CHAPTER 4. DISCUSSION

Overview

The aim of this project was to identify the HF antigens which are targeted in the presumptive autoimmune HF disorder alopecia areata (AA). A flow chart of the steps I employed in this project is shown in Figure 52. The main hindrance for our understanding of the etiology of AA has been the gap in our knowledge of the HF-specific antigens responsible for the observed immunoreactivity to the HF. Previous attempts in identifying AA-relevant antigens included the identification of melanocytes specific proteins capable of activating T cells in causing AA in SCID mice (Gilhar *et al*., 2001), the binding of human AA sera antibodies in to the hair specific 44/46kDa keratins by Western Blotting (Tobin & Bystryn, 1996), immunoprecipitation of trichohyalin (THH) by AA sera in canine and equine AA (Tobin *et al*., 1998b; Tobin *et al*., 1998a) and more recently the identification of 8 antigens using protein microarray during an attempt in the generation of protein biochip for AA diagnosis (Lueking *et al*., 2005).

A significant proportion of this project was focused on developing methods for antigen identification. Methods were developed to first isolate AA-relevant antigens from normal human HF extracts using human AA sera. As the antibodies implicated in AA are mainly IgG (Tobin *et al*., 1994b), I began by attempting to purify IgG from AA/normal serum (Figure 52, Step 1) and to use this for the immunoprecipitation of relevant antigens from normal HF extract (Figure 52, Step 2). However, this approach was not without its problems. Due to the small amount of starting material, the yield of purified IgG was low and

also the end product was often contaminated with albumin. The immunoprecipitation assays using these so-called purified IgG products were unsuccessful. Removal of this purification step was deemed to likely increase the amount of HF antigen that would be precipitated, as IgG loss during purification would now be avoided. Therefore, instead of trying to purity IgG from AA/normal serum, the whole serum was used for the immunoprecipitation assays (Figure 52, Step 3).

The original aim was to separate the mixture of HF-associated antigens in the immunoprecipitate using 2D electrophoresis. The presence of IgG, which co-eluted with the HF antigens as they were removed from the solid support during the boiling step, likely masked the presence of any potential HF antigens of similar Mwt and p*I* in the gel or the Western Blot. Thus, I modified the immunoprecipitation protocol by cross-linking IgG permanently onto the Protein G beads (Figure 52, Step 4). After separation of the antigens in the immunoprecipitate by 2D gel electrophoresis, very few protein spots were detected (Figure 52, Step 5). Indeed, analysis with MALDI-TOF identified some of them as contaminants. So instead of doing immunoprecipitation assays, whole normal HF extract was separated first by 2D gel electrophoresis followed by Western Blotting with probing by AA serum (Figure 52, Step 6). A comrade SDS-PAGE gel was run in parallel and stained with SimplyBlue Safestain. In this way, spots from this gel which matched the positions/locations of the positive spots on the Western Blot, were excised and analysed by MALDI-TOF/TOF. Although some of these spots were positively identified as keratins, one could not be 100% sure of the accuracy of mirroring the gel and the

Western Blot. Also, this method restricted the amount of protein that could be separated by the 2D gel electrophoresis and thus the amount of protein available for subsequent analysis. Moreover, not all potentially relevant proteins are analysed as only the excised spots were progressed to MALDI-TOF/TOF analysis. To include all the potentially relevant proteins in the identification process, immunoprecipitation was carried out as before using normal HF extract and human serum, but now the whole immunoprecipitate was analysed directly using LC-MALDI-TOF/TOF (Figure 52, Step 7). Using this approach THH and K16 were identified to be AA-relevant. Antibodies to these HF-associated proteins were then used in functional tests. Antibodies to both proteins appeared to impede hair shaft growth in *ex vivo* culture (Figure 52, Step 8) and by indirect immunofluorescence analysis were shown to bind to the IRS (antibody to THH) and ORS (antibody to K16) of the HF (Figure 52, Step 9).

