University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student:

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2. Supervisor:

   Surname: **Milling** Forename: **Simon**

   E-mail address: **simon.milling@glasgow.ac.uk**

3. Research Project Report

   3.1 Project Title (maximum 20 words):

   Investigation of cytokine profiles in alopecia areata patients

   3.2 Project Lay Summary (copied from application):

   Alopecia areata (AA) is the most prevalent autoimmune disease and has a lifetime risk of 1.7%. Close examination of the genetics of people with AA has recently revealed that this disease has much in common with other forms of autoimmunity. In particular, the immune system in AA patients is altered. Cells that normally respond to infections are not properly controlled and attack hair follicles, causing alopecia. Treatments that inhibit these immune responses have been shown to limit the disease in AA patients and in animals with similar symptoms. However, little is known about why these immune responses lose control, and begin to cause hair loss. This study will involve analysing blood samples from AA patients and healthy controls to identify important changes in the blood that are associated with this condition. We will be measuring the levels of specific proteins in the blood that will tell us about the type of immune response in individual AA patients. We will then associate these changes in the blood with clinical features such as the type of AA a patient has, how long they have had hair loss for and whether they are in a period of active disease or not.

This will help us to understand how the disease varies between patients and how the immune response varies depending on how severe the symptoms are.

3.3 Start Date: **15/05/2017** Finish Date: **23/06/2017**

3.4 Original project aims and objectives (100 words max):

The project is intended to collect circulating immune cells (Th1 and Th17) and cytokine (IFNγ) blood stream levels of six AA patients to analyse and compare with a similar number of healthy control (HC) individuals. We hypothesize, hence, that it would be feasible to establish significant changes in cytokines, circulating immune cells and their functional protein machinery that characterize the onset of AA.

3.5 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

Peripheral blood collection and processing

18mL of peripheral blood was collected from six consented participants with AA at a research clinic based at the Queen Elizabeth University Hospital. Healthy peripheral blood for comparison purposes was collected from volunteers (Glasgow University staff).

At room temperature (RT), under a sterile hood, blood was diluted 1:1 in PBS, and layered on top of 4 mL of histopaque (Sigma), in a 15mL centrifuge tube. Samples were centrifuged at 2100rpm for 25 minutes at RT, with no brake applied. Plasma was collected and stored for cytokine profiling. The peripheral blood mononuclear cells (PBMCS) layer was extracted and washed twice in PBS, once at 400g and then slowly at 200g, each for 10 minutes at 4°C. Cells were counted using a haemocytometer and the number of cells per mL of blood was calculated and recorded.

Multicolour flow cytometry analysis (FCA)

Cells were stained for viability for 30 minutes at 4°C. Cells were washed with FACS buffer (FB), i.e. PBS supplemented with 2mM EDTA and 2% FCS, at 400g for 5 minutes, and subsequently resuspended in 1 mL FB. Antibody mixes were prepared in FACS tubes for each sample, as illustrated in table 1. Cells were added to the antibody mixes and incubated at RT for 30 minutes. Cells were washed with FB, fixed and run on the BD LSR Fortessa the following day.

Sandwich ELISA

IFNγ ELISAs (ELISA max deluxe kit, Biolegend) were carried out according to the supplier's protocol. The protocol was amended slightly by using a 50µL of samples rather than the recommended 100µl.

3.6 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

The analysed cohort was very varied biologically because the six AA patients were affected by different AA conditions, as shown in table 2. Hence, the PBMCs

