defrosted. Concentration of cytokines were measured using the U-plex proinflammatory combo 1 and U-plex Th17 combo 1 human assay kits according to Manufacturer's protocol (Meso Scale Diagnostics, US). Biotinylated target antibodies were mixed

with their associated linkers and pooled. The pooled antibody-linker cocktail (50 µL) was added to each well of a 96 well plate and incubated with shaking for 1 hour at RT. The plate was washed three times with PBST (PBS with 0.05% TWEEN-20, ThermoFisher UK). Plasma diluted (1:1) with diluent 43 (MSD, US) or calibrator was added to each well. The plate was sealed and incubated with shaking for 1 hour at RT. The plate was washed three times with PBST, and then 50 µL of the detection antibody solution was added to each well. Plate was sealed and incubated with shaking for 1 hour at RT. The plate was washed again three times with PBST, and then 150 µL of GOLD read buffer (MSD, US) was added to each well. The plate was immediately read on an MSD instrument.

### 2.6.2 ELISA

Zonulin (Elabscience, US) and anti-tissue transglutaminase IgA (BioRad Laboratories, US) levels were measured by ELISA in plasma and serum, respectively, according to manufacturer's protocols. Plasma or serum were defrosted and then centrifuged. The samples and standards were added in 100 µL aliquots to a pre-coated 96-well plate and incubated for the specified time. Liquid was removed from the wells and washed 3 times with the appropriate wash buffer. Conjugated detection antibodies (100 µL) were added and incubated for the specified time. The plate was washed again 3 times, and then the substrate was added for the specified time. Incubation steps were carried out at RT or 37°C as specified by manufacturer's protocol. The specified volume of the stop solution was added and the optical density (OD) was measured using a plate reader (Dynex Technologies, US) set to 450nm. Concentration of zonulin or anti-TTG IgA were interpolated from the standard curve in GraphPad Prism7 (US).

Calprotectin was extracted from 100mg of stool by adding 4.9 mL of faecal extraction buffer (Calpro AS, Norway). The samples were placed in a shaker for 30 minutes at 1000 rpm, and then centrifuged for 3 minutes at 6000 G. The supernatant was removed and frozen at -20°C prior to measuring calprotectin. Levels of calprotectin were measured in duplicate by ELISA according to manufacturer's protocol (CALP0170 ALP kit, Calpro AS, Norway). Calprotectin extracts were diluted 1:100 with the appropriate dilution buffer, and ELISA steps

above were followed to measure calprotectin. ODs were measured using a plate reader (Dynex Technologies, US) set to 405 nm. Concentrations of calprotectin were interpolated from the standard curve and values were converted to obtain calprotectin values in mg/kg.

## 2.7 Histology

Skin biopsies were fixed in 10% formalin for 24 hours. Tissues were processed and embedded in paraffin by Fiona McMonagle at the University of Glasgow, or by Morag McNulty and colleagues at the Core Histology Facility at the Laboratory Medicine Building, Queen Elizabeth University Hospital. Tissue sections were cut using a microtome and stained with Harris haematoxylin and eosin (H&E) using standard protocol.

### 2.7.1 Immunohistochemistry

Tissue sections were dewaxed using xylene and rehydrated using different concentrations of ethanol (100%, 90%, 70%), prior to rinsing in water. Antigen retrieval was performed by microwaving sections immersed in Tris EDTA buffer (pH8) for 5 minutes in a pressure cooker. Sections were cooled, rinsed in water and then treated with 3% $H_20_2$ for 10 minutes to quench endogenous peroxidase. Sections were rinsed in water, and then incubated with casein blocking solution (Vector Laboratories, UK) for 1 hour at RT. Blocking solution was removed, and primary antibodies diluted in antibody diluent (Agilent DAKO, UK) were added to sections for 1 hour at RT. Primary antibodies were mouse anti-human CD68 (clone KP1, 1/200, Agilent DAKO, UK) or mouse anti-human CD3 (clone F7.2.38, 1/100, Agilent DAKO, UK). Isotype antibodies were used as controls (IgG1, kappa, Agilent DAKO, UK). Sections were washed twice in TBST (Tris-buffered saline, 0.05% Tween-20) for 5 minutes. ImmPRESS (anti-mouse Polymer kit, Vector Laboratories, UK) was then added to sections for 30 minutes at RT. Sections were washed again in TBST, and then DAB peroxidase substrate (Vector laboratories, UK) was added and incubated in the dark for 5 minutes. Sections were washed in water for 10 minutes, counterstained with Harris haematoxylin, dehydrated and then mounted using DPX solution. IHC and H&E stains were visualised using a light microscope and images were captured using the Olympus cell^B software.

## 2.8 Subset linear regression

The model, $Y_i = ß_0 + ß_1X_{1i} + ß_2X_{2i} + ß_3X_{3i} + ε_i$, was fitted where $Y_i$ represents the dependent variable and X represents the independent variable. The scripts used for this analyses were edited from codes developed by Umer Ijaz, University of Glasgow. Regression models were generated in R using the leaps package (https://cran.r-project.org/web/packages/leaps/leaps.pdf) (D'Amore et al., 2016). The leaps package performs a search for the best subsets of the variables in x for predicting y in linear regression. Cross validation was used to assess the predictive accuracy of each model (https://cran.r-project.org/web/packages/DAAG/DAAG.pdf). Models were ranked according to cross validation error (CVE) which is a value indicating the mean difference between the model's estimated values and the real values. Adjusted $R^2$ values were calculated for each model to indicate the proportion of variation in the dependent variable explained by independent variable(s). Variables selected in the models with the lowest CVE were presented in a heatmaps, where positive and negative predictors were indicated in red and blue, respectively, with associated adjusted p values.

## 2.9 Statistical analysis

Flow cytometry and protein concentration data are individually represented with means. Statistical differences between AA patients and HCs were identified using a Mann-Whitney U test (GraphPad Prism8, U.S). Statistical differences between three or more groups were identified using a Kruskal-Wallis test with a Dunn's test for multiple comparisons (GraphPad Prism8). Correlation coefficients were determined using Pearson correlation with a two-tailed test (GraphPad Prism8, U.S). P values are shown as *<0.05, **<0.01 and ***<0.005.

Statistical analysis of stratified data presented in chapter 5 was performed in R (v 3.6.3). Flow cytometry data were first normalised using the hyperbolic Arcsinh method with a scaling factor of 5 (Amir et al., 2013). This function is similar to biexponential transformation but integrates a scaling factor which accounts for potential negative values obtained in flow cytometry data. Statistical differences between groups were calculated using a one-way ANOVA with Bonferroni correction. Schematic diagrams were made using Biorender.com.

# 3 The systemic cytokine signature of AA patients, and the association with comorbidities

## 3.1 Introduction

Cytokines are protein communicators that dictate immune responses. They are key in driving protective responses against pathogens, promoting immune memory and immune regulation. Their potency can make cytokines dangerous in specific circumstances, such as during aberrant immune responses against self-antigen. Thus, blockade of cytokines is a successful therapeutic strategy for many autoimmune diseases by dampening harmful responses to limit tissue pathology (Rosman et al., 2013).

IFN$\gamma$ and IL-15 are important cytokines in AA pathogenesis because of their role in driving the cytotoxic function of $CD8^+$ T cells against hair follicles (Xing et al., 2014). Systemically, people with AA have increased levels of Th17-assocaited cytokines, namely IL-17A, IL-23, IL-21 and IL-22, in addition to classical pro-inflammatory cytokines, TNF and IL-6 (Tembhre & Sharma, 2013; Bilgic et al., 2015; Atwa et al., 2015). Transcriptome profiling of AA skin indicates an increase in expression of type-1 cytokines, in addition to an increase in IL-23 (Suarez-Farinas et al., 2015). Together, these studies indicate involvement of type-1 and type-17 cytokine responses in AA pathogenesis.

The complexity of the systemic cytokine signature may be attributed to the relationship between AA and incidence of additional inflammatory comorbidities, most commonly affecting the thyroid and skin (Huang et al., 2013). Psychological illness is also common and poses a huge problem for many with AA (Aghaei et al., 2014; Hunt, 2005). Psychological morbidity is associated with many autoimmune diseases, most notably rheumatic and inflammatory bowel diseases (Nerurkar et al., 2018; Martin-Subero et al., 2015); conditions that are also associated with increased systemic cytokines. These observations are being clinically exploited to identify whether blocking inflammatory cytokines could be effective in immune-mediated depression (Kappelmann et al., 2018). The association between inflammation and depression had not been studied in the context of AA until we published these data (Bain et al., 2019).

The aims of the experiments described in this chapter were to:

1. Further elucidate the systemic cytokine signature associated with AA, and compare levels of cytokines in AA with those observed in psoriatic arthritis (PsA).

2. Identify associations between circulating inflammatory cytokines, depression and anxiety.

## 3.2 Participant characteristics

Samples collected from 39 AA patients, 23 PsA patients and 26 healthy controls were used for cytokine analyses (Table 3.1). The AA patient cohort included 4 individuals in remission, 13 with <50% hair loss, 5 with >50% hair loss and 17 with severe hair loss (>95%). Patients experienced AA for between 1 and 55 years, and 20 patients were atopic and 6 had a thyroid-related illness. Additionally, 1 patient had vitiligo, 1 had epilepsy and 1 had pernicious anaemia. One patient had female pattern baldness in addition to AA. Importantly, AA patients had no diagnosis of a major inflammatory disease, including Crohn's disease (CD), ulcerative colitis (UC), rheumatoid arthritis (RA), PsA, psoriasis (PsO), systemic lupus erythematosus (SLE) or ankylosing spondylitis (AS). Approximately 50% of the AA patients were not receiving treatment at the time of recruitment; 8 were receiving diphenylcyclopropenone (DCP), 8 were receiving intralesional steroid injections and 1 patient was receiving methotrexate. Psychological morbidity was assessed in all AA participants using the Hospital Anxiety and Depression Scale.

PsA patients were recruited as part of a cross-sectional study ran by the Universities of Manchester and Glasgow. 23 participants from this cohort were included in these analyses for comparison purposes. None of the PsA patients were on biologic treatment. Healthy controls were recruited at the University of Glasgow based on their age and gender to match the patient cohorts.

**Table 3.1 Characteristics of participants with alopecia areata, psoriatic arthritis and healthy controls.**
Plasma samples collected from these individuals were used for analyses of circulating cytokines. IL steroids; intralesional steroids, SD; standard deviation.

| | Alopecia areata | Psoriatic arthritis | Healthy controls |
|---|---|---|---|
| Total | 39 | 23 | 26 |
| Gender F/M | 30/9 | 16/7 | 16/9 |
| Age (years) ± SD | Female 45 ± 13.1<br>Male 37 ± 10.2 | Female 40 ± 14.4<br>Male 43 ± 9.2 | Female 46 ± 9.7<br>Male 37 ± 13.4 |
| Disease duration (years)<br>Average ± SD | 1-55<br>16.6 ± 12.9 | | |
| **Disease severity**<br>Inactive<br><50%<br>>50%<br>Total loss | <br>4<br>13<br>5<br>17 | | |
| **Comorbidities**<br>Atopic<br>Thyroid illness<br>Vitiligo<br>Epilepsy<br>Pattern baldness<br>Pernicious anaemia | <br>20<br>6<br>1<br>1<br>1<br>1 | | |
| **Treatment**<br>None<br>Diphenylcyclopropenone<br>IL steroids<br>Methotrexate | <br>19<br>8<br>8<br>1 | | |

## 3.3 Pro-inflammatory and type-17 cytokines are increased in AA

The potent pro-inflammatory cytokines, IL-6 and TNF, are increased in other cohorts of AA patients (Atwa et al., 2015). Here, plasma levels of TNF and IL-6 were modestly, but significantly increased in AA patients compared to HCs. IL-1ß was also elevated, whereas, IL-8 was significantly decreased in AA (Figure 3.1A).

Type-1 cytokine responses are proven to be critical in driving hair loss in the skin, but no change was observed in the levels of circulating IFNγ or IL-12p70 (Figure 3.1B). Some patients did have higher levels of IFNγ and IL-12, but the concentrations were not different to a proportion of HCs. IL-15, a driver of cytotoxic T cell activity, was also unchanged in AA patients compared to HCs. Levels of IL-27, a member of the IL-12 family, were also not different from HCs (Figure 3.1B).

Previous reports indicate that type-17 cytokines including IL-17A, IL-21, IL-22 and IL-23 are increased in the circulation of people with AA (Atwa et al., 2015). In our cohort, circulating levels of IL-17A, IL-23 and IL-21 were also significantly elevated (Figure 3.2A, 3.2B). IL-22 was not increased in AA compared to HCs, however the concentration of IL-17F was significantly increased (Figure 3.2A). In attempt to understand the biological relevance of these data, the concentrations of type-17 cytokines in AA plasma were compared to cytokine levels in plasma from PsA patients. PsA is an autoimmune disease affecting the joints and skin where the IL-17/-23 axis is critical for pathology (Suzuki et al., 2014). The levels of IL-17A, IL-17F and IL-23 were not increased in PsA patients, compared to HCs (Figure 3.2A). However, the mean level of IL-17A observed in PsA patients was higher than in the AA cohort, thus a proportion of PsA patients have high levels of IL-17A (Figure 3.2A). IL-21 levels were increased in PsA patients compared to HCs, but the increase was more pronounced in AA (Figure 3.2B).

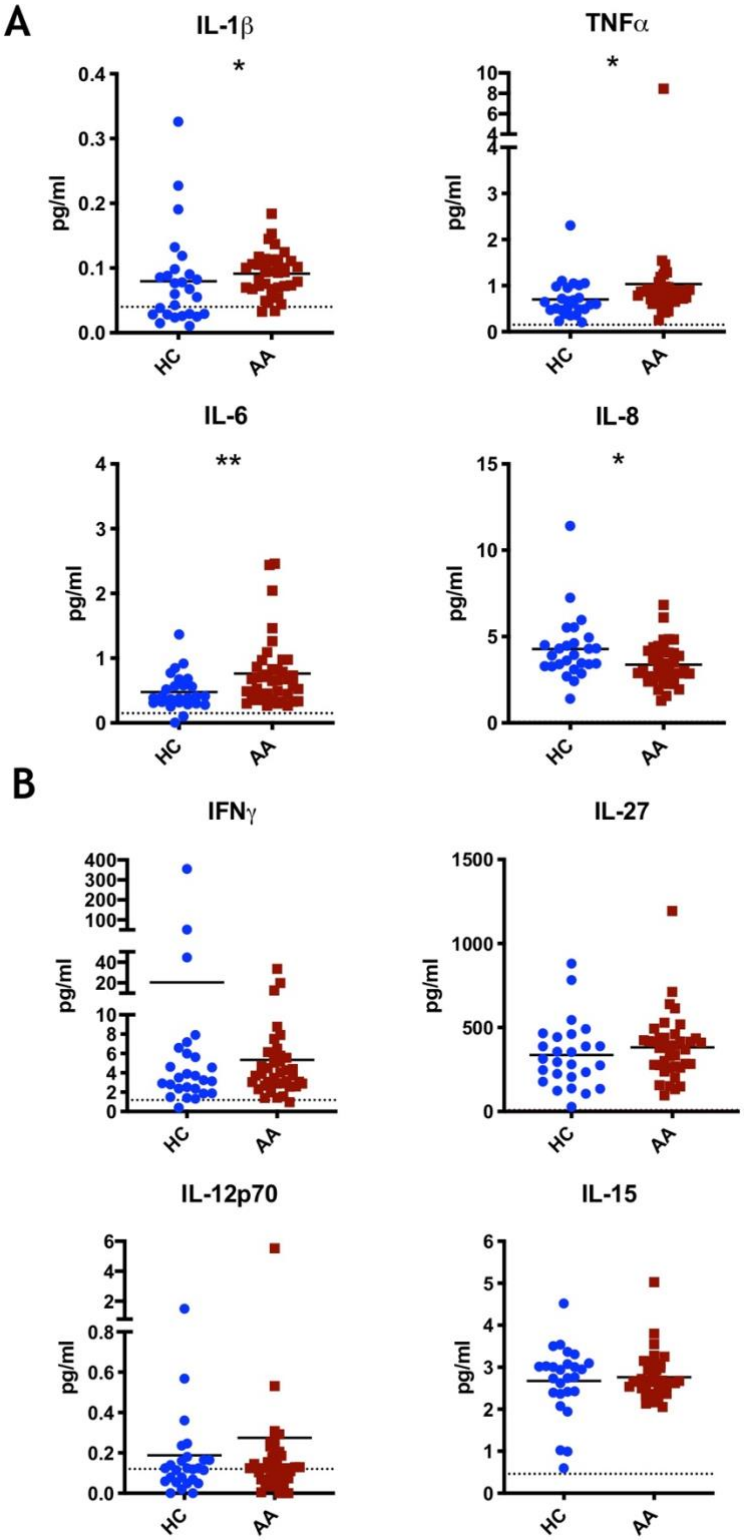


**Figure 3.1 Inflammatory and type 1 cytokine levels.**
Cytokines were measured in plasma from alopecia areata (AA) patients and healthy controls (HCs) using a multiplex assay. Means are plotted (pg/mL) and black dotted lines represent the lower limit of detection. Statistical differences between groups were determined using a Mann-Whitney U test, *p<0.05, **p<0.01.

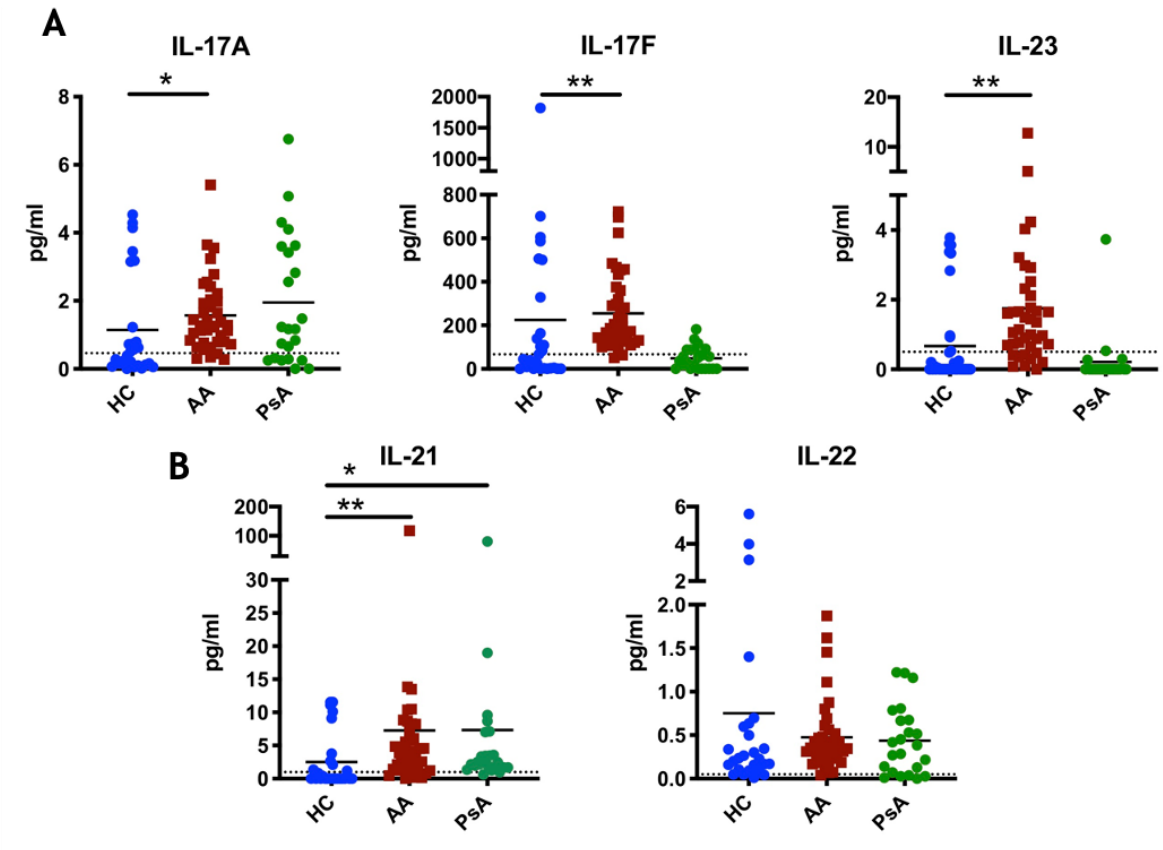


**Figure 3.2 Type-17 associated cytokine levels.**
Cytokines were measured in plasma from alopecia areata (AA) patients, psoriatic arthritis (PsA) patients and healthy controls (HCs) using a multiplex assay. Means are plotted (pg/mL) and black dotted lines represent the lower limit of detection. Statistical differences between groups were determined using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test, *p<0.05, **p<0.01.

## 3.4 Type-2 associated cytokines are increased in AA

Atopic dermatitis and allergic rhinitis are common comorbidities in people with AA (Huang et al, 2013), and analysis of AA skin has indicated differential expression of type-2 associated genes (Suarez-Farinas et al., 2015). We observed that the levels of the tissue alarmins IL-31 and IL-33 were significantly increased in AA compared to HCs (Figure 3.3A). IL-17E, also known as IL-25, was also raised in AA circulation in comparison to HCs. Levels of IL-31, IL-33 and IL-17E were not altered in PsA patients. IL-13 and IL-4 concentrations were measured but the vast majority of samples were below the limit of detection of the assay (data not shown). The anti-inflammatory cytokine IL-10 was also measured but was not significantly different from HCs, despite a proportion of AA patients having high circulating levels (Figure 3.3B).

Approximately 50% of this cohort had a history of atopy (eczema, hayfever, asthma). It was hypothesised that the overall increase in type-2 cytokines observed in Figure 3.3A is attributed to patients who are atopic. Thus, patients were stratified to compare levels of cytokines in atopic and non-atopic AA patients (Figure 3.4A). The circulating levels of IL-31, IL-33, IL-17E were significantly elevated in both AA non-atopic and AA atopic patients compared to HCs (Figure 3.4A). Notably, AA patients with the highest levels of IL-31, IL-33 and IL-17E were in the atopic group. The levels of IL-17A and IL-23 were also significantly increased in AA non-atopic and AA atopic patients compared to HCs (Figure 3.4B).

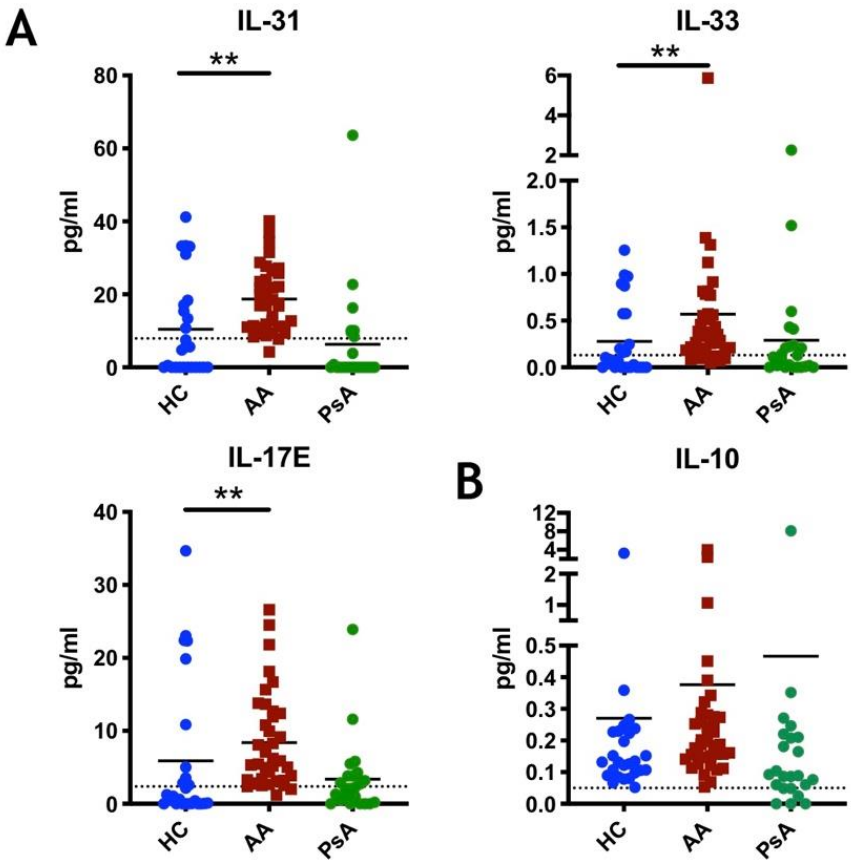


**Figure 3.3 Type-2 associated and interleukin-10 cytokine levels**
Cytokines were measured in plasma from alopecia areata (AA) patients, psoriatic arthritis (PsA) patients and healthy controls (HCs) using a multiplex assay. Means are plotted (pg/mL) and black dotted lines represent the lower limit of detection. Statistical differences between groups were determined using a Kruskal-Wallis test followed by a Dunn's multiple comparison test, **p<0.01.

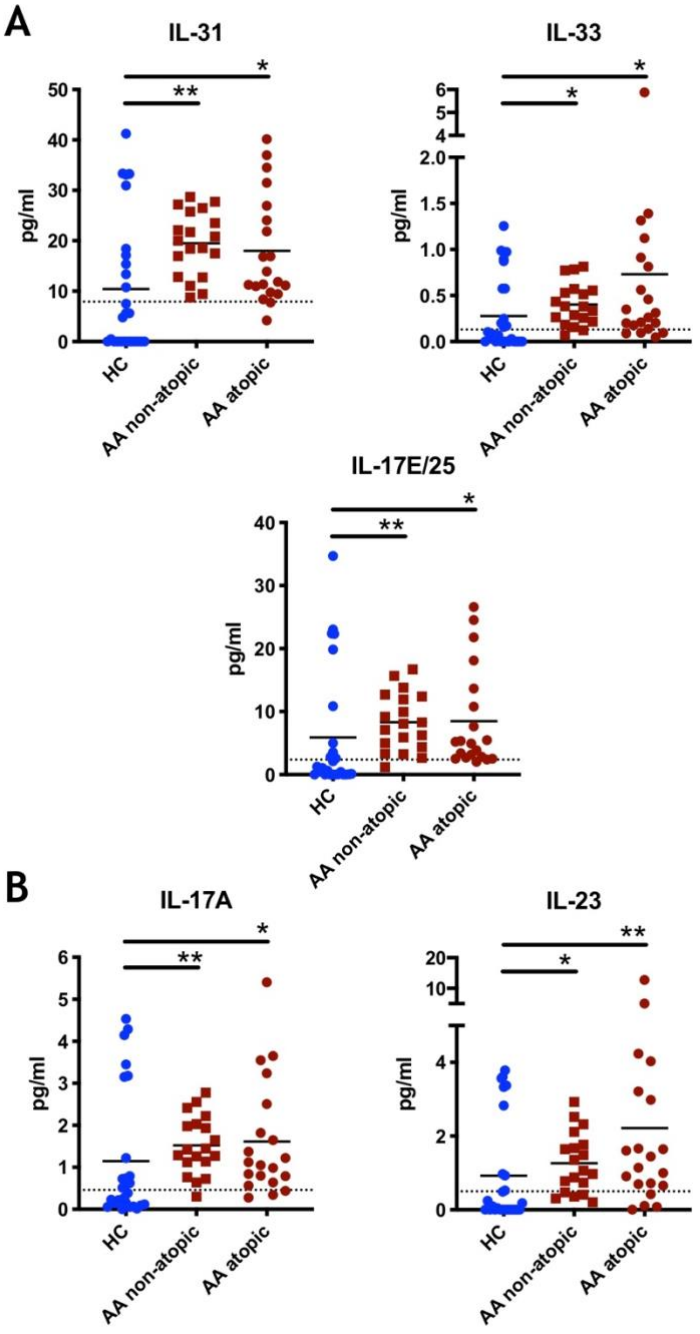


**Figure 3.4 Circulating cytokine levels in atopic and non-atopic AA patients.**
Cytokines were measured in plasma from alopecia areata patients who were atopic (AA atopic) or non-atopic (AA) and healthy controls (HCs) using a multiplex assay. Mean are plotted (pg/mL) and black dotted lines represent the lower limit of detection. Statistical differences between groups were determined using a Kruskal-Wallis test followed by a Dunn's multiple comparisons, *p<0.05, **p<0.01.

## 3.5 Cytokine concentration and disease severity

The AA cohort includes patients with inactive, patchy and severe disease which we hypothesised might contribute to differences in plasma cytokine levels. Severity of hair loss was measured using the severity of alopecia tool (SALT) score which calculates the percentage of hair lost from the scalp. AA patients

were then stratified into four groups based on their SALT score: inactive, <50% hair loss, >50% hair loss and total loss (>95% hair loss) (Figure 3.5).

Concentrations of IL-17A and IL-17F were significantly increased in the total loss category compared to HCs (Figure 3.5A), whereas groups of patients with patchy hair loss were not significantly elevated compared to HCs. Notably, the mean levels of IL-17A in those with total loss (1.71pg/mL) and >50% loss (1.76pg/mL) are similar, and the >50% loss cohort lacks sufficient power (n=5). The levels of IL-23 were unchanged between all AA groups and HCs (Figure 3.5A). IL-21 concentration was significantly increased in patients with >50% loss and total loss compared to HCs (Figure 3.5B).

Levels of IL-31 and IL-17E were also significantly elevated in the total loss category compared to HCs (Figure 3.6A), but no change was observed in the other categories compared to HCs. We also observed that the concentration of IL-33 was significantly raised in patients with >50% hair loss compared to HCs, however we reiterate that this group lacks sufficient power (Figure 3.6A). The concentration of IL-10 was unchanged between all groups of patients and HCs (Figure 3.6B).

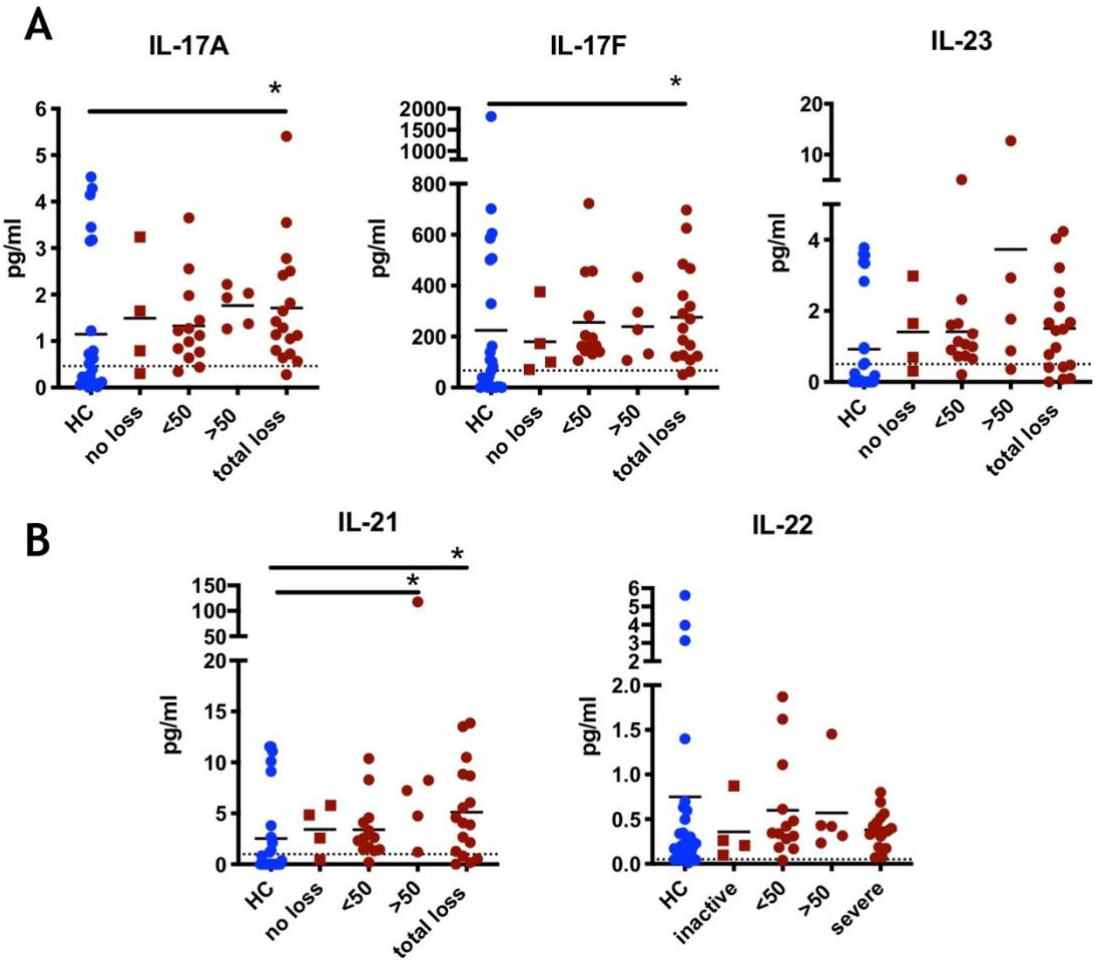


**Figure 3.5 Type-17 associated cytokine levels and disease severity.**
Cytokines were measured in plasma from alopecia areata (AA) patients and healthy controls (HCs) using a multiplex assay. AA patients were stratified based on severity of alopecia tool (SALT) scores; no loss/inactive, <50% hair loss, >50% hair loss and severe >95% hair loss. Means are plotted (pg/mL) and black dotted lines represent the lower limit of detection. Statistical differences between groups were determined using a Kruskal-Wallis test followed by a Dunn's multiple comparison test, *p<0.05.

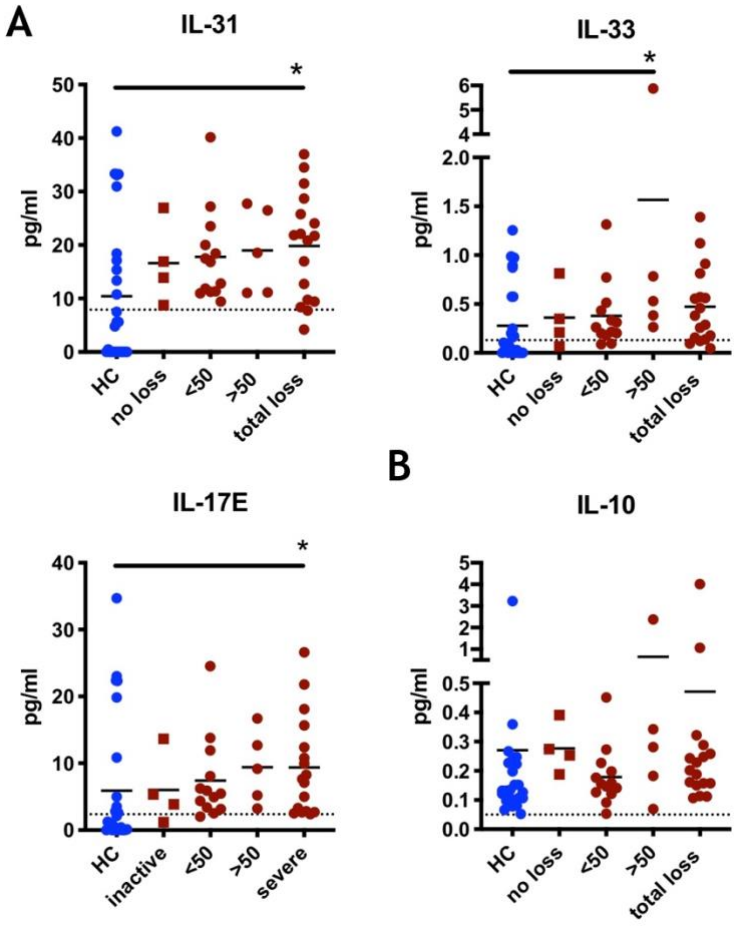


**Figure 3.6 Type-2 associated cytokine levels and disease severity.**
Cytokines were measured in plasma from alopecia areata (AA) patients and healthy controls (HCs) using a multiplex assay. AA patients were stratified based on severity of alopecia tool (SALT) scores; no loss/inactive, <50% hair loss, >50% hair loss and severe >95% hair loss. Means are plotted (pg/mL) and black dotted lines represent the lower limit of detection. Statistical differences between groups were determined using a Kruskal-Wallis test followed by a Dunn's multiple comparison test, *p<0.05.

## 3.6 Cytokines and the relationship with psychological distress

### 3.6.1 Depression and anxiety are common in individuals with AA

Depression, anxiety and related conditions affect between 60-70 % of people with AA which can have severe detrimental effects on an individual's well-being (Ruiz-Doblado et al., 2003; Colón et al., 1991). Psychological distress of AA patients enrolled in this study was assessed using the Hospital Anxiety and Depression Scale (Zigmond & Snaith, 1983). Depression and anxiety scores were grouped into four categories: no depression/anxiety (0-7), mild (8-10), moderate (11-14) and severe (15-20).

Within the total cohort of 39 individuals, 18% of patients were experiencing signs of depression (Figure 3.7A, 8% mild, 3% moderate, 8% severe). Of these individuals, 51% indicated behaviours of anxiety (Figure 3.8A, 28% mild, 10% moderate, 13% severe). As in Figures 3.5 and 3.6, AA patients were stratified based on hair loss severity to identify associations between psychological morbidity and severity of hair loss. A higher proportion of individuals with patchy hair loss (Figure 3.7B, 16.7% mild, 5.6% moderate, 11.1% severe) indicated signs of depression, with 55% experiencing anxiety (Figure 3.8B). In contrast, individuals with severe AA were less effected by depressive symptoms (Figure 3.7D). One patient indicated severe signs of anxiety and depression, but the other individuals were not affected by depression despite some mild indication of anxiety (Figure 3.8D). In the clinic, it is apparent that disease duration can associate with incidence of psychological distress. 50% of patients with patchy hair loss with a disease duration of ≤10 years experienced depressive symptoms (Figure 3.7C), with 75% experiencing anxiety (Figure 3.8C).

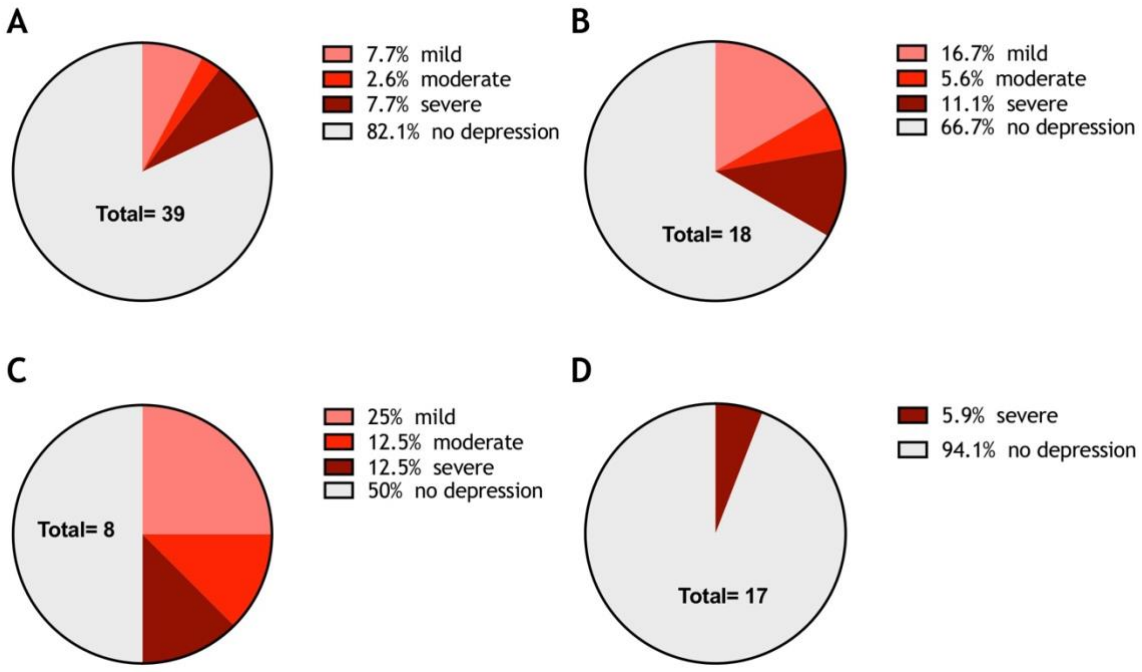


**Figure 3.7 Depression scores of AA patients.**
Depression scores were determined using the Hospital Anxiety and Depression Scale, HADS questionnaire. (A) depression scores for total AA participants. (B) depression scores for AA participants with patchy hair loss (<95%). (C) depression scores for AA participants with patchy hair loss and disease duration of <10 years. (D) depression scores for AA participants with severe hair loss (>95%). Categories were determined as no depression (0-7), mild (8-10), moderate (11-14) and severe (15-20).

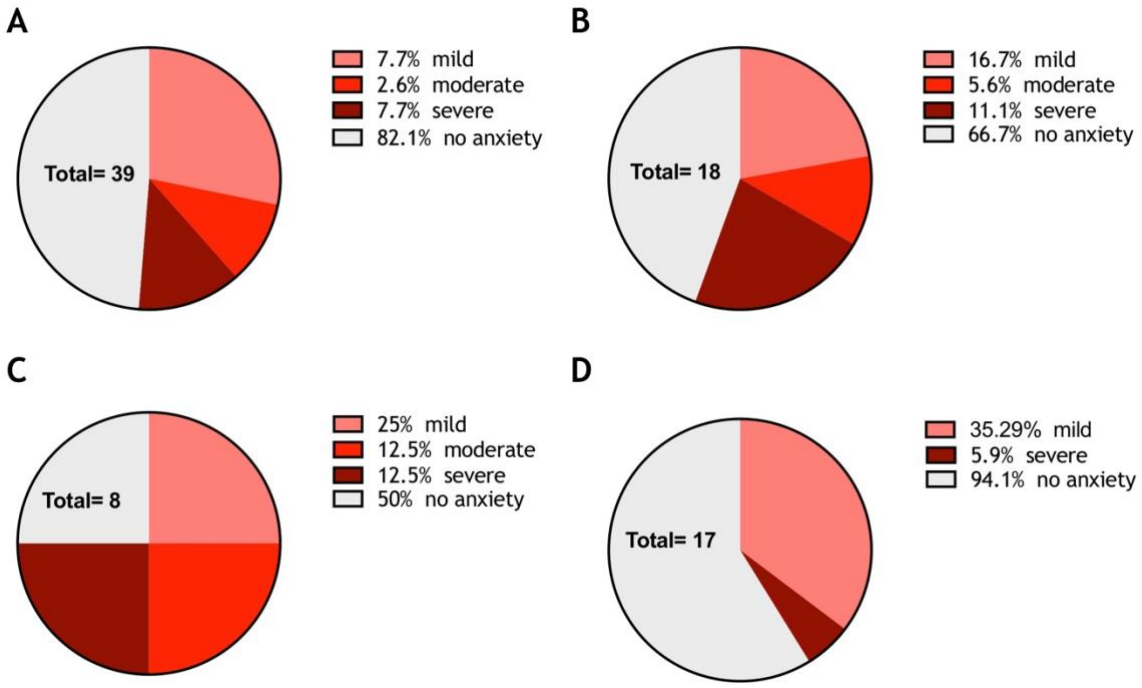


**Figure 3.8 Anxiety scores of AA patients.**
Anxiety scores were determined using the Hospital Anxiety and Depression Scale, HADS questionnaire. (A) anxiety scores for total AA participants. (B) anxiety scores for AA participants with patchy hair loss (<95%). (C) anxiety scores for AA participants with patchy hair loss and disease duration of <10 years. (D) anxiety scores for AA participants with severe hair loss (>95%). Categories were determined as no anxiety (0-7), mild (8-10), moderate (11-14) and severe (15-20).

### 3.6.2 Levels of IL-22 and IL-17E positively predict depression score

AA impacts physical appearance which contributes to distress, but depression is multifactorial and inflammatory mediators are now implicated as potential drivers (Dowlati et al., 2010; Farooq et al., 2016). Here, it was hypothesised that the systemic cytokine signature of AA patients is associated with incidence of psychological morbidity. Subset linear regression analysis was therefore used to identify inflammatory cytokine predictors of depression and anxiety scores (Figure 3.9). Subset regression identified IL-22 and IL-17E as positive predictors of depression score. In contrast, IL-31 was a negative predictor of depression score. As shown in Figure 3.6A, IL-17E levels were increased in patients with severe hair loss. However, this finding is not robust as the other groups of AA patients are too small.

Anxiety and depression are often experienced together, and linear regression indicated that IL-22 was also a positive predictor of anxiety score, but the model

had a low adjusted correlation coefficient (data not shown, adj$R^2$=0.156). Disease severity and disease duration were negatively associated with anxiety score (data not shown), but no associations were observed between these parameters and depression. No associations between age and depression or anxiety scores were observed.