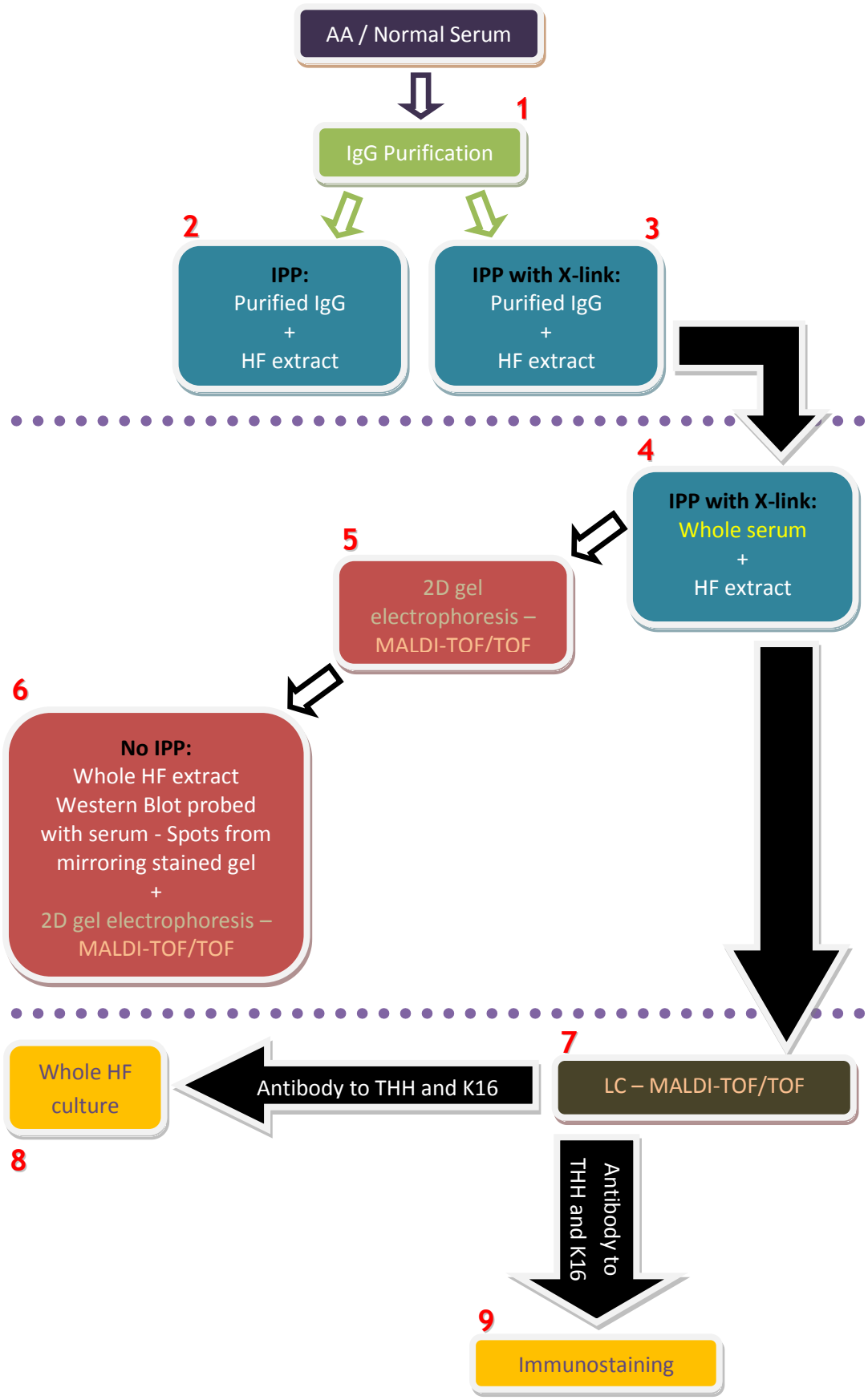

Figure 52. Flow chart showing the flow of experimental events from beginning to end of project. IPP = Immunoprecipitation; X-link = cross-linking.

4.1 LOW IgG RECOVERY IN PURIFICATION OF HUMAN SERA IgG BY PROTEIN G AFFINIITY CHROMATOGRAPHY

The final yield of purified IgG from both normal control and AA serum was lower than expected (Steward, 1984) as determined using radial immunodiffusion. In reviewing the method used, it was noted that the sample was transferred between different reaction containers during the many steps involved. There were also experimental steps which used filter units with membranes as a sample concentrating device. This process led to the exposure of the IgG to large surface areas to which some of the IgG may have adsorbed. This loss of IgG during the purification process together with the relatively low volumes of starting serum available (0.5 mL to 1.0 mL) may explain the low yield of IgG obtained. Moreover, at least some albumin was detected to be present in the purified product, and this could have been caused by residual albumin in the chromatography column and co-eluted with the IgG. Both scenarios would therefore decrease the purity of the IgG. Thus, this so-called 'purified IgG' was therefore unsuitable for use in immunoprecipitation and Western Blotting due not only to the low concentration of IgG but also the concomitant presence of contaminants.

4.2 IMMUNOPRECIPITATION OF HAIR FOLLICLE ANTIGENS WITH AND WITHOUT CROSS-LINKING BETWEEN IgG AND PROTEIN G

Without the introduction of the cross-linking step the immunoprecipitates contained IgG which co-eluted with the target antigens during boiling. As the original strategy was to separate proteins in the immunoprecipitate by SDS-PAGE and then excise them for analysis, the presence of this relatively large amount of IgG contaminated the gels and appeared as two broad protein bands (heavy and light chains of ~50 kDa and 25 kDa) in the Western Blots and the stained gels. Thus, potential target antigens with similar Mwt as the heavy and light chains of IgG may have been masked and so not visualised in this assay. However, with the cross-linking step, IgG was cross-linked to the Protein G permanently and therefore would not break off from the Protein G during the boiling step. This was evidenced by the finding that 1D SDS-PAGE followed by Western Blotting of these immunoprecipitates using ECL as the visualisation method, showed little or no IgG in the immunoprecipitates. Thus, all remaining assays were conducted using cross-linking. Albumin was detected in the immunoprecipitate but only in low quantity. It should be noted that the Protein G solid supports used for the IgG purification and immunoprecipitation assays and were supplied in different forms from different suppliers. The Protein G used for IgG purification was supplied in the form of a pre-packed chromatography column (Millipore Corporation, Massachusetts) and the one used for immunoprecipitation was supplied as a suspension in 20% ethanol

(Sigma-Aldrich Company Ltd., Dorset). Although specification for both Protein G products indicated that these were recombinant proteins with their albumin binding sites deleted (see References section, Montage® Antibody Purification Kit and Protein G Immobilized On Agarose), albumin contamination was observed in both the 'purified IgG' and the immunoprecipitates. However, the level of contamination was observed to be higher in the 'purified IgG' (intense signal detected using the relatively less sensitive chromogen detection method) than in the immunoprecipitates (only moderate signals detected despite using the highly sensitive ECL method). This difference in contamination level may have be caused by the different approaches these two procedures were conducted, even though both make use of recombinant Protein G on solid support.

4.3 PROTEOMICS METHOD ONE – 2D GEL ELECTROPHORESIS AND MALDI-TOF/TOF ANALYSIS OF IMMUNOPRECIPITATES USING ALOPECIA AREATA SERUM

Seven HF antigen spots were analysed by MALDI-TOF/TOF. Of these, three of them were identified as the common contaminants K2, K9 and K10 (Parker *et al*., 1998). Two others proteins were identified as K14 and one as K16. The small number of protein spots observed in each gel was perhaps due to the very low amount of protein present in these immunoprecipitates. This was likely

determined by 1) concentration of starting IgG and HF antigens, 2) affinity or titre of anti-HF antibodies. In addition, keratins are common contaminants in 2D gel electrophoresis (Parker *et al*., 1998) and for a project such as the current one these may further hinder HF antigen identification due to their potential sequences homology, as some possible target antigens are also likely to be keratins (Tobin & Bystryn, 1996).