count here reported was not used to infer the total number ofimmune cells from their percentage level obtained at Fortessa.
PBMCs were isolated from debris using the FCA by gating FSC and SSC - hence according to their size, shape and height. Dead cells were excluded thanks to the viability stain and $CD4^+$ T cells were identified as shown in Figure 1a. Th1 and Th17 cells were identified using CXCR3 and CCR6 as surrogate markers (figure 1a). FCA revealed no significant differences between the relative percentages of gated PBMCs between HC and AA samples (figure 1b, c, d, e, f). In particular, whereas the frequency of total $CD4^+$ cells is unvaried, a slight increase (2.5%) of the $CCR6^+$ percentage in AA samples can be noted. In the CXCR3 graph, then, one of the AT patient (AA102) presents the highest AA value while the other AT sample collected (AA104) shows exactly the opposite behaviour. Similar patterns are observable in CLA graph. Moreover, overall circulating Th1 and/or skin homing (CLA^+) T cells show more scattered blood frequencies among individuals than Th17 or gut homing ($CCR9^+$) T cells, independently from the HC/AA categories.
Since AA is reported to be a Th1-related autoimmune disease and naïve T cells activation and differentiation in Th1 is enhanced by the release of IFNγ from activated T cells, an IFNγ sandwich ELISA was performed with four AA and HC samples. Again, there was no substantial increases in the concentration levels of IFNγ in AA plasmas but the fashion of data was more spreaded than those in HCs (figure 2). Furthermore, AA104, i.e. the only Alopecia Totalis (AT) sample assayed, presents the highest concentration of IFNγ in its plasma. This is immediately preceded by AA105 that shows regrowth under Dyphencyprone treatment. Their concentrations, then, is roughly two-fold that of patchy AA plasmas.

3.7 Discussion (500 words max):

The results illustrate above do not confirm our hypothesis. However, they are most definitely insufficient either to accept or to reject our hypothesis. A larger cohort of AA patients would have probably allowed us to establish significant changes in their cytokine levels and to characterize the quantity and the percentages of the immunophenotyped PBMCs in peripheral blood of AA individuals (Suarez-Farinas et al., 2015). The six AA blood samples collected have overly various clinical pictures to constitute a suitable cohort for our hypothesis (table 2). Moreover, the fact that no significant changes - in terms of inflammatory cytokines and relevant PBMCs – were associated with the AA blood samples collected is mainly related to the few numbers of samples collected. Indeed, differences, even if not significant, can be already signalled between the well clustered $CCR6^+$ cell populations of figure 1c. On the other hand, the contradictory behaviour of the two AT with $CXCR3^+$ and CLA^+ cells demands more specimens to be understood. AT patients present a complete hair loss of the scalp, implying a broader inflammation that should be detected systemically (Tembhre and Sharma, 2013). In figure 1, some frequency values for AA103, AA104 (AT) and AA105 were not reported because not reliable. Since these three samples have been run at the Fortessa in the same day, it is plausible that errors have occurred either in the antibody mix addition or in the FCA. Incorrect

implementations of IFNγ ELISA are responsible for the further waste of useful data – one of which was AT - whose replacement was impeded from time constraints (table 2). It is however interesting for our hypothesis that AA104 (AT) has the highest IFNγ concentration (figure 2). It is worth noting that the high IFNγ measured in AA105, in spite of the regrowth stage, could be related to the diphencyprone (DCP) treatment itself (Hoffmann et al., 1994).
Nevertheless, the absence of statistically significant results might suggest that the blood cytokines and PBMCs are not altered systemically in the active phase of AA. However, the cardinal signs of inflammation in the hairless patches are trivial (Ma et al., 2017). Therefore, it is plausible that an increase in Th1, Th17 skin homing cells and proinflammatory cytokines is only detectable before the onset of hair loss, for example at the start of the loss of immune privilege by the hair follicle (Xing et al., 2014). This would be followed by the consequent enhancement of a CD8+ T cells response driven by the production of cytokine-dependant immune response elicited by Th1 cells, Th17 cells and, to a minor extent, also by Th2 cells (Ma et al., 2017). This inflammation would tend to become chronic with potential deterioration of AA in AT or Universalis – completely hairless patients (Renert-Yuval and Guttman-Yassky, 2016). Hence, the best place where to search for elicited cytokines and immune T cells, is not the blood but the affected scalp (Suarez-Farinas et al., 2015).
In conclusion, results neither support nor bury our hypothesis of characterizing the onset of AA by collecting, analysing and comparing Th1, Th17 and IFNγ from peripheral blood of HC and AA individuals. The continuation of the same protocols with a greater awareness towards manual or other technical errors, the enlargement of the cohort and the implementation of skin biopsies from the blood donor AA patients, is suggested for the hypothesis to be entirely demonstrated, or confuted.