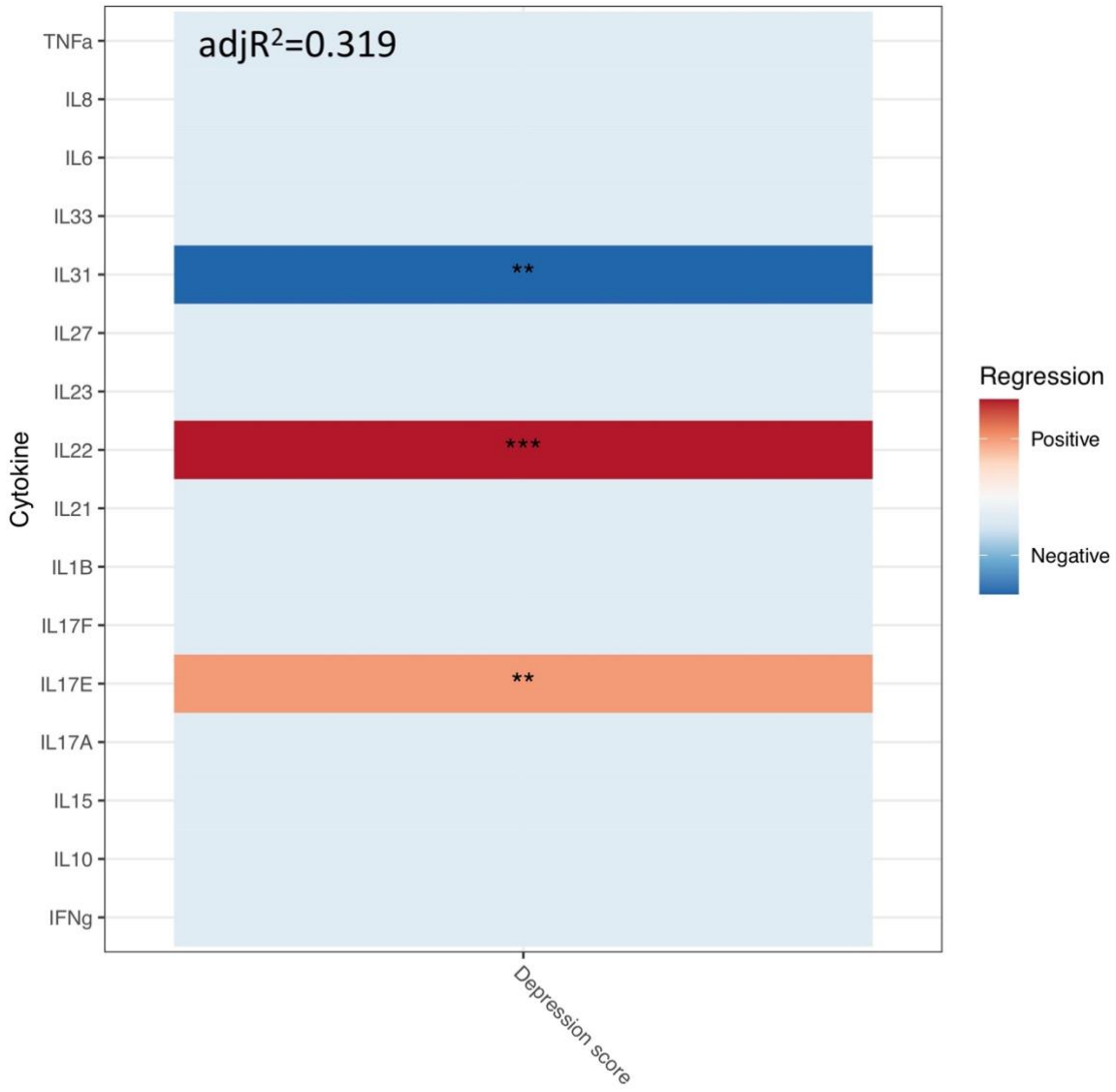


**Figure 3.9 Significant cytokine predictors of depression score**
A regression model was fitted where y represents HADS depression score and x represents the circulating cytokine concentration data set. The heatmap represents significant positive (red) and negative (blue) predictors, adj$R^2$=0.319, **p<0.01, ***p<0.001.

## 3.7 Discussion

### 3.7.1 The cytokine signature of people with AA

We sought to fully characterise the circulating cytokine phenotype of individuals with AA. These data indicate that a plethora of cytokines are dysregulated in AA, implicating both type-17 and type-2 immune responses.

Consistent with other studies (Atwa et al., 2015), the levels of TNF, IL-1ß and IL-6 were increased in AA plasma. These cytokines are potent pro-inflammatory proteins primarily produced by innate immune cells and are classically involved in acute responses against pathogens. Collectively, they drive apoptosis, recruitment of leukocytes and contribute to the differentiation of specific adaptive responses, particularly Th17 cells (Zhou et al., 2007; Sedger & McDermott, 2014). TNF is widely implicated in autoimmune diseases, particularly where monocytes and macrophages are involved, and blocking TNF or the receptor is successful in the treatment of diseases including rheumatoid arthritis (Elliott et al., 1994) and IBD (Targan et al., 1997).

In AA, the increases in plasma TNF, IL-1ß and IL-6 were modest; on average the concentrations were below 2pg/mL, and limited data exist on the role of TNF, IL-1ß and IL-6 in the immune response driving hair loss. Classically-activated macrophages, responding to IFN$\gamma$ and toll-like receptor stimulation, are strong producers of these cytokines (Murray & Wynn, 2011). Macrophages are known to be important in normal cycling of the hair follicle (Muneeb et al., 2019), but there are limited data describing macrophages in AA pathogenesis. Clinical observations have reported development of AA in patients receiving anti-TNF therapy (Tauber et al., 2014) suggesting that disrupting TNF signalling may be detrimental to hair growth. However, it has been shown that keratinocytes secrete TNF in response to ultraviolet or bacterial stimulus (Nakamura et al., 2002; Bashir et al., 2009), thus damage caused by $CD8^+$ T cells may stimulate TNF release. TNF can synergise with IFN$\gamma$ to drive upregulation of IL-15 in dermal sheath cells, and this cytokine strongly drives $CD8^+$ T cell cytotoxicity (Xing et al., 2014). IL-6 can also be released by keratinocytes and its expression is increased in psoriasis plaques. However, in psoriasis IL-6 drives epidermal hyperplasia which is not associated with AA (Goodman et al., 2009). IL-6, in

combination with TGFß, potently drives differentiation of IL-17$^{+}$ CD4 T cells (Zhou et al., 2007); the potential role of Th17 cells in AA pathogenesis is discussed later in this section.

In this cohort, levels of circulating IFNγ and IL-15 were not increased in AA circulation. Other publications report elevated IFNγ in AA patients (Tembhre & Sharma, 2013; Kasumagic-Halilovic et al., 2010). In the C3H Hej mouse model of alopecia areata, NKG2D$^{+}$ CD8$^{+}$ T cells are necessary for inducing hair loss. This study also indicated that blockade of IFNγ or IL-15Rß prevented hair loss (Xing et al., 2014). In humans, JAK inhibitors are effective in promoting hair regrowth, but they suppress multiple gamma chain cytokines in addition to IFNγ and IL-15 signalling pathways (Liu et al., 2017). In our study, a proportion of patients have IFNγ plasma levels above 10pg/mL; these concentrations are consistent with another study (Kasumagic-Halilovic et al., 2010). Patients with high levels did not consistently have more severe disease. The IFNγ concentration was above 40pg/mL for a proportion of the HC cohort, which reduced our ability to detect statistically-significant differences between AA patients and HCs. Additionally, the concentration of IL-12, a key driver of Th1-differentiaion was also unchanged in this AA cohort. These data clearly indicate that type-1 cytokines are not systemically dysregulated in AA despite their importance in the local inflammatory response against hair follicles.

Our data indicate a robust type-17 cytokine response associated with AA patients. IL-17A and IL-23 are significantly elevated in this cohort and these data replicate findings by others. IL-23 is important for the survival and differentiation of Th17 cells (McGeachy et al., 2009), whereas IL-17A is an effector cytokine that recruits neutrophils and monocytes to tissues. In the skin, IL-23 can be produced by immune cells, including dermal dendritic cells and also by keratinocytes in response to toll-like receptor 4 stimulation (Yoon et al., 2016). At present, the role of these cytokines in AA pathogenesis is not well understood. Results from a double-blind randomised pilot study (n=11) indicated that inhibition of IL-17A using Secukinumab is not effective in promoting hair regrowth (Guttman-Yassky et al., 2018). There have also been a handful of small studies assessing the effect of blocking the IL-12/IL-23 p40 subunit, using Ustekinumab, on hair regrowth but the results are inconclusive. A small open-

label study (n=3) indicated that blockade of the IL-12/IL-23 p40 subunit was effective in a patient with severe AA who was treated for one year (Guttman-Yassky et al., 2016), but the same drug was ineffective in promoting hair regrowth in another study of 4 patients with severe AA (Ortolan et al., 2019). Notably, AA has even developed in patients receiving Ustekinumab for treatment of plaque psoriasis (Verros et al., 2012). Micro array profiling indicated that IL-23 transcripts are increased in hairless skin but there were no associated changes in genes specifically associated with Th17 cells (Suarez-Farinas et al., 2015).

To extend on previous studies, we compared concentrations of type-17 cytokines in AA patients with those measured in PsA patients, a disease that is dominated by IL-17 and IL-23 pathology. Interestingly, these analyses revealed that the mean levels of IL-17A in the PsA cohort were not significantly higher than observed in AA despite some PsA patients having high concentrations. Additionally, the levels of IL-23 were not increased in PsA compared to AA or HCs. Taken together, our data and others indicate that IL-17A and IL-23 are increased in AA circulation, and that the concentrations may be clinically relevant based on comparison with those measured in PsA. The results from clinical studies described above are limited because they are extremely small. However the varied response to Ustekinumab (IL-12/IL-23 p40 blocker) may indicate that AA patients of a specific pathotype will respond. The efficacy of Ustekinumab for specific AA patients may be attributed to blockade of IL-12 which contributes to $CD8^+$ T cell and Th1 cell responses. In contrast to IL-17A, IL-12 and IL-23 are vital for the generation of Th1 and Th17 cell responses, highlighting that targeting cytokines which drive T cell differentiation may be a better therapeutic strategy for AA, rather than targeting downstream cytokines.

AA patients also had elevated levels of circulating IL-17F and IL-21. Changes in the expression of IL-17F in AA sera have not been reported previously. IL-17F and IL-17A share the same receptor and are 50% homologous (Sarma et al., 2009), but emerging evidence suggests they have different functions in specific situations. They are both produced by Th17 cells, innate lymphoid cells and natural killer cells, but only IL-17F is expressed by mast cells, activated monocytes and specific epithelial cells (Ishigame et al., 2009). Recently, it was

shown that blocking IL-17F is protective in a mouse model of colitis, whereas inhibiting IL-17A can exacerbate inflammation (Tang et al., 2018). Similar to AA, IL-17F is increased in serum of psoriatic arthritis and atopic dermatitis patients (Soderstrom et al., 2017; Park et al., 2015). In the skin, IL-17F potently induces human keratinocytes to express IL-6 and IL-8 (Fujishima et al., 2010), but its role in AA has not been studied. The functions of IL-21 are well-understood, and its key functions are in Th17 and follicular T cell development, promoting survival of CD8 T cells and plasma B cell maturation (Spolski & Leonard, 2014). Interestingly, IL-21, in synergy with IL-15, strongly promotes cytotoxic responses of $CD8^+$ T cells which may be relevant in AA (Zeng et al., 2005). Notably, IL-21 signals via JAK-STAT signalling pathways (Deenick et al., 2013). In AA serum, autoantibodies against hair follicle antigens are reported to be increased (Tobin et al., 1994), and treatment of AA with DCP reduces the levels of circulating antibodies (Tobin et al., 2002). However, the importance of B cells and potential autoantibodies in mechanisms driving hair loss has not been investigated. Because IL-21 is increased in circulation, it would be interesting to understand if systemic IL-21 is contributing to a potential B cell response in AA patients. In the following chapter, we investigate frequencies of circulating B cell populations in AA patients.

For the first time, we indicate that the concentration of IL-31, IL-33 and IL-17E are increased in AA circulation compared to HCs. These cytokines are associated with type-2 responses, but we did not detect changes in IL-13 or IL-4, however these cytokines were mainly undetected. The high incidence of atopy in AA patients indicates that type 1 and type 2 responses can co-exist in the skin, but we were concerned that our findings may be due to the high levels of atopic individuals in our cohort. Stratification of AA patients led to the rejection of this hypothesis as the levels of IL-31, IL-33 and IL-17E were significantly increased in both atopic and non-atopic AA patients compared to HCs.

IL-31 is released by various immune cells and is involved in the pathogenic mechanisms driving atopic disease (Singh et al., 2016; Dillon et al., 2004). IL-31 is a pruritic cytokine that has been shown to disrupt keratinocyte differentiation (Sonkoly et al., 2006; Cornelissen et al., 2012), but to our knowledge has not been studied in the context of AA. Some studies involving IL-31 have reported

interesting observations; transgenic mice overexpressing IL-31 develop severe pruritis in combination with patchy hair loss (Dillon et al., 2004). Also, perhaps not surprisingly, IL-31 levels are higher in atopic dermatitis lesions compared to AA skin (Hofbauer et al., 2012).

IL-17E/IL-25 is also a driver of type 2 responses, but is a member of the IL-17 family that binds a heteromeric receptor complex composed of IL-17RA and IL-17RB (Rickel et al., 2008). IL-17E is produced by cells associated with atopy, including Th2 and mast cells, and in the skin has been shown to reduce filaggrin expression by keratinocytes which negatively impacts barrier function (Hvid et al., 2011).

On the other hand, IL-33 is a tissue alarmin that is constitutively expressed by epithelial cells and is only released in an activated form when a cell dies via necrosis, and not programmed cell-death. The increased level of IL-33 in AA sera compared to HCs is very modest, especially compared to levels observed in asthma sufferers (Guo et al., 2014). During asthmatic responses, IL-33 drives the expression of fibrotic factors by fibroblasts which causes tissue remodelling. The inflammation caused by T cells against hair follicles is very localised and follows a relapsing-remitting pattern which may explain the low levels of IL-33 observed. Nevertheless, understanding the role of IL-33 in AA may be therapeutically beneficial. The repetitive attack of the hair follicle could be causing fibrosis to the hair follicle, which may be why patients with established long-term disease are less likely to respond to treatment (Renert-Yuval & Guttman-Yassky, 2016).

Previous studies have indicated relationships between circulating cytokines and the severity of hair loss. This cohort were stratified based on their SALT score to compare cytokine levels; no current loss, <50% loss, >50% loss and total loss (>95%). IL-17A, IL-17F, IL-21 IL-31 and IL-17E were significantly increased in patients with severe disease compared to controls. These changes were not observed in the other categories, apart from an increase in IL-21 in the >50% group compared to HCs. As indicated previously, the >50% group was underpowered (n=4). Notably, the mean levels of IL-17A, IL-17F, IL-31 and IL-17E were very similar between the severe and the >50% hair loss category, and there

were also patients in the <50% category with high levels. The severe category was the largest in this cohort (n=17) which influenced our ability to detect significance. We did not identify changes in the levels of IL-23 between the different groups of AA patients and HCs. In another cohort of AA patients, the levels of IL-17A were found to correlate with SALT score (Atwa et al., 2015). However, these findings are not reliable because the categories of patients with more severe disease were not efficiently powered (S3=4, S4a=1, S4b=1, S5=3). These patients had a disease duration of between 0.25 to 60 months, whereas the cohort in our study included a high proportion of patients with a longer disease duration (range, 1-55 years). It is therefore difficult to compare these cohorts because it is anticipated that disease duration will influence changes in the cytokine immunophenotype. More recently, it was reported that the levels of IL-15 correlate with disease severity (Ebrahim et al., 2019). However, we didn't observe IL-15 levels to be different between AA and HCs. In summary, it is not possible to draw conclusions about the relationship between disease severity and circulating cytokines without increasing the size of our cohort. We utilised a power calculation to determine how many samples would be required to determine a significant difference between HCs and the patchy hair loss cohorts. For example, a power calculation based on the IL-31 data set, assuming power of 0.8, indicated that a minimum of 22 patients in the >50% patchy group would be required to determine a significant difference from HCs.

### 3.7.2 AA and depression

We found that 18% of this cohort experienced depressive symptoms at time of enrolment. This is higher than the point prevalence of depression in the general population which is approximately 12.9% (Lim et al., 2018). Depression is also common in other AA cohorts (Aghaei et al., 2014; Sellami et al., 2014). The known causes of depression are multifactorial; implicating genetics, environmental factors, and in some cases, inflammation (Malhi & Mann, 2018). For decades, peripheral inflammation has been associated with inducing 'sickness behaviour' and more recently, understanding the mechanisms underpinning this relationship have led to targeting major depressive disorder with anti-inflammatory therapies (Raison et al., 2013; Kappelmann et al., 2018). Autoimmune diseases, most notably rheumatic diseases, are highly associated

with depression (Nerurkar et al., 2018). To date, circulating IL-1B, TNF and IL-6 have been the main focus of research (Haapakoski et al., 2015; Dowlati et al., 2010).

Circulating cytokines can interfere with neural pathways by crossing the blood brain barrier or by interacting with peripheral nerve endings, including the vagus nerve (Miller & Raison, 2016). Understanding the causative mechanisms between peripheral inflammation and depression is extremely challenging, therefore the current understanding is mainly derived from clinical trials or murine behavioural models of depression. Nevertheless, it's been shown that TNF can disrupt the balance of the neurotransmitter, glutamate, in the brain. TNF does this in many different ways that include inhibiting uptake of glutamate, leading to increased extracellular levels, and by increasing levels of glutaminase (Bortolato et al., 2015). Ultimately, these effects disrupt the balance of glutamate neurotransmission leading to glutamate excitotoxicity (Davies et al., 2011).

The physical aspect of AA is likely a major contributor to depression for patients, but we hypothesised that the inflammatory cytokines elevated in the periphery could also contribute. Linear regression modelling indicated that IL-17E and IL-22 are positive predictors depression score. These cytokines have not been associated with depression, but other type-17 cytokines have been shown to induce depressive behaviour in mouse models of inflammation and in patients (Li et al., 2019). IL-17A release, caused by imiquimod administration, caused depressive behaviours in animals (Nadeem et al., 2017), and the IL-17RA is expressed in the brain during inflammation in humans (Kebir et al., 2007). IL-22 is associated with neuroinflammatory diseases, including multiple sclerosis (Jadidi-Niaragh & Mirshafiey, 2011), and has been shown to disrupt the blood brain barrier, but its potential role in depression has not been studied. Notably, the receptor for IL-22 is expressed by brain endothelial cells (Kebir et al., 2007).

Despite these interesting observations, it is important to emphasise that our data are not able to make causative links between the levels of circulating cytokines and incidence of depression in people with AA. We refer to the cytokines selected in the model as 'predictors' from a mathematical modelling

perspective, but we cannot indicate that these cytokines are able to predict incidence of depression. Therefore, these data only indicate an association between cytokine levels and depression score, and at this stage we do not have any causative evidence.

### 3.7.3 Conclusion

This chapter describes the broad cytokine dysregulation associated with AA, characterised by an increase in both type-17 and type-2 cytokines. Our data complement previous studies, but also extend on the current literature by implicating changes in levels of circulating IL-33, IL-31, IL-17E and IL-17F.

Levels of IL-17A, IL-23, IL-31 and IL-17E appeared to be increased in patients with severe disease, but our study lacked power to successfully determine the association between circulating cytokines and disease severity. At present, no reliable clinical tool other than measuring extent of hair loss exists to measure disease activity in patients, thus making identification of connections between immune parameters and AA disease activity challenging. By stratifying AA patients, we identified that changes in the levels of type-2 associated cytokines are not specifically linked to incidence of atopy, indicating that type 2 responses may be important in the immune-mechanisms driving hair loss.

In this chapter, we also discuss the potentially wider implications of elevated circulating inflammatory cytokines. Depression was common in this cohort, and the levels of IL-22 and IL-17E positively predicted incidence of depressive symptoms. Whilst our data do not directly link circulating cytokines with incidence of depression, it would be interesting to measure the future impact of immune-suppressive treatment on psychological well-being of individuals with AA.

# 4 Cellular and molecular mechanisms associated with AA pathogenesis

## 4.1 Introduction

The understanding of the immune mechanisms driving AA is predominantly focused on aberrant $CD8^+$ T cell responses (Xing et al., 2014; Petukhova et al., 2010). Immune responses driving organ specific autoimmune diseases may be dominated by a specific immune cell subset, however cells of the immune system do not function in isolation. Identifying how other cells drive and support the T cell response will provide potential avenues for therapeutic investigation. This is particularly important for heterogeneous diseases, such as AA, where investigating alternative aspects of the immune response may reveal different disease pathotypes.

$CD4^+$ T cells are critical in the generation of successful $CD8^+$ T cell responses. They provide 'help' by licensing dendritic cells to activate cytotoxic T cells (Smith et al., 2004), and are important for robust recall responses (Laidlaw et al., 2016; Janssen et al., 2003). The numbers of $CD4^+$ T cells are increased in AA lesions (Ito et al., 2008) and numbers of circulating Th17 cells are elevated in AA patients compared to HCs (Han et al., 2015), but their relevance in AA pathogenesis is yet to be elucidated. Natural killer (NK) cells have also been an area of interest because of their cytotoxic effector function. NK cells do not recognise specific antigen, but instead kill cells which do not express MHC class I and by recognising 'stress' signals using receptors that include NKG2D (Vivier et al., 2008). NK cells are present in AA perifollicular infiltrates alongside $CD8^+$ T cells (Ito et al., 2008), however ablation of NK cells led to worsening of hair loss in a mouse model of AA (Kaufman et al., 2010). NK cells are potent producers of IFN$\gamma$ and act as an important link between innate and adaptive type-1 immune responses (Martín-Fontecha et al., 2004), thus their involvement in AA warrants further investigation.

Having shown that AA is associated with elevated type-17 and type-2 cytokines, we now aimed to characterise the cellular immunophenotype of individuals with AA. To build on previous studies, subsets of circulating $CD8^+$ T cells, $CD4^+$ T cells

and NK cells were analysed. In addition, the proportions of circulating dendritic cells (DCs) and B cell subsets we determined. DCs activate T cells or induce tolerogenic responses (Probst et al., 2005; Jones et al., 2016; Esterházy et al., 2016), and are therefore implicated in T cell driven autoimmunity (Ohnmacht et al., 2009). At present, conventional DCs have not been studied in the context of AA. B cells have also not been a focus of AA research, however aberrant B cell function and particularly the generation of auto-antibodies are implicated in other organ-specific autoimmune responses that share immune mechanisms with AA (Orban et al., 2009). Notably, auto-antibodies specific for follicular antigens are reported to be elevated in AA circulation (Tobin et al., 1994).

One of the objectives of my project was to investigate the link between AA and subclinical intestinal inflammation. AA and intestinal diseases such as coeliac disease (CeD), Crohn's disease (CD) and ulcerative colitis (UC) share genetic risk alleles (Petukhova et al., 2010), and anecdotal evidence describes intestinal discomfort in some individuals with AA. In the clinic, the occurrence of intestinal inflammation is determined by measuring the levels of faecal calprotectin, an inflammatory protein that is released by neutrophils (Voganatsi et al., 2001). Zonulin is an experimental marker of increased intestinal permeability that is elevated in sera of patients with CeD (Fasano et al., 2000) and type 1 diabetes (T1D) (Sapone et al., 2006).

Of note, there is considerable overlap between the immune mechanisms driving CeD and AA (Hüe et al., 2004). Based on this observation, it was hypothesised that AA may be associated with increased incidence of immune-reactivity to gluten. Clinically, occurrence of gluten reactivity is determined by measuring circulating levels of anti-tissue transglutaminase (anti-TTG) IgA (Kaswala et al., 2015).

The aims of the experiments described in this chapter were:

1. To assess the proportions and numbers of circulating $CD8^+$ T cells, $CD4^+$ T cells, B cells, NK cells and DCs in peripheral blood of AA patients and healthy controls (HCs).

2. To assess the proportions and numbers of skin and gut homing T cell subsets in AA patients and HCs.

3. To measure circulating levels of zonulin and anti-TTG IgA, and faecal calprotectin levels in AA patients.

## 4.2 Participant characteristics

Peripheral blood was collected from 38 AA patients and 20 HCs for flow cytometric analysis of circulating $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells and DCs (Table 4.1). A proportion of these samples were used for each flow cytometry panel as indicated in individual figure legends. The AA cohort included 3 patients in remission, 12 with <50% hair loss, 5 with >50% hair loss and 18 with severe hair loss (>95%). The average disease duration of this AA cohort was 16 years, with a range between 1 and 46 years. 22 patients had a history of atopy, 6 reported thyroid related conditions, 1 had vitiligo, 1 had epilepsy, and 1 had pernicious anaemia. One patient also had female pattern baldness in addition to AA. Importantly, none of the patients had a diagnosis of a major inflammatory disease, including CD, UC, rheumatoid arthritis (RA), psoriatic arthritis (PsA), psoriasis (PsO), systemic lupus erythematosus (SLE) or ankylosing spondylitis (AS). At the time of recruitment, 17 patients were receiving treatment: 9 patients were receiving intralesional steroid injections, 7 were receiving diphenylcyclopropenone (DCP) treatment and 1 patient was receiving methotrexate. Healthy controls were recruited based on their age and gender to match the AA patient cohort.

**Table 4.1 Characteristics of participants with alopecia areata and healthy controls.**
Peripheral blood collected from these individuals were used for analyses of circulating immune cells. Subsets of these samples were used for each flow cytometry panel. SD; standard deviation, IL steroids; intralesional steroids.

| | Alopecia areata | Healthy controls |
|---|---|---|
| Total | 38 | 20 |
| Gender F/M | 32/6 | 16/4 |
| Age (years) ± SD | Female, 45 ± 11.5<br>Male, 32 ± 7.6 | Female, 42 ± 9.5<br>Male, 28 ± 6.5 |
| Disease duration (years)<br>Average ± SD | 1-46<br>16 ± 11.5 | |
| **Disease severity**<br>Inactive<br><50%<br>>50%<br>Total loss >95% | <br>3<br>12<br>5<br>18 | |
| **Comorbidities**<br>Atopic<br>Thyroid illness<br>Vitiligo<br>Epilepsy<br>Pattern baldness<br>Pernicious anaemia | <br>22<br>6<br>1<br>1<br>1<br>1 | |
| **Treatment**<br>None<br>Diphenylcyclopropenone<br>IL steroids<br>Methotrexate | <br>21<br>7<br>9<br>1 | |

## 4.3 Design and optimisation of flow cytometry panels

Flow cytometry antibody panels were designed to quantify and phenotype $CD8^+$ T cells, $CD4^+$ T cells, B cells, DCs and NK cells. In total, 74 immune cell subsets were identified as summarised in Figure 4.1. The T cell and NK panels were designed specifically for this study, whereas the B cell and DC panels were acquired from previous studies in the Milling and Goodyear laboratories. Optimisation of the T and NK cell panels was carried out using peripheral blood mononuclear cells (PBMCs) collected from healthy control donors. Antibodies were tested on different fluorochromes and their concentrations were titrated

to achieve optimal staining. Isotype or fluorescent minus one (FMO) controls were used for specific markers across all samples to ensure accurate gating throughout the patient and HC cohort. Following successful optimisation, the panels were used to identify immune cells in the cohorts described in Table 4.1. Cell populations were quantified as a percentage of $CD45^+$ live cells or as number of cells per ml of peripheral blood. Where appropriate, cell subsets were also analysed as a frequency of the parent population.

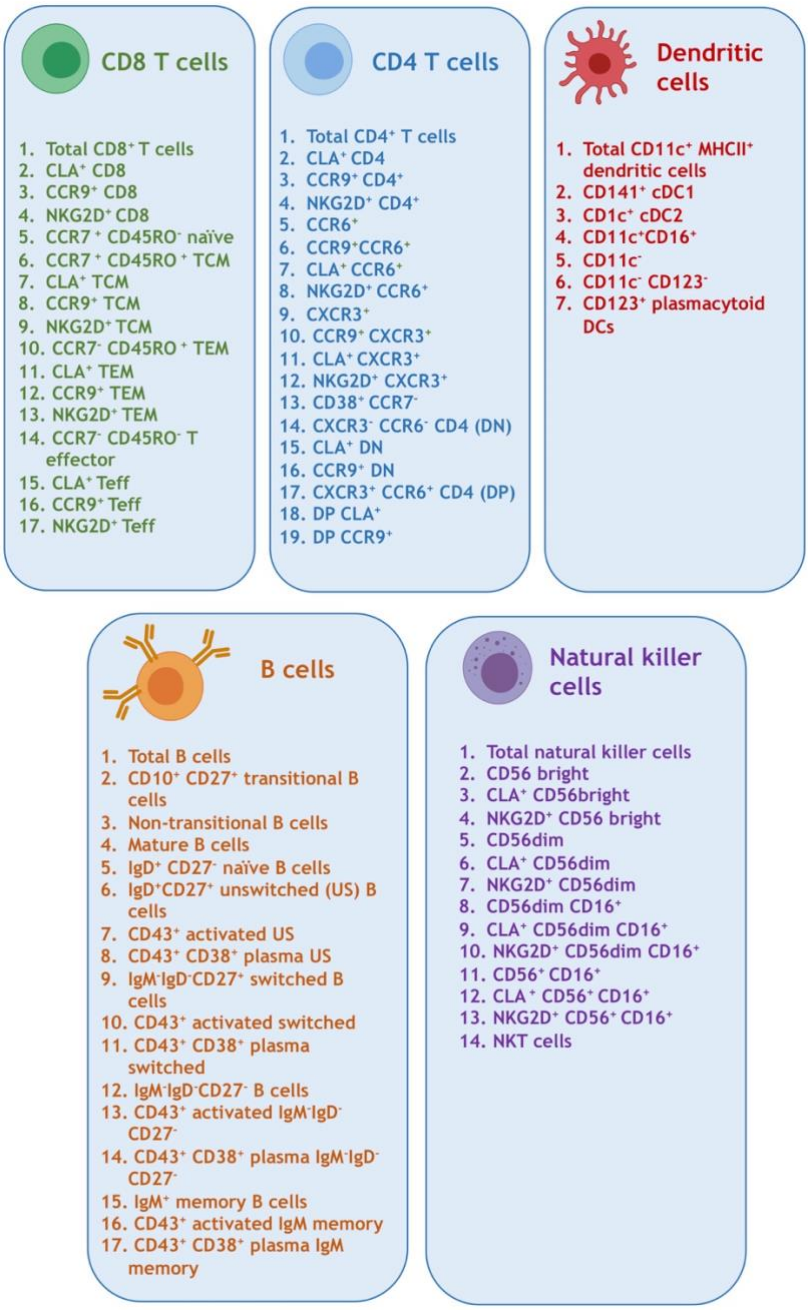


**Figure 4.1 Overview of the immune cells subsets identified in peripheral blood**
Five flow cytometry panels were used to phenotype 74 immune cell populations in peripheral blood of AA patients and HCs.

## 4.4 Circulating T cell subsets

### 4.4.1 CD8$^+$ T cells

CD8$^+$ T cells were identified using the gating strategy depicted in Figure 4.2. Side scatter and forward scatter were used to remove debris and doublets from the analysis. Live CD45$^+$ leukocytes were gated on prior to identification of CD8$^+$ T cells and CD8$^+$ T cells expressing the NKG2D receptor. Consistent with previous observations (Prajapati et al., 2018), the majority of CD8 T cells (91.8 – 99.6%) were NKG2D positive. Memory and effector subsets were identified using the lymph-node homing chemokine receptor 7 (CCR7) and CD45RO, to gate naïve, central memory (TCM), effector memory (TEM) and effector subsets (Figure 4.2A).

CD8$^+$ T cells were analysed further to assess their tissue homing potential. Upon activation in a lymph node by DCs, T cells upregulate receptors that allow them to preferentially traffic to the tissue where they are required to carry out their effector function (Iwata et al., 2004; Picker et al., 1993). To identify T cells that may be involved in AA pathogenesis, the expression of the skin homing receptor, cutaneous lymphocyte antigen (CLA), was measured (Figure 4.2B). In relation to the hypothesis that AA is linked to the intestine, the expression of the gut-homing chemokine receptor, CCR9, on T cells was also analysed (Figure 4.2B) (Mora et al., 2003).

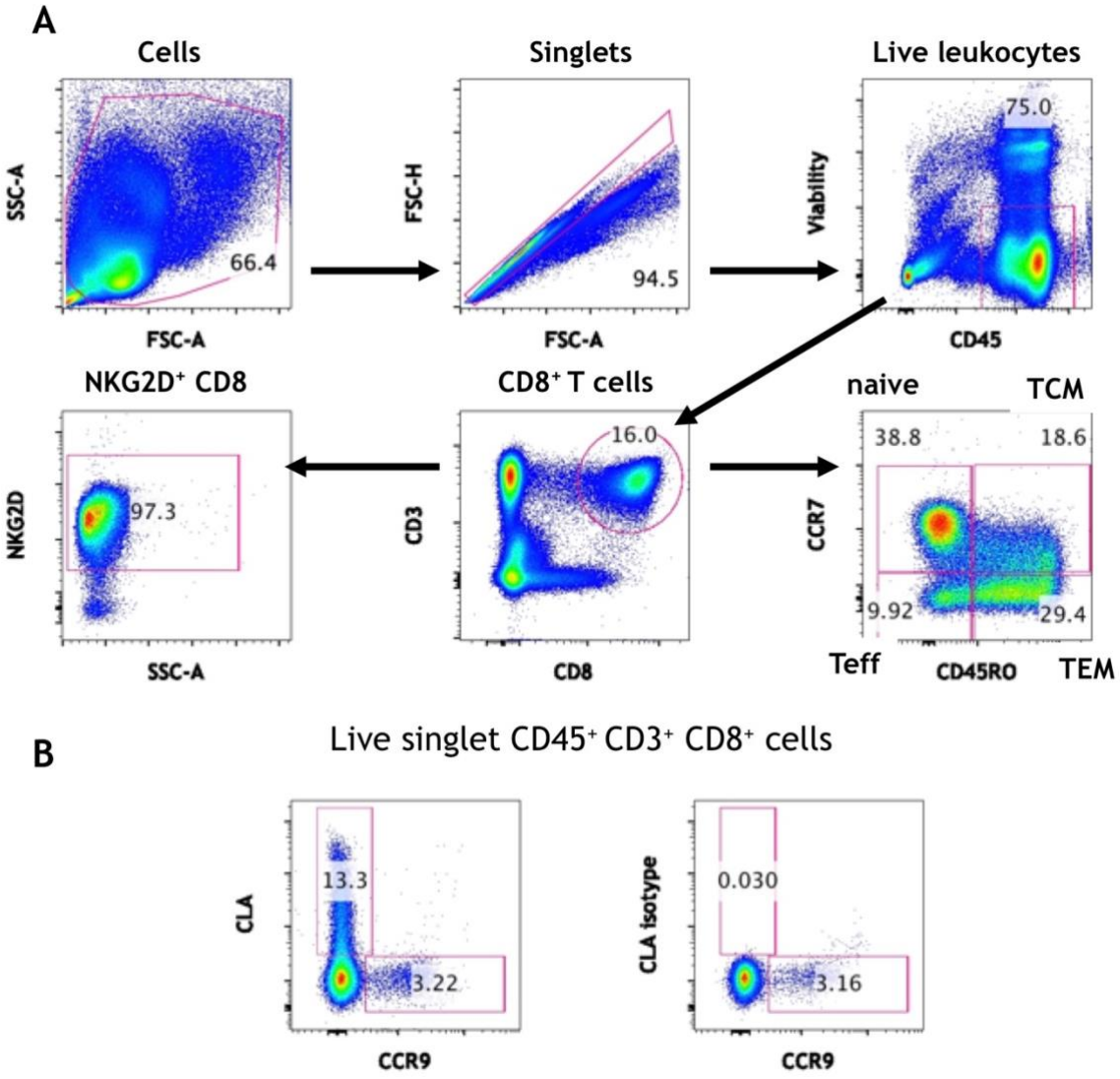


**Figure 4.2 Identification of CD8⁺ T cells**
Peripheral blood mononuclear cells were isolated and stained with the CD8⁺ T cell panel. (A) CD8⁺ T cells were identified as single live CD45⁺ CD3⁺ CD8⁺ cells, NKG2D⁺ CD8 T cells, and naïve, central memory (TCM), effector memory (TEM) and effector (Teff) subsets. (B) Representative plots of cutaneous lymphocyte antigen (CLA) and CCR9 staining of single live CD45⁺ CD3⁺ CD8⁺ cells. Representative staining for the CLA isotype (Rat IgMk) is illustrated.

The numbers of PBMCs per mL of blood from AA patients and HCs were compared, but no difference was observed (Figure 4.3A). There was also no change in the total number of live CD45⁺ leukocytes between AA patients and HCs (Figure 4.3B).

The frequency and number of total CD8⁺ T cells was not changed between AA patients and HCs (Figure 4.4A). It was hypothesised that the proportion of circulating NKG2D⁺ CD8⁺ T cells may be altered due to their importance in driving AA, but no differences were observed (Figure 4.4B).

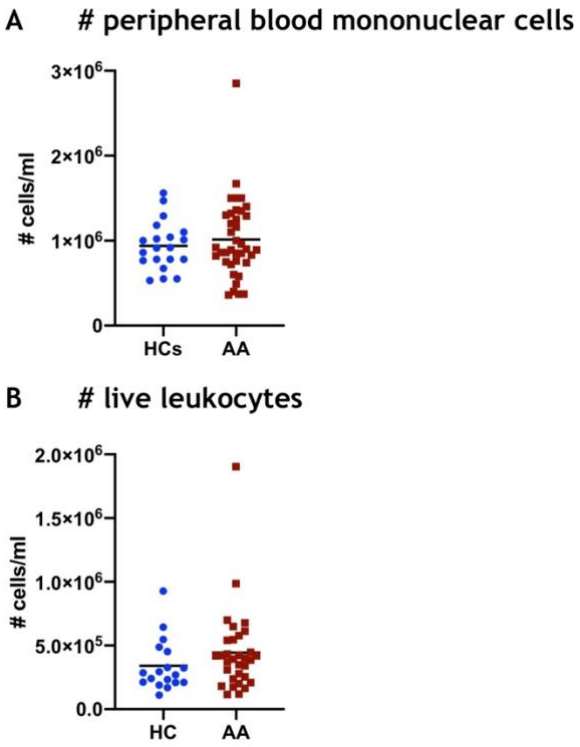


**Figure 4.3 Comparison of total circulating peripheral blood mononuclear cells between HC and AA cohorts.**
(A) Number of total peripheral blood mononuclear cells per mL of blood in AA patients (n=38) and HCs (n=20). (B) Number of live leukocytes ($CD45^+$) per mL of blood in AA patients (n=32) and HCs (n=18).

Further analysis of $CD8^+$ T cells revealed that there were also no significant changes in the proportion or numbers of circulating naïve, TCM, TEM or effector cell subsets between AA patients and HCs (Figure 4.4C, 4.5). The proportion and number of $NKG2D^+$ CD8 memory and effector subsets were also analysed but no differences were found (Figure 4.6). Analysis of skin and gut homing $CD8^+$ T cell subsets also revealed no changes (Figure 4.7).

In summary, the proportions and numbers of $CD8^+$ T cell subsets are not changed in the circulation of AA patients in this cohort compared to our HC cohort.