4.4 PROTEOMICS METHOD TWO – 2D GEL ELECTROPHORESIS, WESTERN BLOTTING USING ALOPECIA AREATA SERUM AND MALDI-TOF/TOF ANALYSIS OF WHOLE HAIR FOLLICLE EXTRACT

Because of the low number of antigens visualized and identified in the 2D gels in Proteomics Method One, instead of using immunoprecipitates, whole HF extract was separated by 2D gel electrophoresis followed by Western Blotting. AA-relevant antigens were detected by probing the membrane with AA serum. A second 2D gel was run in parallel and the proteins in the gel detected using SimplyBlue SafeStain. The proteins detected in this gel, which mirrors the positive signals in the Western Blot, were excised for MALDI-TOF/TOF analysis. By using the whole serum instead of the immunoprecipitate I could increase the amount of potential target antigens in the gel, which were then subsequently excised for MALDI-TOF/TOF analysis.

More HF antigen protein spots were observed using this method than the previous one, but like in Proteomics Method One all were keratins including the presence of common keratin contaminants K1, K9 and K10. The total number of proteins observed was still less than expected. One of the possible explanations for this is that the low protein content available for analysis resulted in MALDI-TOF not always being able to assign positive identities to the proteins. Data from tandem MS/MS analysis, which contains information of the sequence of the peptides digested from the proteins, was therefore very important for confirming protein identities as the database searches are more specific using these data (Mann *et al*., 2001). Proteomics Method Two also include a desalting step before the MALDI-TOF/TOF analysis. This was an important enhancement as it improved the signal-to-noise ratio of the target peptides in the spectra by removing the high salt content in the digested sample (Bagshaw *et al*., 2000). However, in terms of the amount of proteins excised from gel and then analysed by MALDI-TOF/TOF, it is difficult to see how this could be improved, as increasing the concentration of the starting sample used is not straightforward. In our 2D gel electrophoresis system the amount of HF protein that can be loaded onto the IEF strip is limited as excess protein leads to poor separation during IEF, and causes horizontal streaking after the second dimension is completed. Overloading can also reduce accuracy when excising protein spots from the stained gel by mirroring it with the Western Blot.

The above limitations of using 2D gel electrophoresis are however not new to researchers. Proteins in low abundance are often not detected by the 2D gel electrophoresis-MS approach (Gygi *et al*., 2000) and proteins which are very

basic or very acidic, or with very high or very low Mwt are also often not resolved by this system (c.f. Peng & Gygi, 2001; Mann *et al*., 2001). THH is a large protein with Mwt of ~220kDa. This limitation of the 2D gel electrophoresis system could be an explanation for why THH was not being observed during Proteomics Method One and Two, but was then identified by LC-MALDI-TOF/TOF analysis later in the project. Moreover, the limited resolving power of this system means proteins which are very similar in Mwt and p*I* co-migrates (c.f. Peng & Gygi, 2001) and thus further complicating the protein identification process.

4.5 PROTEOMICS METHOD THREE – LC-MALDI-TOF/TOF ANALYSIS OF IMMUNOPRECIPITATION PRODUCTS USING ALOPECIA AREATA SERUM WITH CROSS-LINKING

Given the limitations of the 2D gel electrophoresis system as discussed above, this was no longer used for the remaining of the project. Instead, HF antigens were isolated by human sera using immunoprecipitation assays. These isolated HF antigens were then separated by LC and followed by analysis using MALDI-TOF/TOF. This method was standardized tested by analysing HF antigens immunoprecipitated by one AA serum (AA5).

This method resulted in the identification of several proteins, including 14 keratins. The common contaminants K1, K9 and K10 were again observed.

In the previous studies by Tobin *et al.* using AA serum antibodies for the identification of target antigens (Tobin & Bystryn, 1996; Tobin *et al*., 1998b; Tobin *et al*., 1998a), AA serum exhibited a much greater reactivity against normal HF extracted proteins compared to the normal serum reactivity. Indeed, natural antibodies against autoantigens are present in all individuals (Avrameas, 1991; Atassi & Casali, 2008). However, normally these antibodies are not disease-causing, as their avidity to self antigens is low (Atassi & Casali, 2008). Although, changes in, for example, antigen expression, or in the regulation of the expression of these normal antigens, could lead to antibody switching such that pathogenic antibodies may be generated, that are still based on these existing 'normal' antibodies (Atassi & Casali, 2008). Control healthy individuals have 'natural' antibodies against proteins in HF extracts, but these were found to be of much lower titre than AA serum antibodies to HF extract proteins (Tobin *et al*., 1994a). Therefore, when studying the AA-relevant antigens, it is important to compare reactivities of normal and AA serum antibodies in order to identify proteins which may be 'natural antigens' and part of the normal autoantibody pool and so are not likely to be not involved in the pathogenesis of AA.

Substantially more proteins were identified in Proteomics Method Three comparing to Proteomics Method One and Two. Although most of the identified

were keratins, other types of proteins were detected. The proteins identified in Proteomics Method One, Two and Three, however, can not yet be concluded as AA-relevant antigens. As they were carried out as part of my method development steps, only HF antigens immunoprecipitated (Proteomics Method One and Three) or detected in Western Blotting (Proteomics Method Two) by a single AA serum was analysed in each of these studies. As discussed above, it is important to compare results obtained from normal serum because of the presence of low titre natural antibodies to HF. Now with the of isolation HF antigens using human sera and protein identification methods established, both AA sera and normal sera were tested for their reactivities to normal human scalp HF antigens, in this way it was possible to identify the HF antigens which are identified because of their relevance to AA and which are identified simply because they are reactive with the normal antibody pool.