4. Reflection by the student on the experience and value of the studentship (300 words max):

The value of this studentship for me, both as scientist and – above all – human being, has been inestimable. I have been kindly hosted by Dr. Milling in a high-quality laboratory, for research equipment and commitment, at the fourth level of the Glasgow Biomedical Research Centre, a leading institution in biomedical research. Here I have been welcomed by a wonderful group of people both for humanity and professionality. Thanks to the weekly alternate journal clubs and lab meetings, I could learn how to critically read a scientific paper, i.e. by carefully analysing its figures, and how to elegantly present the results obtained in the own research project and its future objectives. By ruthlessly hassling the people of the team with my curiosity, I also had the fantastic opportunity to learn about several other research projects and experiments, gathering the countless difficulties of their design and implementation. For the commendable time and breath wasted in this sense, I owe a special mention to the affable postdoc Alberto Bravo who, thanks to the sacrifice of a laboratory animal, has taught me as well how to dissect a mouse and how to identify and extract the different inner anatomical parts.
However, my most profound sense of gratitude and affection deservingly goes to my actual supervisor, instructor, advisor, patient listener and reviewer as well as brilliant PhD student, Kym Bain without who this experience would have not been the same. She taught me everything: from blood samples processing to flow cytometry analysis,

from cytokine ELISAs to cell immunostaining, from safe sterile laboratory practices to the hallmarks of an appropriate scientific writing.
In conclusion, the Mucosal Immunology research people are joined by a robust mutual sense of respect and esteem that is wielded by a sincere feeling of amiability and friendship. Inevitably, this peaceful environment has largely contributed to make this experience unforgettable.
I will always be grateful to these people for having given me the chance to live it for these amazing six weeks.