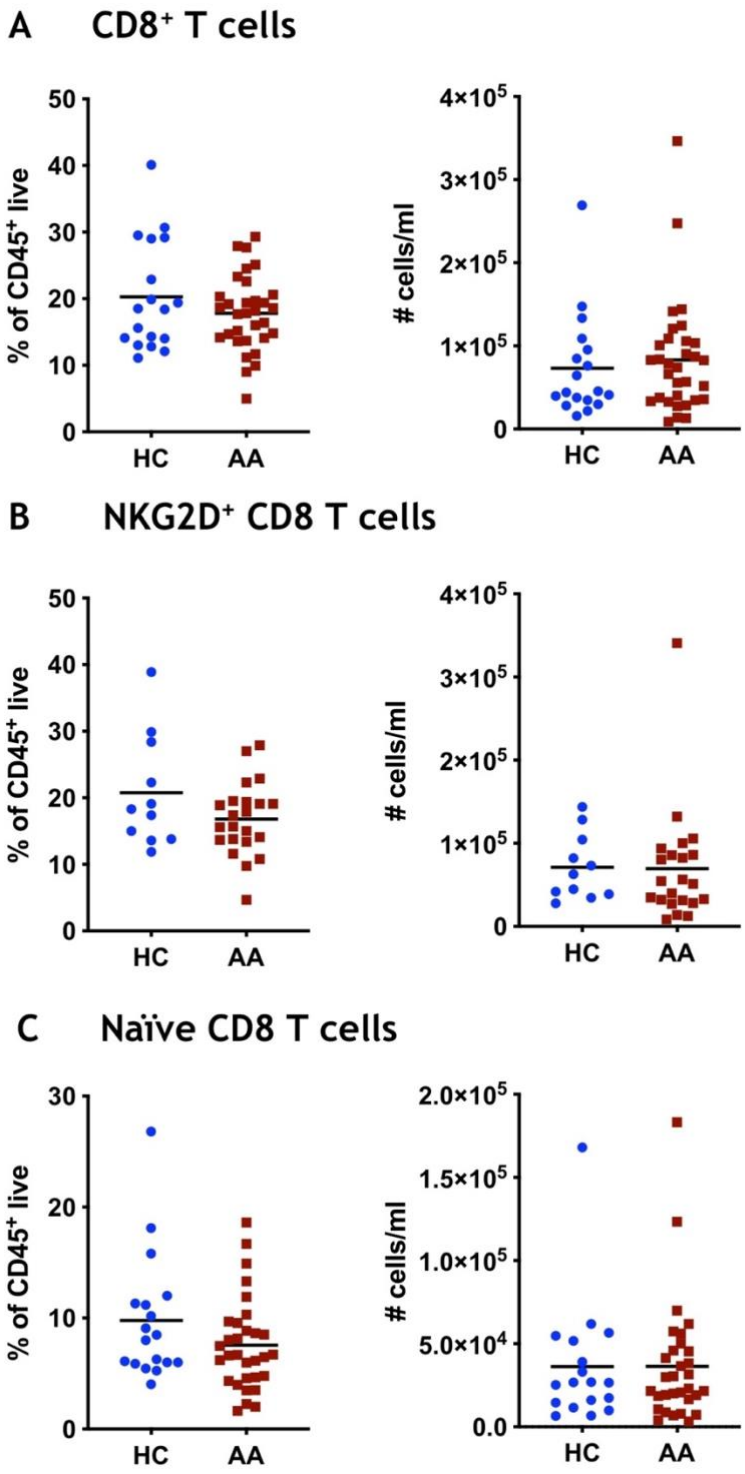


**Figure 4.4 Comparison of circulating CD8⁺ T cell subsets between HC and AA cohorts.** Frequencies as a % of CD45⁺ live cells (left) and numbers per mL of blood (right) of (A) CD8⁺ T cells and (C) naïve CCR7⁺ CD45RO⁻ CD8 T cells in AA patients (n=32) and HCs (n=18). B) Frequencies as a % of CD45⁺ live cells (left) and numbers per mL of blood (right) of CD8⁺ T NKG2D⁺ CD8 T cells in AA patients (n=22) and HCs (n=11). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

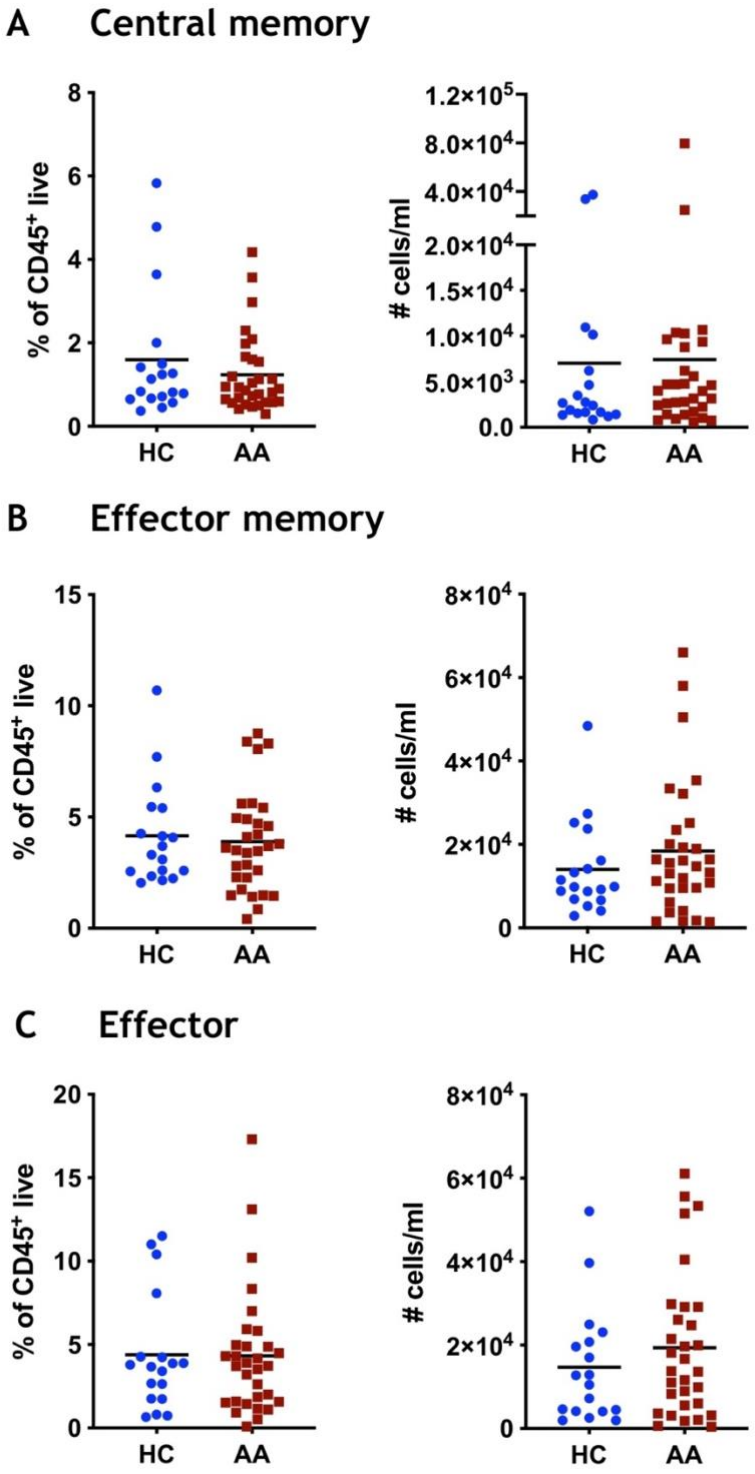


**Figure 4.5 Comparison of circulating effector and memory CD8⁺ T cell subsets between HC and AA cohorts.**
Frequencies as a % of CD45⁺ live cells (left) and numbers per mL of blood (right) of (A) central memory (CCR7⁺CD45RO⁺), (B) effector memory (CCR7⁻CD45RO⁺) and (C) effector (CCR7⁻ CD45RO⁻) CD8 T cells in AA patients (n=31) and HCs (n=18). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

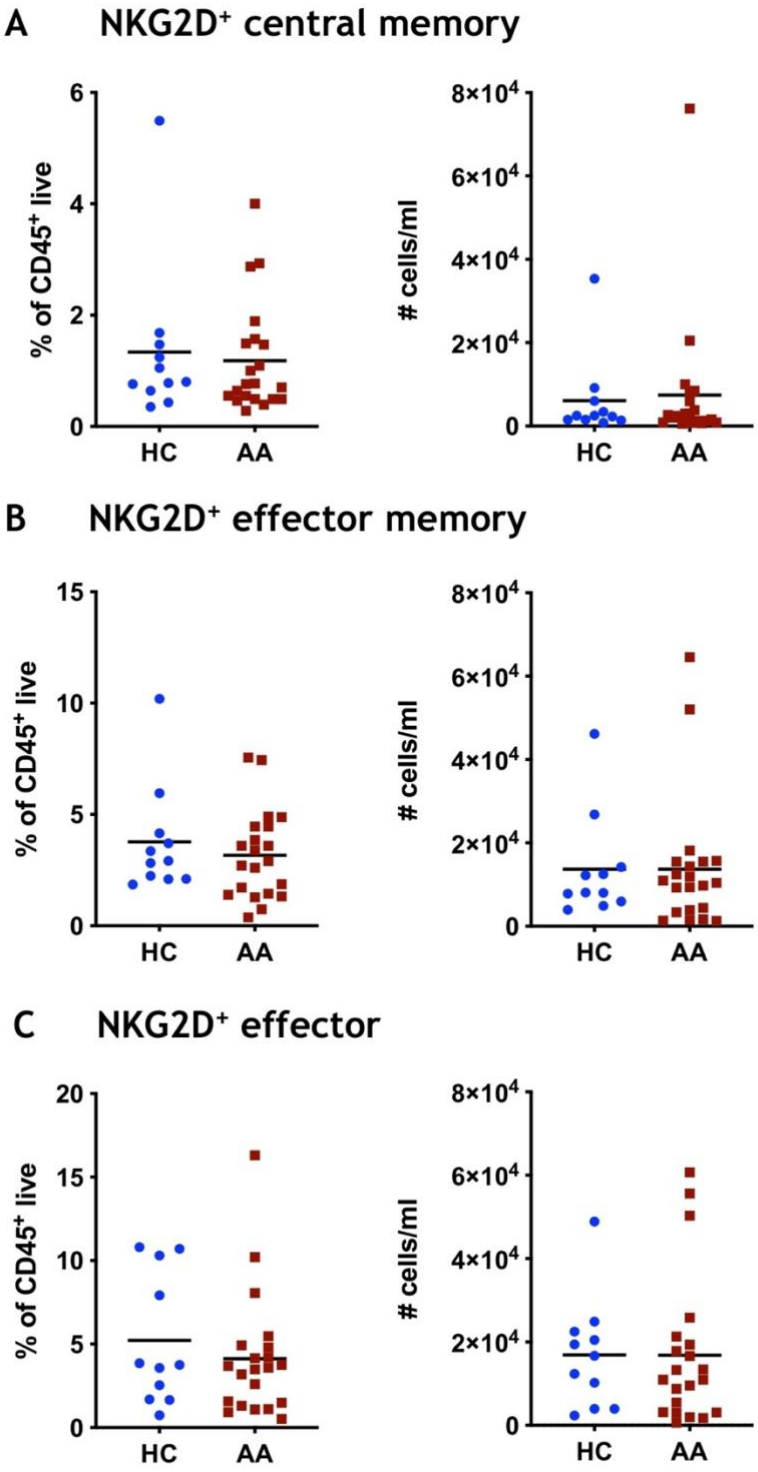


**Figure 4.6 Comparison of circulating NKG2D⁺ CD8 T cell subsets between HC and AA cohorts.** Frequencies as a % of CD45⁺ live cells (left) and numbers per mL of blood (right) of (A) NKG2D⁺ central memory, (B) NKG2D⁺ effector memory and (C) NKG2D⁺ effector CD8 T cells in AA patients (n=21) and HCs (n=11). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

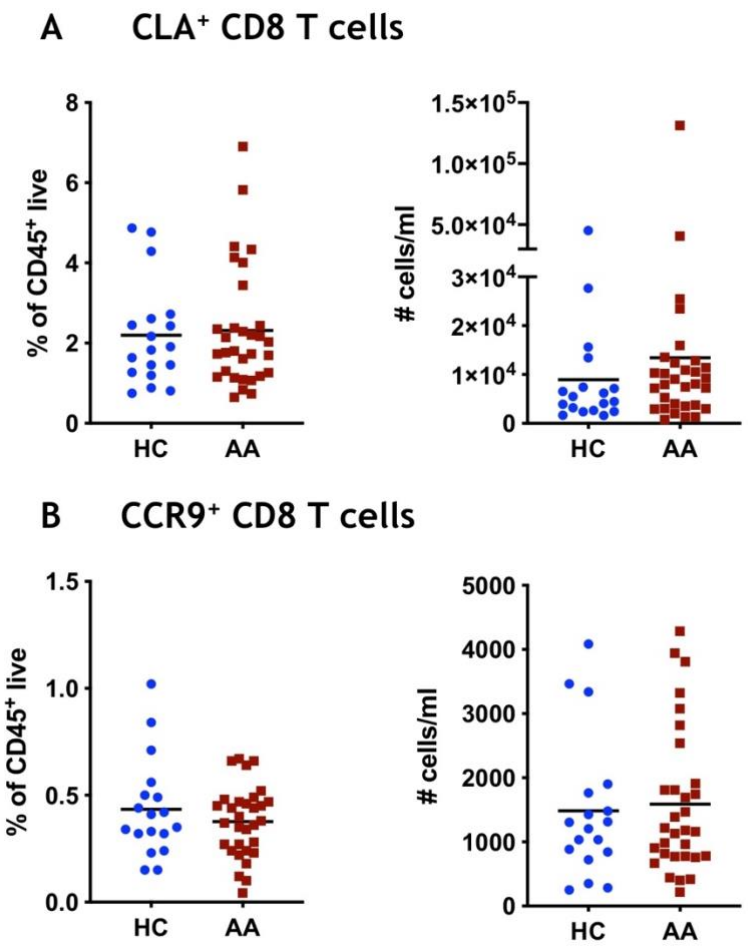


**Figure 4.7 Comparison of circulating CLA$^+$ and CCR9$^+$ CD8 T cell subsets between HC and AA cohorts.**
Frequencies as a % of CD45$^+$ live cells (left) and numbers per mL of blood (right) of (A) CLA$^+$ CD8 T cells and (B) CCR9$^+$ CD8 T cells in AA patients (n=31) and HCs (n=18). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

## 4.4.2 CD4$^+$ T cells

CD4$^+$ T cells were identified using the gating strategy depicted in Figure 4.8. Side scatter and forward scatter were used to remove debris and doublets from the analysis. Live CD45$^+$ leukocytes were gated on prior to identification of CD3$^+$ CD4$^+$ T cells. Although usually associated with NK and CD8$^+$ T cells, the NKG2D receptor can also be expressed by CD4$^+$ T cells. In this cohort, 0.62 – 10.8% of CD4$^+$ T cells expressed NKG2D. To identify whether AA is associated with specific changes in T-helper subsets, the chemokine receptors CCR6 and CXCR3 were used as surrogate markers to identify Th17 (Hirota et al., 2007) and Th1 (Syrbe et al., 1999) cells, respectively. As before, CLA and CCR9 were used to identify CD4$^+$ T cells with potential to home to the skin and gut, respectively (Figure 4.8B). Another CD4$^+$ T cell subset (CCR7$^-$CD38$^+$), shown to be enriched for cells

that recognise antigen of mucosal origin (Preacute et al., 2011), was also analysed (Figure 4.8A).

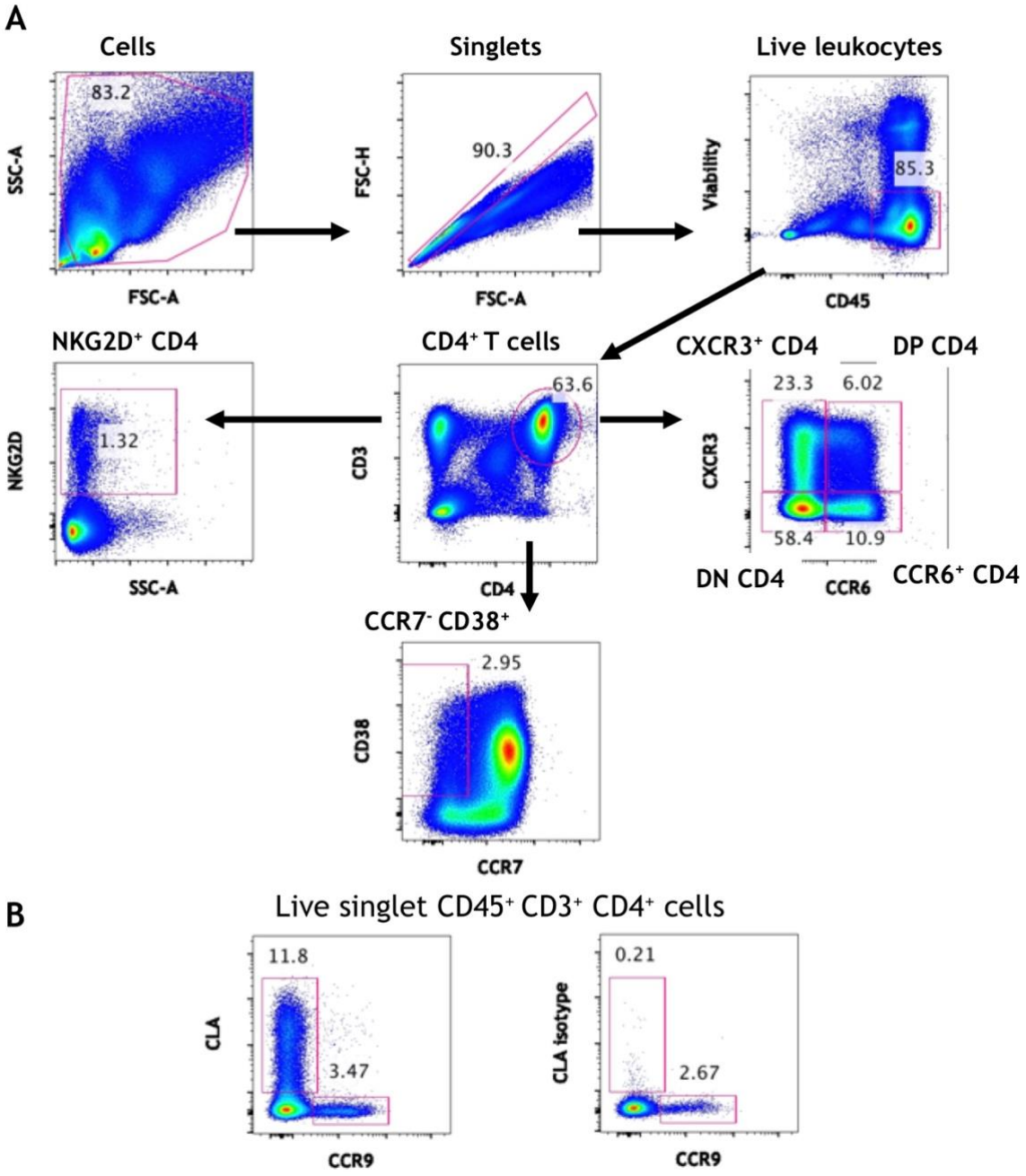


**Figure 4.8 Identification of CD4⁺ T cells**
Peripheral blood mononuclear cells were isolated and stained with the CD4⁺ T cell panel. (A) Cells were identified as single live CD45⁺ CD3⁺ CD4⁺ cells and NKG2D⁺ CD4 T cells. T-helper CD4 subsets were identified as CXCR3⁺, CCR6⁺, double positive (CXCR3⁺CCR6⁺) and double negative (CXCR3⁻CCR6⁻). CD4⁺ T cells reported to recognise antigen of mucosal origin were identified as CCR7⁻CD38⁺. (B) Representative plots of cutaneous lymphocyte antigen (CLA) and CCR9 staining of live CD45⁺ CD3⁺ CD4⁺ cells. Representative staining for the CLA isotype (Rat IgMk) is shown.

The total frequency and numbers of CD4⁺ T cells were unchanged between AA patients and HCs (Figure 4.9A). The proportions and numbers of CXCR3⁺ CD4 T

cells were also unchanged (Figure 4.9B), but the proportion of circulating $CCR6^+$ CD4 T cells were increased in AA patients compared to HCs (Figure 4.9C). However, the numbers of $CCR6^+$ CD4 T cells were not significantly different between AA patients and HC cohorts ($p=0.055$). $CD4^+$ T cells with dual Th1 and Th17 effector function co-express CXCR3 and CCR6 (Duhen & Campbell, 2014; Kamali et al., 2019). However, the frequency or numbers of this population were not altered in AA circulation (plots not shown).

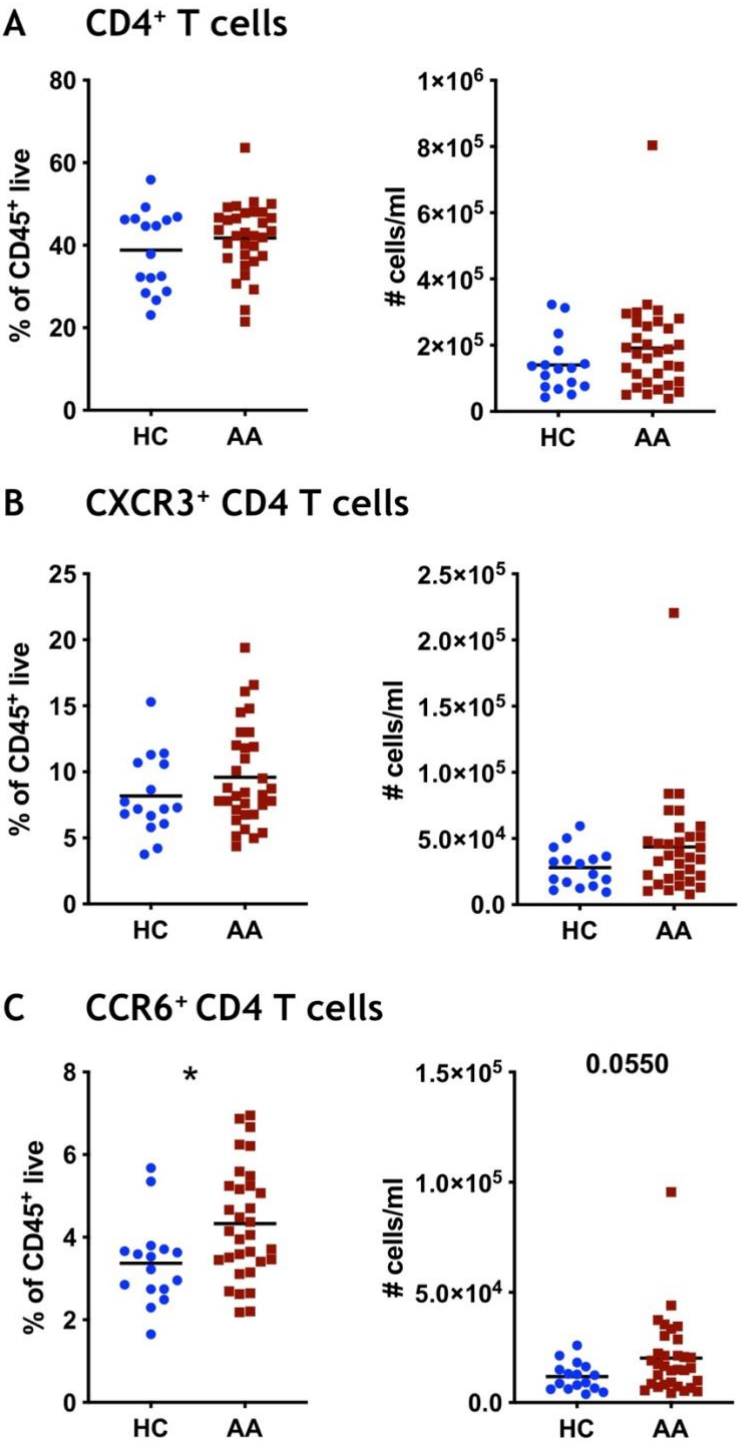


**Figure 4.9 Comparison of circulating CD4⁺ T cell and specific T-helper subsets between HC and AA cohorts.**
Frequencies as a % of CD45⁺ live cells (left) and numbers per mL of blood (right) of (A) CD4⁺ T cells, (B) CXCR3⁺ CD4 T cells and (C) CCR6⁺ CD4 T cells in AA patients (n=32) and HCs (n=16). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test, *p<0.05.

To analyse the CCR6⁺ T cell subset further, the proportions of CCR6⁺ T cells expressing skin or gut homing markers was determined. On average, 48% and 7% of CCR6⁺ CD4 T cells expressed CLA and CCR9, respectively. In contrast, 20% and

4% of CXCR3$^+$ CD4 T cells expressed CLA and CCR9, respectively. No differences were observed in the proportions of skin or gut homing CCR6$^+$ CD4 T cells between AA patients and HCs (Figure 4.10), despite a trend towards increased CCR9$^+$ CCR6$^+$ CD4 T cells in AA patients. The frequency of CCR7$^-$CD38$^+$ CD4 T cells were also not altered in circulation (Figure 4.11).

In summary, CD4$^+$ T cell subsets were modestly altered in AA. A significant increase in the proportion of CCR6$^+$ CD4 T cells in AA patients was identified, but this increase was not significant (p=0.055) when cell numbers per ml of blood were compared.

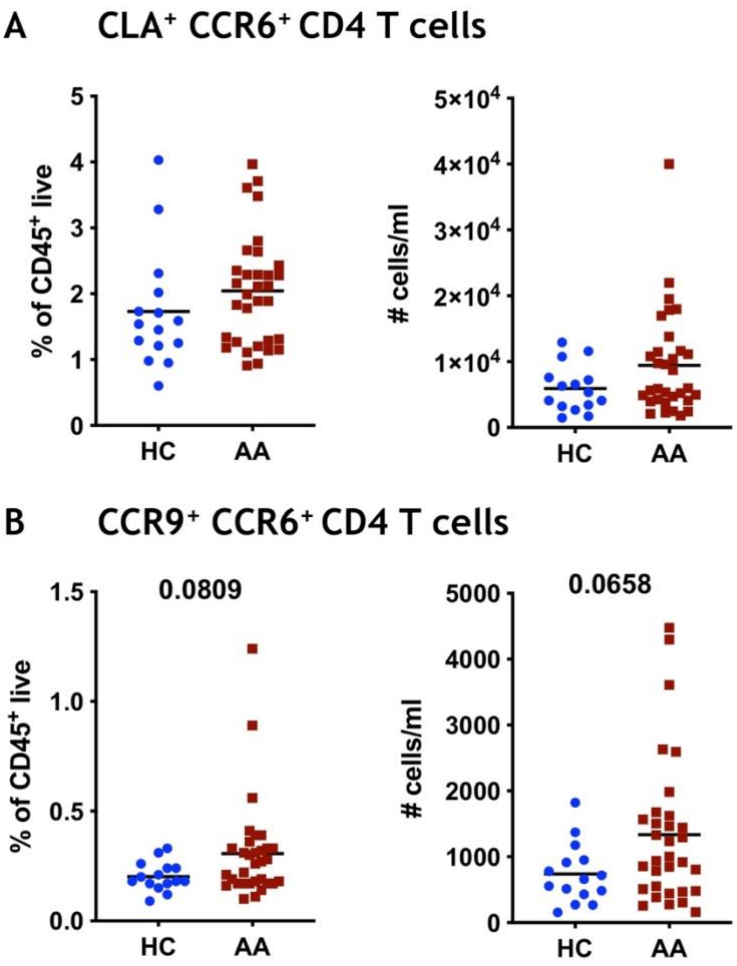


**Figure 4.10 Comparison of circulating skin and gut homing CCR6+ CD4 T cell subsets between HC and AA cohorts.**
Frequencies as a % of CD45$^+$ live cells (left) and numbers per mL of blood (right) of (A) CLA$^+$ CCR6$^+$ CD4 T cells and (B) CCR9$^+$ CCR6$^+$ CD4 T cells in AA patients (n=32) and HCs (n=15). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

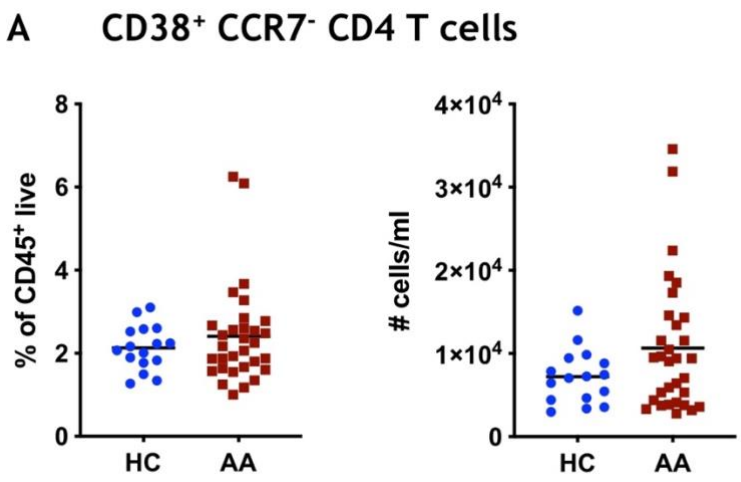


**Figure 4.11 Comparison of CD38$^+$ CCR7$^-$ CD4 T cells between HC and AA cohorts.** Frequency as a % of CD45$^+$ live cells (left) and numbers per mL of blood (right) of (A) CD38$^+$ CCR7$^-$ CD4 T cells in AA patients and HCs. Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

## 4.5 Circulating B cells

B cell subsets were identified using the gating strategy depicted in Figure 4.12. Side scatter and forward scatter were used to remove debris and doublets, prior to gating of live CD19$^+$ B cells. First, non-transitional B cells were identified by removal of immature B cells that have recently emigrated from the bone marrow (Sims et al., 2005), referred to as transitional B cells (CD10$^+$CD38$^+$). Mature naïve B cells express IgM and IgD antibody isotypes prior to activation-induced class-switching (Geisberger et al., 2006). Consistent with other studies (Seifert et al., 2015), IgM$^+$ IgD$^{lo}$ B cells express the memory marker, CD27, as illustrated in Figure 4.11B. This population also contains CD27$^+$ IgM-only B cells. For the purpose of this analysis, this subset will be referred to as IgM memory B cells. The remaining B cells, referred to here as mature B cells, include cells that are naïve (IgD$^+$IgM$^+$CD27$^-$), class-switched (IgD$^-$IgM$^-$CD27$^+$), unswitched B cells (IgD$^+$ IgM$^+$) that also express CD27, similar to the IgM memory subset and double negative B cells (DN, IgD$^-$ CD27$^-$). The DN subset are a small population of switched B cells that have previously been described to exhibit an exhausted phenotype in HIV patients (Moir et al., 2008). Subsequently, the proportion of plasma cells (CD38$^+$CD43$^+$) and activated cells (CD43$^+$) within these subsets were identified.

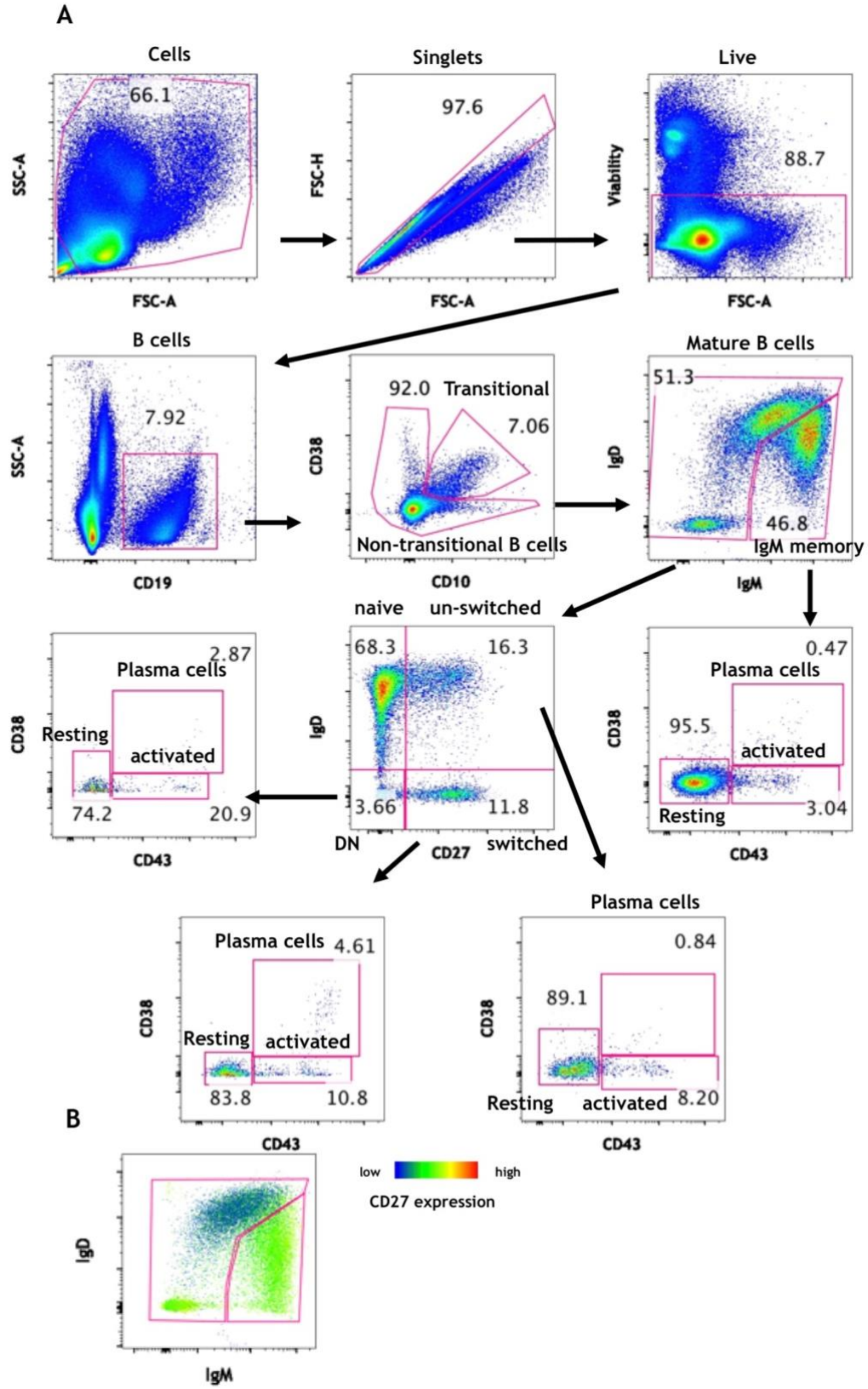


**Figure 4.12 Identification of B cells**
Peripheral blood mononuclear cells were isolated and stained with the B cell panel. (A) Cells are identified as single live $CD19^+$ B cells. Transitional and non-transitional B cells were identified, prior to gating of IgM memory and mature B cells. Naïve, un-switched, class-switched and $IgD^-$ $CD27^-$ were gated prior to identification of memory, activated or plasma B cells. (B) Representative plot highlighting expression of CD27 on mature B cells relative to IgD and IgM expression.

The proportion of total circulating B cells were significantly increased in AA patients compared to HCs (Figure 4.13A), however this increase was not significant in terms of numbers of B cells. A significant increase in the proportion of transitional and non-transitional B cells was also observed (Figure 4.13B, C). The numbers of transitional B cells were also increased, however the change in non-transitional B cells was not reflected in terms of numbers. To analyse the proportional changes further, the frequencies of non-transitional and transitional B cells were analysed as a proportion of total B cells. These data indicate that the proportions of non-transitional B cells are in fact reduced (Figure 4.13D), whereas transitional B cells are increased (Figure 4.13E).

No changes in the proportions or numbers of IgM memory cells were observed (Figure 4.14A). However, the proportion of mature B cells were significantly elevated in AA circulation (Figure 4.14B). This change was not reflected when analysed in cell numbers. As before, we also analysed these subsets as a proportion of total B cells, but no significant differences were indicated (Figure 4.14C,D).

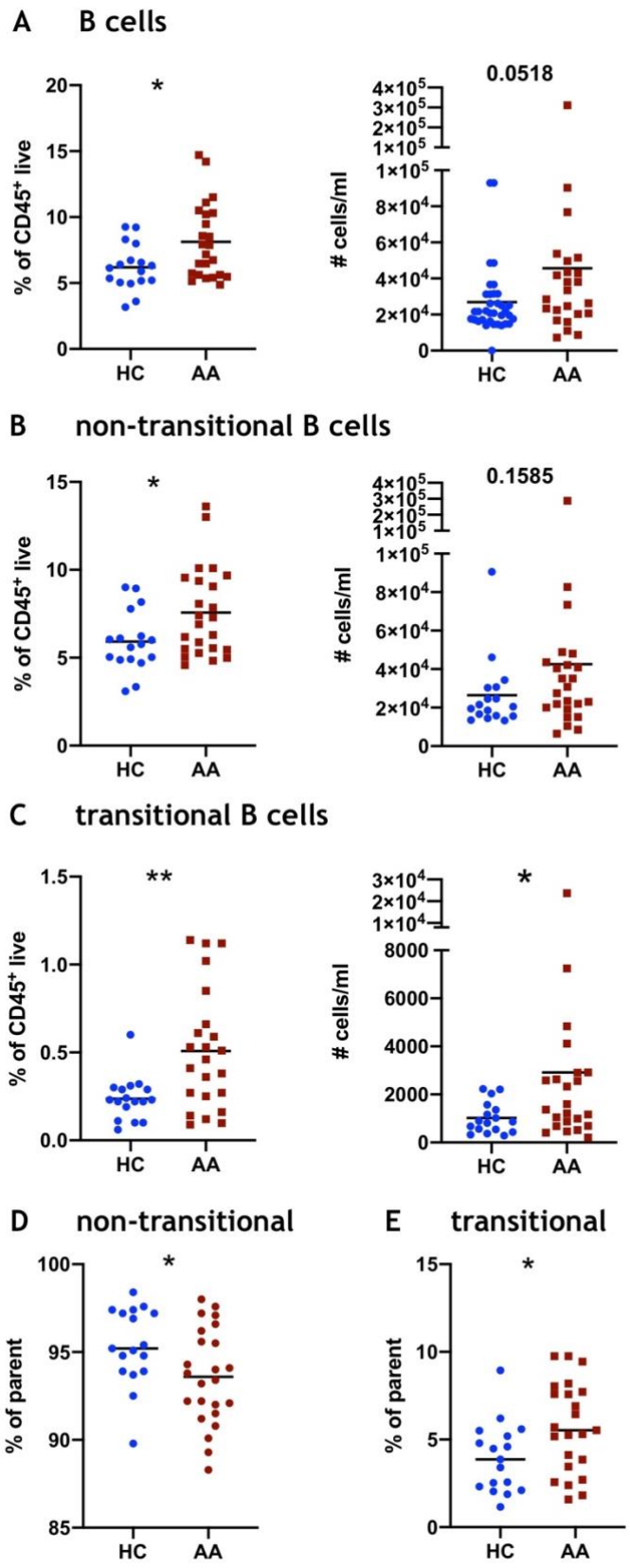


**Figure 4.13 Comparison of circulating B cell subsets between HC and AA cohorts.**
Frequencies as a % of live cells (left) and numbers per mL of blood (right) of (A) B cells, (B) non-transitional and (C) transitional B cells in AA patients (n=24) and HCs (n=17). Frequency as a % of parent population of (D) non-transitional and (E) transitional B cells. Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test, *p<0.05, **p<0.01.

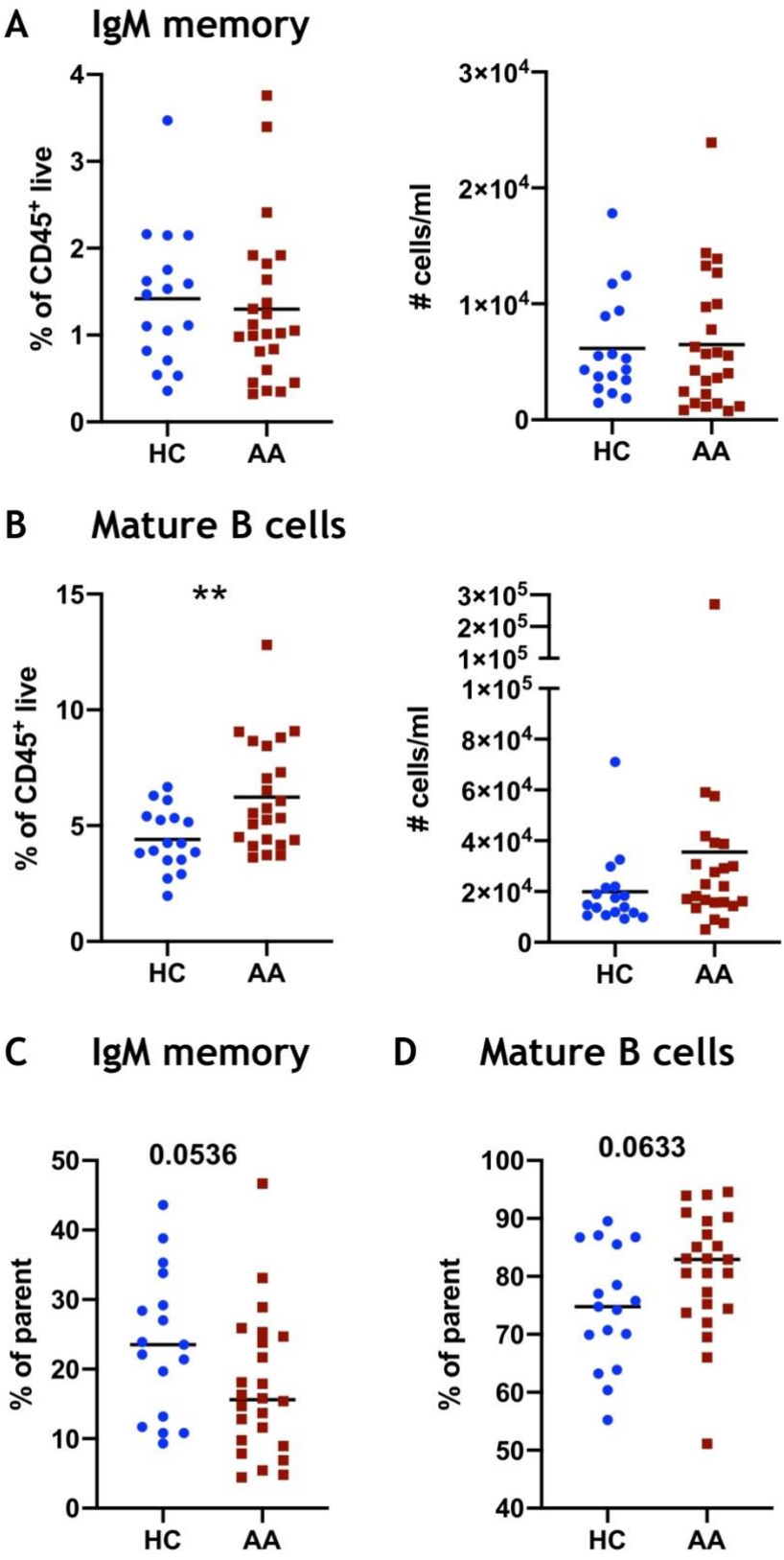


**Figure 4.14 Comparison of IgM memory and IgM non-memory B cell subsets between HC and AA cohorts.**
Frequencies as a % of live cells (left) and numbers per mL of blood (right) of (A) IgM memory B cells and (B) mature B cells and in AA patients (n=24) and HCs (n=17). Frequency as a % of parent population of (C) IgM memory and (D) mature B cells. Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

The IgM non-memory population was then divided into naïve, unswitched and class-switched cell subsets. There was also a population that did not express IgD, IgM or CD27 ($IgM^-IgD^-CD27^-$). The proportion of naïve B cells were significantly increased in AA circulation compared to HCs (Figure 4.15A), but again this change was not significant when analysed as cell numbers (p=0.0548). There were no changes in the frequency or number of unswitched, class-switched, or $IgD^-IgM^-CD27^-$ populations (Figure 4.15B,C,D). Analysis of naïve B cells, as a proportion of the parent population (IgM non-memory) also revealed a significant increase (Figure 4.16A). However, the proportions of resting memory, plasma and activated B cells within each of these four populations indicated no differences between AA and HCs (plots not shown).

In summary, the frequencies of specific B cell subsets are altered in AA circulation. The proportions of total B cells are increased, and this change is associated with an increase in the proportion of transitional B cells and naïve B cells, but with no alterations in the frequencies of plasma or activated B cells.

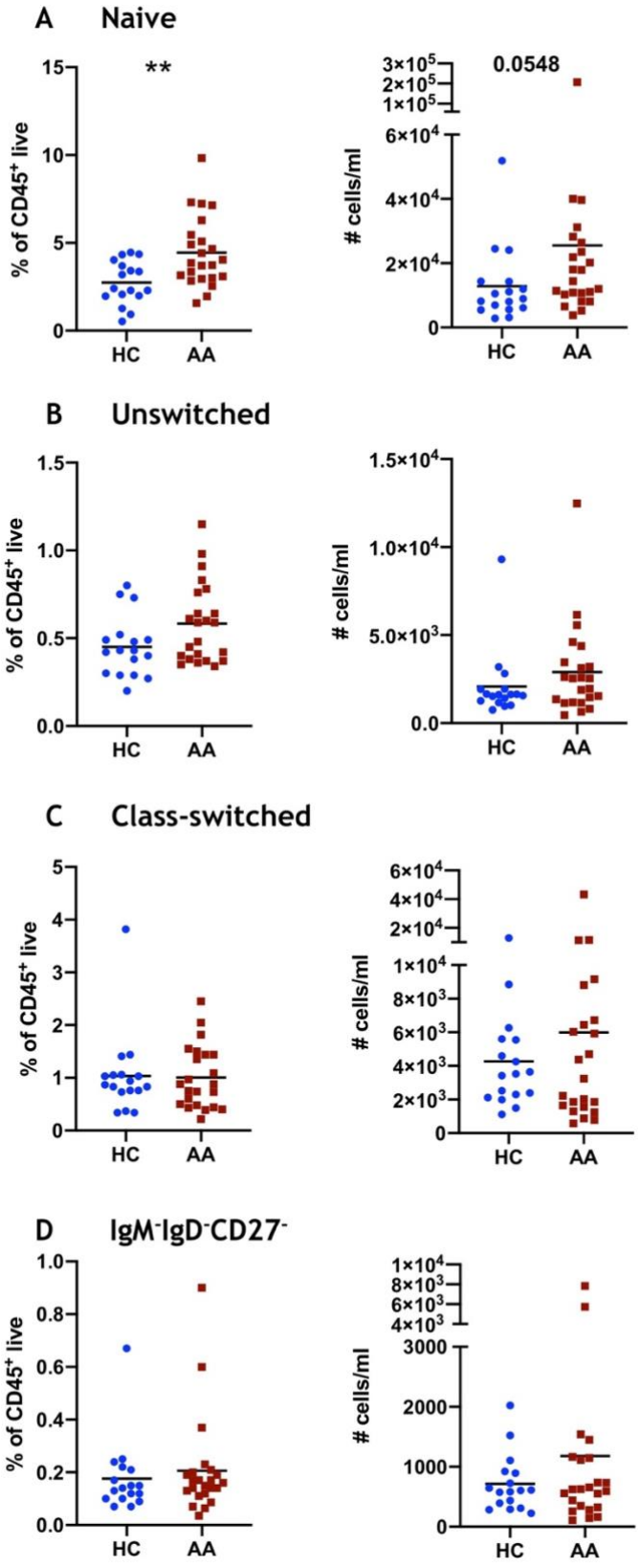


**Figure 4.15 Comparison of naive, class-switched and un-switched B cell subsets between HC and AA cohorts.**
Frequencies as a % of live cells (left) and numbers per mL of blood (right) of (A) naïve, (B) unswitched, (C) switched and (D) $IgM^{-}IgD^{-}CD27^{-}$ B cells in AA patients (n=23) and HCs (n=17). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test, **p<0.01.

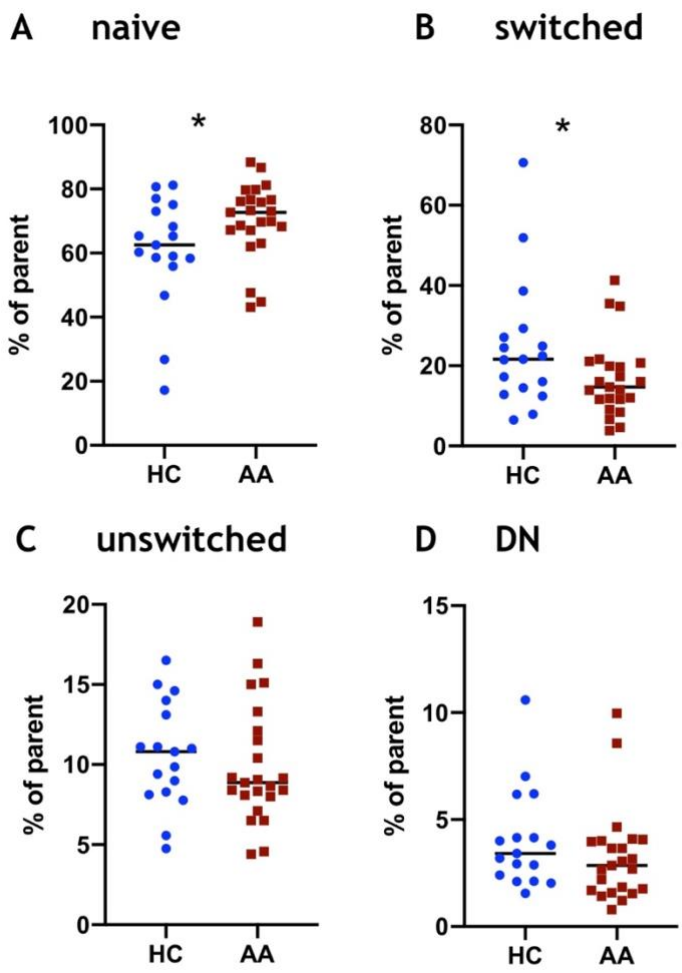


**Figure 4.16 Comparison of naive, class-switched and un-switched B cell subsets between HC and AA cohorts.**
Frequency as a % of parent population of (A) naïve, (B) class-switched, (C) unswitched and (D) $IgM^{-}IgD^{-}CD27^{-}$ B cells in AA patients (n=23) and HCs (n=17). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test, *p<0.05.