4.6 IDENTIFICATION OF POTENTIAL ALOPECIA AREATA-RELEVANT HAIR FOLLICLE ANTIGENS TARGETS

Immunoprecipitations of proteins extracted from normal anagen HFs (plucked) were conducted with a group of 10 AA and 10 normal sera. Negative controls were carried out by omitting either the serum or the HF extract from the experimental process. The proteins immunoprecipitated were regarded as AA relevant if they were immunoprecipitated only by antibodies present in AA sera

(i.e. not also in normal sera (and negative controls). An association with AA was also inferred if HF antigens were immunoprecipitated by both AA and normal serum, but if the prevalence and affinity of these was higher in the AA group than the normal group, and even more importantly if they were not observed in the negative controls.

For the proteins identified by LC-MALDI-TOF/TOF, whether they were chosen for further functional study was determined most importantly by their frequency of occurrence in the AA serum and normal serum group. THH was identified in all 10 of the immunoprecipitates of AA sera but in only in 5 of the normal sera group. A second protein, K16, was identified in 8 of the AA serum immunoprecipitates but in only 4 of the normal control group.

Mascot scores from MALDI-TOF/TOF analysis cannot be used to quantify proteins in absolute terms as it is an algorithm designed only for calculating the probability of whether a protein match with the database is random (or in other words the probably of a match not being a random event) (Perkins *et al*., 1999). The Mascot score for an identified protein would be affected by the size of the protein database being searched, size of the protein identified and quality of the mass spectra (Perkins *et al*., 1999). Even though the Mascot score cannot be used quantitatively, for the purpose of this project, the Mascot scores were used as an additional guidance. All the immunoprecipitates (from 10 AA sera and 10 normal sera) were prepared in the same way and were treated the same during sample preparation for LC-MALDI-TOF/TOF analysis. These were then

analysed with LC-MALDI-TOF/TOF (including database searches) under the same conditions, as well as in the closest possible temporal succession to avoid fluctuations in instrument performances. These samples were also prepared and analysed randomly in order to remove bias. By using these experimental conditions, the quality of the mass spectra generated should not be affected by factors other than the composition of the samples themselves. As the presence of a higher quantity of a particular protein in the sample results in a more favourable signal-to-noise ratio, this gives a higher probability for the protein to be identified with a higher Mascot score, than if this protein was present in a lower quantity. Therefore, Mascot scores awarded for a particular positively identified protein in the different immunoprecipitates could be compared to reflect the relative quantity of this protein.

For the immunoprecipitates with a positive identification of the presence of THH, the THH Mascot scores were averaged among the AA group (1276/10 = 127.6) and among the normal group (401/5 = 80.2). The difference between the average THH Mascot scores of the two groups was statistically highly significant ($p = 0.005$). This suggests that there was a relatively higher amount of THH present in AA immunoprecipitates than in the 5 THH positive normal immunoprecipitates. The higher amount of THH protein present in AA immunoprecipitates reflects either (1) the higher avidity of AA serum IgG antibodies to THH compared to those that occur in normal serum IgG, or (2) the amount of serum IgG which was reactive against THH was higher in AA than in normal sera. Either or both of these possibilities are likely, as the starting amount of normal HF extract and IgG used in all immunoprecipitations was the

same. It is important to note that both AA and normal serum IgG are reactive against THH in *normal* HF extracts. THH is a 'normal' protein present in the IRS of normal HFs with an important structural role (Rothnagel & Rogers, 1986). If THH, or other such 'normal' proteins are target antigens in AA, the attack of these antigens during disease pathogenesis would indicate that it is an inappropriate immune response against a 'normal' antigen, but not an abnormally expressed protein targeted by a 'normal' immune response.

When interpreting the normal samples in which THH was not positively identified by MALDI-TOF/TOF, it cannot be confirmed that there was complete absence of THH in the immunoprecipitates. It is possible that the level of THH in these samples was too low and outside the sensitivity of the instrument. However, this still indicates that the normal sera which were used to generate these immunoprecipitates had either very little IgG to THH, or had IgG with very low avidity to THH.

The high levels of both frequency/incidence of anti-THH antibodies in AA sera provides good evidence that the AA sera contained antibodies with abnormal IgG activity towards epitopes in THH from normal HF extract. Using immunoprecipitation as the assay also provide support that these THH-reactive IgG antibodies can bind to a conformation peptide, as the proteins were not denatured during the process. In this way the observed reactivity may be more *in vivo*-like by comparison with Western Blotting analysis in which protein epitopes are denatured by SDS-PAGE separation.

When comparing the mean Mascot scores of K16 identified in the 8 AA serum samples (595/8 = 74.4) and in the 4 normal serum samples (263/4 = 65.8), the difference was only p = 0.097. However, the higher mean score for the AA patient group could be interpreted as the possible presence of higher amount of IgG or higher avidity of IgG to normal HF K16 compared to the normal group. And given the two-fold differences in incidence between AA and control groups, antibodies to this antigen was also tested in functional studies.

While K5 was observed in one immunoprecipitate of each group, K14, K17 and heat-shock protein 27 were identified with higher frequency in the AA group. However, as these proteins were also identified in the negative controls, a case for their relevance in AA could not be made.