5. Dissemination: (note any presentations/publications submitted/planned from the work):
None.

6. Signatures:

Supervisor Date 19th July 2017

Student Date 20th July 2017

Appendix

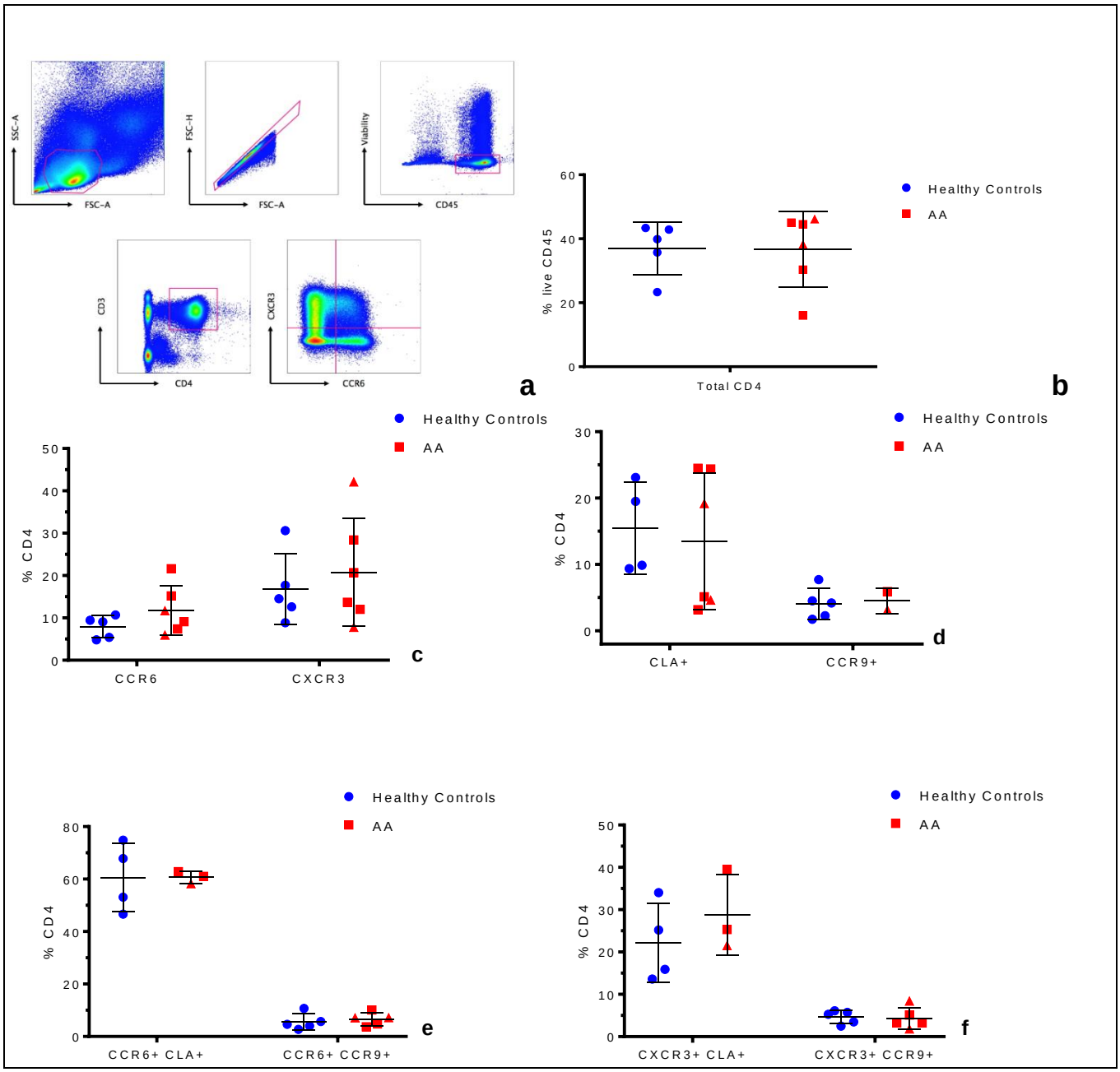

Figure 1. Frequencies of T cell subsets in peripheral blood of six Alopecia Areata (AA) patients and five healthy controls (HC). *(A) CXCR3+ T cells and CCR6+ T cells were isolated through the illustrated gating strategy. (B) Frequency of CD4 T cells as a percentage of CD45+ live cells. (C) Frequency of CCR6+ and CXCR3+ CD4 T cells as a percentage of total CD4 T cells. (D) Frequency of CD4 T cells expressing cutaneous lymphocyte antigen (CLA - skin homing marker) or CCR9 (gut homing marker) as a percentage of total CD4 T cells. Frequency of CCR6 (E) and CXCR3 (F) cells expressing CLA and CCR9. The red triangle points are used throughout the entire figure to indicate the results relative to the Alopecia Totalis (AT) samples. Due to technical errors with the staining, a HC and three AA sample miss some marker measurement, vanishing from the graphs. Error bars represent +/- standard deviation. Welch's t test was used to calculate P values for statistically significant concentration differences between HC and AA unpaired groups.*