## 4.6 Circulating natural killer cells

NK cell subsets were identified using the gating strategy depicted in Figure 4.17A. Side scatter and forward scatter were used to remove debris and doublets from the analysis prior to gating of live cells. NK cells were identified as $CD56^{+}$ and negative for CD3 expression, whereas NKT cells were $CD3^{+}CD56^{+}$. Four NK cell subsets were identified by differential expression of CD56 and CD16: $CD56^{dim}$, $CD56^{bright}$, $CD56^{+}CD16^{+}$ and $CD56^{dim}CD16^{+}$. Consistent with other studies (Poli et al., 2009), the average frequency of $CD56^{dim}CD16^{+}$ NK cells as a proportion of live cells was 8.4%, making them the largest NK cell subset in peripheral blood. Cells within the $CD56^{dim}$, $CD56^{bright}$ and $CD56^{+}CD16^{+}$ gates, but not $CD56^{dim}CD16^{+}$ cells, expressed the pan innate lymphoid cell (ILC) marker, CD127 (as illustrated in Figure 4.16B). Thus, these gates likely include some ILCs

because CD127 is known to be expressed by immature NKs cells, and CD56 can be expressed by ILCs (Nagasawa et al., 2019).

NK cells express inhibitory and activation receptors on their surface that include NKG2D, NKp46 and NKp44 (Vivier et al., 2008). NKG2D expression on NK cell subsets was assessed, alongside expression of CLA to identify skin homing NKG2D$^{+}$ NK cells. As reported previously, and illustrated in Figure 4.17C, the majority of mature CD56dimCD16$^{+}$ NK cells express the NKG2D receptor. Whereas, the CD56bright and CD56$^{+}$CD16$^{+}$ populations were found to be most enriched for cells that co-expressed CLA and NKG2D (Figure 4.17C).

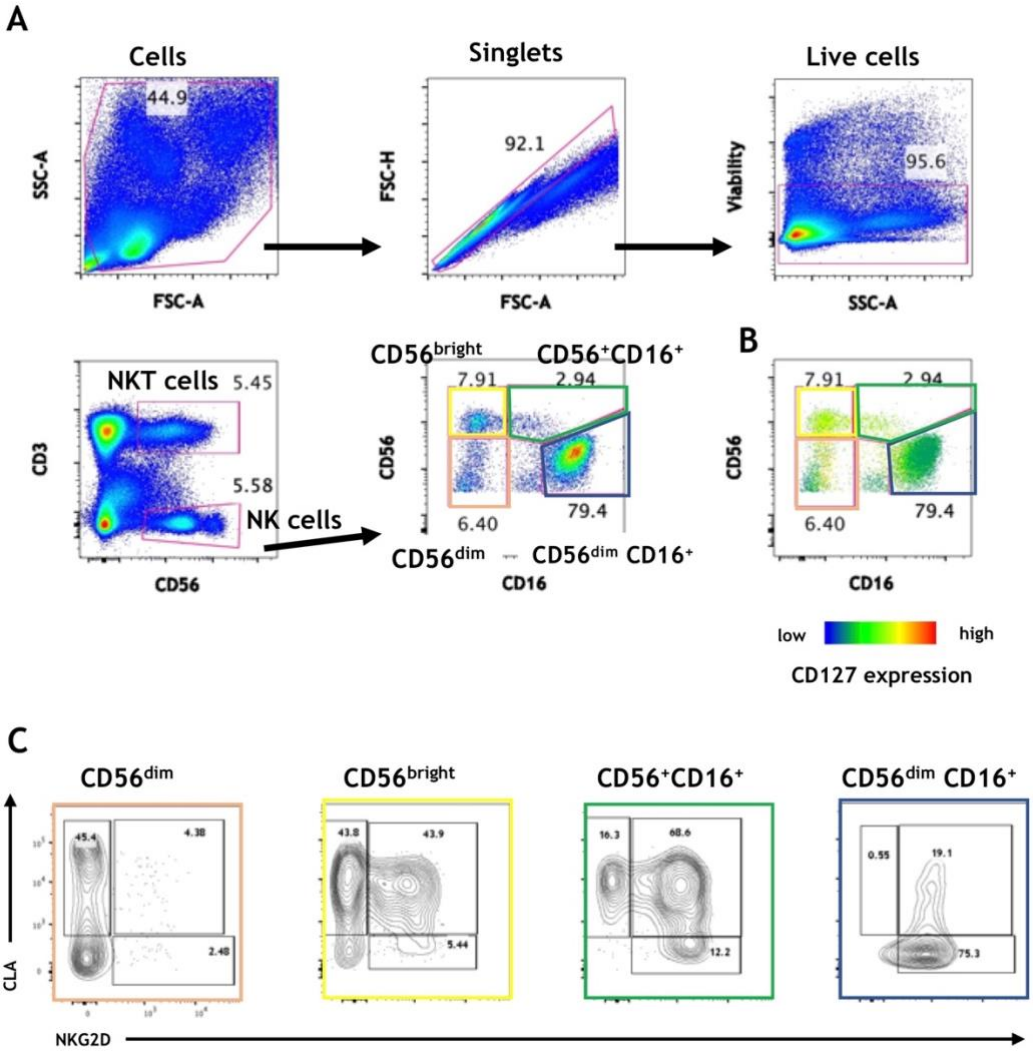


**Figure 4.17 Identification of natural killer cell populations**
Peripheral blood mononuclear cells were isolated and stained with the NK cell panel. (A) NK cells were identified as single live CD3$^{-}$ CD56$^{+}$ cells and NKT cells as CD3$^{+}$CD56$^{+}$ cells. NK cell subsets were identified as CD56$^{dim}$, CD56$^{bright}$, CD56$^{+}$CD16$^{+}$ and CD56$^{dim}$CD16$^{+}$ cells. (B) Representative plot highlighting expression of C1D27 on NK cells relative to CD56 and CD16 expression. (C) Representative plots highlighting CLA and NKG2D expression on each subset of NK cells.

Analysis revealed that there were no differences in the proportions or numbers of circulating NK cells or NKT cells in AA patients compared to HCs (Figure 4.18A, B). In addition, the frequencies and numbers of the four NK cell subsets were unchanged in circulation (Figure 4.19). Furthermore, analysis of subsets expressing NKG2D and CLA did not identify any changes (plots not shown). In summary, NK cell subsets are not different between our HC cohort and this cohort of AA patients.

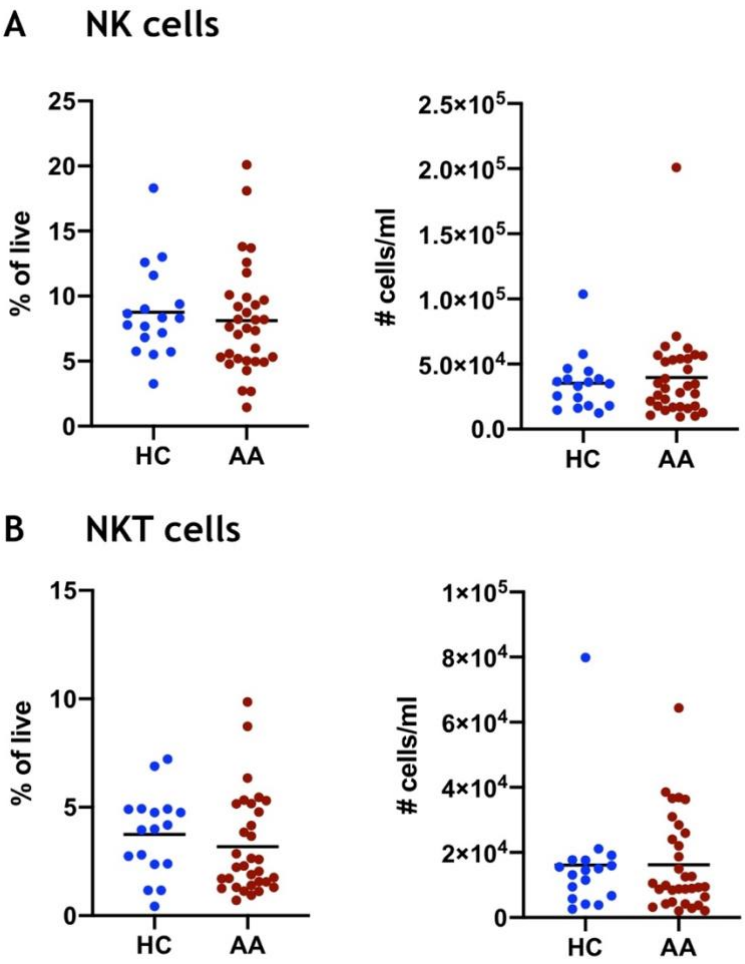


**Figure 4.18 Comparison of natural killer cells and NKT cells between HC and AA cohorts.** Frequencies as a % of live cells (left) and numbers per mL of blood (right) of (A) NK cells and (B) NKT cells in AA patients (n=32) and HCs (n=17). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

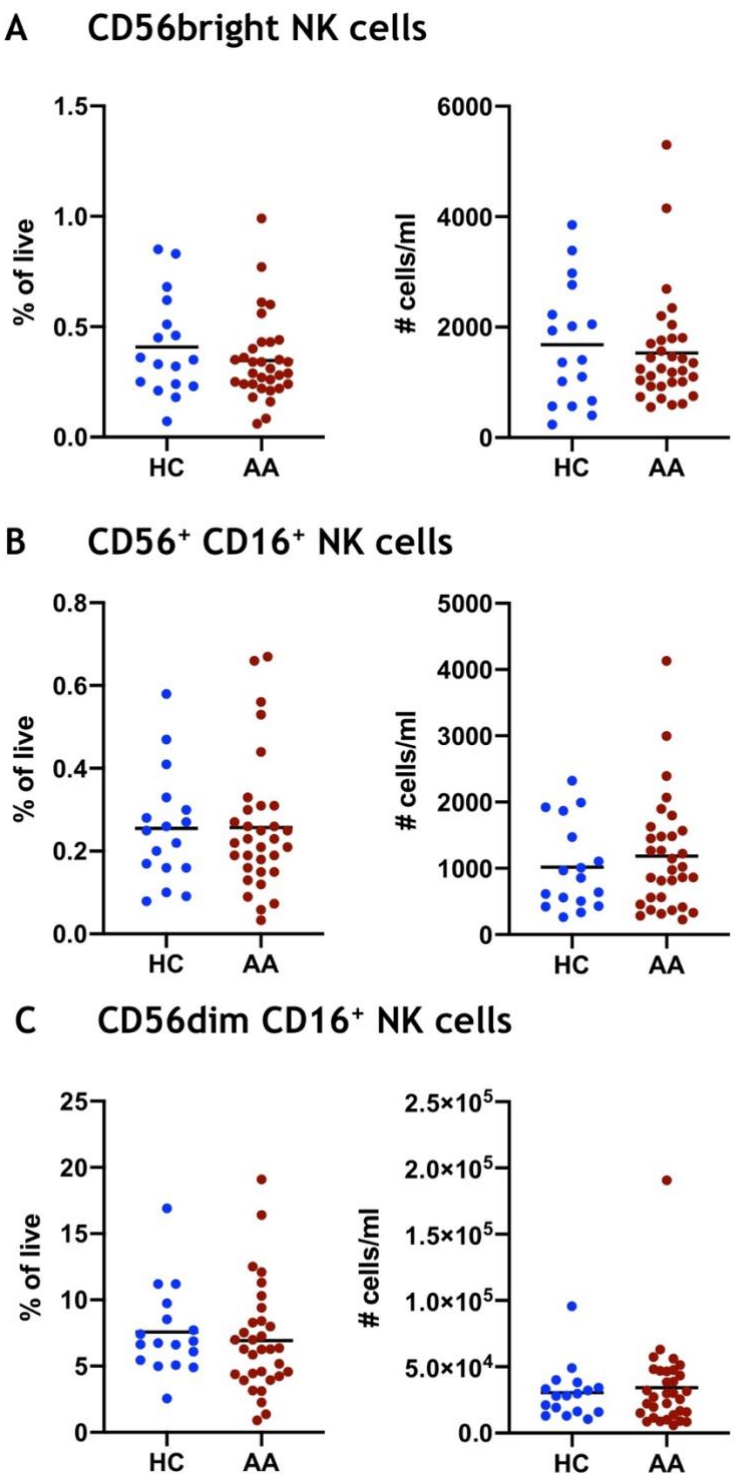


**Figure 4.19 Comparison of NK cell subsets between HC and AA cohorts.**
Frequencies as a % of live cells (left) and numbers per mL of blood (right) of (A) CD56bright, (B) $CD56^{+}CD16^{+}$ and (C) $CD56dimCD16^{+}$ cells in AA patients (n=32) and HCs (n=17). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

## 4.7 Circulating dendritic cells

Dendritic cells were identified using the gating strategy depicted in Figure 4.20. Side scatter and forward scatter were used to remove debris and doublet cells

from the analysis prior to gating of live cells. DCs were identified as $MHCII^{+}CD11c^{+}$ and negative for lineage markers of other cells: CD3 (T cells), CD19 (B cells), CD15 (neutrophils), CD14 (monocytes) and CD56 (NK cells). Conventional DCs (cDCs) consist of two distinct populations, known as cDC1s and cDC2s, that can be identified by expression of CD141 and CD1c, respectively. Within the $MHCII^{+}$ $CD11c^{+}$ DC population, a myeloid subset exists that expresses CD16 as illustrated in Figure 4.20B. Subsequently, plasmacytoid DCs (pDCs) were identified within the $CD11c^{-}MHCII^{+}$ gate as $CD123^{+}$ cells.

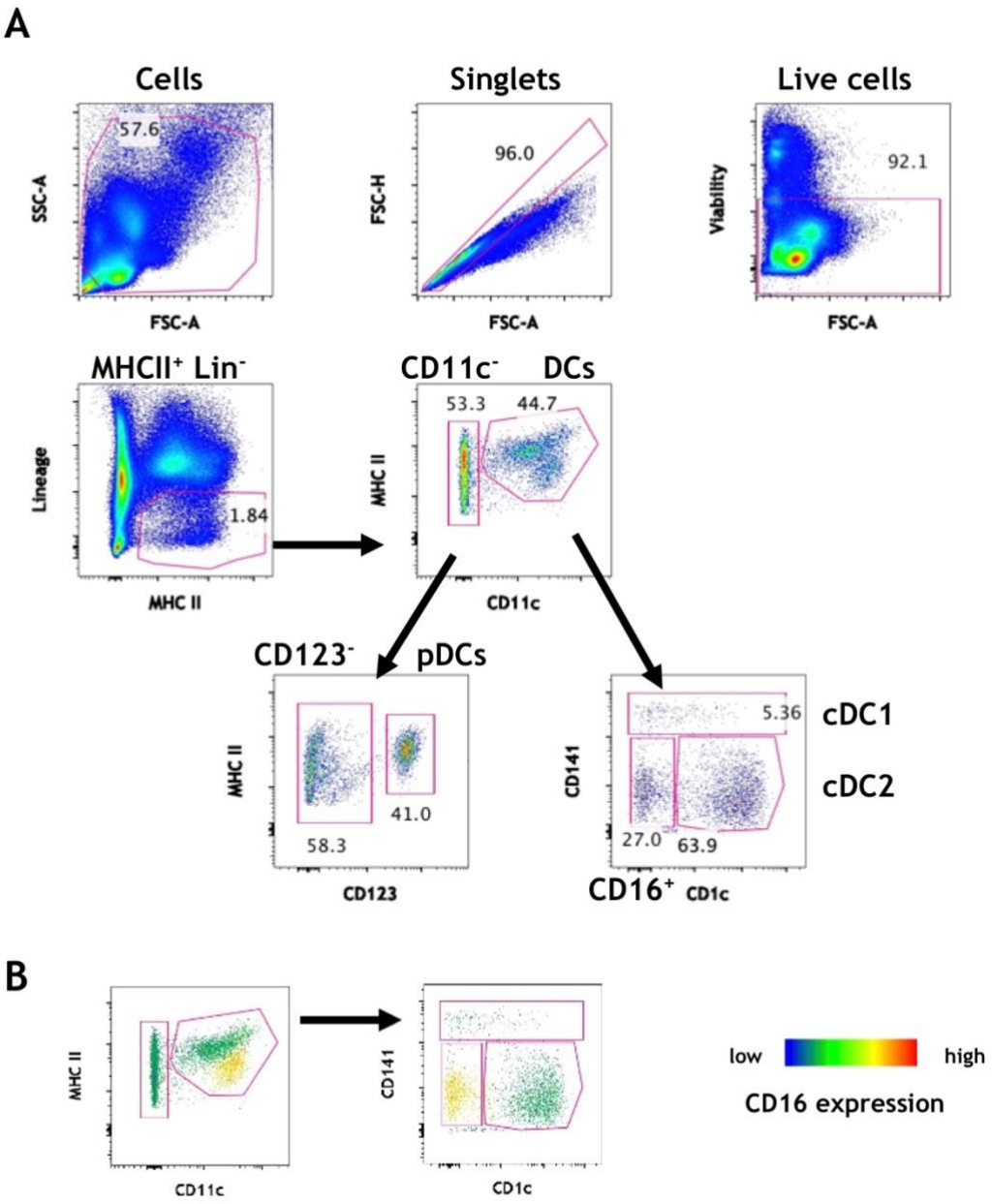


**Figure 4.20 Identification of dendritic cells**
Peripheral blood mononuclear cells were isolated and stained with the DC panel. (A) DCs were identified as single live lineage negative ($CD3^-CD19^-CD15^-CD14^-CD56^-$) $CD11c^+MHCII^+$ cells. Conventional DC subsets were identified as $CD141^+$ (cDC1) or $CD1c^+$ (cDC2). Plasmacytoid DCs were identified as $CD11c^-MHCII^+CD123^+$ cells. (B) Representative plot highlighting expression of CD16 relative to CD11c, CD141 and CD1c expression.

No difference was observed in the proportion or number of circulating total cDCs in AA patients compared to HCs (Figure 4.21A). The proportions and number of cDC1 and cDC2 subsets were also unchanged (Figure 4.21B, C). In addition to cDCs, the $CD11c^-$ fraction were analysed. No changes in the proportions or numbers of $CD123^+$ pDCs were observed (Figure 4.22B), however the proportion of total $MHCII^+$ $CD11c^-$ cells was significantly decreased in AA in comparison to HCs (Figure 4.22A). This change was not significant in terms of numbers of cells.

The CD11c$^+$MHCII$^+$CD16$^+$ population was also quantified, but no differences were identified (Figure 4.22C). In summary, conventional and plasmacytoid DCs are not altered in AA circulation.

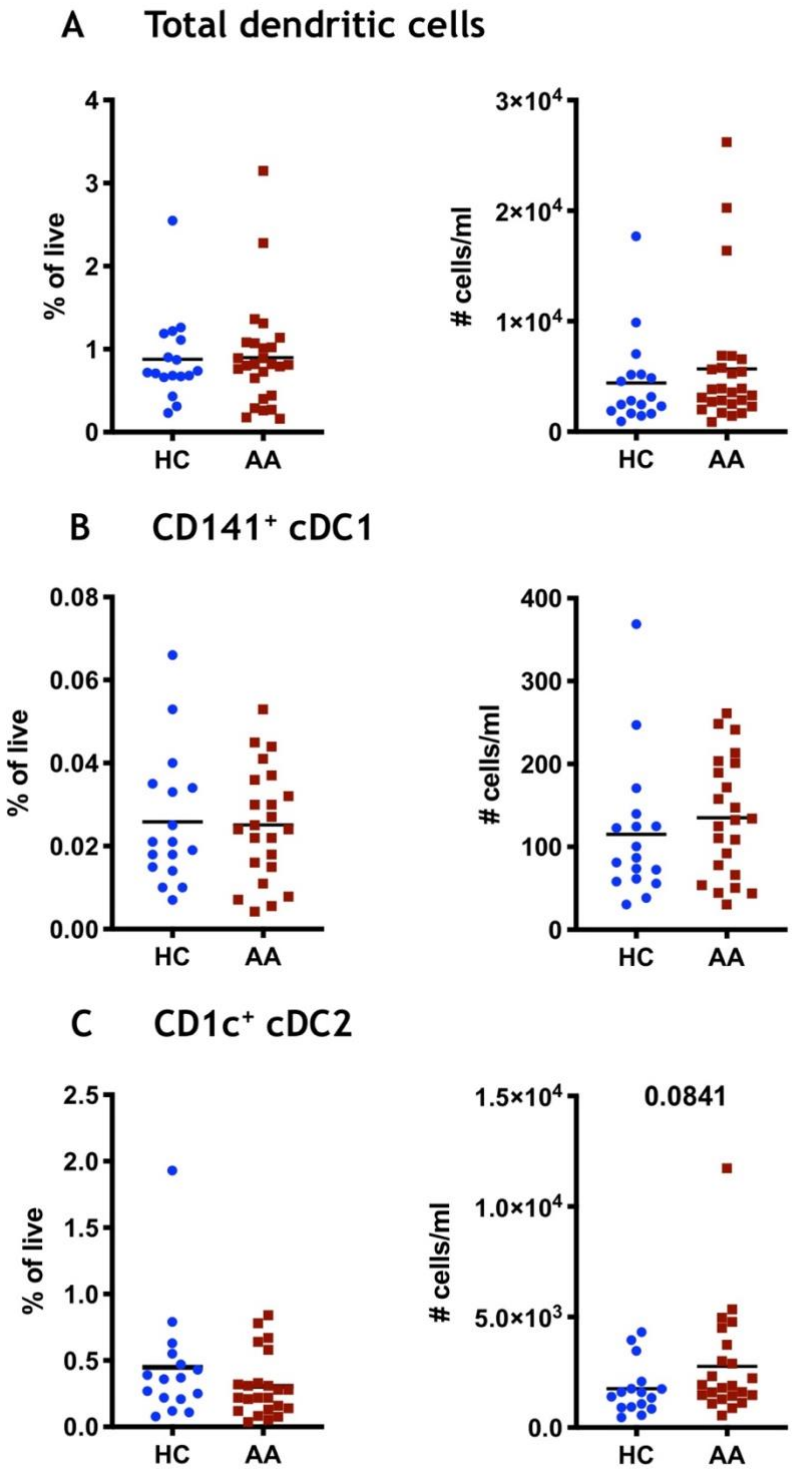


**Figure 4.21 Comparison of cDC subsets between HC and AA cohorts.**
Frequencies as a % of live cells (left) and numbers per mL of blood (right) of (A) total DCs, (B) cDC1s and (C) cDC2s in AA patients (n=26) and HCs (n=17). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test.

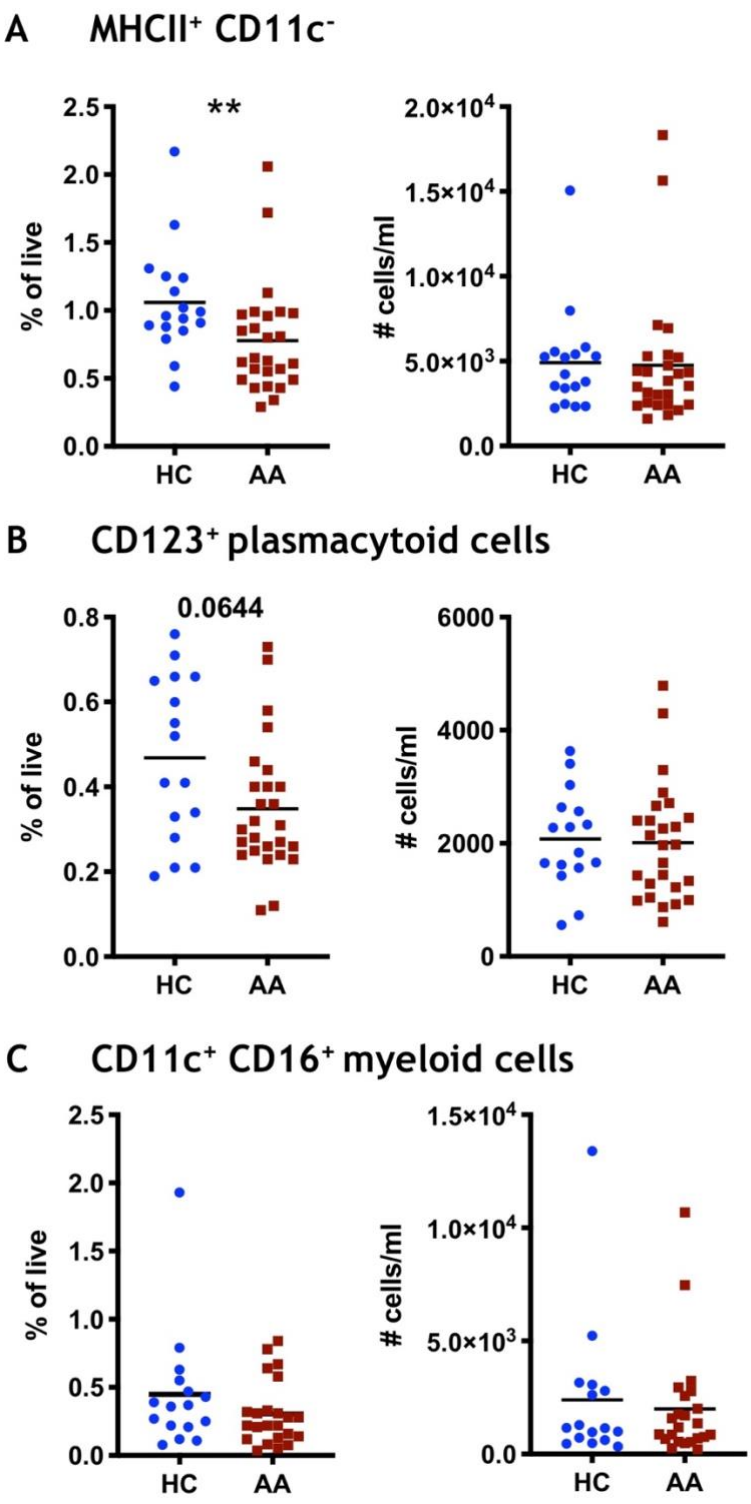


**Figure 4.22 Comparison of pDCs and CD16$^+$ myeloid subsets between HC and AA cohorts.** Frequencies as a % of live cells (left) and numbers per mL of blood (right) of (A) MHCII$^+$CD11c$^-$ cells, (B) pDCs and (C) CD11c$^+$CD16$^+$ myeloid cells in AA patients (n=26) and HCs (n=17). Means are plotted. Statistical differences between groups were determined using a Mann-Whitney U test, *p<0.05, **p<0.01.

## 4.8 Markers of intestinal inflammation

The levels of faecal calprotectin and serum zonulin were measured to determine whether AA is associated with raised markers of intestinal inflammation or intestinal permeability, respectively. The samples were collected using the same criteria as described in section 4.2, and the cohorts used for each assay overlapped with samples used for the phenotyping experiments (Table 4.2). For comparison purposes, faecal calprotectin was also measured in a cohort of paediatric CD patients. This data set was obtained from a study led by Professor Konstantinos Gerasimidis at the University of Glasgow.

**Table 4.2 Participant cohorts used for protein assays**
Stool samples and plasma or serum extracted from peripheral blood collected from alopecia areata patients and healthy controls were utilised to measure inflammatory mediators. AA; alopecia areata, HC; healthy controls, CD; Crohn's disease.

| Assay | Number of samples | Gender F/M | Age ± SD |
|---|---|---|---|
| **Faecal calprotectin** | AA: 21<br>HC: 3 | 20/1<br>3/0 | 47 ± 12<br>45 ± 15.7 |
| **Plasma zonulin** | AA: 31<br>HC:27 | 27/4<br>22/5 | 47 ± 12<br>40 ± 13.5 |
| **Serum TTG IgA** | AA: 38 | 30/8 | 43.6 ± 13.3 |

Calprotectin levels in AA patient plasma were not indicative of intestinal inflammation (Figure 4.23A). On average, the concentration in AA sera was 58mg/kg. In comparison, the average concentration observed in sera from CD patients was 989 mg/kg. Notably, a small proportion of individuals with AA did have higher levels than the majority of AA patients; 153mg/kg, 158mg/kg and 292mg/kg. Comparison of calprotectin in AA patients with HCs was not possible due to lack of power (n=3).

Zonulin levels were also measured, however the concentrations of zonulin could not be interpolated because the majority of samples were above the maximum range of the manufacturer's standard curve. To provide preliminary evidence, the optical density measurements of zonulin detection were analysed. These

data indicate that there is an increased detection of zonulin in AA samples in comparison to HCs (Figure 4.23B).

Additionally, the levels of serum anti-tissue transglutaminase IgA (anti-TTG IgA) were measured in relation to the hypothesis that AA is associated with increased incidence of CeD. These data indicate that 5 patients had TTG IgA levels above the manufacturer's cut-off, indicating that 13% of this cohort have a positive TTG result (Figure 4.23C). Only 4 positive samples are represented in Figure 4.22C because 1 sample was above the limit of detection. To verify these data, patients were contacted by clinical colleagues and TTG-IgA levels were measured clinically in the NHS labs. In contrast to our results, only 1 patient was determined as a positive test result, and the remaining patients were deemed negative (data not shown).

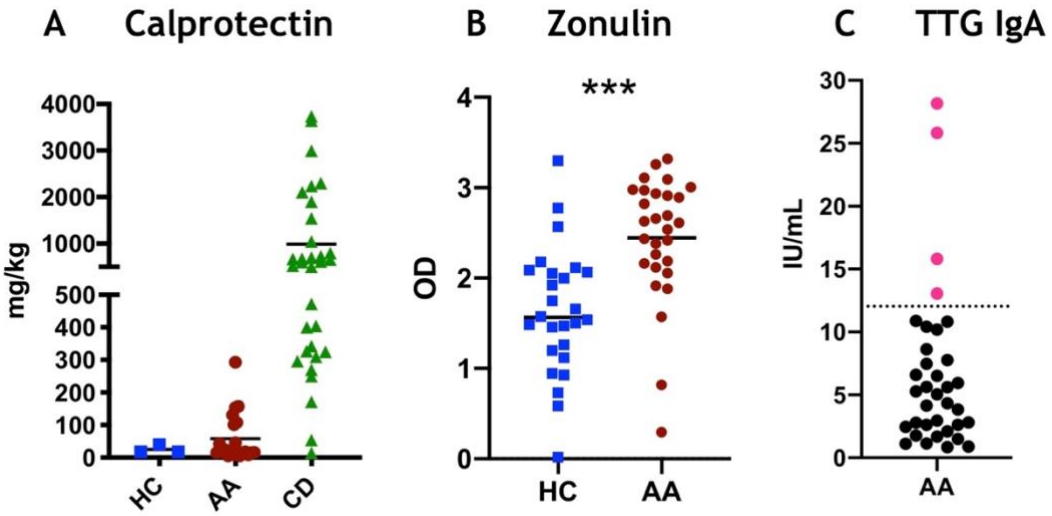


**Figure 4.23 Levels of peripheral zonulin and TTG IgA, and faecal calprotectin**
(A) Levels of calprotectin (mg/kg) were measured in stool samples from AA patients (n=21), HCs (n=3) and pediatric Crohn's disease (CD) patients (n=32) using an ELISA assay. (B) Levels of zonulin were measured in plasma from AA patients (n=31) and HCs (n=27) using an ELISA assay. Data represents optical density due to a large fraction of readings measuring above the limit of detection. (C) Levels of anti-tissue transglutaminase (TTG) IgA (IU/mL) were measured in serum of AA patients (n=38). Dotted line represents the cut-off for a positive result, and positive samples are represented as pink circles. One sample was above the maximum range of the manufacturer's standard curve and is not shown. Means are plotted. Statistical differences between groups were determined using a Kruskal-Wallis test, ***$p<0.001$.

# 4.9 Discussion

## 4.9.1 The immunophenotype of AA patients

In recent years, AA research has mainly been focused on understanding the mechanisms that drive $CD8^+$ T cell cytotoxic activity against hair follicles (Xing et al., 2014; Jabbari et al., 2016). However, at present there is very limited understanding of how other subsets of lymphocytes and myeloid cells contribute to AA pathogenesis. A handful of studies have begun to investigate the role of alternative cell types that are present in follicular infiltrates, but further investigation is required to identify their importance in AA pathology. In this chapter, we sought to characterise the peripheral immunophenotype associated with AA to identify novel immune cell targets. We focused on five key subsets of immune cells; $CD8^+$ T cells, $CD4^+$ T cells, B cells, NK cells and DCs, to generate extensive insight into 74 circulating cell subsets.

Activation of a T cell in a lymph node leads to differentiation of T cells with the same antigen specificity but with different functions in terms of effector and memory capacity (Chang et al., 2014). Effector T cells migrate to tissues, whereas effector memory cells migrate between lymphoid nodes and tissue, and central memory cells circulate between lymph nodes. In this chapter, because of the involvement of CD8 T cells in AA pathogenesis, it was hypothesised that the proportions of $CD8^+$ T cell subsets may be altered in the circulation of individuals with AA. However, no differences in the proportions or numbers of total $CD8^+$ T cells, or memory and effector subsets were observed. There were also no changes in proportions of $NKG2D^+$ CD8 T cells. CLA is upregulated by T cells that have been activated in skin-draining lymph nodes, and thus allows them to migrate into the skin by binding E-selectin expressed by endothelial cells (Picker et al., 1993). It was hypothesised that the circulating skin homing CD8 T cell compartment may be altered in AA patients due to migration of CD8 T cells to the skin. However, analysis of skin-homing $CD8^+$ T cell subsets revealed no differences between AA patients and HCs. Few studies analysing peripheral $CD8^+$ T cell subsets in other cohorts of AA patients have been conducted. However, a previous study reported increased frequency of TCM $CD8^+$ T cells and decreased frequencies of TEM $CD8^+$ T cells in AA circulation compared to HCs (Czarnowicki

et al., 2017). This study also identified a decrease in the proportion of circulating skin homing TCM $CD8^+$ T cells in AA patients compared to HCs.

Clearly, there are disparities between our study and the study conducted by Czarnowicki *et al* (2017) as we did not identify changes in the frequencies of CD8 T cell populations between our AA cohort and HCs. Importantly, Czarnowicki *et al* (2017) characterised TEM and TCM subsets using the same markers as our study, and the size of the AA patient cohorts used in both studies are the same, despite Czarnowicki *et al* (2017) having more HCs (n=30). Furthermore, the AA cohorts appear to be similar between both studies in terms of disease severity. However, we did observe that the AA cohort utilised by Czarnowicki *et al* (2017) had a higher proportion of males than our study, and the study did not describe the proportion of AA participants with a history of atopy. Importantly, the AA cohort in our study has a mean disease duration of 16 years, whereas the cohort in the Czarnowicki study had low mean duration of 8 years. Taken together, it may be hypothesised that changes in the proportions of circulating CD8 T cell populations may not be associated with chronic AA.

Collectively, our data indicate that circulating CD8 T cell populations are not altered in AA patients. These data are somewhat surprising when considering the importance of $CD8^+$ T cells in AA pathogenesis. However, these data only allow us to conclude that the size of the overall peripheral $CD8^+$ T cell effector and memory populations are not altered in this cohort of patients, and do not rule out the importance of memory populations in the immune responses taking place in lymph nodes or in the skin. It may be hypothesised that the $CD8^+$ T cell compartment responsible for driving AA may be partly composed of a resident T cell population (Cheuk et al., 2017). Interestingly, it the context of psoriasis, it has been shown that resident T cell populations can generate psoriasis lesions without infiltration of memory T cells. It was demonstrated that transferring non-lesional skin from psoriasis patients onto immunodeficient mice led to development of psoriasis lesions (Boyman et al., 2004), whereas transfer of normal skin did not lead to inflammation. Additionally, this study indicates that resident memory cells with capacity to drive inflammation can be located in non-lesional skin. To our knowledge, the importance of resident memory T cells in AA pathogenesis has not been studied.

$CD4^+$ T cells are vital for generating robust $CD8^+$ T cells responses. In a mouse model of AA, for instance, $CD4^+$ T cells are required to induce severe AA, whereas subcutaneous transfer of $CD8^+$ T cells alone induces only localised hair loss (Gilhar et al., 2002; McElwee et al., 2005). $CD4^+$ T cells are required for priming of $CD8^+$ T cells, by licensing DCs (Smith et al., 2004) and providing IL-2 to drive proliferation. However, once activated, $CD8^+$ T cell effector function is not dependent on $CD4^+$ T cell help. The results above indicate that $CD4^+$ T cell help is required for the progression of hair loss in the C3H Hej mouse model. In humans, $CD4^+$ T cells are present in the perifollicular region of affected follicles (Ito et al., 2008; Hong et al., 2017), and transcriptome analysis indicates a mixed $CD4^+$ T cell profile with upregulation in genes associated with Th2 and Th1 responses (Suarez-Farinas et al., 2015). IL-23 was also upregulated, but no increases in Th17-associated genes were observed. (Suarez-Farinas et al., 2015). However, other studies have reported IL-$17^+$ CD4 T cells in close proximity to affected HF (Hong et al., 2017; Tanemura et al., 2013). Together, these data indicate potential involvement of Th1, Th2 and Th17 cells.

To expand on these data, we assessed the levels of circulating $CD4^+$ T cells, and the frequencies of Th1 and Th17 subsets using CXCR3 and CCR6 as surrogate markers, respectively. We identified no changes in the total frequency of $CD4^+$ T cells or in the proportions or numbers of $CXCR3^+$ CD4 T cells in AA patients compared to HCs. However, the proportion of $CCR6^+$ CD4 T cells was elevated in AA circulation compared to HCs. This increase was not significant in terms of numbers of $CCR6^+$ CD4 T cells ($p=0.055$). Nevertheless, these data corroborate another study that reported increased proportions of IL-$17^+$ CD4 T cells in AA circulation (Han et al., 2015). In contrast, a more recent study did not identify changes in IL-$17^+$ CD4 T cells, but reported altered frequencies within the TEM and TCM $CD4^+$ T cell compartment (Czarnowicki et al., 2017). It is important to note that the $CCR6^+$ CD4 T cell population does not solely represent Th17 cells, but will also include a proportion of regulatory T cells (Tregs) (Lee et al., 2017). We did not assess Treg proportions here. However two separate studies reported a reduction in the frequencies of circulating Tregs in AA patients (Han et al., 2015; Hamed et al., 2019). Thus, the Treg compartment, if reduced in size, may have decreased the proportion of $CCR6^+$ CD4 T cells. We did not quantify Tregs in our analyses.

Our data and others suggest that CCR6$^+$ CD4 T cells are differentially represented in AA circulation. These data complement our findings in chapter 3 which indicated elevated levels of type-17 cytokines in AA circulation compared to HCs, however the necessity of these cells in AA pathology is yet to be addressed. Th1 cells are classically associated with driving type 1 cytotoxic responses, however responses to infections and those associated with other autoimmune diseases often involve both Th1 and Th17 responses. For example, IL-21, a Th17 derived cytokine is important for CD8$^+$ T cell effector function in the context of chronic viral infections (Xin et al., 2015). We reported a systemic increase in IL-21 levels in the previous chapter, and the IL-2/IL-21 locus is significantly associated with AA (Petukhova et al., 2010). In contrast, studies using the AA mouse model indicate that IL-21 blockade was not effective in promoting hair regrowth (Xing et al., 2014). Collectively, these data indicate that Th17-associated responses are often dysregulated in AA, but that they may not be vital for driving hair loss.

We were able to analyse the CCR6$^+$ CD4 T cell population further by analysing expression of CLA. However, no changes between AA patients and HCs were observed. One of the aims of this chapter was to assess whether gut homing T cells were altered in AA circulation. Th17 cells are implicated in IBD (Kleinschek et al., 2009), thus it was hypothesised that intestinal involvement may result in alterations in the size of the population of gut-homing CCR6$^+$ CD4 T cells. However, no significant differences were identified in the proportions or numbers of gut-homing CCR6$^+$ CD4 T cells. However, a fraction of AA patients did have increased proportions and numbers of CCR9$^+$ CCR6$^+$ CD4 T cells. This observation may indicate that a subset of AA patients are associated with changes in T cell migration to the intestine, suggesting that some patients may be associated with altered intestinal immune responses.

Whilst these data are interesting, our analysis of CD4 T cell populations is somewhat limited due to the use of surrogate chemokine markers to identify Th1 and Th17 cells. Previous studies in our lab and others have indicated that circulating IL-17$^+$ CD4 T cells express CCR6 (Hirota et al., 2007). However, we cannot confirm that CCR6$^+$ CD4 T cells are *bona fide* Th17 cells without staining for IL-17 expression. These analyses were not conducted as part of our original

phenotyping experiments, however we are now measuring expression of IFNγ and IL-17 in CD4 T cell subsets to validate and support our results.

In addition, the frequencies of $CCR7^{-}CD38^{+}$ CD4 T cells, a population shown to be enriched for T cells specific for antigen of intestinal origin, were analysed (Preacute et al., 2011). Consistent with other studies, this population represented approximately 6% of circulating $CD4^{+}$ T cells. No differences in the frequencies or numbers of this cell type were observed between AA patients and HCs. However, this finding is perhaps not surprising, because the frequency of this subset was also not altered in CeD patients, despite this population being enriched for cells that were specific for the gluten peptide (Preacute et al., 2011). Thus, the sole quantification of this subset does not appear to be useful and the potential involvement of this population is the context of AA requires functional investigation.

We examined B cell populations in AA circulation. B cells primarily produce antibodies but they can also function as antigen presenting cells (Marino et al., 2012) and secrete cytokines (Barr et al., 2012). B cells are widely implicated in autoimmune diseases (Barr et al., 2012; Brink et al., 2013; Ilonen et al., 2013), typically due to their production of autoreactive antibodies which are often measured clinically for diagnosis and disease monitoring purposes (Hanly et al., 2010). In this cohort of AA patients, the total frequency of B cells was increased in comparison to HCs. Similar to previous analysis in this chapter, the increase in absolute cell numbers did not reach significance. There were also significant increases in the proportions of non-transitional and transitional B cells in AA circulation compared to HCs. However, the increase in transitional B cells was most prominent as we also observed a significant increase when analysing absolute numbers of transitional B cells between AA and HCs. We next assessed B cell subsets within the mature B cell population. The proportion of naïve mature B cells was increased in AA circulation compared to HCs, but again this increase was not significant in terms of absolute numbers ($p=0.0548$). There were no differences in the frequencies or numbers of unswitched, switched or DN B cell subsets. Analysis of these four subsets (naïve, switched, unswitched and DN) as a frequency of the parent population (IgM non-memory) also indicated an significant increase in the proportion of naïve B cells. Collectively,

analysis of B cells in AA circulation indicates changes in the transitional and naïve B cell compartments. To our knowledge, these observations have not been reported in association with AA.

To date, B cell responses have not been an area of interest in the context of immune responses targeting HFs. However, a collection of studies support the notion of potential B cell involvement. Case studies have reported severe AA in patients with common variable immunodeficiency (CVID) which represent a heterogeneous family of primary deficiencies that are associated with low or absent antibody responses (Bonilla et al., 2016). Furthermore, AA is associated with high incidence of thyroid-related autoimmune disease (Lee et al., 2019) where autoantibodies are known to drive pathology (Chazenbalk et al., 2002). Auto-antibodies against follicular antigen have also been reported to be increased in circulation of AA patients compared to HCs (Tobin et al., 1994).