While interpreting the difference between the AA group and the normal group, it is also important to take into account the clinical features of the serum donors. The average age of serum donor was higher in the AA group than in the normal group (39 years versus 28 years). In addition, while 90% of AA donors were female, 60% of the control group were female. As age and sex are related to the level of natural autoantibodies and susceptibility to autoimmune diseases in general (Andersen-Ranberg *et al*., 2004; Fairweather *et al*., 2008), it is possible that the level of autoantibody present in the AA group would be higher than in the normal group, whether or not these autoantibodies are AA-relevant. Another factor affecting the amount of AA-relevant IgG in the AA sera is the disease status of the AA patients when the blood samples were taken. The half-

life of IgG in blood is less than 1 month (Table 2). While all AA patients had long standing disease, some were experiencing hair regrowth and hair loss either separately or contemporaneously. Therefore, it is difficult to conclude firmly whether the most disease relevant IgG antibodies were present in the AA patient serum at the time of collection.

Given the aforementioned caveats associated with this experimental approach, it is important that the results should not be interpreted to indicate that AA sera contain antibodies that have the highest reactivity against THH and keratin 16 compared to other potential AA-relevant antigens, which are yet to be identified. As mentioned before, it is not possible to use these data to compare between different antigen proteins. However, the fact that THH was observed in all 10 of the AA sera does suggest that it is one of the major HF antigens that AA patient sera IgG can react to.

Thus, only two HF-associated proteins were identified from the final analysis. This was less than expected. With the suspicion that melanocytes are targeted in AA (Gilhar *et al*., 2001), and previous observations that keratins are also targeted, one would expect proteins related to melanocytes and some other keratins would be identified by this route. It is possible that the level and nature of antigens associated with melanocytes is reduced in these plucked HF extracts compared to whole and intact HFs. This may explains why melanocyte target antigens were not identified in this study. A similar case can be made for the absence of potential AA target antigens associated with the CTS and the FP,

which would not be present in plucked HF extracts. In previous studies by Tobin *et al*. (Tobin *et al*., 1994b) using Western Blotting, there were usually more than two protein bands for most AA sera when they were used for probing HF antigens separated by 1D SDS-PAGE and then transblotted onto the membrane. When looking at several AA sera collectively and then comparing them with normal sera, some of those protein bands in their Western Blots could have been those that were excluded here due to their occurrence in the normal group.

Another important consideration is that the number of proteins identified was fewer than expected possibly because of the very low amount of proteins present in the immunoprecipitates. They may be so low in amount to be outside the lowest detection sensitivity of the instrument. The LC-MALDI-TOF/TOF analysis of normal HF antigens immunoprecipitated by serum AA5 in Proteomics Method Three resulted in positive identifications of 22 proteins (Table 7). However, in this final proteomics experiment, for the immunoprecipitate generated using the same serum AA5, the number of positive protein identifications was only 4 (Table 8). This lower number of proteins identified from immunoprecipitates generated under the same samples and conditions could be caused by a change in the sensitivity of the instruments, as Proteomics Method Three and this final proteomics experiment were conducted 8 months apart, during which the instruments underwent technical services. Another possible explanation for low number of proteins in the immunoprecipitate is the number of fractions into which the LC separated the digested peptides. In Proteomics Method Three this was separated to 336

fractions, but here in this final proteomics experiment it was separated to only 48 fractions. The latter will have therefore a higher number of distinct peptides making the mass spectra more complicated and thus more difficult ultimately for protein identification. If there are proteins with low Mwt present in the sample, or proteins present in low quantity, they would therefore become harder to detect compared to when the peptides were separated into larger number of fractions by the LC.

Given my sometimes limited access to the mass spectrometry instruments, it was not possible to do analysis with 336 fractions for all the 20 sera samples. Also, for this number of fractions, a maximum of two immunoprecipitate samples could be analysed per week. This means it would take at least 10 weeks to complete all the analysis, comparing to three weeks as was done here. Thus such a long experimental time interval may render results of samples analysed less comparable due to possible fluctuations in instrument performance.

These considerations suggest that more potential AA-relevant antigens may be present in the immunoprecipitates, although they were not identified using the assays conducted here in this project. These yet-to-be identified target proteins may be present in the immunoprecipitates in very small quantities. However, IgG antibodies to these HF antigens *in vivo* may still have a role in the disorder, because of the amplifying effect of the immune system once an immune reaction has started (Section 1.7). Some of these HF proteins may also have very low Mwt and so the number of their post-digested peptides will be relatively

small. This will therefore complicate the identification of these low Mwt proteins. In the mixture of all the many proteins, they may not be detected at all or if so, have very low Mascot scores, and provide a small number of peptides for matching with the database.

While Proteomics Method Two was carried out using serum AA5 antibodies as a probe for Western Blotting, one may question why some of the keratins identified in Proteomics Method Two (Table 6) were not observed here in this final proteomics experiment (Table 8), as the normal HF antigen immunoprecipitate of the same serum AA5 was also analysed here. One of the possible explanations is, as mentioned above, that HF antigens can exist being in different conformations for antibody binding during both immunoprecipitation and SDS-PAGE analysis/assays. And thus it is possible for the same antibodies to have different avidity to the same antigens because of conformational changes in their binding epitopes.

Another comparison one may try is the proteins positively identified by LC-MALDI-TOF/TOF (Table 8) and proteins detected using 1D SDS-PAGE of the immunoprecipitate when checking whether the immunoprecipitation and IgG-Protein G cross-linking was successful (Figure 36 (a) to (t)). It is of note that when no signal was detected by the AE1/AE3 antibody this may not infer an absolute absence of keratins in the immunoprecipitated sample. As this AE1/AE3 antibody can detect only 15 types of keratins (see References section, Mouse anti-cytokeratin AE1/AE3 monoclonal antibody), the presence of other

types of keratins would therefore not have been detected. The transblot of proteins from the SDS-PAGE gel to the PVDF membrane is rarely 100% efficient. Some proteins may have been left behind in the gel or lost in the buffer during transblotting and so may help explain why IgG and/or keratins were detected by LC-MALDI-TOF/TOF but not in the Western Blot in some cases (for example, IgG in immunoprecipitates of AA6, and AA7 detected by LC-MALDI-TOF/TOF but not by Western Blotting). Moreover, protein loss is also possible during sample preparation for LC-MALDI-TOF/TOF analysis (e.g. during the desalting step). Therefore, a protein that was detected by the antibodies in Western Blotting could be lost before reaching LC-MALDI-TOF/TOF analysis (for example, albumin was detected in the AA1 immunoprecipitate by Western Blotting but not by LC-MALDI-TOF/TOF).