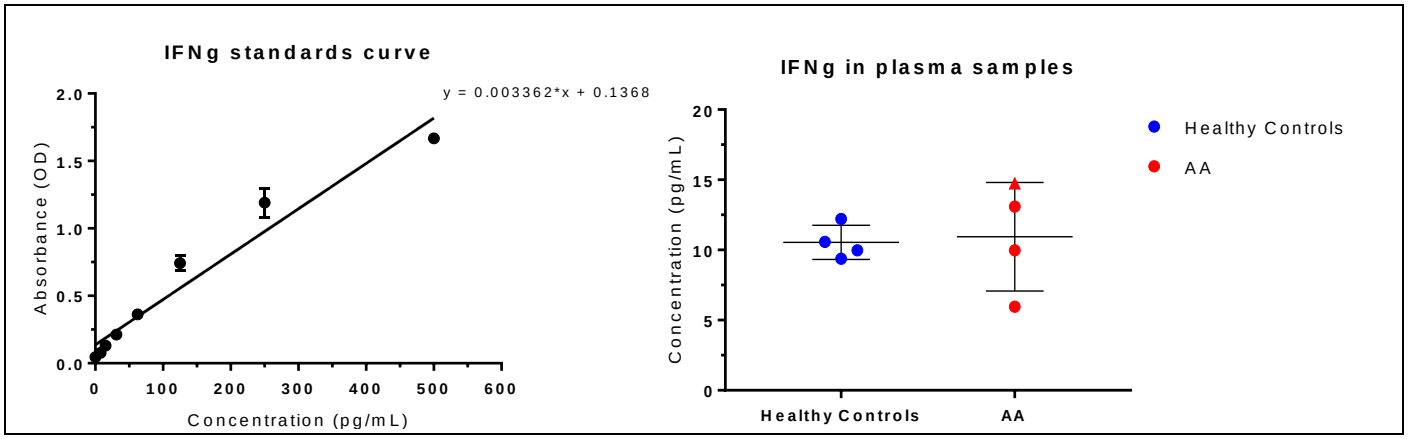

Figure 2. Standard curve and samples concentration of IFNγ sandwich ELISA. *The IFNγ levels in the plasma of four AA and HC blood samples were tested to detect any increase in systemic inflammation in AA with respect to HCs. Two AA and one HC plasma samples, previously collected, could not be used due to errors in the handling procedure. The standard curve, on the left, allowed us to collect reliable measurements for all the samples, plotted on the right. In the samples graph the inferred concentrations are plotted against the cohort group to which they belong. Red triangle represents again AT sample. Error bars represent +/- standard deviation. Welch's t test was used to calculate P values for statistically significant concentration differences between HC and AA unpaired groups.*

T cells antibody staining strategy for flow cytometry analysis			
Markers	T cells tube	ISO tube	Unstained tube
CD3	AF700[2]	AF700[2]	None
CD8	PacBlue/BV421[2]	PacBlue/BV421[2]	None
CD4	BUV395[2+1]	BUV395[2+1]	None
CXCR3	PerCP[4]	mIgG1k[2]	None
CCR6	BV605[4]	mIgG2bk[2]	None
CLA	PE[3]	RatIgMk[1]	None
CCR9	APC[5]	APC[5]	None
Cells (µL)	200	200	100

Table 1. Summary of the antibody staining for immunophenotyping of PBMC samples for T cells flow cytometry analysis. *The names of the chromophore antibodies are written under the label of each tube and their aliquot volume is at their apex. For CD4 stating a UV biotin secondary antibody system was used. Unstained tube was used to help with the flow cytometry machine compensation, hence just half of the cell solution was used. Isotype (ISO) tube was used to detect 'noise' in the measurement of CXCR3, CCR6 and CLA.*

Samples	State of AA condition	PBMCs count (10^6 cells/mL)	Flow cytometry analysis	IFNγ ELISA
AA100	Past onset currently subsided	1.2	Implemented	Not implemented
AA101	Patchy	1.15	Implemented	Not implemented
AA102	Totalis	0.76	Implemented	Not implemented
AA103	Patchy	0.36	Implemented	Implemented
AA104	Totalis	0.6	Implemented	Implemented
AA105	Regrowth with DCP treatment	0.4	Implemented	Implemented
AA106	Patchy	2.14	Not implemented	Implemented

Table 2. Summary of clinical conditions, PBMCs count and analyses implemented for each collected sample. *The table illustrates the current AA condition of each patient at sample collection. The estimated concentration of PBMCs in the blood samples is reported together with those tests that have been actually implemented, and hence reported. Whereas flow cytometry analysis for AA106 could not be implemented because of time constraints, the incorrect implementation of ELISA for AA100-102 plasmas has caused their consumption without any reliable result.*

References

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