Transitional B cells represent developmental precursors of mature B cells, that have emigrated from the bone marrow into circulation to migrate to secondary lymphoid organs (Sims et al., 2005). A large proportion of transitional B cells do not reach full maturity because many are deleted during the developmental selection process. Transitional B cells have been studied in the context of other autoimmune diseases, and are found to be increased in the circulation of SLE patients (Sims et al., 2005) and in individuals with immunodeficiency, including X-linked lymphoproliferative disease (Cuss et al., 2006). In normal controls, transitional B cells represent approximately 2-3% of circulating B cells, whereas 6.7% of B cells in SLE circulation have a transitional phenotype (Sims et al., 2005). In our study, the average proportion of transitional B cells in AA and HC circulation were 5.7% and 4%, respectively. It can therefore be concluded that the increase in proportions of circulatory transitional cells observed in AA patients may be immunologically relevant, due to similarities with SLE.

Notably, we also observed an increase in the proportion of naïve B cells. In SLE patients, expansion of peripheral transitional B cells is reported to be linked to defects in B cell tolerance that lead to increased levels of circulating autoreactive naïve B cells (Yurasov et al., 2005). Thus, it can be hypothesised that the increase in transitional and naïve B cells indicates an increase in the

autoreactive B cell pool in AA patients. Autoreactive naïve B cells may not be pathogenic, but activation of autoreactive B cells can lead to generation of high affinity antibodies which could contribute to autoimmunity. Notably, we did not identify changes in the frequencies of switched B cell subsets in AA patients. Whilst these data indicate that AA is not associated with increased frequency of activated B cells, our data cannot determine if there is a change in the frequency of activated autoreactive B cells with the switched B cell pool. In the context of SLE, meta-analysis of genetic risk factors indicated a significant association between B cell signaling pathways and SLE risk (Julià et al., 2018). Specific B cell genes have not been associated with AA in genetic wide association studies (GWAS), however the locus containing CTLA-4 is significantly associated with AA (Petukhova et al., 2010), and CTLA-4 is crucial for controlling B cell responses (Sage et al., 2014). The cytokine signature associated with AA, as described in chapter 3 may also play a role in the generation of increased transitional and naïve B cells. Notably, IL-21 was significantly increased in AA circulation compared to HCs, and IL-21 has been shown to influence proliferation and survival of B cells. Interestingly, overexpression of IL-21 *in vivo* caused increased frequency of transitional splenic B cells in mice (Ozaki et al., 2004). We can speculate that the cytokine milieu associated with AA may impact the autoreactive B cell pool, however further investigation is required to understand the pathological role of B cells in the context of AA.

To complement the analyses of peripheral $CD8^+$ T cells subsets, circulating subsets of NK cells were analysed. NK cells are innate lymphocytes that represent the innate cytotoxic counterpart of type 1 responses (Moretta et al., 2008). NK cells mediate cytotoxic activity against abnormal cells and are important during viral infections and for removing malignant cells. The involvement of NK cells in AA pathogenesis has generated curiosity because immune privileged hair follicles are reported to express low levels of MHCI (Ito et al., 2008), which would normally induce NK cell killing of target cells. Additionally, NK cells recognise stress ligands using receptors including NKG2D, and this receptor is strongly associated with AA development (Petukhova et al., 2010). Following collapse of immune privilege, NK cells are present in follicular infiltrates in AA (Ito et al., 2008) and are likely involved in initial IFN$\gamma$ production prior to $CD8^+$ T cell involvement. Furthermore, IL-15 is crucial for NK cells

survival, and blocking IL-15Rß in the C3H Hej model was successful in promoting hair regrowth (Xing et al., 2014). However, in contrast, it was shown in the AA mouse model that hair loss is exacerbated when NK cells are depleted (Kaufman et al., 2010). Thus, NK cells may play a range of roles in AA pathogenesis.

In human peripheral blood, NK cell subsets are identified by expression of CD56 and CD16. The main subsets are $CD56^{bright}$ and $CD56^{dim}CD16^{+}$ (Angelo et al., 2015). $CD56^{dim}$ NK cells are the largest subset in peripheral blood and exhibit potent cytotoxic activity, whereas the $CD56^{bright}$ population, thought to represent the less mature subset of NK cells are potent cytokine producers (Poli et al., 2009). We measured the proportions and numbers of NK cells but observed no differences between AA and HCs. Specific subsets of NK cells were identified using CD56 and CD16 to identify 4 populations; these included the main $CD56^{bright}$ and $CD56^{dim}CD16^{+}$ populations and the smaller, $CD56^{+}CD16^{+}$ and $CD56^{dim}$ subsets. $CD56^{+}CD16^{+}$ NK cells represent approximately 0.3% of the peripheral NK cell population, and are most likely cells that are transitioning from the $CD56^{bright}$ to the large $CD56^{dim}CD16^{+}$ population. Whereas, $CD56^{dim}$ cells that do not express CD16 represent approximately 0.4% of the peripheral NK cells population as have been associated with better outcome of melanoma patients (Vujanovic et al., 2019). However, we did not observe changes in the proportions or numbers of any of the NK cells subsets between AA patients and HCs.

To analyse NK cells further, we assessed whether AA was associated with changes in frequencies of skin homing NK cells or differential expression of the NKG2D receptor. These data indicated that the NK cells that expressed high levels of CD56 ($CD56^{bright}$ and $CD56^{+}CD16^{+}$) were most enriched for cells co-expressing CLA and NKG2D. Whereas, the $CD56^{dim}$ subset, the largest subset in peripheral blood with most effective cytolytic capacity, is most enriched with cells that solely express NKG2D. However, we observed no changes in the proportions or numbers of skin homing NK cells, or $NKG2D^{+}$ cells in AA patients compared to HCs.

Finally, the proportions of circulating DCs were assessed in AA circulation. Dendritic cells represent a critical link between the innate and adaptive immune

system, and are therefore in a prime position to dictate the flavour of the ensuing immune response. By communicating with T cells, DCs have the capacity to either induce an immune response or tolerogenic responses (Probst et al., 2005; Esterházy et al., 2016; Persson et al., 2013), which are especially relevant for avoiding autoimmune reactions. For these reasons, DCs are thought to play key roles in autoimmune diseases (Ganguly et al., 2013). Conventional DCs are now commonly divided into two populations based on their ontogeny; cDC1s are important for cross-presentation to $CD8^+$ T cells (Haan et al., 2000), whereas cDC2s seem more important for driving $CD4^+$ helper T cell responses (Gao et al., 2013).

To our knowledge, conventional DCs have not been studied in the context of AA. As previously discussed, genetic locus containing the co-stimulatory receptors, CTLA-4 and ICOS, have been significantly associated with AA (Petukhova et al., 2010). CTLA-4 and ICOS are receptors expressed by T cells and Tregs that bind ligands on the surface of DCs, and contribute to the inhibition or activation of T cells, respectively (Riley et al., 2002; Bour-Jordan et al., 2011). Thus, we hypothesised that DC and T cell interaction is compromised in AA. In this cohort, conventional DC subsets, and plasmacytoid DCs were analysed in blood. A subset of $CD16^+$ cells within the $CD11c^+MHCII^+$ population, that did not express CD141 or CD1c, were also analysed. These cells were previously reported to represent a population of DCs characterised by FCGR3A (CD16) expression that are enriched for an IFN signature (Villani et al., 2017). However, more recent analysis has confirmed that these cells are not DCs, and represent a CD14dim monocyte population (Calzetti et al., 2018). Our analyses revealed that total DCs, cDC1s, cDC2s, and $MHCII^+CD16^+$ cells were not found at different frequencies in AA patients compared to our HCs. However, there was a significant decrease in the proportion the $MHCII^+CD11c^-$ compartment, but we observed no change in frequency or numbers of $CD123^+$ pDCs.

These data indicate that the proportion and numbers of conventional DCs are not altered in AA circulation. There are also no changes in pDCs, despite a decrease in the frequency of CD11c- cells. The pDC fraction is known to be heterogeneous (Villani et al., 2017) and in this study we could not interrogate this population further due to a lack of markers. Notably, it was found that the

$CD11c^-$ $CD123^+$ compartment includes a proportion of DC precursors (See et al., 2017) Nevertheless, it may be interesting to investigate the pDC population further in future studies. pDCs are potent producers of type 1 interferon (IFN) and are implicated in the pathogenesis of psoriasis (Erkek et al., 2000) and T1D (Ferreira et al., 2014; Stewart et al., 1993). In the context of T1D, activation of pDCs and subsequent release of IFNa is associated with immune complexes composed of DNA from dying ß-islet cells and autoantibodies (Diana et al., 2012). Interestingly, pDCs have been observed in close proximity to affected HF in AA lesions (Rahal et al., 2016) but their role has not been investigated. These data indicate that pDCs may be important for potentiating immune activation of autoimmune responses, however their role in chronic disease maintenance is less clear.

It is important to discuss why some of the significant changes in frequencies of specific cell subsets cells were not reflected when calculated as numbers of cells. Possible explanations for this include variability in sample processing that may impact the number of recovered viable cells, that may not impact analysis of frequencies to the same extent. For example, one AA patient had a cell count of $4.9x10^5$ cells/mL of blood, which was considerably lower than the average cell count in the AA cohort ($1x10^6$ cells/mL of blood). This low cell count was reflected when assessing the numbers of specific cell populations. However, the proportions of cell subsets was not affected; 5.24% of this patients leukocytes were $CCR6^+$ CD4 T cells, which was similar to the mean frequency of this population in the AA cohort (4.3%).

Based on our data, it is clear that specific B cell and CD4 T cell populations require further investigation to understand their involvement in AA pathogenesis. However, it is important to discuss the limitations of our current analysis. We reported that we did not identify a change in the frequency of plasma B cells between AA patients and HCs. However, this result is not accurate because we did not consider the loss of CD19 expression in the plasma population. Plasma B cells are rare in blood, however future analysis should consider the loss of CD19 expression and incorporate CD138 to accurately identify plasma cells (Halliley et al., 2015).

Additionally, our panel was not able to assess specific populations of B cells, including B-1 cells. B-1 cells represent an innate-like B cell population that produce a large amount of polyreactive IgM, referred to as natural antibodies, and these antibodies are vital during the early stages of infection (Choi et al., 2012). In the context of autoimmunity, B-1 cells are of interest because they produce autoantibodies, cytokines and can function as antigen presenting cells. Compared to regular B cells, B-1 cells express high levels of costimulatory ligands, including CD80, and produce large amounts of IL-10 (Duan & Morel, 2006). We therefore propose that future studies should assess the role of B-1 cells in AA pathogenesis because of their potential importance in contributing to autoreactive T cell responses, and autoantibody generation.

In addition, we did not analyse the frequencies of anergic B cells in AA circulation. Here, we have indicated that the proportion of transitional B cells is increased in AA circulation, suggesting an increased egress of immature B cells from the bone marrow which may indicate an increase in circulating autoreactive B cells. It would therefore be interesting to assess the proportions of anergic circulating B cells to investigate the possibility that mechanisms controlling B cell anergy are disrupted in AA patients, as seen in other autoimmune diseases. This could be achieved using our current data by gating $IgM^{lo}$ naïve B cells to identify anergic B cells as indicated in previous studies (Quach et al., 2012).

Throughout this chapter, details have been provided on the choice of markers for assessing specific populations of immune cells, however there are immune cell populations that we did not analyse that are of interest for future studies. Aside from NK cells, we did not analyse other populations of innate lymphocytes, including $\gamma\delta$ T cells. $\gamma\delta$ T cells express a $\gamma\delta$ heterodimeric receptor which recognise a specific repertoire of self and non-self antigen. Unlike conventional T cells, they are not MHC-restricted, and can respond rapidly to antigenic challenge in an unrestricted manner. $\gamma\delta$ T cells predominantly reside at mucosal surfaces and their effector functions include cytokine production and direct cytotoxic killing of target cells.

In the context of AA, $\gamma\delta$ T cells are of interest because they respond to 'stress' signals, including MICA (Xu et al., 2011), and are potent producers of pro-inflammatory cytokines including IFN$\gamma$. In the skin, $\gamma\delta$ T cells are essential for maintaining barrier function by functioning as a surveillance system for damaged keratinocytes and for driving keratinocyte differentiation during wound healing (Jameson et al., 2002). In circulation, $\gamma\delta$ T cells are rare, representing approximately 1-10% of circulating lymphocytes which was one of the reasons why we did not focus on this population for our phenotyping experiments. However, it has been shown that a distinct population of CLA+ $\gamma\delta$ T cells exist in peripheral blood, and that this population is reduced in numbers in the circulation of psoriasis patients, but increased in lesional skin (Laggner et al., 2011). Interestingly, this population produce IL-17A. In addition to $\gamma\delta$ T cells, mucosal-associated variant T (MAIT) cells are present in psoriatic skin and are enriched for IL-17 expression (Teunissen et al., 2014). MAIT cells differ from $\gamma\delta$ T cells in that their activation is restricted to major histocompatibility complex class I-related (MR1) gene. MAIT cells can respond to bacterial metabolites, and therefore play an important role in barrier function via interaction with the skin's microbiome (Constantinides et al., 2017). Collectively, these studies provide a rational for investigating innate lymphocytes in circulation and in the skin of AA patients.

### 4.9.2AA and intestinal-associated markers

People with AA report ongoing intestinal discomfort but there has been limited investigation into a potential link between AA and intestinal inflammation. Beyond anecdotal patient-reported connections, a relationship between AA and the intestine is plausible because AA shares genetic risk factors with inflammatory diseases of intestinal origin including ulcerative colitis (UC) and Crohn's disease (CD) (Petukhova et al., 2010). Single nucleotide polymorphisms (SNPs) in the inhibitory receptor gene, BTNL2, have been significantly associated with AA (Petukhova et al., 2010), UC (Silverberg et al., 2009) and CD (Yang et al., 2014) in GWAS. HLA regions are also associated with risk of developing these diseases, for example HLA-DQA1 SNPs are significantly associated with both AA and UC (Asano et al., 2009). Notably, faecal microbial transplant induced hair regrowth in two AA patients (Rebello et al., 2017), however analysis of bacterial

DNA in stool samples from a small cohort of AA patients did not reveal significant changes in the composition of the microbiome (Arrones et al., 2020).

In this chapter, we report that AA is not associated with significant changes to the proportions or numbers of gut homing T cells. However, the levels of zonulin were increased in AA circulation compared to HCs. Zonulin is a protein that regulates intestinal permeability by modulating tight junctions (Fasano et al., 2000; Wang et al., 2000), and is implicated in autoimmune diseases that are associated with intestinal permeability, including T1D (Sapone et al., 2006) and CeD (Drago et al., 2009). Zonulin is released by intestinal epithelial cells in response to gliadin or bacterial antigen binding the CXCR3 receptor (Lammers et al., 2008; Asmar et al., 2002). This causes the release of zonulin into the lumen, which transactivates the epidermal growth factor receptor (EGFR) in an autocrine manner, leading to tight junction disassembly (Tripathi et al., 2009). Ultimately, these events lead to increased antigen exposure of the immune system which can cause aberrant immune responses. Connecting intestinal permeability with autoimmunity in remote tissues is complex. However it is accepted that aberrant intestinal responses, particularly against the microbiota, impact peripheral tolerance mechanisms and thus can perpetuate reactions to self-antigen (Lee et al., 2011; Wu et al., 2010). Whilst our data provide preliminary evidence that there may be increased intestinal permeability, interpolation of the concentrations of zonulin were not possible, thus the experiment must be repeated.

Faecal calprotectin was also measured, but was not found to be raised in AA patients. These results were expected as none of the AA patients had a diagnosis of IBD. However, a fraction of AA patients did have levels of faecal calprotectin above 150mg/kg; 153mg/kg, 158mg/kg and 292mg/kg. These data may indicate low levels of intestinal inflammation. However, due to the lack of HCs in this data set, it is not possible to understand whether these levels of calprotectin are also observed in the general population.

The observed increase in zonulin led to the hypothesis that AA may be associated with increased reactivity to food antigen. CeD is a disease characterised by immune reactivity to the gliadin peptide and the core immune

mechanisms are very similar to AA, including NKG2D activation, IL-15 signalling and cytotoxic lymphocytes (Meresse et al., 2004). Furthermore, AA and CeD disease share genetic risk factors (Petukhova et al., 2010). Regions containing CTLA-4 and IL-21/-2 SNPs are shared genetic risk loci for both AA and CeD (van Heel et al., 2007; Dubois et al., 2010). A small study involving 12 children with AA indicated that five patients had a positive TTG test, and that a gluten-free diet resulted in hair regrowth (Ertekin et al., 2014). However, gluten free diet in adults with CeD and AA had no effect on hair loss (Bardella et al., 2000). We hypothesised that AA may be associated with increased incidence of CeD. Our initial data indicated that 13% of the AA cohort had increased levels of TTG IgA, as indicated by the assay's cut-off. This is approximately 10-fold higher than the global incidence rate (0.5-1%) of CeD (Gujral, 2012).

The TTG IgA assay is used clinically to diagnose CeD, alongside an endoscopy to confirm small intestinal inflammation. To verify our findings, TTG IgA levels were measured clinically in the five patients with high levels of antibodies. These data indicated that only 1 patient had clinically relevant levels of anti-TTG IgA, whereas the other 4 had normal levels. It can be concluded that a fraction of AA patients have raised levels of anti-TTG IgA, but these data are not indicative of levels associated with CeD. Thus, our data indicate that AA may not associated with increased incidence of CeD.

In addition to measuring circulating markers of intestinal inflammation, we sought to investigate the intestinal microbiome of AA patients. Unfortunately, these data could not be included in my thesis, however we can provide detail of our preliminary results. Stool samples were collected from AA patients (n=41) and HCs (n=19), bacterial DNA was extracted and the 16s regions were sequenced to identify the bacterial species in each sample. Our analyses indicated that the composition of the faecal microbiome is not significantly different in comparison to HCs. These data are somewhat disappointing because we hypothesised that the composition of the intestinal microbiome would be altered in AA, as observed in other autoimmune diseases, notably those affecting the skin including psoriasis (Hidalgo-Cantabrana et al., 2019). Despite no change in microbiota diversity, we did identify a significant reduction in Shannon Diversity Index in the AA cohort compared to HCs, indicating that the abundance

of specific bacterial species may be altered. We are now analysing these data further to understand how changes in abundance of specific bacteria are associated with AA, and incorporating clinical data to understand if disease length, disease severity and comorbidity status are associated with changes in bacterial composition.

### 4.9.3 Conclusion

Extensive analysis of the immunophenotype of individuals with AA has revealed changes in the $CD4^+$ T cell and B cell compartments. In agreement with previous studies, AA is associated with an increase in the frequency of circulating $CCR6^+$ CD4 T cells. These data also complement our findings in chapter 3 describing the increase in type 17 cytokines in peripheral blood.

This chapter also describes novel findings identifying changes in specific subsets of B cells in AA circulation. Frequencies of B cells in transitional and naïve compartments are elevated, which indicates an increased egress of immature B cells from the bone marrow into the mature naïve B cell pool. Transitional B cell frequencies are raised in patients with other autoimmune diseases and are reported to be enriched for cells that produce autoantibodies (Yurasov et al., 2005; Wang et al., 2019). Although one study has reported that AA is associated with increased levels of follicular autoantibodies (Tobin et al., 1994), the importance of autoantibodies in driving hair loss has not been reported. As discussed, it can by hypothesised that the systemic inflammatory environment, associated with the signature described in chapter 3, may contribute to the observed increase in transitional B cells.

The latter experiments described in this chapter reveal that AA is associated with raised levels of circulating zonulin. Whilst these data are preliminary, they provide novel evidence that intestinal permeability may be increased in people with AA. We also indicated that 13% (5/38) of AA patients have raised levels of anti-TTG IgA, but further investigation revealed that only 1 patient had clinically relevant levels of antibodies. Thus, our data indicate that AA is not associated with increased incidence of clinical CeD.

While examination of peripheral blood has generated some novel observations, it has not enabled us to substantially deepen our understanding of AA pathogenesis. We therefore decided on two approaches to explore this further, patient stratification (Chapter 5), and transcriptome analysis of AA skin (Chapter 6).

# 5 The relationship between distinct features of the peripheral immunophenotype and clinical parameters

## 5.1 Introduction

The presentation of hair loss caused by AA is extremely variable amongst affected individuals (Gilhar et al., 2012). The condition can cause a singular patch of hair loss, multiple patches or can become severe to affect the entire scalp. AA can also follow a relapsing-remitting pattern, involve facial and body hair loss, and is commonly associated with the development of other inflammatory diseases affecting the skin.

In the previous chapter, the cellular immunophenotype associated with AA was described in detail highlighting increases in the frequencies of $CCR6^+$ CD4 T cells and specific B cell subsets. In chapter 3, it was reported that type 17 cytokines, in addition to type 2 cytokines are elevated in AA circulation. Due to the heterogeneity of AA, we hypothesised that changes in the frequencies of specific cell subsets and systemic cytokine concentrations may be associated with specific clinical features. It was anticipated that these analyses would reveal novel aspects of AA pathogenesis, that could inform future clinical studies and treatment strategies. Thus, the aim of the experiments described in this chapter was:

> To investigate the relationship between the systemic immunophenotype associated with AA and specific clinical parameters.

## 5.2 Participant characteristics

The cohort used for the analyses in this chapter was the same as chapter 4, as described in Table 5.1. However, a small proportion of these participants could not be included in the analysis where immunophenotyping datasets were partially incomplete. The AA cohort was stratified based on multiple clinical parameters including extent of hair loss, history of atopy and current treatment. The details of the cohorts used for each analysis are described in the figure legends.

The overall cohort included 39 AA patients: 4 in remission, 11 with <50% hair loss, 6 with >50% hair loss and 18 with total loss (>95% hair loss), and 20 HCs. 22 of the AA patients were atopic (history of hayfever, asthma and/or eczema), 7 had thyroid related illness, 2 had vitiligo, 1 had epilepsy and 1 had pernicious anaemia at time of recruitment. One of the female participants had pattern baldness in addition to a diagnosis of AA. None of the patients had a diagnosis of a major inflammatory disease, including CD, UC, rheumatoid arthritis (RA), psoriatic arthritis (PsA), psoriasis (PsO), systemic lupus erythematosus (SLE) or ankylosing spondylitis (AS). A proportion of patients were receiving treatment when recruited: 8 patients were receiving intralesional steroid injections, 9 were receiving diphenylcyclopropenone (DCP) treatment and 1 patient was receiving methotrexate. 21 patients (54%) were not receiving any treatment. Healthy controls were recruited to age and gender match the AA patient cohort.

**Table 5.1 Characteristics of participants with alopecia areata and healthy controls.** Peripheral blood collected from these individuals were used for analyses of circulating immune cells and cytokines. The cohort was stratified based on specific clinical parameters to assess the relationship between changes in the peripheral immunophenotype and subgroups of AA patients. SD; standard deviation, IL steroids; intralesional steroids.

| | Alopecia areata | Healthy controls |
|---|---|---|
| Total | 39 | 20 |
| Gender F/M | 31/8 | 16/4 |
| Age (years) ± SD | Female 46 ± 11.6<br>Male 37 ± 10.3 | Female 42 ± 9.5<br>Male 28 ± 7 |
| **Disease duration** (years)<br>Average ± SD | 1-46<br>16 ± 11.4 | |
| **Disease severity**<br>Inactive<br>AP1, <50% scalp hair loss<br>AP2, >50% scalp hair loss<br>AT, >95% scalp hair loss | <br>4<br>11<br>6<br>18 | |
| **Comorbidities**<br>Atopic<br>Thyroid illness<br>Vitiligo<br>Epilepsy<br>Pattern baldness<br>Pernicious anaemia | <br>22<br>7<br>2<br>1<br>1<br>1 | |
| **Treatment**<br>None<br>Diphencyprone<br>IL steroids<br>Methotrexate | <br>21<br>9<br>8<br>1 | |

## 5.3 Processing of data for comparative and subset regression analysis

Frequencies of CD4 T cell, CD8 T cell and B cell subsets as described in chapter 4 were used for stratification analysis conducted in R. Prior to analysis, the frequency data were normalised using 'edgeR' and 'DESeq' packages, and then transformed using the hyperbolic Arcsinh method with a cofactor of 5. This Arcsinh function transforms the flow cytometry data on a linear scale, and then displays data which is beyond the threshold (cofactor) in a log scale (Amir et al.,

2013). This is useful when comparing multiple variables. These data were then analysed using one-way ANOVA to identify significant differences between patient sub-groups.

Subset regression analysis was used to identify positive and negative relationships between cell subsets, cytokines and clinical parameters as described in the following sections. The model, $Y_i = ß_0 + ß_1X_{1i} + ß_2X_{2i} + ß_3X_{3i} + \varepsilon_i$, was fitted where $Y_i$ represents the chosen clinical parameter (dependent variable) and X represents the cell frequency or cytokine concentration data (independent variable). Models were generated using the 'leaps' package (https://cran.r-project.org/web/packages/leaps/leaps.pdf) which identifies the best variables in X for predicting Y (D'Amore et al., 2016). Models were then tested for their predictive accuracy and ranked according to cross-validation error (CVE). The CVE represents the average difference between the model's estimated values and the true values. The p-values associated with positive and negative predictors selected by the most accurate model, as indicated by the lowest CVE, are presented in heat maps.

## 5.4 Relationship between frequencies of circulating CD4 T cells, cytokines and clinical parameters

In chapter 4, it was reported that the frequency of $CCR6^+$ CD4 T cells was increased in AA circulation compared to HCs. Here we investigate if this observation is associated with extent of scalp hair loss. Patients were stratified based on the Severity of Alopecia Tool (SALT) score into four groups as described in Table 5.1; inactive (SALT 0), AP1 (<50% loss), AP2 (>50% loss) and AT (>95% loss). Only two patients in the CD4 T cell dataset had inactive disease and were therefore removed from the analysis. Stratification indicated that the frequency of $CCR6^+$ CD4 T cells is specifically increased in the AP1 group in comparison to HCs (Figure 5.1). $CXCR3^+$ $CCR6^+$ (DP) CD4 T cells and skin homing DP CD4 T cells were significantly increased in the AP1 group compared to HCs and the AT cohort. Whereas, the proportion of CD4 T cells were elevated in AP1 patients compared to HCs. Stratification also indicated a significant increase in the frequencies of skin homing CD4 T cells and gut homing DP CD4 T cells in the AP1 group compared to the AT cohort (Figure 5.1).

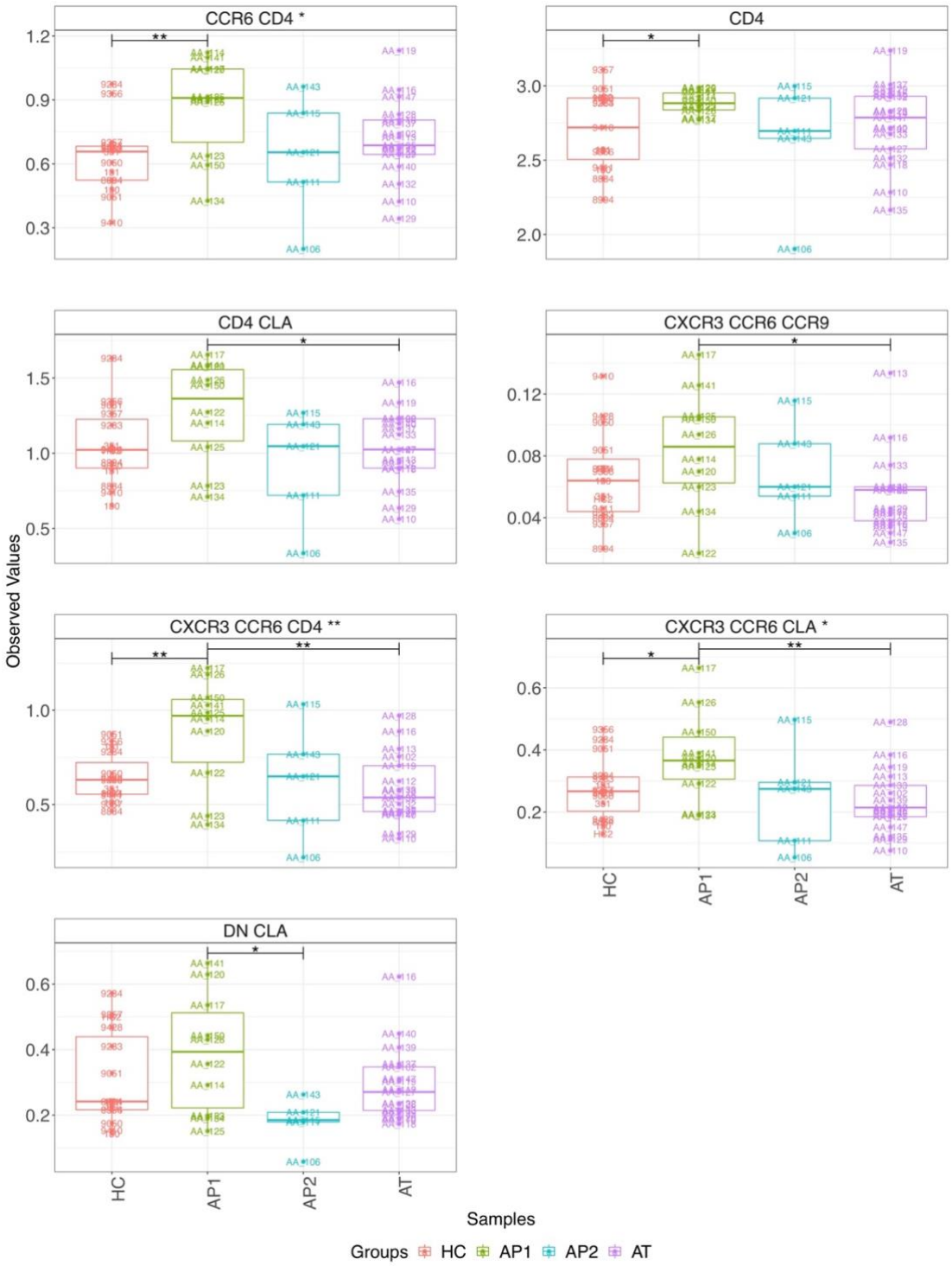


**Figure 5.1 Comparison of CD4 T cell subsets between HCs and AA cohorts stratified by extent of hair loss**
Normalised observed values of frequencies of CD4 T cell subsets in HCs (n=15) and subgroups of AA patients with <50% hair loss (AP1, n=10), >50% hair loss (AP2, n=5), >95% total hair loss (AT, n=17). Means are plotted. DN, double negative $CXCR3^-$ $CCR6^-$ CD4 T cells. Data were normalised using 'edgeR' and 'DESeq' packages, and then transformed using the hyperbolic Arcsinh method. Statistical differences between groups were determined by one-way ANOVA with Bonferroni correction, *p<0.05, **p<0.01.

Using subset regression analysis, we tested the strength of the relationship between changes in the frequencies of circulating CD4 T cell subsets and patchy

hair loss (AP1 group). The most accurate model indicated that the frequency of CXCR3$^{+}$ CCR6$^{+}$ CD4 T cells positively and significantly predicted AP1 status (adjR$^{2}$=0.281, Figure 5.2). This model also indicated a weaker, but significant negative association between AP1 status and the frequency of gut homing (CCR9$^{+}$) CD4 T cells.

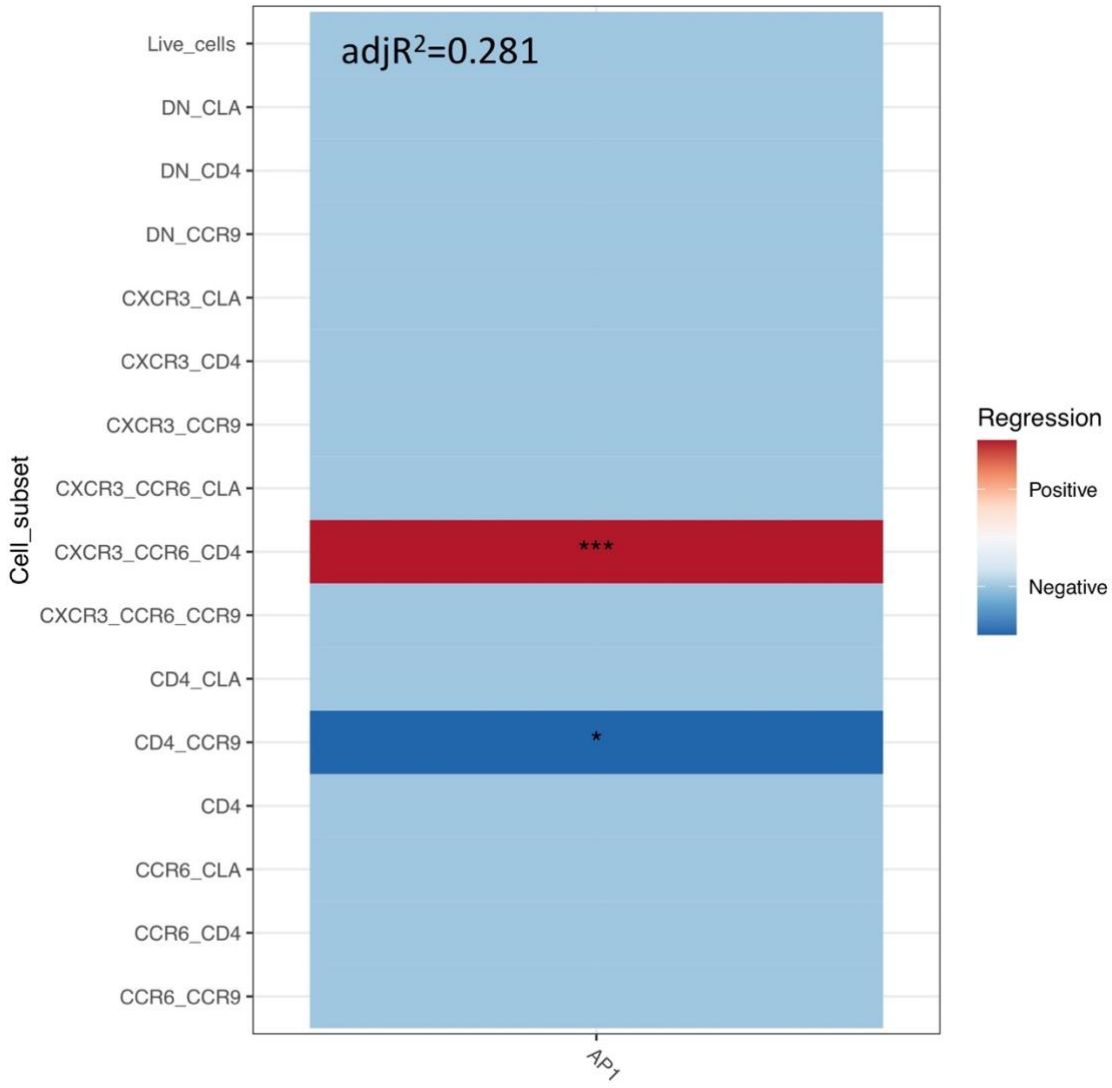


**Figure 5.2 Significant CD4 T cell predictors of AP1 status**
A regression model was fitted where Yi represents AP1 diagnosis and independent variables represent the CD4 T cell data set. The heatmap represents significant positive (red) and negative (blue) predictors indicated by the model with the lowest cross-validated error (0.337), adjR$^{2}$=0.281, *p<0.05, ***p<0.001.

Collectively, these data indicate an association between patchy AA (AP1) and increased frequency of CCR6$^{+}$ CD4 T cell populations, particularly with the double positive, CXCR3$^{+}$ CCR6$^{+}$ CD4 T cells. We hypothesised that there may also be a relationship between patchy AA and circulating type 1 or type 2 cytokines.

However, in chapter 3 it was reported that increased levels of systemic cytokines do not associate with patchy hair loss (Figures 3.5 and 3.6). There was also no association between history of atopy and changes in the proportions of CD4 T cell subsets.

A proportion of participants were receiving treatment when recruited to the study (Table 5.1). Stratification of the cohort based on different treatment types indicated that IL steroid therapy is associated with a significant increase in the frequency of skin homing CD4 T cells, DP CD4 T cells, skin homing DP CD4 T cells and skin homing $CXCR3^+$ CD4 T cells compared to HCs and the AA no treatment group (Figure 5.3). There was also a significant increase in the frequency of $CCR6^+$ CD4 T cells in the IL steroid group, compared to HCs (Figure 5.3).

To investigate the relationship between this therapy and CD4 T cells subsets further, the AP1 cohort was stratified based on IL steroid or no treatment. These analyses indicate that an increase in the frequency of $CCR6^+$ CD4 T cells is associated with participants with or without IL steroid treatment (Figure 5.4). However, the increase in the frequency of skin homing CD4 T cells, DP CD4 T cells, skin homing DP CD4 T cells, skin homing $CXCR3^+$ CD4 T and skin homing DN CD4 T cells were only associated with those receiving IL steroids. There was also a significant decrease in the frequency of DN CD4 T cells and gut homing DN CD4 T cells in AP1 IL steroid group compared to the AP1 no treatment group, however this was not observed when compared to HCs.

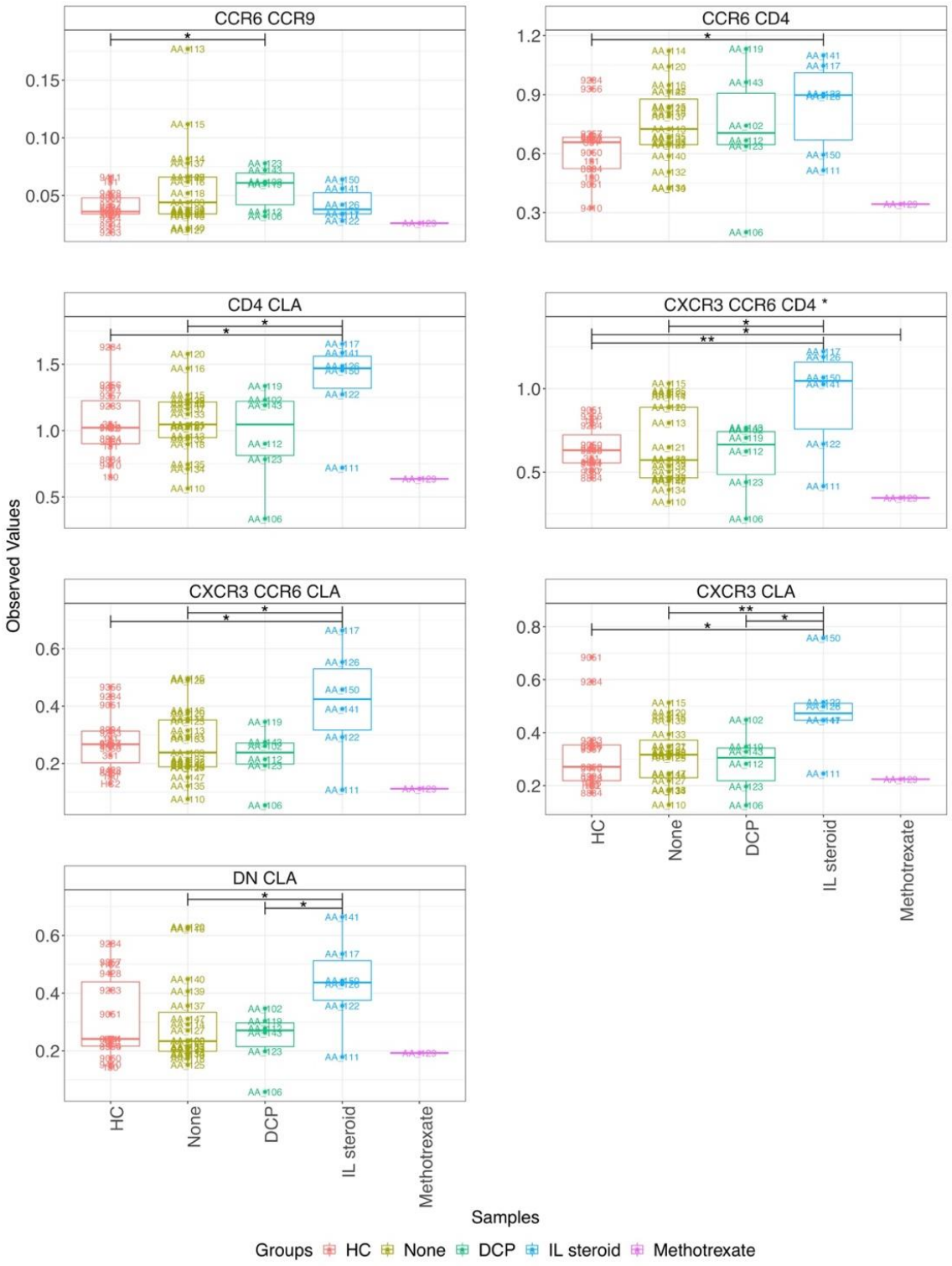


**Figure 5.3 Comparison of CD4 T cell subsets between HCs and AA cohorts stratified by treatment**

Normalised observed values of frequencies of CD4 T cell subsets in HCs (n=15) and subgroups of AA patients with no treatment or receiving diphencyprone (DCP, n=6), intralesional steroid (IL steroid, n=6) or methotrexate (n=1) treatment. DN, double negative $CXCR3^{-}$ $CCR6^{-}$ CD4 T cells. Means are plotted. Data were normalised using 'edgeR' and 'DESeq' packages, and then transformed using the hyperbolic Arcsinh method. Statistical differences between groups were determined by one-way ANOVA with Bonferroni correction, *p<0.05, **p<0.01.

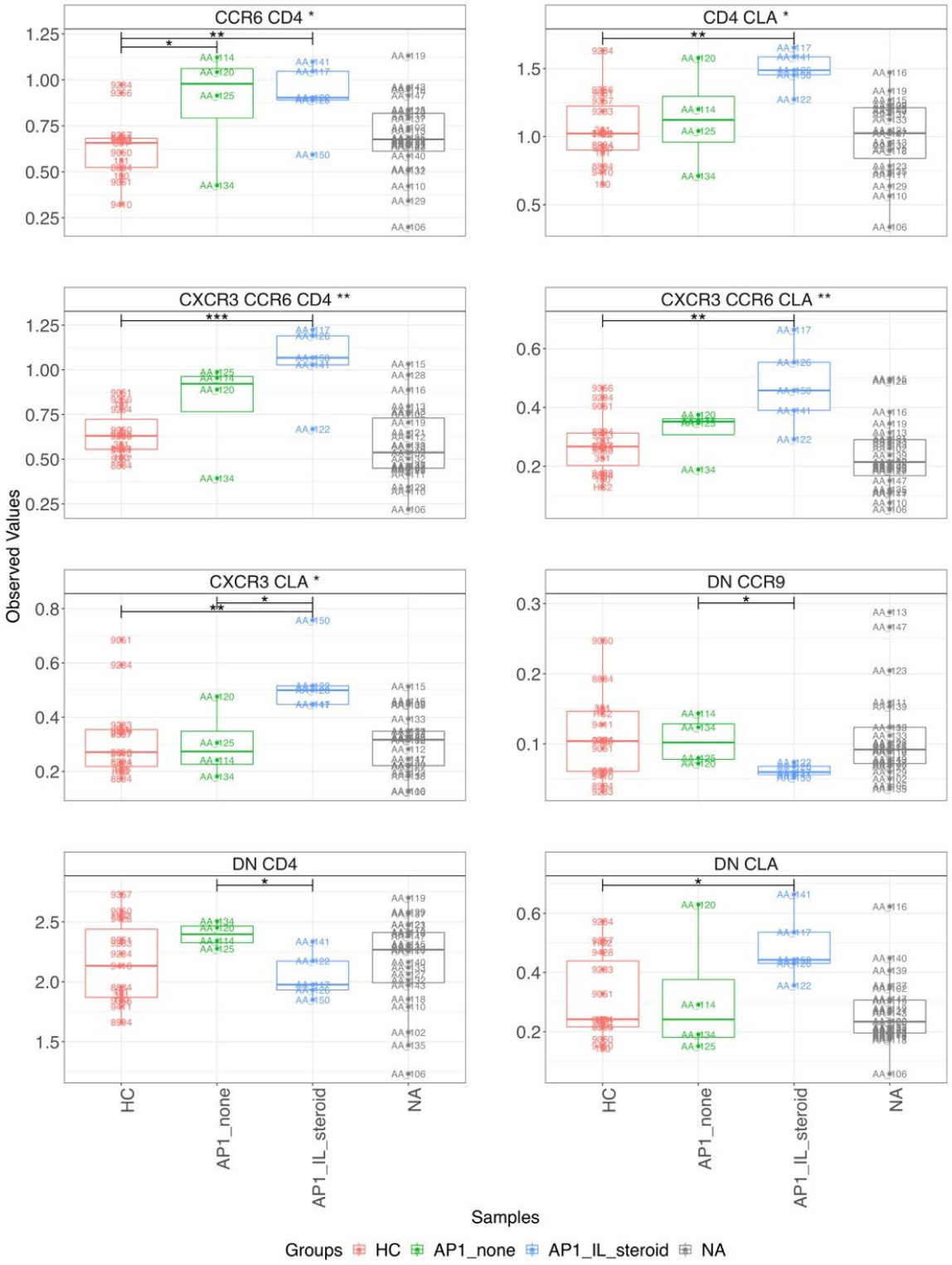


**Figure 5.4 Comparison of CD4 T cell subsets between HCs and AP1 cohorts stratified by IL steroid treatment**

Normalised observed values of frequencies of CD4 T cell subsets in HCs (n=15) and subgroups of AA patients with <50% hair loss with IL steroid treatment (AP1_IL_steroid, n=5) or without treatment (AP1_none, n=4). NA group represents remaining AA patients in the AA cohort. Means are plotted. DN, double negative $CXCR3^-$ $CCR6^-$ CD4 T cells. Data were normalised using 'edgeR' and 'DESeq' packages, and then transformed using the hyperbolic Arcsinh method. Statistical differences between groups were determined by one-way ANOVA with Bonferroni correction, *p<0.05, **p<0.01, **p<0.001.