While proteomics using MALDI-TOF/TOF is often used for protein profiling because of its sensitivity and ability to aid identification of known proteins (c.f. Albrethsen, 2007), its effectiveness in this project may have been somewhat hampered by the very limited availability of antigens within the immunoprecipitates. It was also appreciated during this project that the application of proteomics alone is not enough in identifying AA-the relevant antigens. Prior knowledge of the condition as well as the methodology helps with data interpretation. For example, knowing that these are proteins samples extracted from HFs allowed one to contextualize the data. Researchers not familiar with hair research may have considered all the keratins observed as contaminants. Even so, the identified proteins needs to be confirmed as AA target antigens and their role addressed using functional studies. However,

functional studies are required to determine whether AA is associated with autoimmune reactivities to THH and K16 identified using proteomics. For this organ culture of human scalp anagen HF may also a valuable approach.

Lueking and colleagues used protein microarrays for the development of a protein biochip for AA diagnosis and AA autoantigen profiling (Lueking *et al*., 2005). Similar to this project, they found that AA sera reacted more intensely than control sera to only a very restricted number of antigen targets. However, the Lueking *et al* used target antigens derived from a library of recombinant proteins which were derived from human foetal brain whereas the current project sources antigens from normal human scalp follicles. Therefore, it is unclear how informative human foetal brain protein could be in the search for AA relevant target antigens.

4.7 INHIBITION OF HAIR FIBRE ELONGATION BY ANTIBODIES TO TRICHOHYALIN AND KERATIN 16 IN *EX VIVO* WHOLE HAIR FOLLICLE CULTURE

HFs isolated from skin samples can be cultured for at least 9 days using a well established method (Philpott *et al*., 1996). *Ex vivo* culture of whole human scalp HF was therefore conducted using this method as a functional test of the effect of AA sera, normal sera, anti-THH antibodies and anti-K16 antibodies on

HF fibre elongation. Complement from guinea pig serum was also added to the culture as complement activation is a possible mechanism through which the antibodies can attack the cells in culture (Cui *et al*., 1993; Norris *et al*., 1988). However, the presence of even just 1% of serum in the culture medium can inhibit HF growth (Westgate *et al*., 1993). Anticipating this, HFs were cultured with the same amount of total serum (human or guinea pig) to normalise the effect of the presence of serum in the cultures, so that any difference in hair fibre elongation measured comparing to the control (which also has the same percentage of added serum) can be attributed to the effect of the test serum/test antibody. Moreover, it was shown that having serum in HF culture medium causes hair growth decline after 5 days (Westgate *et al*., 1993). Therefore, HFs were only maintained for 5 days in this study.

Average hair shaft elongation was less for HFs that were cultured with antibodies to THH and K16 for 5 days (Experiment 1), when compared with the negative control which was also cultured with presence of serum. Both of these differences were significant, to $p = 0.009$ for culture with THH antibody and $p = 0.002$ for culture with keratin 16 antibody. As mentioned above the serum level was kept the same including in the negative control, the difference of hair shaft elongation did not appear to be due to the presence of serum in the culture.

Serum has striking morphological effects on HF/hair shaft growth in *ex vivo* cultures (Westgate *et al*., 1993). Comparing serum-supplemented to control

serum-free cultures revealed that the HFs in serum became dystrophic over 5 days in culture.

In freshly isolated anagen HF Ki67-positive cells were mainly observed in the bulb and some in the ORS. However, none or only a few proliferative cells were seen in the bulb for all the cultured HFs after 5 days in *ex vivo* culture. Some scattered proliferative keratinocytes were detected in the ORS however. As the growth of all HFs was coming to an end by day 5 (Figure 43 (a) and (b)), all HFs gave broadly similar morphologies and proliferation levels. The peak in HF elongation at day 5 is probably because of the presence of serum in the culture medium, as HFs have been observed to continue to show hair shaft elongation up to at least 9 days in serum free medium (Philpott *et al*., 1996). To see how the presence and distribution of proliferating cells was affected by the different culture conditions, the HFs would need to be sectioned at multiple time points.

The results suggest that IgG antibodies to THH and K16 antibodies may inhibit HF/hair shaft growth *in vitro* if in the presence of a complement source. Thus, the possible mechanism may be by complement activation as has been demonstrated in other cutaneous cell systems (Cui *et al*., 1993; Norris *et al*., 1988; Arteaga *et al*., 2002). The formation of antibody-antigen complexes can activate the complement components, which subsequently form the membrane attack complex (MAC) (as discussed in Section 1.7). The MAC can then form pores in cell membranes, causing the cells to lyse (Kolb et al., 1972; Podack & Tschopp, 1982).

The second experiment was set up to repeat the first. However, a problem with the use of L-glutamine source was encountered after completion of the study. When reviewing the methods used, this is one possible explanation why the results in Experiment 2 did not concur with those of Experiment 1. The inclusion of L-glutamine used in the culture medium is important for HF metabolism (Philpott *et al*., 1994). Although the same bottle of L-glutamine stored at 4°C was used for both experiments, at this temperature L-glutamine decomposes (Hardy *et al*., 1993). The time gap between the first and second experiment was nearly 1 month. Therefore, the bio-availability of L-glutamine is likely to have been reduced in the second experiment, and may explain why the overall growth of all HFs in the second experiment is considerably less than that of the first experiment (mean of 0.43 mm in the first experiment versus 0.29 mm in the second experiment). This impediment therefore makes it more difficult to distinguish the effects of different treatments over the limited number of days. Unfortunately, this experiment was not repeated due to a shortage of face-lift skin availability at the period of the project.