## 5.5 Relationship between frequencies of circulating B cells, cytokines and clinical parameters

In chapter 4, it was reported that frequencies of total B cells, naïve B cells and transitional B cells are elevated in AA circulation compared to HCs. Stratification of AA patients based on extent of hair loss indicated a significant increase in the frequency of total B cells, naïve B cells, total mature B cells and transitional B cells in the AP1 and AT groups in comparison to HCs (Figure 5.5). Thus, in contrast to the CD4 T cell data, the increase in the proportions of B cell subsets are associated with both patchy and severe hair loss, and not specific to a subgroup of patients. Stratification also indicated that the AP1 group is specifically associated with an increase in the frequency of $CD43^{+}$ class-switched B cells, and the AT group is associated with an increase in the frequency of total non-transitional B cells in comparison to HCs (Figure 5.5).

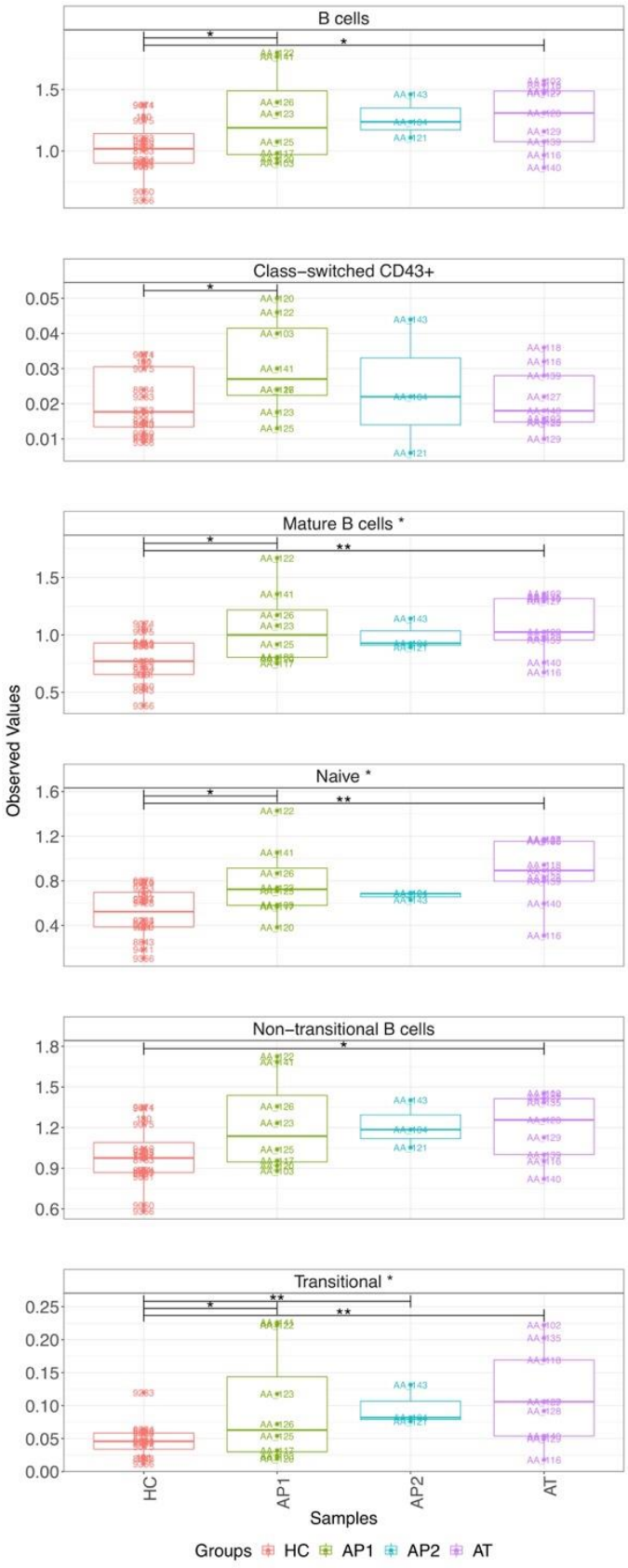


**Figure 5.5 Comparison of B cell subsets between HCs and AA cohorts stratified by extent of hair loss**

Normalised observed values of frequencies of B cell subsets in HCs (n=16) and subgroups of AA patients with <50% hair loss (AP1, n=8), >50% hair loss (AP2, n=3) and >95% total hair loss (AT, n=9). Means are plotted. Data were normalised using 'edgeR' and 'DESeq' packages, and then transformed using the hyperbolic Arcsinh method. Statistical differences between groups were determined by one-way ANOVA with Bonferroni correction, *p<0.05, **p<0.01.

The known pathogenic link between aberrant B cell responses and allergy, and the high incidence of atopy in AA cohorts, led us to hypothesise that the changes in frequencies of B cell subsets may be attributed to presence of atopy. Stratification of the cohort based on history of atopy indicated that the frequency of B cells, mature B cells, naïve B cells, non-transitional B cells and transitional B cells are significantly elevated in atopic AA patients in comparison to HCs (Figure 5.6). The frequencies of these subsets were not raised in non-atopic AA patients, but there was a significant increase in the frequency of unswitched resting B cells compared to HCs (Figure 5.6).

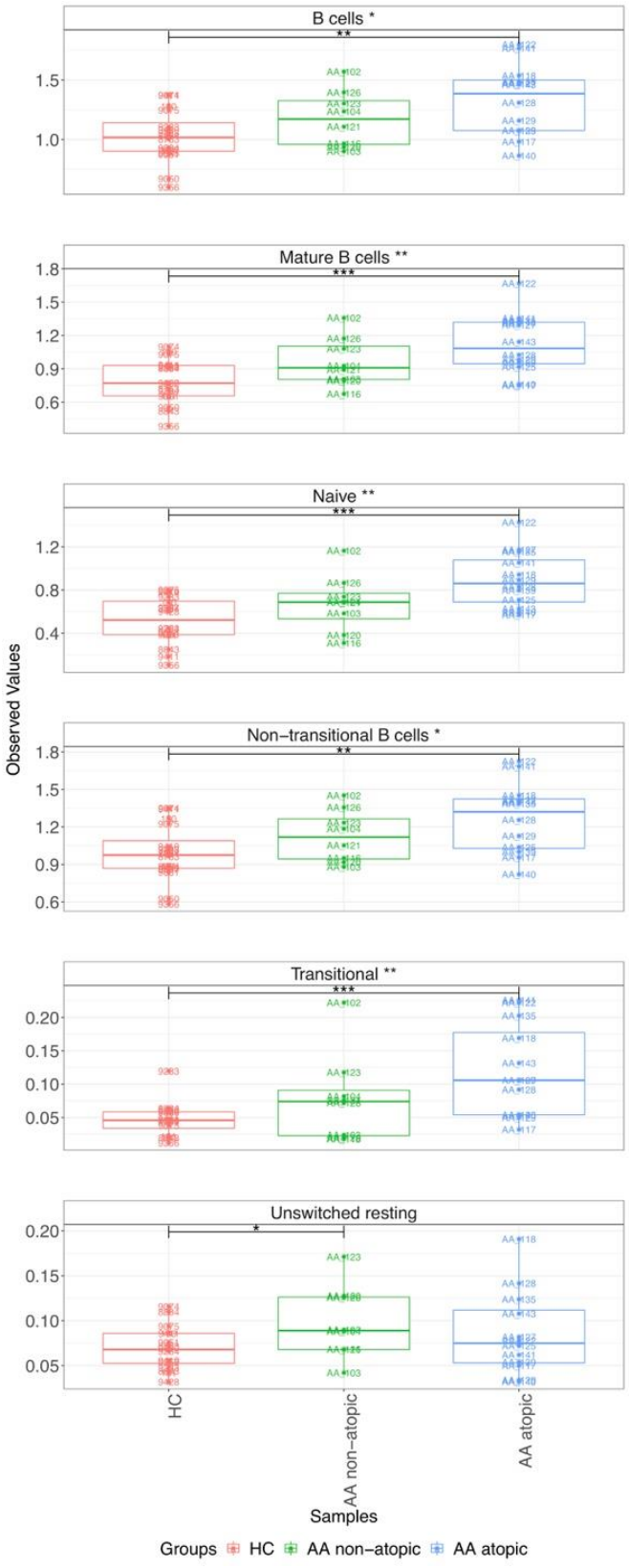


**Figure 5.6 Comparison of B cell subsets between HCs and AA cohorts stratified by history of atopy**

Normalised observed values of frequencies of B cell subsets in HCs (n=16) and subgroups of AA patients with AA only (AA non-atopic, n=8) and AA in addition to atopy (atopic, n=12). Data were normalised using 'edgeR' and 'DESeq' packages, and then transformed using the hyperbolic Arcsinh method. Means are plotted. Statistical differences between groups were determined by one-way ANOVA with Bonferroni correction, *p<0.05, **p<0.01, ***p<0.001.

We sought to test the relationship between circulating B cell frequencies and atopy further using subset regression analysis (Figure 5.7). The most accurate model implicated nine B cell populations as significant predictors of atopy status (adj$R^2$=0.513). The most significant positive predictors were naïve and IgM memory B cells, whereas the most significant negative predictors were IgM memory resting, $CD43^+$ IgM memory and class-switched plasma B cells. 10 B cell populations were implicated as significant predictors of non-atopic AA status (adj$R^2$=0.440). As expected, 6 of the independent variables selected in the non-atopic model overlapped with the atopic model, but in the opposite direction.

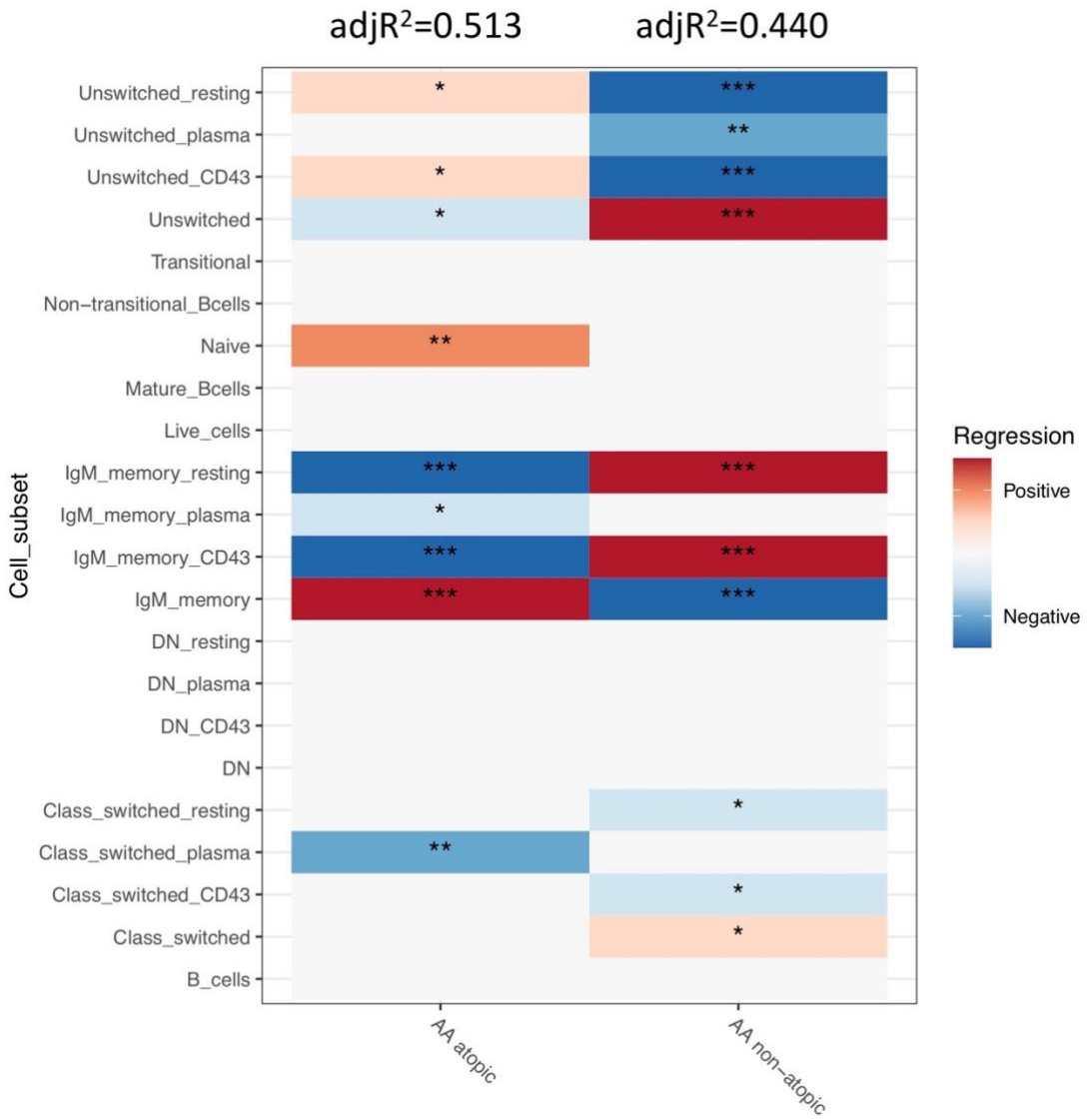


**Figure 5.7 Significant B cell predictors of atopy and non-atopy**
Regression models were fitted where Yi represents AA atopic or AA non-atopic status, and independent variables represent the B cell data set. The heatmap represents significant positive (red) and negative (blue) predictors indicated by the model with the lowest cross-validated error. AA atopic adj$R^2$ =0.513 (CVE=0.40), non-atopic adj$R^2$=0.440 (CVE=0.357) *p<0.05, **p<0.01, ***p<0.001.

The B cell data were stratified based on current treatment (Figure 5.8). The frequency of naïve, total mature and transitional B cells were elevated in groups receiving DCP or IL steroid treatment, and in patients who were not receiving treatment in comparison to HCs. Thus indicating that changes in proportions of naïve, mature and transitional B cells are independent of treatment. However, the frequencies of total B cells and non-transitional B cells were elevated in both the IL steroid and DCP groups, but not in un-treated patients. Furthermore, DCP treatment was specifically associated with increased proportions of DN plasma, switched plasma and unswitched resting populations.

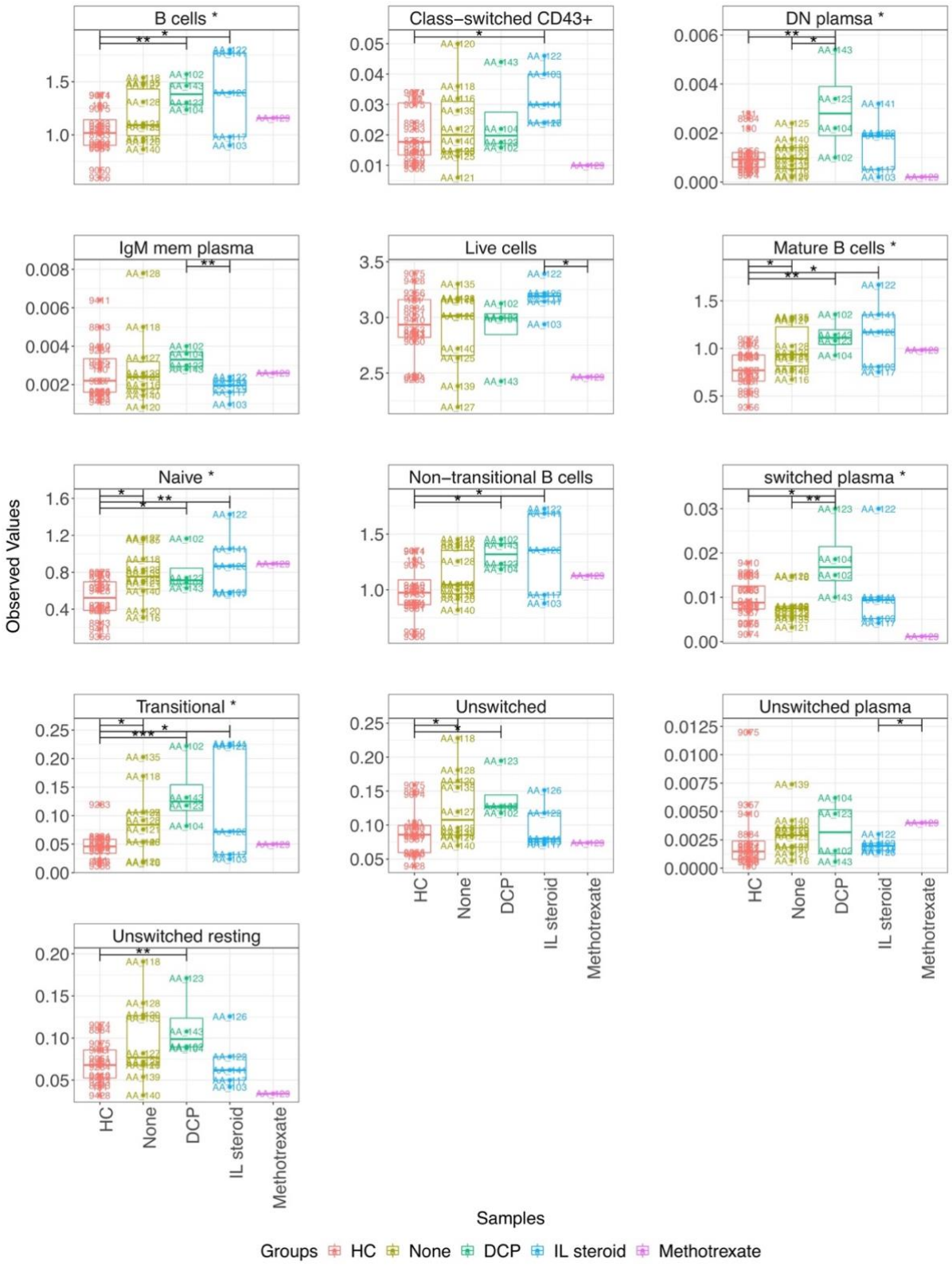


**Figure 5.8 Comparison of B cell subsets between HCs and AA cohorts stratified by treatment**
Normalised observed values of frequencies of B cell subsets in HCs (n=16) and subgroups of AA patients with no treatment (n=10) or receiving diphencyprone (DCP, n=4), intralesional steroid (IL steroid, n=5) or methotrexate (n=1) treatment. DN plasma; $CD27^{-}IgD^{-}$ plasma B cells. Means are plotted. Data were normalised using 'edgeR' and 'DESeq' packages, and then transformed using the hyperbolic Arcsinh method.Statistical differences between groups were determined by one-way ANOVA with Bonferroni correction, *p<0.05, **p<0.01, ***p<0.001

## 5.6 Relationship between frequencies of circulating CD8 T cells, cytokines and clinical parameters

It was reported in chapter 4 that the frequencies of circulating $CD8^+$ T cells are not changed in this AA cohort compared to HCs. This was somewhat surprising given the important role of CD8 T cells in driving AA pathogenesis. It was hypothesised that changes in the frequencies of CD8 T cell populations may exist within specific subsets of patients. Stratification of patients based on extent of hair loss revealed that the frequency of skin homing CD8 T cells and skin homing effector memory (TEM) CD8 T cells are significantly reduced in the AT group in comparison to those with patchy hair loss (AP1). However, this decrease was not apparent when compared with HCs (Figure 5.9). These analyses also indicated that the frequency of gut-homing central memory (TCM) CD8 T cells are reduced in the AP1 group when compared to HCs.

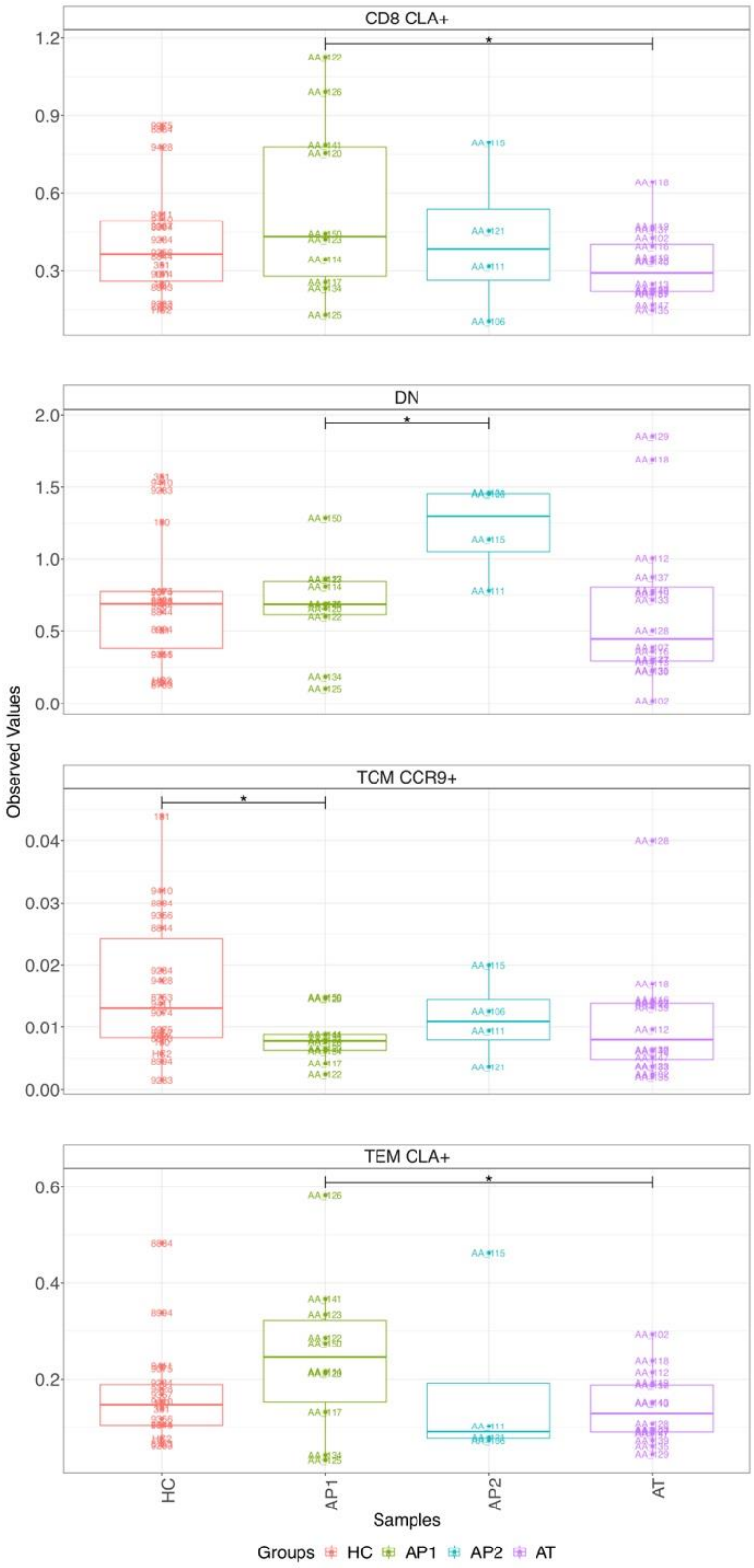


**Figure 5.9 Comparison of CD8 T cell subsets between HCs and AA cohorts stratified by extent of hair loss**

Normalised observed values of frequencies of CD8 T cell subsets in HCs (n=18) and subgroups of AA patients with <50% hair loss (AP1, n=10), >50% hair loss (AP2, n=4) and >95% total hair loss (AT, n=16). Means are plotted. DN, double negative $CCR7^{-}CD45RO^{-}$ CD8 T cells; TCM, central memory; TEM, effector memory. Data were normalised using 'edgeR' and 'DESeq' packages, and then transformed using the hyperbolic Arcsinh method. Statistical differences between groups were determined by one-way ANOVA with Bonferroni correction, *p<0.05, **p<0.01.

It was hypothesised that a decrease in the frequency of circulating CD8 T cells in individuals with severe AA is caused by increased egress of cells from blood due to greater surface area of skin involvement. To test this hypothesis, we used subset regression to identify CD8 T cell subsets that are associated with individuals who have lost all body hair. This group of patients had varied SALT scores but had no hair on their face and body. The strongest model implicated total CD8 T cells and gut-homing CD8 TEM cells as negative predictors of total body involvement status. However, this model had a low adjusted correlation coefficient and only described approximately 18% of the variance (data not shown, adj$R^2$=0.182). Importantly, subset regression indicated that changes in these CD8 T cell populations were not associated with having partial or no body hair loss (data not shown).

It was hypothesised that circulating cytokines may also be associated with total body hair loss. However, subset regression did not identify significant cytokine predictors of body involvement.

## 5.7 Discussion

The data described in chapters 3 and 4 indicated that AA is associated with a complex peripheral cytokine and cellular signature, characterised by elevated concentrations of type 17 and type 2 cytokines, and changes in the frequencies of circulating CD4 T cells and B cell subsets. In this chapter, these data are described in more depth by assessing how changes in the circulating immunophenotype relate to different subgroups of AA patients.

Patient stratification is now used in various fields of medicine to improve clinical care by using molecular biomarkers to predict treatment response, and for identifying patients who are likely to relapse or continue in a state of remission. In the context of autoimmune disease research, stratification is useful for dissecting pathological mechanisms that drive specific disease pathotypes, and often facilitates better translation of findings from basic research to a clinical setting (Delhalle et al., 2018). AA can be stratified based on extent of hair loss into patchy (AP) and severe disease (AT), including alopecia totalis and alopecia universalis. At a cellular level, the current understanding indicates that all forms of acute AA are associated with peribulbar infiltration of lymphocytes (Alkhalifah

et al., 2010). However, there is still a limited understanding of immune mechanisms that define differences between patchy and severe disease states. This has limited development of therapies, especially for refractory AT and AU.

In recent years, a number of studies have begun to characterise differences between AA subgroups. At a histopathological level, chronic and acute AA may be defined by the number of follicles, and the ratio of terminal-vellus hairs (Whiting, 2003). Acute AA is associated with dense follicular infiltration of lymphocytes surrounding anagen hair bulbs. Whereas, in chronic AA, the terminal-vellus ratio is reduced to approximately 1:1 from the normal 7:1, and follicular infiltration is reduced (Whiting, 2003). Microarray analysis of AP and AT biopsies indicates that AT/AU is associated with greater differential expression in immune-related genes when compared to AP disease, and that specific immune gene sets are down regulated in patients of more than 5 years disease duration compared to those of shorter disease duration (Jabbari et al., 2016).

To expand on current knowledge, we sought to investigate how circulating immune cells and cytokines associate with different AA disease states. We first stratified our AA cohort based on extent of hair loss to determine if the changes in frequencies of immune cell subsets are associated with AP or AT. In the previous chapter, it was reported that the frequency of $CCR6^+$ CD4 T cells are increased in AA patients compared to HCs. Here, we report that the increased proportion of $CCR6^+$ CD4 T cells is specifically associated with the AP1 group (<50% hair loss). Additionally, AP1 is associated with an increase in the frequency of total CD4 T cells, DP ($CCR6^+$ $CXCR3^+$) CD4 T cells and skin homing DP ($CLA^+$ $CCR6^+$ $CXCR3^+$) CD4 T cells in comparison to HCs. Furthermore, subset regression indicated that the DP CD4 T cell population is significantly and positively associated with AP1 status.

A published study reported that the frequency of $IFN\gamma^+$ $CLA^-$ CD4 T cells are decreased and $IL\text{-}13^+$ $CLA^-$ CD4 T cells are increased in people with patchy hair loss when compared to HCs (Czarnowicki et al., 2017), but this study did not compare $CCR6^+$ or $IL\text{-}17^+$ CD4 T cell populations between different AA cohorts and HCs. Another study found that the frequency of Th17 ($IL\text{-}17^+$) cells were unchanged when comparing mild (<49% loss) with severe AA cohorts (Han et al.,

2015), but this study did not compare mild AA with HCs, thus we cannot directly compare their findings with our results. Interestingly, Han et al (2015) did identify an increase in the frequency of Th17 cells in patients described as having 'active' disease with a positive hair pull test (Han et al., 2015), and in those with a disease duration of <6 months compared to >6 months. The hair pull test involves applying force to 10-15 hairs at the margin of a patch, and a positive result is indicated when more than 10% of hairs fall out. In our study, only 9% (3/32) of the AA cohort had a disease duration of 1 year or less, thus it wasn't possible to ascertain if we also see a significant association between frequencies of $CCR6^+$ CD4 T cells and a short disease duration.

To our knowledge, DP CD4 T cells have not been studied in the context of AA. In this chapter, we report that increased proportions of DP CD4 T cells and skin homing DP CD4 T cells are specifically associated with AP1 status. CD4 T cells that co-express CXCR3 and CCR6 are associated with dual Th1 and Th17 effector function, and therefore have the capacity to produce both IFN$\gamma$ and IL-17A (Acosta-Rodriguez et al., 2007). Usually, the direction of Th17/1 cell plasticity involves Th17 cells acquiring a Th1 effector function (Nistala et al., 2010; Hirota et al., 2011). Effector T cell plasticity is considered to be important in tissue specific immune responses against pathogens (DuPage & Bluestone, 2016; Geginat et al., 2016), but is also implicated in the pathogenesis of a variety of chronic inflammatory diseases (Harbour et al., 2015; Bending et al., 2009).

In CD patients, a specific population of intestinal memory $CXCR3^+$ $CCR6^+$ CD4 T cells have been identified, that are hyper-responsive to IL-23 and are resistant to glucocorticoids, highlighting the pathogenic potential of this subset (Ramesh et al., 2014). As discussed previously, blocking IL-17A does not seem to be useful in driving hair regrowth in AA patients (Guttman-Yassky et al., 2018). Therefore, the DP subset is perhaps not interesting from a IL-17A production perspective. However, Th1/17 cells could be an important contributor of IFN$\gamma$, and therefore contribute to potentiating the CD8 T cell responses against HFs. Further analysis of this DP CD4 T cell subset is required to confirm they have the capacity to produce IL-17A and IFN$\gamma$, and that the cells express Th1 and Th17-associated transcription factors (Wang et al., 2014).

Stratification of cytokine data did not reveal associations between type 17 cytokines and AP1 diagnosis. However, in chapter 3 it was reported that the levels of IL-17A, IL-17F, IL-21, IL-31 and IL-17E are elevated in the AT group, compared to HCs. Collectively, these findings indicate that there is not an obvious correlation between increased proportions of circulating $CCR6^+$ CD4 T cell subsets and higher expression of inflammatory cytokines.

The robust association between the AP1 group and increased frequencies of $CCR6^+$ and DP CD4 T cell populations may indicate distinct differences between patchy and severe disease. Alternatively, it is plausible that this phenotype is not related to extent of hair loss but is indicative of active disease, as indicated by Han et al (2015). In this case, the association between AP1 disease and $CCR6^+$ and DP CD4 T cell populations may be apparent because patients with <50% hair loss are more likely to experience relapsing-remitting AA; these AP1 patients may more closely mirror acute AA in comparison to those with severe disease. It may be the case that $CCR6^+$ CD4 T cell populations are crucial for initiating or maintaining CD8 T cell activity against HFs, but are less important once all scalp hair has been lost.

To investigate the relationship between AA and CD4 T cells further, the AA cohort was stratified based on current treatment. IL steroid treatment was associated with enriched frequencies of skin homing CD4 T cells, DP CD4 T cells, skin homing DP CD4 T cells and skin homing $CXCR3^+$ CD4 T cells, in comparison to HCs. However, the increase in proportion of $CCR6^+$ CD4 T cells was observed in both untreated and IL steroid treated AP1 patients. These observations indicate that the increase in $CCR6^+$ CD4 T cells in the AP1 group is independent of treatment effect. Whilst these data are interesting, they must be regarded as preliminary because the groups involve low numbers of participants (AP1 IL steroid, n= 5, AP1 no treatment, n=4). Nevertheless, initiation of oral glucocorticoid treatment to patients with inflammatory conditions is known to be associated with causing an increase in circulating PBMCs (Shoenfeld et al., 1981). However, this is reported to be specifically associated with neutrophilia (Liles et al., 1995). We did not measure the proportions of circulating neutrophils, however we did not observe changes in the proportions of other live

leukocytes between treatment groups, thus indicating that IL steroid treatment may be associated with specific changes to CD4 T cell proportions.

Triamcinolone intradermal injections are intended to have local effects, however side effects that are associated with systemic administration of glucocorticoids are also reported to be associated with IL injections, suggesting systemic absorption. There is extensive literature indicating that glucocorticoids directly cause apoptosis of immune cells, including T cells (Herold et al., 2005), but more recently it has been shown that Th17 cells can be resistant to glucocorticoids via inhibition of apoptosis by downstream actions of the transcription factors, BCL-2 and RORγt (Banuelos et al., 2016; Ramesh et al., 2014). Furthermore, glucocorticoids have been shown to drive expression of a Th17 cell attractant, CCL20, in the lung epithelium (Zijlstra et al., 2014). Whilst we are unable to confirm that IL steroids used for AA treatment are systemically absorbed, it can be hypothesised that administration of corticosteroids, albeit useful in promoting hair regrowth for some patients, can cause $CCR6^+$ CD4 T cell expansion, particularly the DP compartment. It may also be the case that the observed altered CD4 T cell phenotype is independent of the steroids, and can be attributed to effects caused by induction of hair regrowth or relapsing disease occurring in between treatment administration. Longitudinal analysis following relapsing-remitting AA will be required to indicate an association between CD4 T cell proportions and different disease states.

We next assessed the relationship between changes in B cell proportions and extent of hair loss. In contrast to the CD4 T cell findings, the increase in the proportions of B cells, naive B cells, total mature B cells and transitional B cells were associated with both patchy and severe AA. The increase in proportions of these B cell subsets was also observed in the AP2 group compared to HCs, however this result is not reliable as this group is underpowered (n=3). These data suggest that the majority of changes in the frequencies of B cell subsets are not related to extent of hair loss. However, we did observe that non-transitional B cells were specifically enriched in the AT group compared to HCs, whereas $CD43^+$ class-switched cells were elevated in the AP1 group compared to HCs.

It was hypothesised that changes in proportions of B cells may be attributed to presence of atopic individuals within the AA cohort. ANOVA analysis indicated that elevated proportions of B cells, non-transitional B cells, naïve B cells, total mature B cells and transitional B cells are specifically associated with AA individuals who are atopic in comparison to HCs. Subset regression confirmed this strong relationship, where the model selected 9 B cell subsets as significant predictors of atopic status. The strongest positive predictors of atopy AA were the naive and IgM memory populations. It may be hypothesised that the increased proportion of naïve B cells associated with atopic AA is directly attributed to increased egress of transitional B cells, which may indicate an increased abundance of autoreactive B cells, as discussed in chapter 3. The relationship observed with the increased frequency of the IgM memory population may indicate that atopic AA is associated with elevated T cell-independent B cell responses (Weller, 2004).

Patients with allergic rhinitis also have raised frequencies of B cells in peripheral blood, in addition to $CD27^+$ memory B cells (Luo et al., 2018). Furthermore, atopic dermatitis patients have increased proportions of circulating $CD27^+$ memory subsets (Czarnowicki et al., 2016). Our ANOVA analysis did not identify changes in proportions of $CD27^+$ B cell populations, however regression modelling indicated that $CD27^+$ (both unswitched and switched) subsets are indeed predictors of atopy. Further investigation is required to elucidate the true functional relationship between specific B cell populations and atopic AA.

In chapter 3, stratification of the multiplex cytokine data didn't identify any associations between specific cytokines and atopic AA. Instead, the levels of IL-31, IL-33, IL-17E, IL-17A and IL-23 were significantly increased in both AA atopic and AA non-atopic individuals, compared to HCs. Thus, similar to the CD4 T cell analysis, the data indicate that changes in proportions of circulating B cell subsets and cytokines appear to be independent of each other.

We are unable to conclude whether the changes in B cell phenotype are solely related to atopy, or if they are indicative of a specific population of AA patients. To assess this question, comparison of the AA non-atopic and AA atopic data sets with an atopic population is required. However, it is important to highlight that

the incidence of atopy in the general population is approximately 20% (Kay et al., 1994; Thomsen, 2015), thus our HC cohort will likely include a proportion of atopic individuals. It is plausible that the atopic AA cohort may represent a distinct pathotype of AA, and better characterisation of this cohort may identify alternative therapeutic options for atopic-AA patients. Interestingly, there are a number of studies suggesting that presence of atopy promotes and potentially exacerbates AA (Zhang & McElwee, 2020). Mutations in filaggrin (FLG), an epidermal protein important for maintaining effective barrier function in the skin, are strong risk factors for atopic disease, and FLG mutations are also associated with severe forms of AA (Betz et al., 2007). Furthermore, elevated serum IgE is associated with non-atopic cohorts of AA patients (Bakry et al., 2014; Attia et al., 2010). Taken together, our data and findings from published studies highlight the importance of understanding how atopic mechanisms may contribute to AA pathology in a large proportion of AA patients.

We also assessed the relationship between treatment and the circulating B cell phenotype. The increase in proportions of naïve, mature and transitional B cells were associated with patients receiving DCP, IL steroid or no treatment, indicating that changes in these populations are not associated with a specific therapy. Interestingly, increased proportions of DN plasma, switched plasma and unswitched resting populations were specifically associated with DCP treatment. No associations were observed between changes in the frequencies of CD4 T cell populations and DCP treatment. DCP drives a delayed-type hypersensitivity (DTH) reaction, and is arguably one of the most useful treatments for AA (Spano & Donovan, 2015). The mechanisms responsible for efficacy of DCP treatment are not known, but the treatment is reported to increase IL-10 production (Gulati et al., 2014) and is also described to promote ‘antigenic competition’ which may cause non-specific suppression of AA-associated pathogenic T cells (Singh & Lavanya, 2010). It is particularly interesting that the frequency of switched plasma B cells is elevated in association with DCP treatment. It may be hypothesised that DCP treatment is driving activation and class-switching of B cells. However, based on our data, we cannot conclude whether DCP is specifically driving B cell activation or whether this response may be important for promoting hair regrowth in AA. Further investigation is required in larger AA cohorts to confirm if this B cell phenotype is specifically related to DCP

treatment. Taken together our data indicate that, in contrast to the CD4 T cell data, the changes in proportions of total mature B cells, naïve B cells and transitional B cell subsets appear to be independent of treatment. Whereas, changes in proportions of switched plasma cells are associated with DCP therapy.

Finally, the CD8 T cell data were stratified based on extent of hair loss. The proportions of gut homing TCM CD8 T cells were significantly decreased in the AP1 group in comparison to HCs. It can be hypothesised that this observation indicates increased egress of TCM CD8 T cells from peripheral blood to the intestine. These data are in agreement with the negative association observed between gut homing CD4 T cells and AP1 status by subset regression. Together, these data provide preliminary evidence for potential intestinal involvement in individuals with patchy hair loss.

Interestingly, the proportions of skin homing CD8 T cells and TEM CD8 T cells are significantly reduced in the AT group in comparison to the AP1 cohort. It was hypothesised that increased body involvement, i.e. AA that involves the scalp and the body, is associated with reduction in frequencies of circulating CD8 T cells due to migration of cells from the blood to the skin. Consistent with this hypothesis, regression modelling identified a negative association between CD8 T cells and total body hair loss. However, the model was fairly weak, explaining only 18% of the variability associated with total body involvement.

Based on these data it can be concluded that, as a whole, the proportions of peripheral CD8 T cells are not altered in AA, but there is an association between decreased CD8 T cells and total body involvement. Severe AA is notoriously difficult to treat with available treatments, thus understanding how scalp hair loss develops from a localised CD8 T cell response to effect other areas of the skin will be important for development of novel therapies.

### 5.7.1 Conclusion

In this chapter, stratification of the AA cohort by extent of hair loss, current treatment and history of atopy has facilitated the identification of specific aspects of the immunophenotype that are associated with subgroups of AA patients. Less than 50% scalp involvement (AP1) is associated with increased

proportion of $CCR6^+$ CD4 T cell populations, particularly the DP subset. Whereas, circulating CD8 T cell proportions are not altered when comparing the AA cohorts with HCs, but are significantly reduced in the severe AA subgroup in comparison to the AP1 group. Due to low numbers of samples, it wasn't possible to identify specific changes associated with individuals in the AP2 group. Interestingly, the robust B cell phenotype reported to be associated with AA in chapter 4, is in fact strongly related to individuals with atopic AA. These observations build on pervious published studies that atopic AA represents a distinct form of AA.

Analysis of circulating cytokines in different subgroups of patients indicates that cytokine levels and changes to proportions of immune cell subsets appear to be independent of each other. $CCR6^+$ T cells are elevated in AP1 individuals, whereas the type 17 cytokines, IL-17A, IL-17F and IL-21 are elevated in AT individuals. Furthermore, atopy does not appear to be specifically associated with changes in the concentration of circulating cytokines. Instead, the levels of IL-17A, IL-23, IL-31, IL-33 and IL-17E are associated with both atopic and non-atopic AA patients.

The main challenge associated with identifying immune parameters associated with specific disease states is the lack of a marker for disease activity. A hair pull test can be useful in some cases but is not preferred by most patients or dermatologists because it involves pulling hair from the scalp. A molecular tool, referred to as the ALADIN score, has been developed as an indicator of activity (Jabbari et al., 2016). The score measures transcriptome signatures associated with cytotoxic T cells, IFN signalling and keratin pathways (Jabbari et al., 2016). Whilst useful for analysis of transcriptome datasets, this tool doesn't enable stratification of patients based on activity in a clinical setting. Based on our data, we suggest that to identify peripheral activity markers, longitudinal collection of blood samples may be required. This would allow analysis of immune markers between relapsing episodes, and also to assess changes associated with treatment response and relapse post-treatment.

Nevertheless, the analysis described in this chapter provides an in-depth understanding of how the changes in proportions of CD4 T cells, CD8 T cells and

B cells in peripheral blood relate to different forms of AA. However, the data described so far have not enabled identification of novel pathways critical for AA pathology. To address this, transcriptome analysis of AA scalp biopsies was conducted and is described in the following chapter.

# 6 Identification and validation of enriched transcriptomic signatures in AA skin samples

## 6.1 Introduction

In the previous chapters, the data presented described the complex immunophenotype associated with AA, and how certain features are attributed to specific sub-groups of patients. These data have highlighted novel avenues to explore in AA pathogenesis that may be useful for identifying disease activity markers, and for understanding different AA pathotypes. However, observations obtained from peripheral blood do not necessarily indicate cell types or pathways critical for driving hair loss.

As discussed previously, JAK inhibitors and cytokine-blocking interventions have proven to be useful for some AA patients in clinical studies (Xing et al., 2014; Crispin et al., 2016). However, the current therapeutic landscape for AA remains limited, especially compared to other fields of autoimmunity where immunotherapies are routinely used. To facilitate unbiased identification of key drivers of AA pathology in the affected tissue we performed bulk RNA sequencing of AA scalp biopsies. It was anticipated that this approach would identify important aspects of AA pathology that may have been previously overlooked. Thus, the aims of the experiments described in this chapter were:

1. To identify differentially expressed gene sets in AA compared to HC skin

2. To identify pathways associated with upregulated and downregulated gene sets in AA compared to HC skin

3. To examine enriched immune signatures in AA skin using CIBERSORT

4. To validate enriched signatures using immunohistochemistry

## 6.2 Participant characteristics

Punch biopsies were obtained from 7 AA patients and 3 HCs for transcriptomic analysis using bulk RNA-sequencing (Table 6.1). The AA patients included five with AT disease (>95% hair loss) and two with patchy hair loss (one <50% and the other >50% hair loss). All patient samples were obtained from an AA lesion and not the perilesional region. The age range of the AA participants was between 19 to 70 years, and the disease duration varied greatly, from 1 to 55 years. Three of the AA patients had a diagnosis of a thyroid condition, and four were atopic. No patients had a diagnosis of any major inflammatory disease including CD, UC, rheumatoid arthritis (RA), psoriatic arthritis (PsA), psoriasis (PsO), systemic lupus erythematosus (SLE) or ankylosing spondylitis (AS). At the time of donating a biopsy, two patients were undergoing treatment with diphenylcyclopropenone (DCP).

For the immunohistochemistry studies, sections from a biopsy obtained from an additional patient, AA194, were utilised. Participant AA194 had <50% hair loss, a history of atopy and was receiving IL steroid treatment at the time of recruitment. Three HCs were recruited to donate a scalp biopsy for comparison purposes.

**Table 6.1 Characteristics of AA patients and HCs**
Skin samples collected from eleven individuals were used for bulk RNA-sequencing and/or immunohistochemistry experiments. DCP; diphenylcyclopropenone, AA; alopecia areata, HC; healthy control, F; female, SALT; severity of alopecia tool.

| Participant ID | Age/Gender | SALT score | Disease duration (years) | Treatment | Comorbidities |
|---|---|---|---|---|---|
| AA112 | 64/F | 100 | 7 | DCP | Thyroid condition |
| AA 131 | 19/F | 96.6 | 12 | - | Atopic |
| AA 135 | 55/F | 100 | 30 | - | Atopic |
| AA 136 | 70/F | 32 | 55 | - | - |
| AA 143 | 32/F | 62.6 | 20 | DCP | Atopic |
| AA 151 | 52/F | 100 | 1 | - | Thyroid condition |
| AA 159 | 39/F | 100 | 10 | - | Atopic |
| AA194 | 33/F | 11.33 | 1 | IL steroid | Atopic |
| HC 3 | 27/F | - | - | - | - |
| HC 4 | 66/F | - | - | - | - |
| HC 6 | 23/F | - | - | - | - |

## 6.3 RNA sequencing quality control

The RNA integrity number (RIN) was above 7 for all AA and HC samples, confirming retrieval of high quality RNA. A representative RIN plot is shown in Figure 6.1A and RIN values for each sample are indicated in Table 6.2. The Phred scores were also high for all of the sample libraries indicating that the accuracy of the base selection during sequencing was optimal (Figure 6.1C).

Principal component analysis (PCA) indicated distinct clustering of AA and HC samples, with 20.9% and 20.61% of the variability described by PC1 and PC2, respectively (Figure 6.1B).

**Table 6.2 Quality of extracted RNA from AA and HC scalp biopsies.**
RNA integrity number (RIN) for all samples as indicated by a fragment analyser. RNA obtained from samples was high quality with RIN values between 7.7-9.7.

| Sample ID | Disease group | RIN value |
|---|---|---|
| 112 | AA | 8.9 |
| 131 | AA | 9.0 |
| 135 | AA | 7.7 |
| 136 | AP | 8.6 |
| 143 | AP | 9.3 |
| 151 | AA | 8.7 |
| 159 | AA | 9.7 |
| 3 | HC | 9.0 |
| 4 | HC | 8.9 |
| 6 | HC | 8.9 |

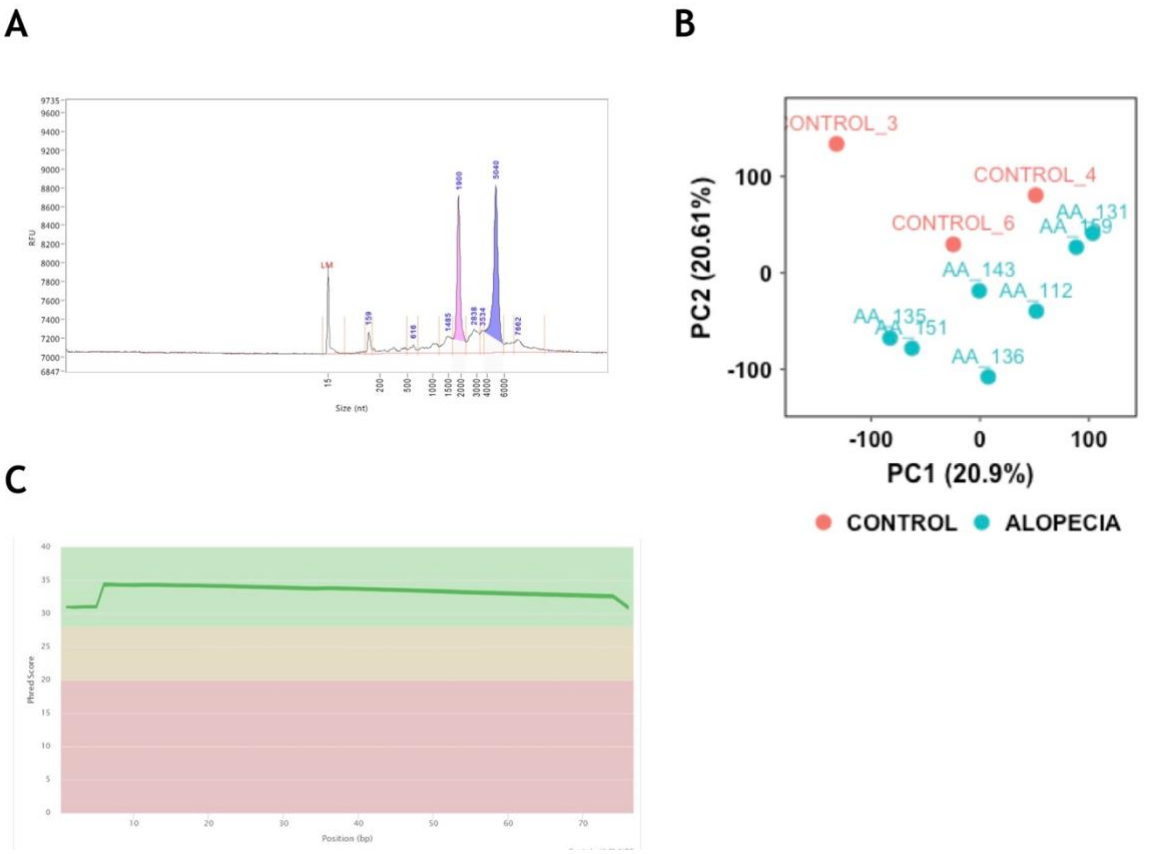


**Figure 6.1 Quality control plots for bulk RNA sequencing data set.**
(A) The quality of RNA extracted from whole skin biopsies was assessed using a fragment analyser. A representative RNA integrity (RIN) plot is shown. (B) Principal component analysis of RNA sequencing data. Gene expression data were scaled using the Z-score transformation. Each dot represents an individual sample, AA (turquoise), healthy control (pink). (C) Representative quality plot indicating performance of base assignment in sequencing experiment for all sample libraries. All samples achieved a score above 30.

## 6.4 Differential expression analysis

Differential expression (DE) analysis revealed upregulation of 162 and downregulation of 417 genes in AA compared to HC skin, as represented in the hierarchically clustered heatmap and volcano plot (log2fold >1, adjP<0.05, Figure 6.2A, B). The heatmap illustrates distinct clustering of the AA samples indicating that the AA transcriptome is consistently different from HC samples. AA samples did not cluster according to hair loss severity (Figure 6.2A). Six of the most significantly upregulated genes in AA skin are depicted (Figure 6.2C). They include *ppargc1a* (transcriptional coactivator*)*, three immune genes (*hladqa2*, *ccl13*, *lsp1), scn7a (sodium channel)* and *padi2* (post-translational modification enzyme).

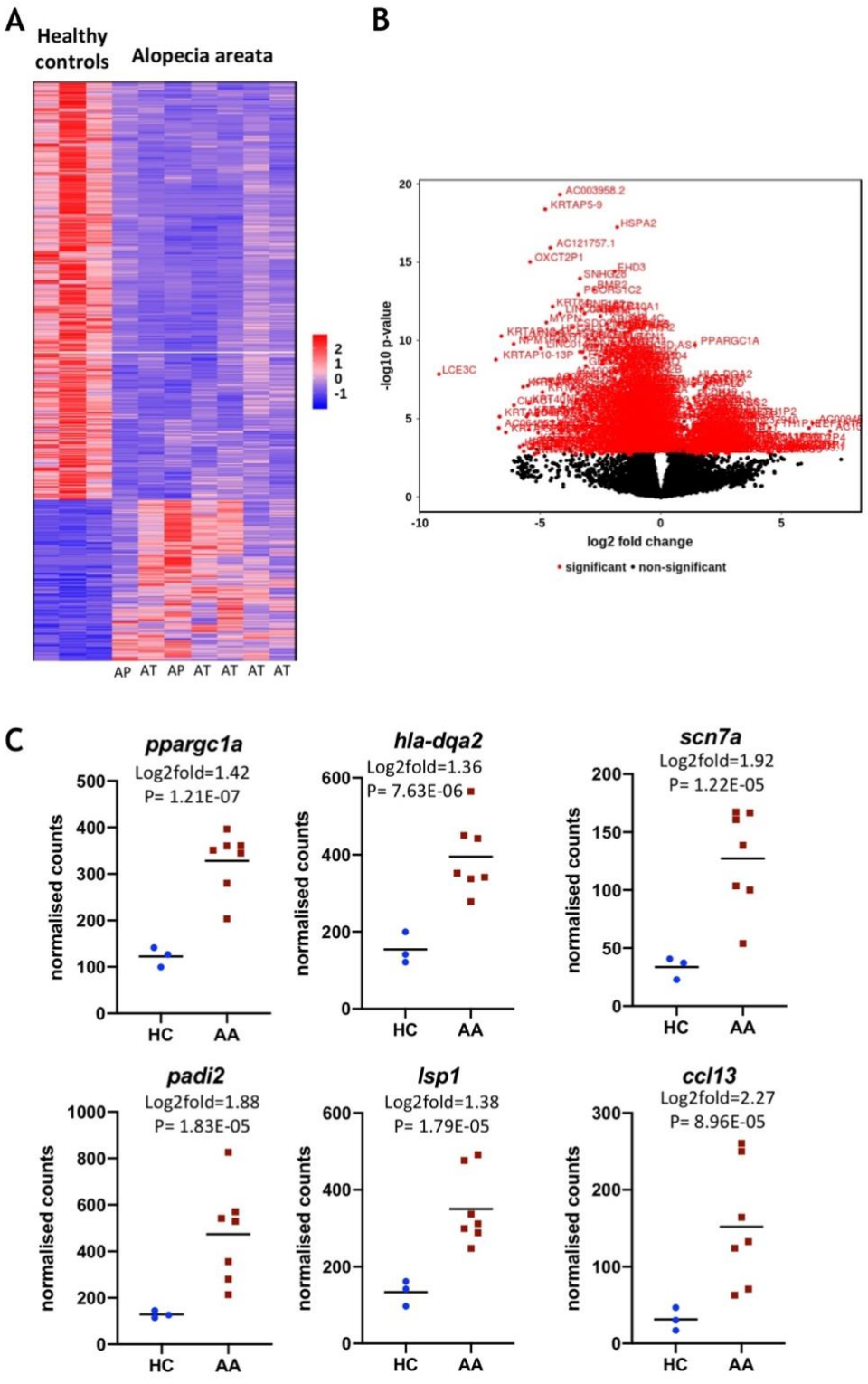


**Figure 6.2 Differential expression analysis of RNA sequencing data**
(A) Hierarchically clustered heatmap of differentially expressed genes identified between AA and HC skin samples (log2fold > 1, adjusted P value < 0.05). The x and y axis were clustered using Spearman distances and UPMGA agglomeration, and expression levels are represented as z-scores. (B) Volcano plot indicating log2fold change and p-values of significantly (red) DE genes between AA and HCs. (C) Normalised expression counts of six most upregulated genes in AA compared to HC samples as identified by DE analysis. Log2fold change and adjusted P values are shown. PPARGC1A; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, HLA-DQA2; major histocompatibility complex, class II, DQ alpha 2, SCN7A; sodium channel protein type 7 subunit alpha, PADI2; Peptidyl Arginine Deiminase 2, LSP1; lymphocyte-specific protein 1, CCL13; chemokine ligand 13.

## 6.5 Hypergeometric gene set enrichment analysis (HGSEA)

HGSEA was performed on upregulated and downregulated genes to identify gene sets significantly dysregulated in AA skin (log2fold>1, adjP<0.05). Five gene sets were significantly enriched including three pathways relating to IFNγ signalling (Figure 6.3A). The IFNγ pathways highlighted similar genes (Figure 6.3B) including chemokines (*ccl13* and *ccl18*), *ciita* (trans-activator of MHC class II), MHC class II genes (*hla-dqa2* and *hla-drb5*) and non-classical MHC class I genes (*hla-g* and *hla-h*). Gene sets involved in homeostatic processes in the skin were also enriched, including those associated with body fluid secretion and actomyosin organisation (Figure 6.3C). The body fluid secretion gene set indicated upregulation of genes expressed by secretory cells of eccrine sweat glands; cholinergic muscarinic receptor 3 (*chrm3*) and potassium calcium-activated channel (*kcnn4*).

To investigate upregulated genes in AA skin further, upstream regulator analysis (UREG) was used to determine changes in gene sets controlled by major transcriptional regulators. UREG indicated that the *irf1* pathway was highly activated in AA samples compared to HCs (Figure 6.3D, log2fold>0, adjP<0.05). 29 genes were associated with IRF1 activation, including genes that are altered in response to IFNγ and those involved in apoptotic pathways; *hla-a*, *ciita*, *cxcl10*, *casp7* and *bcl2*. However, only two genes (CIITA, HLA-A) associated with IRF1 activation were DE (log2fold>1, adjP<0.05).

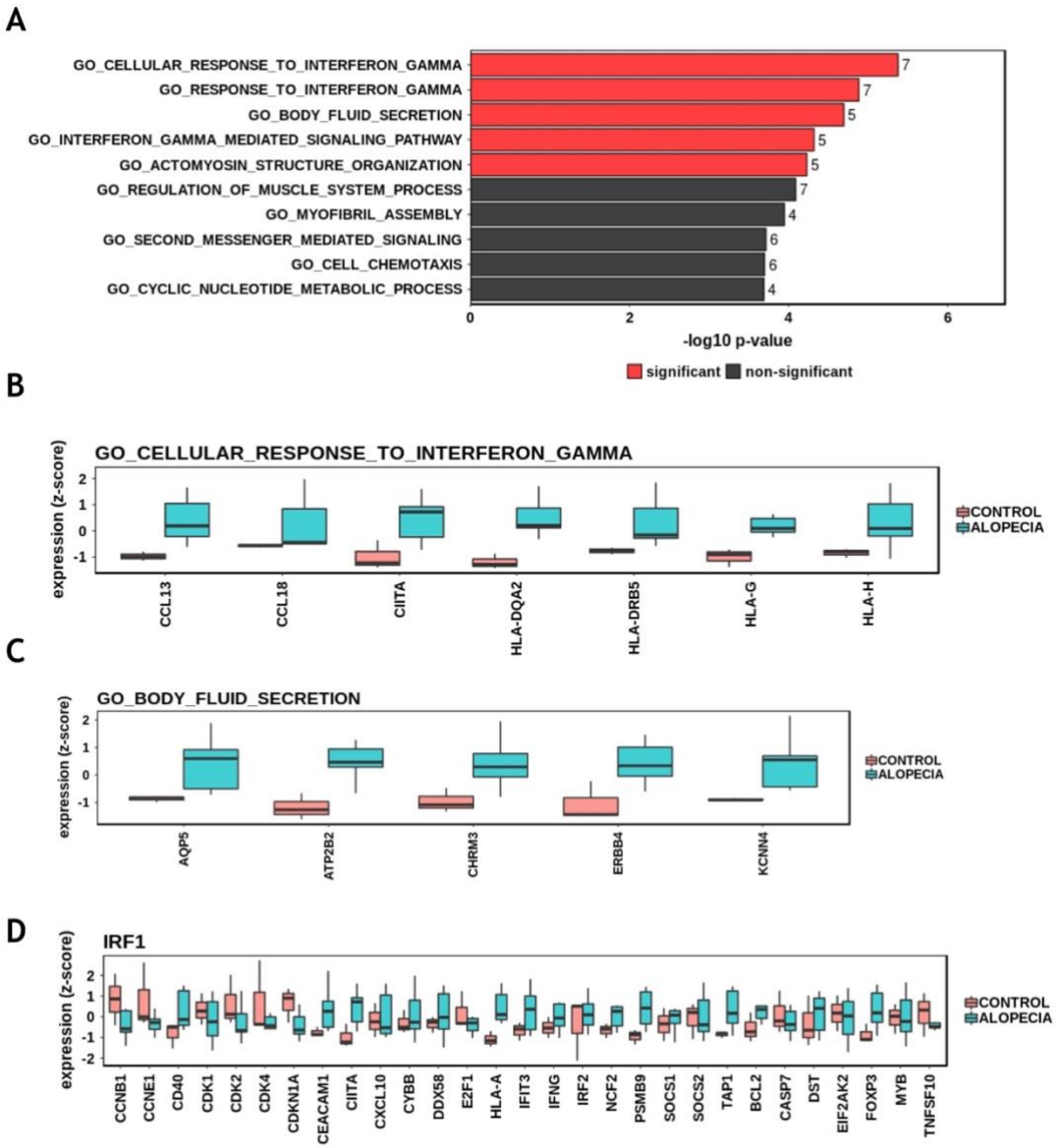


**Figure 6.3 Enrichment analysis of significantly upregulated genes in AA compared to HC skin.** Pathway analysis was performed on upregulated genes using Hypergeometric Gene Set Enrichment analysis using the GO-BP database. Significant gene sets were identified with log2fold > 0, adjusted P value < 0.05. (A) Most enriched gene sets for significantly upregulated genes. Significant pathways are highlighted in red with -log10 enrichment p value, and the number of significant DE genes implicated in each pathway are indicated. (B, C) Significantly upregulated genes in the 'cellular response to interferon gamma' and ''body fluid secretion' GO gene sets. Expression levels are represented as Z-scores. (D) Upstream regulator analysis (UREG) using the TRUSTT database indicated significant activation of IRF1. Significant gene sets were identified with log2fold > 0, adjusted P value < 0.05 and an absolute activation z-score above 2. Only CIITA and HLA-A are significantly upregulated in IRF1 pathway in AA compared to HCs (log2fold >1, adjusted P value <0.05).

We next used HGSEA analysis to identify significantly down regulated gene sets (log2fold>1, adjP<0.05). 10 gene sets were significantly enriched amongst down regulated genes including 6 overlapping pathways involved in transmembrane transport of amino acids, organic acids and anions (Figure 6.4A). These pathways highlighted downregulation of genes coding for multiple solute carriers,

including *slc16a12, slc1a6, slc5a8* and *slc7a4.* The other downregulated pathways highlighted dysregulation in genes involved in developmental and homeostatic pathways. 13 genes were significantly down regulated that are involved in epidermal development (Figure 6.4C). Of note, this gene set highlighted downregulation in *cst6* (cathepsin B) which is involved in HF morphogenesis, and several keratins (*krt16, krt31, krt84, krtap5-9)*. Other genes that play fundamental roles in hair biology were highlighted by the ontogenesis pathway (Figure 6.4B), including bone morphogenetic protein 2 (*bmp2*), lymphoid enhancer binding factor (*lef1*) and distal-less (*dlx*) genes. We also observed robust down regulation of *fgf18* in AA skin (log2fold=-3.33, P=0.011), an important factor maintaining HFSC quiescence.

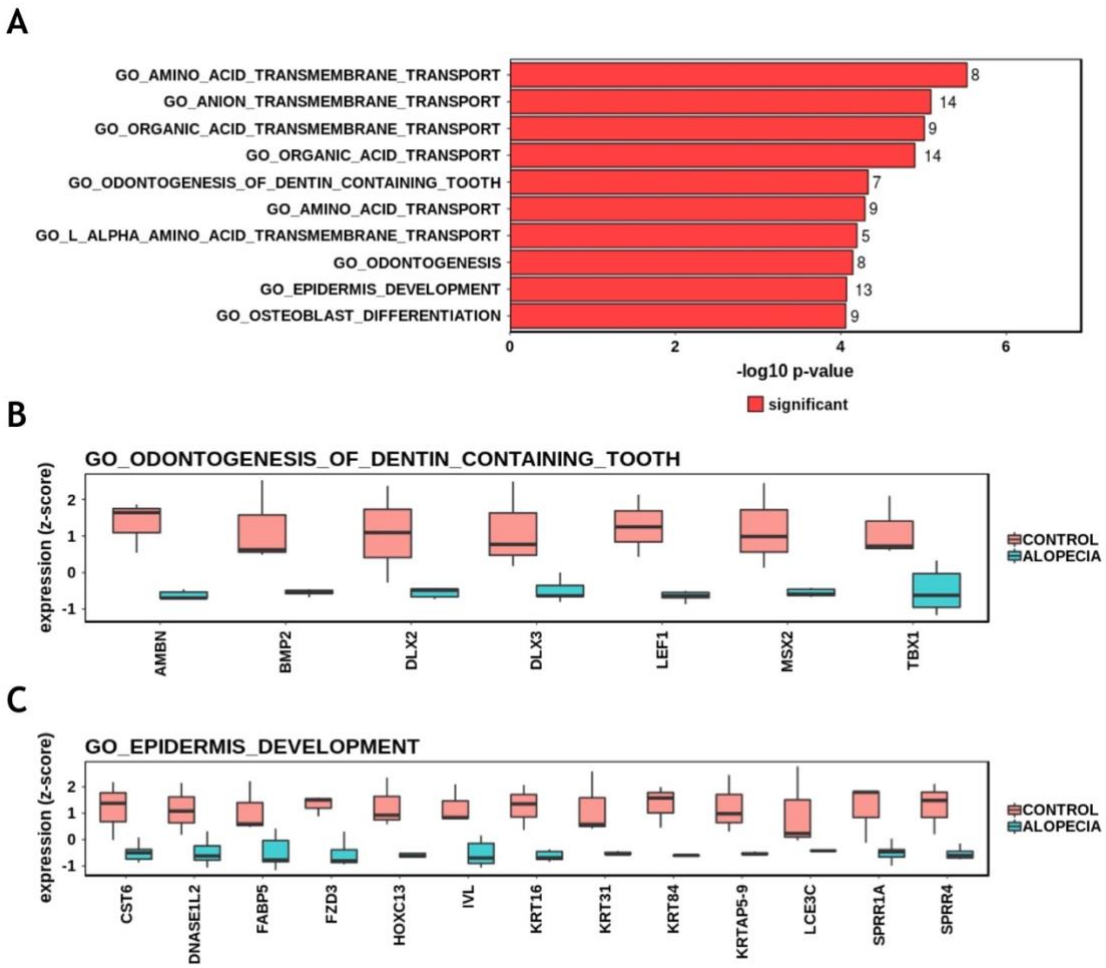


**Figure 6.4 Enrichment analysis of significantly down regulated genes in AA compared to HCs.** Pathway analysis was performed on downregulated genes using Hypergeometric Gene Set Enrichment analysis using the GO-BP database. Significant gene sets were identified with log2fold > 0, adjusted P value < 0.05. (A) Most enriched gene sets for significantly downregulated genes. Significant pathways are highlighted in red with -log10 enrichment p value, and the number of significant DE genes implicated in each pathway are indicated. (B, C) Significantly downregulated genes in the 'odontogenesis of dentine containing tooth' and 'epidermis development' GO gene sets. Expression levels are represented as Z-scores.

Collectively, the HGSEA and UREG gene set analysis indicated that skin from AA patients is enriched for genes involved in regulation of MHC class II expression and those that respond to IFN signalling to drive type 1 responses. Whereas, downregulated pathways are mainly associated with hair follicle processes and skin sub-structures associated with HFs, such as sweat glands.

Expression of genes directly related to CD8 T cell effector function including perforin, granzyme or NKG2D were not DE in AA compared to HC skin. However, DE analysis did indicate that *gnly (*granulysin, log2fold=1.68, adjP=0.035) expression was upregulated in AA compared to HC skin. The NKG2D ligand, *mica,* was also upregulated in AA skin (log2fold=0.78, adjP=0.021). The findings

described in previous chapters, relating to type 17 responses, prompted us to investigate expression of genes related to Th17 effector function. However, we observed no changes in the expression levels of *il-17a, ccr6, rorc* and *il-23r* in AA compared to HC skin.

## 6.6 Identification of an enriched macrophage signature in specific AA samples

We hypothesised that the absence of an enriched CD8 T cell signature in AA skin may be due to two possibilities. Either the CD8 T cell response was not detected by bulk transcriptomic methods, or the immune response at the time of collecting skin samples from this cohort is not dominated by CD8 T cells. Because of the robust IFN signature identified in AA skin, we postulated that classically-activated macrophages may be involved in AA pathology.

We found that macrophage and monocyte markers (*cd80, nos2, fcgr3a, il1b, cxcl8*) were not DE in AA skin compared to HCs (data not shown). However, there were changes in the expression of gene transcripts associated with a tissue repair or 'M2' macrophage phenotype (Figure 6.5A). The expression of the scavenger receptor (*cd163)* and the mannose receptor (*cd206),* were increased above a log2fold change of 0.8, however this increase was not significant (CD163, adjp=0.0895; CD206, adjp=0.206). Other transcripts for genes expressed by dermal macrophages were also elevated, including the C-type lectin receptor, *cd209* (log2fold=1.11, adjP=0.0654), factor XIIIA, *f13a1* (log2fold=0.69, adjP=0.167) and stabilin1, *stab1* (log2fold=0.84, adjP=0.135). There was no increase in expression *arg1* or *trem2* (Figure 6.5B). The expression of CD68 was also unchanged between AA and HC skin (data not shown).

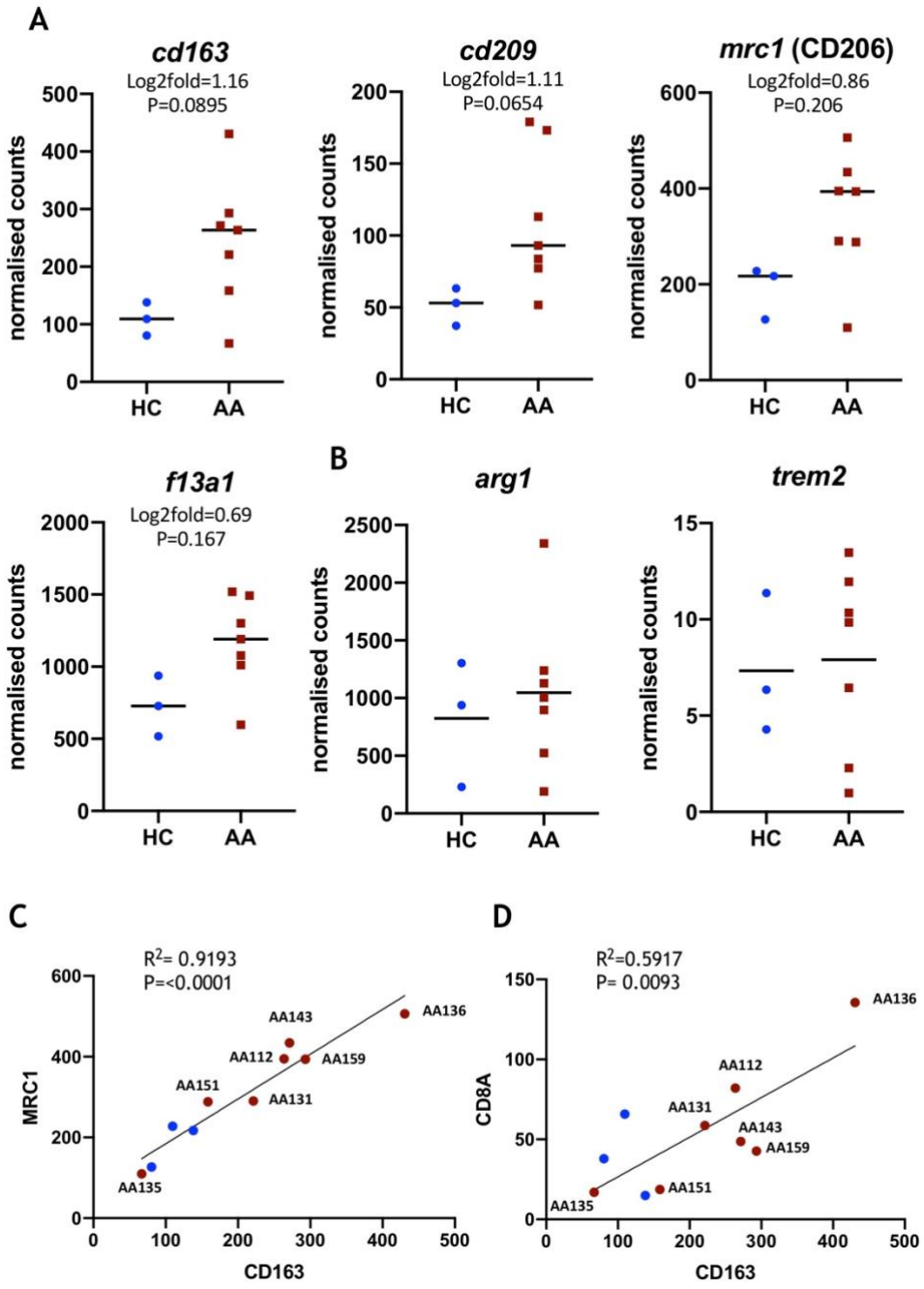


**Figure 6.5 Expression levels of macrophage associated genes in AA and HC skin**
(A, B) Normalised expression counts of macrophage associated genes from RNA-sequencing data set for AA patients and HCs. (C) Pearson correlation of MRC1 and CD163 normalised expression counts. (D) Pearson correlation of CD8A and CD163 normalised expression counts. A two-tailed test was used to measure statistical significance of Pearson correlations. Adjusted $R^2$ and P values are shown.

The expression of *cd163*, *cd209*, *mrc1* and *f13a1* transcripts are variable amongst AA samples (Figure 6.5A) and overall, these markers were not significantly increased in AA compared to HCs. However, the data indicate that this macrophage signature is prominent in some AA samples and not in others. We first confirmed that there is a strong relationship between expression of these markers. Pearson correlation indicated a strong positive relationship between

*mrc1* and *cd163* expression ($R^2$=0.9193, P=<0.0001, Figure 6.5C). There was also a positive correlation between *cd163* and *cd209 expression* ($R^2$=0.6322, P=<0.006, data not shown). Despite no change in expression of *CD8a* between AA and HC skin, Pearson correlation also indicated a positive relationship between the expression of *cd8a* and *cd163* ($R^2$=0.5917, P=0.0093, Figure 6.5D). Collectively, these data indicate that the macrophage signature correlates with CD8a expression, and that this signature appears to vary between samples.

## 6.7 Dysregulation of hair cycling genes

In section 6.5, we highlighted that expression of genes involved in HF morphogenesis are downregulated in AA compared to HC skin (*lef1* and *bmp2*). It was anticipated that the variation of immune signatures described in section 6.6 may also be reflected in expression of HF cycling genes. However, it appears that lef1 and bmp2 are consistently downregulated in skin from all AA participants (Figure 6.6A). Similarly, the expression of *ctnbb1*, another key regulator of HF growth, is consistently downregulated in AA compared to HC skin.

We also observed increased expression of *mica* (log2fold=0.78, adjP=0.021, Figure 6.6B), a gene that codes for a ligand of the NKG2D receptor that is upregulated by stressed epithelial cells. Compared to the expression levels of *lef1*, *bmp2* and *ctnnb1*, *mica* expression varied between the individual AA skin samples (Figure 6.6B). However, we did not observe any correlation between *mica* expression, and *cd163*, *mrc1* or *cd209* (data not shown).

Taken together, these data indicate that genes involved in HF cycling are globally downregulated in all AA patients, whereas the expression of immune-related genes varies between AA samples.

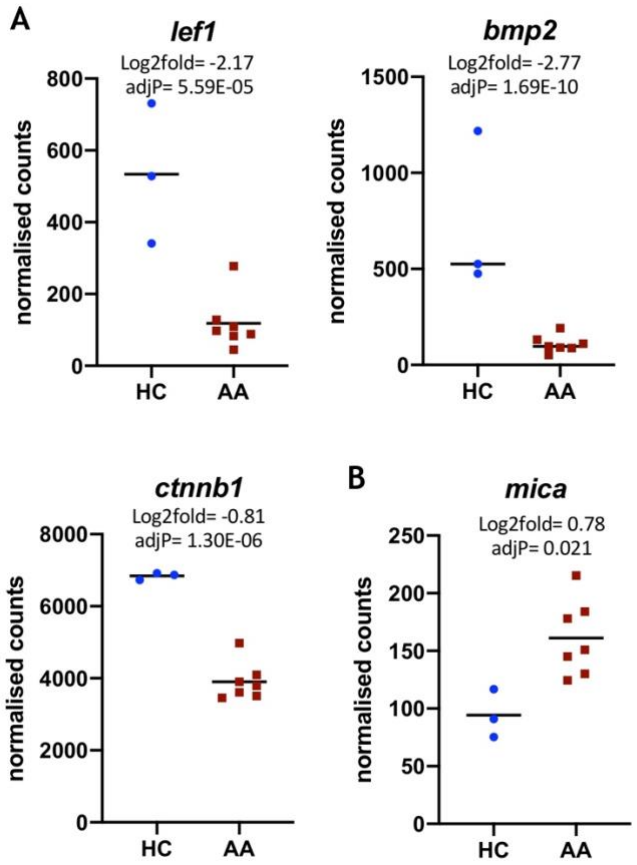


**Figure 6.6 Expression levels of hair follicle cycling genes and stress-induced ligand in AA and HC skin**
(A, B) Normalised expression counts of hair follicle cycling genes from RNA-sequencing data set for AA patients and HCs. Log2fold change and adjusted P values are indicated.

## 6.8 Abundance of immune cell types in AA and HC skin

We next sought to understand our analyses by visualising the RNA-sequencing data relative to immune cell proportions using the online CIBERSORT tool (Newman et al., 2015). CIBERSORT calculates the abundance of specific immune cells within a mixed transcriptomic sequencing data set by using a signature gene set representing 22 different immune cell subsets (Newman et al., 2015). The CIBERSORT results are depicted in Figure 6.7.

The ‘M2’ macrophage signature is not represented in any of the HC samples. However, the relative proportion of the ‘M2’ signature is increased in all AA samples, apart from AA135. The M1 gene set is also detected in three of the AA samples (AA136, AA131 and AA112), but also in HC3 and HC6, and therefore does not appear to differ between AA and HC samples. Interestingly, the AA samples associated with absent or low M2 signature (AA135 and AA159) have increased

abundance of the M0 macrophage gene set, which is also represented in all of the HC samples. The M0 gene set represents un-polarised macrophages and in comparison to the M1 and M2 gene sets is characterised by high expression of *csf1* and *cxcl5*, and low expression of *cd86*, *ccl14* and *ccl8*.

A large proportion of the RNA expression data are associated with DC and CD4 T cell signatures, but the abundance of these cell types does not appear to be different between AA and HC samples. The CD8 T cell signature is detected in HC6, AA112 and AA131 but the signal is very low.

In summary, the CIBERSORT analysis indicated that the 'M2' macrophage signature is enriched in specific AA samples and thus supports our earlier interpretation of the transcriptome data.

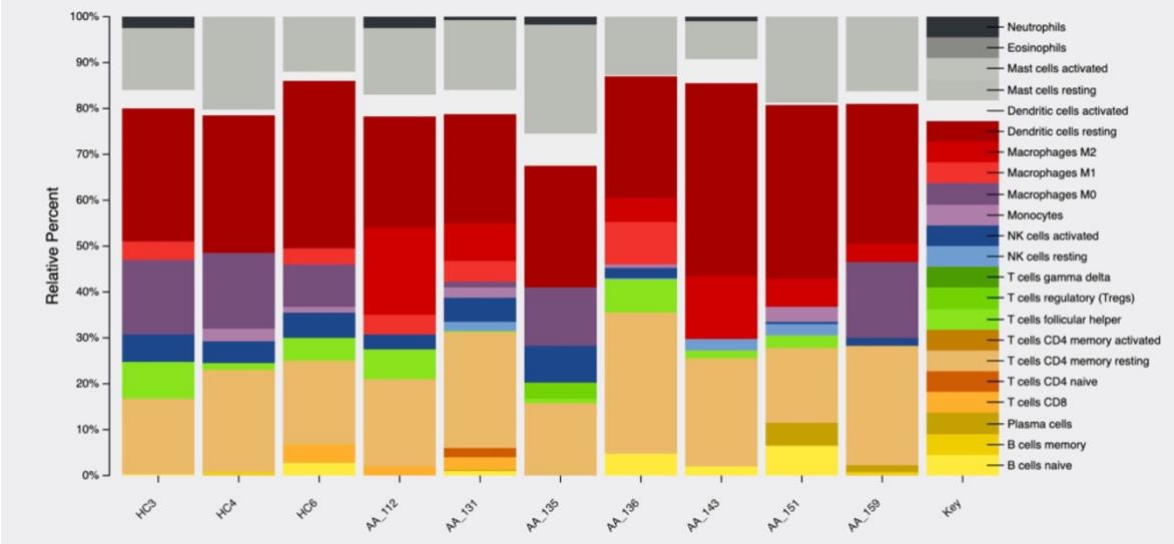


**Figure 6.7 Relative proportions of CIBERSORT immune cell signatures in AA and HC samples.** CIBERSORT results are represented in a stacked bar chart to indicate the relative abundance of immune cell signatures in individual AA samples and HCs. CIBERSORT results were generated by comparing a signature gene set representing gene expression levels for 22 different immune cells with normalised expression counts of expressed genes in AA and HC samples. The y axis indicates the relative percent (left) of each cell type (right, colour key) in individual samples.

## 6.9 Macrophages are in close proximity to HFs

The data described so far indicate modest upregulation of macrophage markers in specific AA samples, but our RNAseq data do not indicate macrophage interaction with HFs. We sought to visualise if macrophages are in close proximity to HFs to investigate the potential involvement of macrophages in AA

pathology. For these experiments, sections from skin biopsies obtained from participants AA143 and AA194 were used.

Immunohistochemical (IHC) staining using the pan-macrophage marker, CD68, revealed that $CD68^+$ cells are located within the perifollicular region of two HFs in AA skin (Figure 6.8A-D, horizontal section, red arrows). $CD68^+$ cells are also visible in close proximity to HFs in a transverse section of an AA skin biopsy (Figure 6.8E, CD68 isotype shown in Figure 6.8F). In addition to follicular regions, $CD68^+$ cells were scattered throughout the upper-dermis and in contact with dermal blood vessels (green arrows, figure 6.8D).

We next sought to visualise if macrophages are located at HFs that are associated with T cell infiltration. To do this, sections from one AA biopsy were stained with CD3 or CD68 antibodies. IHC indicated that both macrophages (Figure 6.9A,B) and T cells (Figure 6.9C,D) are within the perifollicular region of the same HF in AA skin. In comparison to macrophages, T cells tended to form clusters in the dermis beneath the epidermis, or close to HFs (green arrows, figure 6.9C). Importantly, some HFs in AA skin were not associated with immune cell infiltration, with few T cells and macrophages within the perifollicular region (Figure 6.9E,F). Thus, IHC analysis indicates that some HFs in AA skin are associated with mixed infiltration of T cells and macrophages, whereas other HFs appear not to be associated with either cell type.

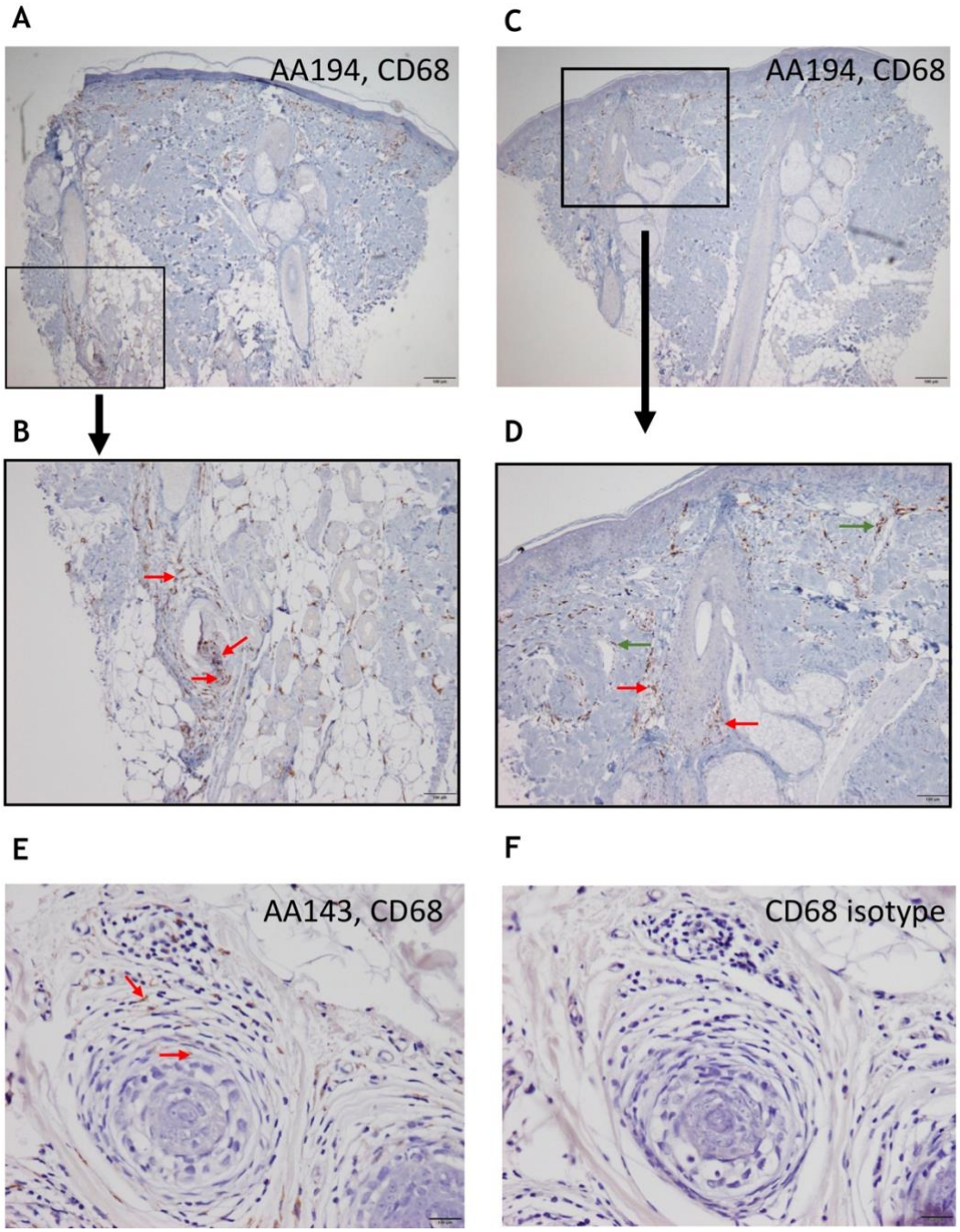


**Figure 6.8 Immunohistochemistry staining of CD68 in AA skin**
AA skin sections were stained with CD68 or isotype antibodies, counterstained with haematoxylin and visualised under the light microscope. CD68 staining of vertical sections from sample AA194 at X4 (A,C) and X10 magnification (B,D). CD68 (E) or isotype (F) staining of transverse sections from sample AA143 at X40 magnification. Red and green arrows indicate CD68 positive cells located at hair follicles and blood vessels, respectively.