The absence of observable effects on hair shaft elongation due to the presence of AA and normal sera in the culture medium maybe due to a low absolute amount of anti-HF IgG in these sera, compared to the affinity-purified commercial antibodies (i.e. anti-THH and anti-K16). *In vivo*, low levels of anti-HF IgG may still be pathogenic as they could generate an amplified immune response via co-operative effects of other immune cells and relevant signalling molecules. In this simplified culture model however, the effects of these IgG antibodies on the HF may be below detection impact over this short culture

period, especially when in the presence of serum. It is possible that additional effects may be seen if the HFs could be kept alive in culture for a longer period of time. However, the presence of serum hinders HF growth as discussed above.

Sodium azide (NaN_3) has long been know to be highly toxic to cells (c.f. Chang & Lamm, 2003), and was shown to hinder hair fibre elongation significantly in *ex vivo* HF culture (at 1.04 mM). This was demonstrated by the difference of mean hair fibre elongation on day 5 between control cultures (with serum) and cultures with NaN_3 ($p = 1.45x10^{-5}$ in Experiment 1, $p = 0.003$ in Experiment 2, Figures 42 (a) and (b)). For the HFs cultured medium supplemented with NaN_3, although their growth curves did not show an obvious cessation of the hair fibre elongation by day 5 (Figure 43 (a) and (b)), no (or only very few) proliferating cells were detected by Ki67 antibody when these HFs were sectioned on day 5 (Figure 46 (a) and (b)).

Overall, these results suggested that antibodies to THH and K16 may have had inhibitory effects on HF/hair shaft growth. However, HF *ex vivo* culture conditions are very different and over-simplified compared to *in vivo* systems. Here, the proposed action of the immune system was limited to complement-mediated cell lysis. Actions of other components of the immune system known to be involved in the pathogenesis of AA (for example, T cells, B cells and other signalling molecules) were not taken into account here.

4.8 INDIRECT IMMUNOFLUORESCENCE ANALYSIS OF ANTIBODIES (ALOPECIA AREATA SERA, NORMA SERA, ANTI-TRICHOHYALIN ANTIBODY AND ANTI-KERATIN 16 ANTIBODY) TO NORMAL HUMAN SCALP HAIR FOLLICLES

The anti-K16 antibody reacted with the ORS and CL cells (Figure 47) (Bernot *et al*., 2002), where the anti-THH antibody (AE15) bound to all three layers of the IRS (Figure 48) (O'Guin *et al*., 1992). When comparing the reactivities of AA serum antibodies and normal serum antibodies to the normal HFs (Figure 49 (a) and (b)), AA serum antibodies showed higher reactivity, as observed by the more intense green fluorescence signals. AA serum antibodies bound most frequently to the IRS and ORS, and less so to the medulla and very weakly towards the FP. While reactivities to the pre-cortex were less common, some AA sera (e.g. AA5) showed very intense binding to this compartment. This AA serum (AA5) also had antibodies which were reactive against the matrix (Table 13). The observations here were similar to a previous study (Tobin *et al*., 1997a), demonstrating the heterogeneity of AA serum antibodies with the very different profiles of reactivity by serum from different AA patients towards normal HFs.

An important finding of this study was that the reactivity of AA serum antibodies to antigens expressed in the ORS and IRS was similar to anti-THH and anti-K16 antibody staining. However, it is very likely that AA sera may contain other

antibody reactivities which bind to other antigens (still unknown) also located in these same HF compartments.

In order to determine whether AA serum antibodies actually co-localised with THH and K16, indirect double immunofluorescence analysis of sera and AE15 antibody was performed (Figure 50 (a) and (b)). The region of antigen co-localisation as evidenced by the overlap of red and green fluorescence to give orange/yellow fluorescence was identified in the lower IRS, nearest to the growing hair bulb. This included mostly reactivity to Henle's layer, and less so in the Huxley's layer and IRS cuticle. To determine how antibodies to THH could affect hair growth in AA, greater knowledge of the structure of the IRS in hair biology is needed. The IRS grows distally during anagen only (c.f. Stenn & Paus, 2001). The three layers of the IRS acquire THH and hyalinise, first in Henle's layer, followed by Huxley's layer and lastly in the IRS cuticle (as discussed in Section 1.2). Disruption in hyalinisation and development of these layers could have a serious effect on the growth of the hair shaft and the HF as a whole, as the anagen-specific IRS is at least an important structural support, i.e. counter the down-growth of the hair bulb and ORS for the growing hair fibre. At the bottom of the IRS where yellow fluorescence were observed in some of the HFs (Figure 50 (a) and (b)), the THH should still be in its granular form, before it leaves the granule and cross-links with other proteins in the IRS (O'Guin *et al*., 1992). Therefore, it is possible that the anti-THH IgG in AA sera are binding the granular form of the THH during IRS differentiation and less to the THH on the surface of the granule or cross-linked THH.

It should be noted that the source of THH antibody used in whole HF culture and in the indirect immunofluorescence studies was different. The version used in whole HF culture was a goat polyclonal antibody raised against a peptide mapping near the N-terminus of human THH (see References section, Trichohyalin (N-16): sc-47517). By contrast the antibody used in indirect immunofluorescence was a mouse monoclonal AE15 raised against a single THH epitope which is expressed in THH of all forms (O'Guin *et al*., 1992). It was felt that a polyclonal anti-THH antibody may be more appropriate for the functional study as it 1) may be more likely to represent anti-THH antibody specificities in human sera and 2) more likely to target a broader range of epitopes on the organ cultured IRS. Anti-K16 antibody used in HF culture was also a goat polyclonal antibody. It was raised against a peptide mapping near the C-terminus of human K16 (see References section, Cytokeratin 16 (C-12): sc-49176).

The identification of THH as an AA-relevant antigen echoed the earlier studies by Tobin *et al.* in canine and equine AA (Tobin *et al*., 1998b; Tobin *et al*., 1998a). The inhibition of *in vitro* hair shaft elongation by anti-THH antibody and the co-localisation of anti-THH antibody with AA serum IgG in the IRS of the HF, further support the findings in these previous studies. K16 has a theoretical Mwt of 51 kDa (SWISS-SPROT database). However, its Mwt would appear differently in a SDS-PAGE gel/Western Blot depending on the conditions applied. Therefore, it is not known yet whether it is one of the keratins which were observed previously by Western Blotting (Tobin *et al*., 1994b). However, the binding of AA serum IgG to the ORS observed both here in this project and

in previous studies (c.f. Tobin & Bystryn, 2000) supports the possibility of K16 being an AA-relevant antigen.

It is possible that antibodies to THH and keratin 16 can lead to the early anagen arrest as observed in disease pathogenesis if these antibodies were functionally active at an anagen-specific site required for the maintenance of the growing state. The formation of the IRS, CL and ORS all occur during early anagen (Stenn & Paus, 2001). An IgG-associated deformation of all of these layers, or even just one of these layers, could lead to loss of support for the whole HF structure.

As HF is reported to be an immune-privileged site (c.f. Paus *et al*., 2005), it is conceivable that if IP collapses in AA then normally expressed normal antigens may be targeted (Paus *et al*., 2004). One of these immune system responses defects could be the upregulated expression of MHC class I and II molecules, leading to the inappropriate presentation of HF proteins to the immune system which subsequently could trigger a full immune response against these HF antigens (Paus *et al*., 2003). The results presented here implicating a role for THH and K16 as AA target antigens is just the beginning of a long journey in further confirming them as functional targets in AA and why these antigens are prone to triggering the IgG response in the pathogenesis of AA. However, by knowing the relevance of THH and K16 in AA, it may be possible to block an immune response to these proteins in AA. Moreover, it should also be possible to determine whether lymphocytes populations in AA are oligoclonal to these

antigen targets, and thereby can induce a cytotoxic response to associated HF cell populations.

CHAPTER 5.

CONCLUSION AND FUTURE WORK

A method of isolating AA-relevant antigens from normal human scalp HF extract using AA sera was developed (i.e. by immunoprecipitation) and for target antigens in these immunoprecipitates identified using proteomics. Two antigens, THH and K16 were identified as particularly important in this regard. Polyclonal antibodies to these two HF-associated antigens were both able to inhibiting hair shaft growth *in vitro* during a whole HF organ culture model (Philpott *et al*., 1996). Indirect double immunofluorescence study of reactivity of AA sera antibodies and anti-THH antibody confirmed co-localisation in the IRS near the anagen hair bulb. A disruption in the functioning of the IRS could have a destructive effect on the growth of the HF.

If greater availability of AA serum from patients with active disease were possible, it would be interesting to test different IgG-purification methods to gain greater purity and recovery of relevant AA autoantibodies. These autoantibodies would be very useful for the potential immunoprecipitation of more AA-relevant antigens both in terms of quantity and identity., they may also be very useful for functional studies.

The use of the 'shot-gun' proteomics approach to identify AA-relevant antigens was more suitable than the use of 2D gel electrophoresis, as it maximised the amount of antigens available by using the whole immunoprecipitate rather than protein spots isolated from gels. Moreover, if it is possible to immunoprecipitate higher quantities of AA-relevant antigens by using a higher amount of starting material, it would be possible to compare quantitatively the amount of these

antigens between each immunoprecipitate analysed, by using isotype-coded affinity tags or isobaric tags. The latter allow for protein quantification in relative and absolute terms (Smith *et al*., 2007).

The identification of THH as an AA-relevant antigen in this project concurs with its identification in previous studies using different approaches from this project (Tobin *et al*., 1998b; Tobin *et al*., 1998a). It would be interesting to see the effect of antibodies to THH in models that more closely resemble the *in vivo* situation. Indirect double immunofluorescence staining of AA sera with AE16 or AE17 antibodies, which are specific for THH on the surface of THH granules THH cross-linked with other intermediate filaments (O'Guin *et al*., 1992), may be able to identify which precise form of THH is preferentially targeted by the AA sera.

It would also be important to repeat the *ex vivo* HF organ culture study. This would consolidate the results obtained from Experiment 1 and thus elaborate on the effect of anti-THH and anti-K16 on hair growth observed here. It may also be possible to test the effect of these antibodies on *ex vivo* HF organ culture in the absence of serum. While it may be resource demanding, commercially-available individual complement components, or complement components purified in house could be used in the HF culture instead of serum. Without the inhibiting effect of serum the HFs could be maintained in culture for more days as well as giving a better demonstration of the effects of the test antibodies on HF growth and morphological changes. Moreover, to further demonstrate the

effects of anti-THH and anti-K16 antibodies in this *ex vivo* HF culture, studies using blocking peptides to these antibodies could also be used. To observe further the relevance of THH and K16 to AA pathogenesis, passive transfer of AA to animal models by injection of T cells activated by these proteins may also be valuable.

Clearly, other AA targets antigens remain to be found. However, in addition to conducting experiments with the AA-relevant antigens (including those already been suggested in various studies), it is important to understand how and what is driving abnormal immune response against these antigens in AA pathogenesis.