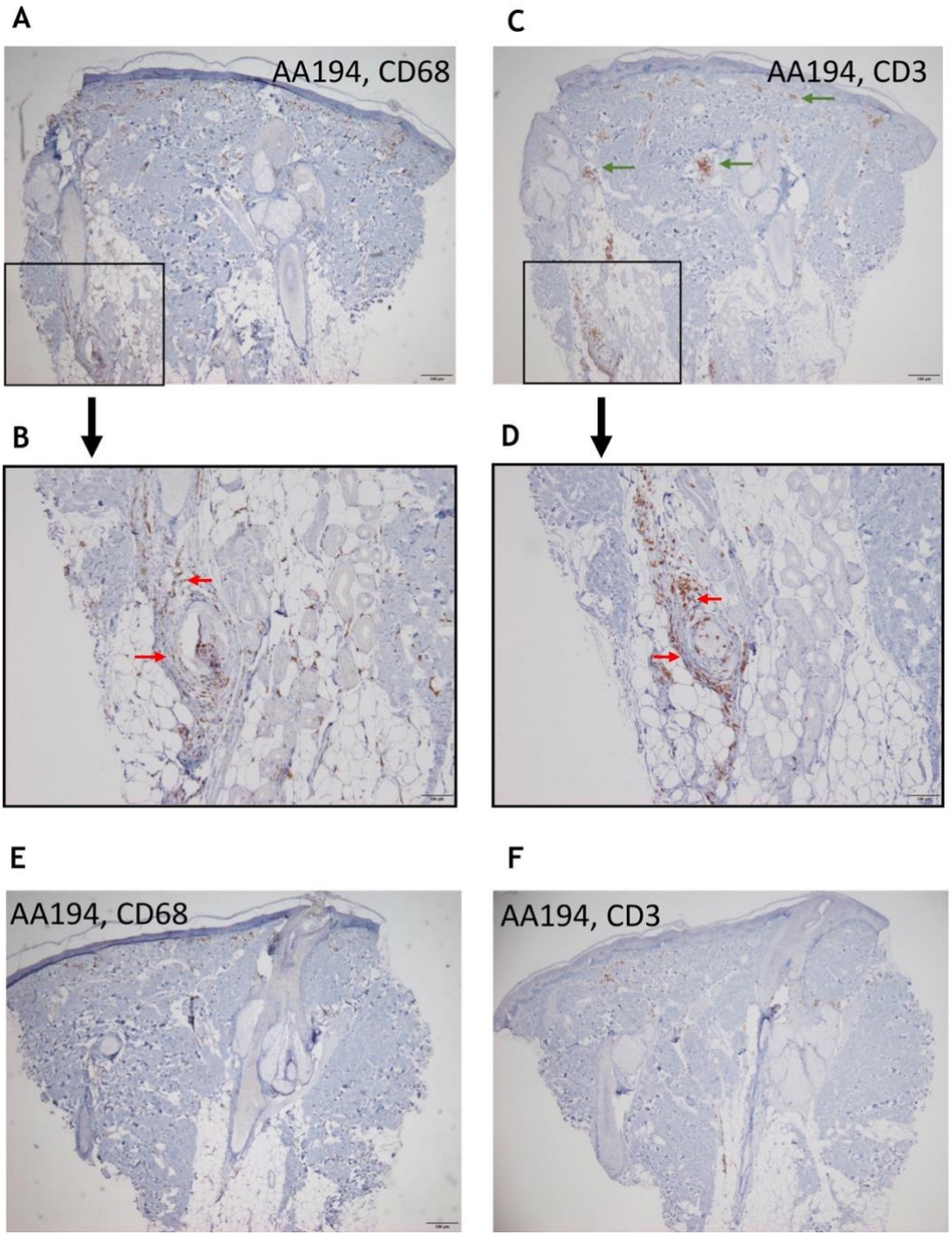


**Figure 6.9 Immunohistochemistry staining of CD68 and CD3 in AA skin**
AA skin sections were stained with CD68 or CD3 antibodies, counterstained with haematoxylin and visualised under the light microscope. CD68 staining of vertical sections from sample AA194 at X4 (A) and X10 magnification (B). CD3 staining of vertical sections from sample AA194 at X4 (C) and X10 magnification (D). CD68 (E) or CD3 (F) staining of vertical sections from alternative region of sample AA194 at X4 magnification. Red arrows indicate CD68 or CD3 positive cells located at hair follicles. Green arrows indicate clusters of CD3 positive cells.

## 6.10 Comparison of AP and AT transcriptomes

In the previous chapters, AA patients were stratified to reveal features of the immunophenotype that associate with patchy (AP) or severe (AT) disease. We sought to compare the AP and AT transcriptomes to identify differences between these disease states at a tissue level. However, the transcriptomic data set is composed of only two AP and five AT samples, therefore the findings discussed in this section are preliminary.

DE analysis revealed that 292 genes are upregulated and 434 genes are downregulated in AP samples compared to HCs (log2fold>0.5, adjP<0.05). Whereas, 147 genes are upregulated and 434 genes are downregulated in AT samples compared to HCs (log2fold>0.5, adjP<0.05). Hierarchical clustering of differentially expressed genes associated with AP compared to HCs indicated that AT samples cluster between AP and HC samples (Figure 6.10A). These data indicate that the dysregulated genes associated with AP behave similarly in AT samples, but less strongly (Figure 6.10A).

Comparison of the gene sets upregulated in AP and AT compared to HC skin indicated that 82 of the upregulated genes are shared (Figure 6.10B). Importantly, shared upregulated genes include many of the genes highlighted in the DE analysis described in sections 6.5 and 6.5, *including ccl13, ccl18, hladqa2, lsp1, padi2* and *ppargc1a*.

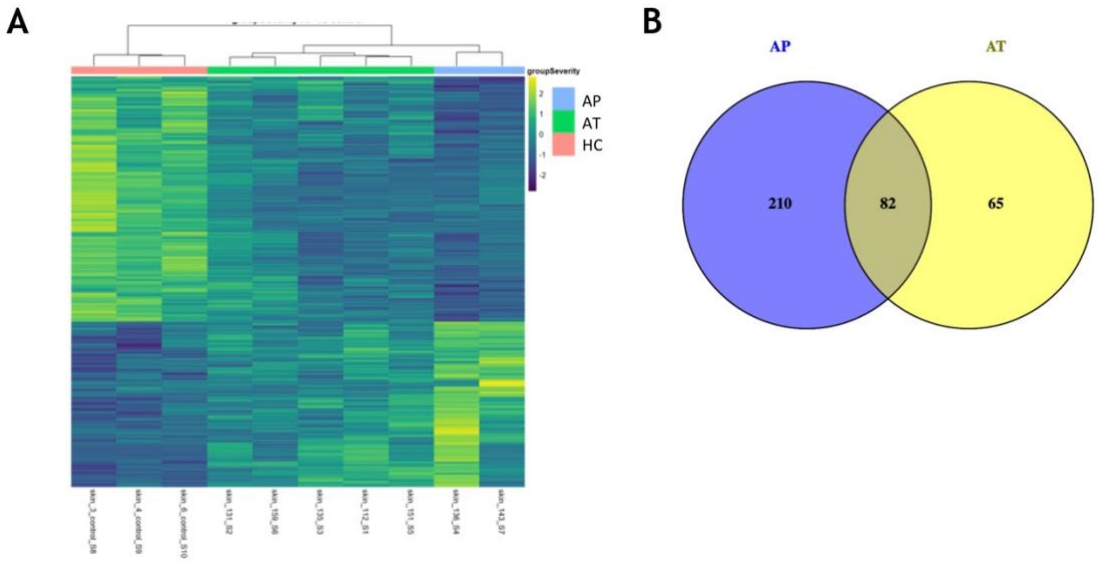


**Figure 6.10 Differential expression analysis of skin from people with AP and AT**
(A) Hierarchically clustered heatmap of patchy (AP, blue), severe (AT, green) and HC (pink) samples based on differentially expressed genes identified between patchy and HC skin samples (log2fold > 0.5, adjusted P value < 0.05). The x and y axis were clustered using Spearman distances and UPMGA agglomeration, and expression levels are represented as z-scores. (B) Venn diagram indicating the number of upregulated genes specifically associated with AP (210) or AT (65) samples, or upregulated genes that are shared between AP and AT samples (82).

## 6.11 Discussion

### 6.11.1 Dysregulated genes and pathways associated with AA

In this chapter, we sought to identify novel drivers of AA at the tissue level using global transcriptome analysis. PCA and hierarchical clustering of DE genes indicated that AA and HC samples cluster separately, confirming that AA skin is transcriptionally distinct from healthy skin. DE analysis indicated that 162 genes are upregulated and 417 genes are downregulated in AA skin compared to HCs. Three of the most upregulated genes in AA skin are involved in immune responses; *hla-dqa2, ccl13* and *lsp1*. HGSEA gene set analysis indicated that AA is associated with enrichment of three overlapping IFNγ response pathways, and pathways involved in body fluid secretion and actomyosin structure organisation. However, IFNγ, NKG2D, CD8a or other CD8 T cell-associated genes were not DE in AA compared to HC skin. Instead, the enrichment of IFNγ–associated pathways indicated upregulation of genes that respond to IFNγ signalling including upregulation of MHC class II expression (*ciita, hla-dqa2, hla-drb5*), non-classical MHC class I expression (*hla-g, hla-h*) and chemokines (*ccl13, ccl18*). Additionally, UREG gene set analysis identified that the IRF1 pathway is significantly

activated, however the majority of the genes highlighted in this pathway were not individually DE between AA and HCs.

The MHC class II genes, *hla*-dqa2 and *hla-drb5*, have both been associated with AA previously. HLA-DQA2 was identified as a genetic risk factor for AA development in the first genome-wide association study (GWAS), alongside other HLA genes including HLA-DRA, HLA-DQA1, and HLA-DQB2 (Petukhova et al., 2010). Whereas, a significant association between HLA-DRB5 and severe AA was identified in a small genetic-association study, but has not been linked with AA at a GWAS level (Lee et al., 2013). The upregulation of MHC class II observed in AA skin could be an artifact of the strong linkage between HLA genes and AA development. However, DE analysis also indicated upregulation of *ciita*, a gene which codes for the co-activator of MHC class II expression, induced by IFN$\gamma$ signalling (Steimle et al., 1994). CIITA is also a genetic locus associated with AA development (Betz et al., 2015). Taken together, these data indicate strong linkage between AA and specific HLA genes, and that lesional AA skin is associated with upregulation of MHC class II. MHCII expression is predominantly associated with dendritic cells, macrophages and Langerhans cells in the skin (Haniffa et al., 2009; Henri et al., 2009). However, keratinocytes can also express MHCII, and it has been shown that MHCII$^+$ keratinocytes are important for generating microbiota-specific CD4 T cell responses in murine skin, indicating a role for keratinocytes in immune surveillance (Tamoutounour et al., 2019). In our data, HGSEA analysis clearly indicated dysregulation of keratin genes, which will mainly be associated with the lack of hair growth. However, it can also be anticipated that MHCII expression by keratinocytes may be important for driving and exacerbating aberrant immune responses against HFs.

Pathway analysis also indicated upregulation of *hla-g* and *hla-h* in AA skin. HLA-G is a non-classical MHC class I gene, known as Qa-2 in mice (Comiskey et al., 2003), that is the ligand for ILT-2, ILT-4 and KIR2DL4 inhibitory receptors (LeMaoult et al., 2005). In normal conditions, HLA-G is reported to be an immuno-inhibitory molecule expressed by trophoblasts (Kovats et al., 1990) and is thought to play an important role in maintaining immune privilege between the mother and foetus (Rizzo et al., 2007). It functions by preventing activation of NK cells (Rouas-Freiss et al., 1997) and CD8 T cells (Contini et al., 2003), and

HLA-G can even be transferred to T cells by trogocytosis to induce tolerogenic responses (LeMaoult et al., 2006). The receptors, ILT-2 and ILT-4, have highest affinity for HLA-G, but they can also compete for MHCI binding, thus directly blocking CD8 T cell activation (Shiroishi et al., 2003). HLA-G expression is also associated with inflammation; virally infected and cancer cells upregulate it to avoid the immune system, and HLA-G expression is upregulated in inflamed tissues of psoriasis (Cardili et al., 2010) and CeD patients (Torres et al., 2006). The murine homolog, Qa-2, is expressed by the HF epithelium (Paus et al., 1994), but to our knowledge HLA-G expression has yet to be investigated in human HFs or in the context of AA pathology. Notably, we observed upregulation in the expression of *mica* in AA skin, another non-classical MHCI molecule that is genetically associated with AA (Petukhova et al., 2010). In contrast to HLA-G, MICA promotes NK and CD8 T cell activation via NKG2D engagement, and is reported to be crucial for driving CD8 T cell activity in AA pathology (Ito et al., 2008).

HLA-H was initially discovered as a non-classical MHCI molecule due to its structure, however further investigation revealed that it does not present peptide, and that specific HLA-H (HFE) mutations cause hereditary haemochromatosis, a condition characterised by excessive iron absorption (Feder et al., 1996). This observation led to studies discovering that under normal circumstances, HFE binds to the transferrin receptor and is involved in production of the iron storage hormone, hepcidin (Gao et al., 2009; Elizabeta Nemeth, 2009). To our knowledge, HLA-H/HFE has not been associated with AA pathology.

Collectively, our data, and findings from other studies indicate a robust connection between the non-classical MHCI locus and AA pathogenesis (Petukhova et al., 2010). Further investigation is required to understand the importance of HLA-G in HF IP mechanisms, and whether the upregulation of this molecule is an important part of AA pathogenesis. The potential involvement of HLA-H in AA is less clear. Interestingly, a meta-analysis study of comorbidities associated with AA indicated high incidence of iron-deficiency anemia (Lee at al., 2019), however a potential relationship between iron storage and hair loss caused by AA has not been investigated.

The expression of specific type-2 associated chemokines, *ccl13* and *ccl18,* were also enriched in AA skin. CCL13 is produced by epithelial and innate cells to induce migration of monocytes, dendritic cells, eosinophils and T cells (Garcia-Zepeda et al., 1996; Uguccioni et al., 1996). Whereas, CCL18 is only expressed by innate cells and causes migration of T and B cells (Vulcano et al., 2003; Hieshima et al., 1997). CCL13 is reported to be increased in serum of asthmatic patients (Kalayci et al., 2004) and cartilage of RA patients (Iwamoto et al., 2005). Interestingly, CCL13 and CCL18 are both upregulated in AD skin lesions, but not in psoriasis lesions, emphasising that these chemokines are specifically important for polarising type-2 driven tissue responses (Günther et al., 2005; Nomura et al., 2003). In the context of AA, CCL13 and CCL18 transcripts have been shown to be upregulated in AA skin by micro array analysis (Suarez-Farinas et al., 2015; Jabbari et al., 2016), and CCL13 is also elevated in non-atopic AA serum (Song et al., 2018). In the previous chapters, the high incidence of atopy in AA cohorts was discussed, suggesting that type-2 responses may be pro-pathogenic for a proportion of AA patients. Notably, a previous study reported that CCL13 mRNA levels in non-lesional AA skin positively correlated with SALT score (Song et al., 2018). These data suggest that type-2 chemokines are upregulated even in un-affected skin of AA patients, and therefore may precede CD8 T cell-driven responses. However, it is also possible that non-lesional scalp represents tissue in remission, and that a type-2 response is associated with hair regrowth.

In chapter 3, we observed an increase in the circulating concentration of type-2 associated cytokines, IL-31 and IL-33 in AA patients. In AA skin, we did not observe DE of il31, il33, il31ra or il1rl1 (ST2) transcripts or in other type-2 associated genes (*il13, gata3*). Thus, our data indicate a robust increase in type-2 chemokines, but not in Th2-associated genes, suggesting that other cells associated with type-2 responses may be involved in AA pathology.

LSP1 (lymphocyte specific protein 1) expression was also highly upregulated in AA compared to HC skin. LSP1 regulates cell motility and its expression requires careful control to ensure successful immune responses. Studies have demonstrated that LSP1 overexpression causes dysfunctional neutrophil responses during infection (Howard et al., 1994), whereas peripheral T cells of

RA patients have reduced expression of LSP1 which is reported to increase migration of T cells to inflamed tissue (Hwang et al., 2015). LSP1 also has a role in macrophage function, specifically during FCγ receptor mediated phagocytosis, because of its pivotal role in rearranging the cytoskeleton for phagocytic cup formation (Maxeiner et al., 2015). In the skin, LSP1, and FMO1, have been identified as markers of fibroblast populations in the human dermis. This subset of fibroblasts express CXCL12 and the authors suggest that they may behave similarly to bone marrow stromal cells to retain $CXCR4^+$ cells in the dermis (Tabib et al., 2018). We are unable to localise the increased expression of LSP1 to a particular cell type, but it is clear that dysregulation of LSP1 impacts multiple immune pathways and therefore this molecule could be an important driver maintaining the inflammatory environment in AA skin.

In addition to immune-related genes*, scn7a, padi2* and *ppargc1a* were amongst the most upregulated genes in AA skin. SCN7A codes for a sodium sensor expressed by neural cells (Hiyama et al., 2002). However, recent studies have shown that SCN7A sensors are also expressed in the epidermis and SCN7A blockade prevents inflammation in the imiquimod-psoriasis mouse model (Zhao et al., 2019). PADI2 is a peptidyl arginine deiminase enzyme that catalyses arginine conversion to citrulline during post-translational modification of proteins (Vossenaar et al., 2003). PAD enzymes are implicated in autoimmune pathologies, particularly RA, because citrullinated proteins are targets of auto-antibodies (Darrah et al., 2018; Foulquier et al., 2007). In addition to upregulation in PADI2, DE analysis indicated significant downregulation PADI1 and PADI3 in AA skin. These findings may be more interesting because PAD1 and PAD3 are expressed by the epidermis and HFs (Nachat et al., 2005; Guerrin et al., 2003). PAD1 and PAD3 co-localise with structural proteins, filaggrin and trichohyalin respectively, which are involved in keratin filament formation (Nachat et al., 2005; Senshu et al., 1996). PAD3 and trichohyalin are expressed specifically by HFs, thus the downregulation of PAD3 is likely a direct result of inactive HFs in AA skin. However, intriguingly it can be hypothesised that dysregulation in PAD1, because of its relationship with filaggrin, could be impacting epidermal barrier function which may be linked to the high incidence of atopy in AA cohorts.

PPARGC1A (PGC-1a) was originally discovered as the co-activator of PPARγ, as a major driver of mitochondrial biogenesis to increase energy expenditure (Puigserver et al., 1998), but has since been shown to co-activate a multitude of transcription factors including thyroid hormone, glucocorticoid, androgen and oestrogen nuclear receptors (Cantó & Auwerx, 2009). Usually, PGC-1a expression is associated with tissues of high energy demand including heart, skeletal muscle and the brain, and therefore dysregulation in its expression is associated with metabolic disorders including type 2 diabetes (Patti et al., 2003; Mootha et al., 2003). PPARγ is expressed by the HF dermal papilla, outer root sheath and matrix during anagen, and follicular stem cell knock-out of PPARγ causes scarring (irreversible damage to HF) alopecia in mice (Karnik et al., 2009). A direct role for PGC-1a in hair pathology has been shown in the context of male-pattern baldness, also known as androgenetic alopecia (AGA), where PGC-1a expression is upregulated by the outer root sheath of miniaturised HFs (Ho et al., 2019). The direct involvement of PGC-1a in AGA pathogenesis makes sense because of the importance of androgen signalling in mechanisms driving AGA, but the involvement of PGC1a in inflammatory driven AA has not been studied.

HGSEA gene set analysis also highlighted DE of genes involved in homeostatic and developmental pathways in AA skin. Many of the downregulated genes, including *cst6*, *bmp2*, *lef1* and *dlx2*, are essential for control of HFSC activity during normal HF cycling (Stenn & Paus, 2001; Rouzankina et al., 2004; Yang et al., 2017). Notably, BMP2 was downregulated in AA skin compared to HCs (log2fold=-2.77, P=1.69E-10), an important regulator of HFSC quiescence during telogen (Plikus et al., 2008). We also observed robust down-regulation of *fgf18* in AA skin. FGF18 is a fibroblast growth factor that is also important for maintaining quiescence during the telogen phase (Kimura-Ueki et al., 2012). In contrast, we did not observe DE of foxp1, an upstream regulator of FGF18 expression (Leishman et al., 2013). Taken together, these data indicate that factors involved in maintaining the quiescence of HFSCs are robustly downregulated compared to HC skin, and therefore appropriate control of HFSCs is not maintained in AA skin.

In previous chapters, specific aspects of the circulating immunophenotype were associated with AP or AT disease. The RNA-sequencing data set consisted of two

AP and five AT samples, thus we sought to compare the transcriptomes of both diseases states. However, due to the low numbers of AP samples, we did not conduct in depth analysis and these findings are considered to be preliminary. DE analysis indicated that AP, in comparison to HCs, was associated with a greater number of upregulated genes than AT compared to HCs. This was somewhat surprising because other gene expression studies have reported severe disease to be associated with greater dysregulation in gene expression (Suarez-Farinas et al., 2015; Jabbari et al., 2016). However, the study conducted by Suarez et al (2015) compared AA patients with <25% hair loss with >25%, with only 3/22 patients with 100% scalp-hair loss. This contrasts our study, where all five AT patients had >96% scalp hair loss. However, a larger micro array data set (20 AP and 20 AT patients) also found that severe disease is associated with a greater number of DE genes (Jabbari et al., 2016). There are also discrepancies between this study and ours because the AP samples collected by Jabbari et al (2016) were perilesional, and ours were collected from the centre of a patch or stable disease area in the context of AT samples. It is clear that our data cannot be used to identify differences between AT and AP samples, however we were able to conclude that genes highly upregulated in AA skin, including *ppargc1a*, *lsp1*, *ccl13, padi2* and *hla-dqa2* were consistently upregulated across all AP and AT skin samples.

### 6.11.2 The potential involvement of macrophages in AA pathology

It was surprising that we did not observe enrichment of a CD8 T cell signature in AA skin, despite clear upregulation of genes downstream of IFN$\gamma$ signalling. To our knowledge, our study is the first to conduct global transcriptome analysis of AA skin biopsies, however other large microarray data sets exist which indicate enrichment of CD8 T cell associated genes (Suarez-Farinas et al., 2015; Jabbari et al., 2016). In section 6.6, we suggest that the lack of enrichment of a CD8 T cell signature in AA skin could be due to two possible scenarios. CD8 T cells localised with affected HFs may comprise a small population and therefore total T cell-associated signatures were unchanged between bulk AA and HC transcriptome data sets, or the mechanisms dominating AA pathogenesis at the time of sample collection are not attributed to T cells. The latter explanation could also be associated with the location that the biopsies were taken, or the

long disease duration associated with the majority of our AA cohort which may impact disease activity. We could not address these questions directly, however we were able to utilise the transcriptome data set to investigate the roles of other immune cells in AA pathology.

We observed that the expression of genes associated with a 'tissue repair' or alternatively activated macrophage phenotype were modestly elevated in AA skin; *cd163, cd209 and mrc1*. We also observed elevated expression of *f13a1*, a marker of dermal macrophages, in specific AA samples (Haniffa et al., 2009). The expression levels of these genes were clearly variable between AA samples, but we confirmed that they are enriched in specific samples. The enrichment of a macrophage signature in specific AA samples was confirmed using the CIBERSORT tool. We also indicate preliminary evidence that macrophages are located in the perifollicular region of HFs in AA skin. However, we must acknowledge that the IHC data were mainly generated using sections from participant AA194. From a clinical perspective, this patient does not correspond to the majority of AA samples used for the transcriptomic part of the study because the participant has a low SALT score (11.33) and was receiving IL steroid treatment at time of recruitment. Although, we were able to identify $CD68^{+}$ cells in the perifollicular region of a sample from participant AA143.

Nevertheless, the IHC data indicate macrophage interaction with HFs in hairless AA skin. Our findings are supported by data presented in a recent open-access preprint which indicated increased abundance of a 'macrophage 2' signature in an AA skin sample, indicated by single cell RNA sequencing (Hughes et al., 2019). This macrophage 2 subset was characterised by expression of CD163, F13A1 and STAB1. Furthermore, we observed a positive correlation between *cd163* and *Cd8a* expression and our IHC data indicate that macrophages are located alongside T cells at affected HFs. We therefore propose that macrophages are involved in AA pathology.

In connection with our findings, we assessed whether the expression of genes associated with HF cycling correlated with expression of macrophage-associated genes. We found that expression of *lef1*, *bmp2* and *ctnnb1* are consistently downregulated in all AA samples, indicating that follicular genes are still

inactivated even in samples with reduced expression of macrophage markers (AA135, AA151). Other mediators discussed earlier, particularly the upregulation of *ccl18* and *ccl13* transcripts in AA skin, support the involvement of macrophages with a tissue-repair phenotype. *In vitro* exposure of monocytes with CCL18 causes upregulation of CD206 (Schraufstatter et al., 2012) and $CD163^+$ $CD209^+$ cells express CCL18 in skin of patients with cutaneous T-cell lymphoma (Günther et al., 2011). Furthermore, *in vitro* polarisation of 'M2' cells using IL-4 and M-CSF is associated with upregulation of CCL13 mRNA levels (Martinez et al., 2006).

To date, macrophages have not been a focus of AA research, however there are a collection of studies describing the involvement of macrophages in normal HF cycling and morphogenesis. In murine skin, it was shown that oncostatin M (OSM) secretion from a specific population of $TREM^+$ macrophages is responsible for maintaining HFSC quiescence, and that these cells reduce in numbers via apoptosis prior to anagen (Wang et al., 2019; Castellana et al., 2014). Notably, OSM signals via the JAK-STAT pathway, indicating another mechanism that may be attributed to the efficacy of JAK-STAT inhibition for driving hair regrowth (Harel et al., 2015). Interestingly, ablation of macrophages during telogen using clodronate was associated with increased secretion of Wnt ligands, and subsequent activation of ß-catenin pathways in HFSCs (Castellana et al., 2014). In the context of inflammation, macrophages also appear to be crucial for driving wound-induced hair regrowth following injury (Rahmani et al., 2018), and hair regeneration induced by mechanical stretch is associated with macrophage production of growth factors, HGF and IGF-1 (Chu et al., 2019). Taken together, these studies illustrate that HF-associated macrophage functions are consistent with the widely recognised homeostatic roles that macrophages play in tissue remodelling and regeneration in multiple areas of the body.

From the data described in this chapter, we cannot determine the phenotype of follicular macrophages in AA skin. Various studies have elegantly shown that macrophages owe their development of specialised functional properties to their surrounding niche (Guilliams & Scott, 2017; van de Laar et al., 2016), and it is clear from the various studies described above that the HF region represents a distinct niche that is associated with cyclic fluctuations in macrophages numbers

that vary in function. It is important to indicate that our bulk RNA sequencing data set represents a mixture of macrophage signatures from multiple niches within the skin, related to both HF and dermal macrophage populations. In contrast to our findings, we expected that genes associated with pro-inflammatory macrophage function would be enriched in AA skin. Interestingly, it has been shown that $CD163^{+}$ macrophages are present in psoriatic skin lesions, and that macrophages with this phenotype can in fact be generated *in vitro* when exposed to IFNγ (Fuentes-Duculan et al., 2010). Therefore, it is feasible that macrophages with a 'M2' phenotype could be generated in AA skin. Alternatively, our data may be indicative of an immune response associated with chronic or established AA, which could be associated with a switch from a type 1 response to a type 2 response, associated with alternatively activated macrophages.

### 6.11.3 Conclusion

The experiments described in this final results chapter sought to identify novel drivers of AA pathology at the tissue level. Upregulated genes sets in AA skin are associated with responses to IFNγ signalling, characterised by upregulation in MHCII genes, non-classical MHCI genes and chemokines associated with type-2 responses. Only 5 pathways, that accounted for 17 genes, were significantly enriched in AA skin. This was surprising given that 162 genes were upregulated in AA skin, and may suggest that changes in gene sets associated with AA are novel and not well-annotated in the gene ontology database. This observation in combination with no change in T cell-associated gene sets led us to investigate other immune cell signatures.

Further interpretation of the transcriptomic data indicated that specific samples in our AA cohort are enriched with a specific macrophage signature, characterised by *cd163, cd209, f13a1 and mrc1.* In light of findings describing the role of macrophages in normal HF cycling (Castellana et al., 2014; Rahmani et al., 2018; Wang et al., 2017), we propose that the inflammatory milieu associated with affected HFs is disrupting the niche that supports the homeostatic function of HF-associated macrophages. It is also probable that the macrophages surrounding the HFs, alongside CD8 T cells, are pro-pathogenic and

may even play a role in fibrotic responses that are detrimental to promoting hair regrowth, especially in patients with severe disease.

In this chapter, the cohort was from a mixture of patients with varied disease severity and disease duration. Four of the AA individuals were also atopic, and one was receiving treatment when recruited to the study. These factors will be likely to influence the transcriptome of AA skin, but due to the size of this cohort we were not able to stratify the patients to elucidate the relationship between clinical parameters and AA pathogenesis. Furthermore, our preliminary IHC analysis indicated that not all HFs in AA skin are associated with infiltration of T cells or macrophages. This is partially attributed to the cycling of HFs, which dictates the expression of antigen required to drive HF-targeted immune responses. This also may be a reason why bulk transcriptome analysis is not ideal for assessing AA skin because it doesn't account for the HF numbers or HFs actively associated with immune infiltration at the time of biopsy collection.

In this chapter, we also provided histological analysis of CD3 and CD68 expression in AA biopsies. This analysis is particularly limited and in future studies we plan to extensively expand on this part of the study. We are specifically interested in understanding how macrophages, in addition to T cells, are interacting with HFs. We therefore plan to expand this analyses in the following ways. First, we aim to better characterise follicular macrophages in control and AA biopsies, by staining for markers identified in the RNAseq. These include the scavenger receptor, CD163, and pathogen recognition receptors, CD209 and CD206. We then aim to characterise the function of these cells, again informed by the transcriptome data sets, by staining for factors produced by these cells, including cytokines (TNF) and enzymes (arginase 1), in addition to pro-fibrotic factors (platelet derived growth factor). To complement these studies, we will also perform co-expression immunofluorescence staining to confirm that macrophages express the markers of interest. It is possible that other antigen presenting cells in the skin, including Langerhans cells, may be responsible for the upregulation of genes of interest identified in the RNAseq data set. Finally, we plan to stain for markers expressed by the follicular epithelium, including MICA, in addition to markers of keratinocyte differentiation to better characterise the HFs which immune cells are interacting

with. Collectively, these experiments will better characterise follicular associated macrophages, and indicate factors expressed by these cells which may be modulating HF behaviour.

In the previous chapters, we described data indicating upregulation of type 17 and type 2 cytokines, and increased frequency of circulating $CCR6^+$ T cells in AA circulation. However, overall, our data clearly indicate that the peripheral and tissue responses associated with AA are distinct, highlighting the complexity of this multifactorial autoimmune disease.

# 7 General discussion

The aim of this study was to identify novel immune mechanisms driving AA. NKG2D$^+$ CD8 T cells are known to be essential for driving hair loss, but other immune cells and mediators which contribute to this response have not been well characterised. This lack of understanding is directly reflected in the limited therapeutic options available for the treatment of AA. To address our aim, we first sought to broadly characterise the circulating immune profile of AA patients. While these data are informative, the systemic immunophenotype does not indicate the responses important for driving AA in the skin. We therefore analysed affected skin at a transcriptome level to identify novel pathways and mediators driving hair loss. To facilitate these experiments, we set up a dedicated research clinic in Glasgow to collect blood and skin samples from volunteers with AA. By doing this, we have generated data sets from peripheral blood and skin that provide an insight into the broad immune dysregulation associated with AA.

Our data obtained from peripheral blood indicates that AA is associated with enriched type 17 and type 2 cytokine responses. In the previous chapters, it was discussed that comorbid atopy is common in AA cohorts, indicating that pathogenic responses which are dominated by type 1 and type 2 responses can coexist in the skin. Based on our data and others, we suggest that atopic AA represents a distinct disease pathotype, and that atopic immune mechanisms may exacerbate AA. This is supported by studies indicating that atopy is associated with severe forms of AA (Betz et al., 2007; Bakry et al., 2014; Zhang & McElwee, 2020).

Increased levels of type 17 and type 2 systemic cytokines were found to be associated with both atopic and non-atopic AA cohorts, however the increase in transitional B cells was specifically associated with atopic AA. Based on current evidence, it is unlikely that pathogenic responses driving AA are attributed to autoantibodies. Instead, B cells may be important for presenting autoreactive antigen, and therefore directly contribute to the generation of autoreactive T cell responses (Rawlings et al., 2017). We suggest that the increase in frequency of circulating transitional B cells is indicative of increased egress of immature B

cells from the bone marrow. Based on data from other diseases, particularly SLE (Yurasov et al., 2005; Sims et al., 2005), this phenotype suggests that atopic AA may be associated with increased levels of circulating autoreactive B cells.

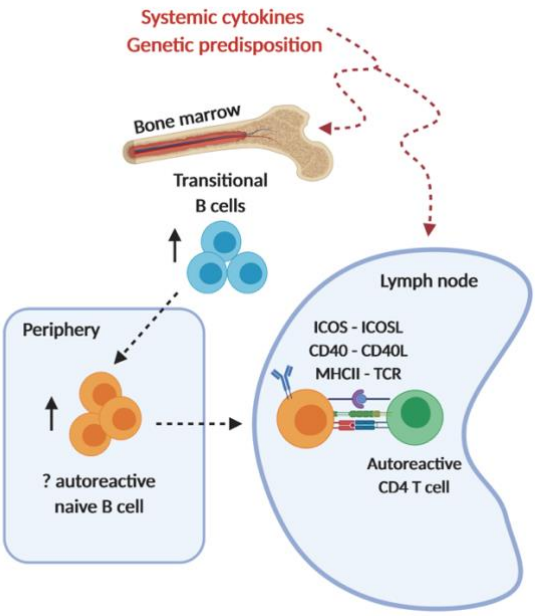


**Figure 7.1 Atopic B cell mechanisms and AA**
B cells are often implicated in autoimmunity. Comorbid atopy is associated with AA, and we propose that atopic AA represents a distinct disease pathotype. The frequency of transitional B cells is elevated in atopic AA individuals, and in cohorts of atopic patients. We propose that transitional B cells feed the autoreactive naïve B cell pool and may contribute to breaking T cell tolerance and therefore contribute to autoreactive T cell responses. These mechanisms may be propagated by increased systemic cytokines and genetic predisposition associated with polymorphisms in B cell stimulating molecules. Diagram adapted from Rawlings et al (2017) and created with Biorender.com.

This phenomenon could be driven by molecular defects in B cell tolerance, which have previously been associated with atopy. In support of this, polymorphisms in ICOS and the IL-2/21 locus are genetic risk loci for AA (Petukhova et al., 2010); molecules which are involved in B cell and T cell interaction. As discussed previously, it is also plausible that increased peripheral cytokines contribute to elevated egress of transitional B cells, including IL-21 (Ozaki et al., 2004). We therefore suggest that B cells contribute to breaking T cell tolerance which drives AA. We did not observe any change in the frequency of switched B cells between atopic AA patients and HCs, but this finding does not indicate that the proportion of activated autoreactive B cells within the switched B cell pool is not altered. In addition, autoreactive B cells may contribute to epitope spreading as hair loss progresses (Rawlings et al., 2017). It is anticipated that stratification of atopic and non-atopic AA patients will be

useful in future clinical studies to elucidate differential immune mechanisms associated with each pathotype. Proposed mechanisms involved in B cell and T cell interactions in the context of AA are represented in Figure 7.1.

The relationship between AA and a type 17 immune response appear to be complex. The levels of type 17 cytokines and frequency of $CCR6^+$ CD4 T cells are increased in AA circulation, indicating a potential role for Th17 cells in AA pathogenesis. Further analyses revealed that changes in $CCR6^+$ CD4 T cell populations, particularly $CCR6^+$ $CXCR3^+$ (DP) CD4 T cells, are associated with mild disease (<50% hair loss). This may indicate differences between extensive and patchy hair loss. However, as suggested in chapter 5, we hypothesise that this T cell phenotype is indicative of active disease. This 'active' phenotype may be observed in this patient group because the mild disease cohort often represent individuals most likely to experience relapsing-remitting hair loss, and therefore may mirror acute hair loss. If this were true, how is a Th17 response contributing to CD8 T cell mediated pathology? If DP CD4 T cells are *bona fide* Th1/17 cells, they may contribute to IFN$\gamma$ production, which directly drives CD8 T cell activity. Thus, CD4 T cells may be important for driving the development of AA. This is supported by studies in the C3H Hej model of AA, which indicated that intradermal transfer of CD4 T cells in addition to CD8 T cells is required to drive extensive AA (McElwee et al., 2005).

Notably, we found that DP CD4 T cells and skin homing DP CD4 T cells appear to be specifically increased in patients with mild disease (<50%) who were on IL steroid treatment. This raises the question of whether $CCR6^+$ T cells are elevated in response to treatment, and are not directly related to AA pathology. In chapter 5, we discussed that Th17 cells can be resistant to glucocorticoids, and it has been proposed that steroid treatment in patients with autoimmune diseases may drive enrichment of pathogenic and drug resistant Th17 cells (Ramesh et al., 2014). Notably, Guttman Yassky et al (2016) noted that the patients (2/3) who responded poorly to Ustekinumab (anti-IL-12/23p40 antibody) treatment were refractory to previous treatment with IL steroids. Thus, it may be speculated that refractory response to IL steroid therapy is associated with a poor response to type-17 cytokine blockade. Further studies with larger cohorts

are now required to identify whether a specific pathotype of AA may benefit from type-17 blocking therapies.

We next used transcriptome analysis to identify novel pathways critical for AA pathogenesis. The data indicated that AA skin is associated with a macrophage signature, characterised by CD163, CD209, CD206 and F13A1 expression, markers which are indicative of an alternatively activated or wound healing phenotype. Initially, this was surprising because AA skin is enriched with genes responding to IFNγ. However, it has been shown that macrophages with an alternatively activated phenotype are present in psoriasis lesions, and that they can be present within an IFNγ-rich environment (Fuentes-Duculan et al., 2010). Macrophages are known to interact with follicles following inflammation to drive HF regeneration (Rahmani et al., 2018), and they secrete molecules which have been shown to influence normal hair cycling (Wang et al., 2019; Castellana et al., 2014). We therefore propose that the inflammatory environment impacts the local niche maintaining follicular macrophage function, and that altered macrophages contribute to mechanisms causing hair loss.

Furthermore, our data and others suggest that the acute response associated with AA appears to differ from that of stable disease. Most of the studies analysing gene expression have been obtained from perilesional skin which is reported to represent active disease (Xing et al., 2014), whereas the samples used for our study were collected from the middle of stable regions. During stable disease, a high proportion of HFs in AA lesions are in telogen or catagen, and because inflammation is associated with anagen associated antigen, inactive follicles are not reported to drive CD8 T cell effector function until they re-enter anagen. Thus, during stable disease we anticipate that macrophages are key pathogenic cells which maintain immune responses preventing hair regrowth. The proposed differences between active and stable AA immune mechanisms are depicted in Figure 7.2.

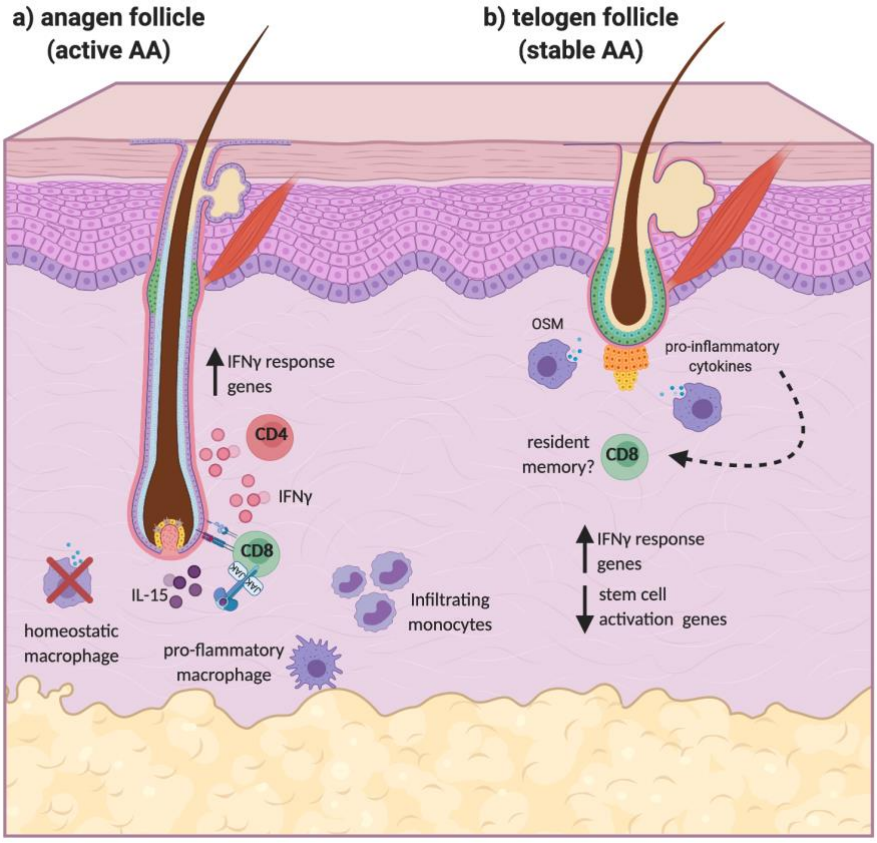


**Figure 7.2 Proposed immune mechanisms driving active and stable AA**
AA is known to be driven by NKG2D$^+$ CD8 T cells which express IFNγ. Follicular inflammation is generated against hair follicles in anagen (a), which leads to premature catagen and telogen entry. We propose that this environment disrupts the activity of homeostatic follicular-associated macrophages. Transcriptomic profiling of stable AA skin indicated enrichment of a alternatively activated macrophage signature (b). We propose that pathogenic macrophages contribute to preventing hair loss by secreting molecules which inhibit anagen entry and promote T cell mediated inflammation. Created with Biorender.com.

### *Future therapeutic strategies for AA*

The field of dermatology is gradually moving from the use of broad spectrum immune suppressants towards using targeted therapies, most notably for the treatment of psoriasis. In recent years, clinical studies have indicated that AA may be targeted with currently available immune-modulating therapies, and JAK inhibitors are proven to be particularly useful (Jabbari et al., 2018; Wang et al., 2018). From a mechanistic point of view, the broad targeting of JAK inhibition does not facilitate better understanding of the specific cytokines important for driving AA. Interestingly, it is becoming apparent that successful treatment of AA requires promotion of anagen in addition to blocking harmful inflammation. This may be especially true for severe and refractory AA, and these patients

represent the cohort who are most likely to be eligible to receive targeted therapies.

Notably, it has been shown that JAK inhibition induces anagen by activating hair follicle progenitor cells (Harel et al., 2015). These data indicate that JAK inhibitor therapy, in the context of AA, has a dual function, and may indicate why this therapeutic strategy is particularly effective for a large proportion of patients. Aside from the side effect profile, a major problem associated with JAK therapy is that disease relapse is common following treatment cessation (Yael Renert-Yuval, 2017). Thus, better understanding of the mechanisms modulating JAK-STAT mediated HF control are required to design novel therapeutic strategies. Recent investigation has indicated that macrophages present during telogen can inhibit anagen entry, and that this mechanism involves JAK-STAT signalling, via OSM secretion (Wang et al., 2019). This mechanism appears to be important during normal hair cycling. We now ask how this knowledge can be exploited to better target mechanisms driving AA?

We propose that targeting macrophages offers a tangible therapeutic option for AA. In normal human skin, macrophage numbers decline during early catagen, however during late catagen follicular macrophages adopt a phenotype similar ($CD206^+$) to the signature observed in lesional AA skin (Hardman et al., 2019). Furthermore, the OSM secreting macrophage population observed in mice, which inhibit anagen entry, express CD163 (Wang et al., 2019). We therefore hypothesise that $CD206^+$ macrophages in AA skin contribute to AA pathogenesis and inhibit hair growth. These functions may involve secretion of factors that have already been associated with macrophages, including OSM or cytokines (Figure 7.2). Further investigation is required to characterise the macrophage phenotype in lesional AA skin, and to identify functions of macrophage behaviour which contribute to hair loss.

Our data clearly highlight that AA represents a heterogenous autoimmune disease, and that AA is associated with distinct tissue and systemic inflammatory signatures. From a clinical perspective, AA is not directly life threatening and affected individuals are generally regarded as healthy by the dermatology community. However, the increased incidence of inflammatory comorbidities

among patient cohorts may indicate that the immune dysregulation associated with AA should be taken more seriously. Importantly, affected individuals are often concerned about how AA-driven hair loss may lead to the development of other inflammatory illnesses. Notably, we observed that the number of circulating $CCR9^{+}$ CCR6 CD4 T cells are elevated in a selection of AA patients, and preliminary evidence indicates that zonulin levels are raised. Thus, the type 17 signature could be related to sub-clinical intestinal inflammation, which would corroborate with patient reported intestinal discomfort. Furthermore, we found that levels of circulating IL-22 and IL-17E are positive predictors of depression score. Whilst these data do not indicate that cytokines drive depression in AA, we suggest that they may contribute based on studies describing the link between peripheral inflammation and depressive effects on the brain (Bain et al., 2019). Based on these observations, we propose that the systemic inflammatory signature associated with AA provides a rationale connecting follicular associated pathology and wider tissue involvement.

## 7.1 Final conclusions

The data described in this thesis provide better understanding of the immune dysregulation associated with AA. The systemic signature of AA is characteristic of a polygenic autoimmune condition, and provides insight into different disease pathotypes that will be useful for informing future mechanistic and clinical studies. We also suggest that macrophages represent a novel pathogenic driver of AA, and that future investigations are required to identify mechanisms which could be therapeutically targeted in the future.

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