5. Conclusions and future perspectives

5.1 Conclusions

Throughout this thesis I presented the results of a transcriptional characterisation of cytokine induced cell states. For this study, we focused on human $CD4^+$ T cells and macrophages, assessing their response to cytokines associated to autoimmunity. At first, we optimised experimental protocols to isolate, stimulate and polarise naive $CD4^+$ T cells and monocyte derived macrophages, ensuring that the phenotypes obtained agreed with their description in the literature. These findings were confirmed by low coverage RNA-seq, where we identified expression of known markers such as T-bet and IL-12 in Th1 cells, GATA3 and IL-4 in Th2 cells, IL-17F and RORC in Th17 cells, FoxP3 and CTLA-4 in iTregs, COX-1 in M1 macrophages and COX-2 in M2 macrophages. Furthermore, we also proposed possible surface markers for $CD4^+$ T cell subsets using an unbiased approach that combines gene ontology annotation, GO term overrepresentation analysis and differential gene expression.

We performed differential expression analysis and identified combinations of cytokines and time points which might cause transcriptional changes in each cell type. Based on this analysis, we hypothesise that polarisation of $CD4^+$ T cells to known lineages is long lasting, with steadily increasing changes only slightly apparent at 16 hours. On the other hand, polarisation with IFN-β seemed to cause more immediate and transitory changes.

We proposed functional implications of transcriptional changes. For example, we observed similarity between the Th0 and Th1 transcriptomes, which suggested that $CD4^+$ T cells stimulated with our method might acquire a state similar to Th1. We also hypothesise that Th2 cells downregulate the type I IFN signalling pathway, and proposed that this downregulation could be mediated by IRFs. Moreover, we observed that downregulation of the PI3K pathway might be involved in differentiation of iTreg and Th17 cells. Using gene co-expression network analysis, we hypothesised that this might involve suppression of the Th2 differentiation program by the TF Sox4.

We also seemed to confirm the proinflammatory phenotype of M1 and the anti-inflammatory phenotype of M2 macrophages. Furthermore, we observed that M1 differentiation might be mediated by STAT1, and M2 differentiation by STAT6. When studying the effect of cytokines associated to autoimmunity, we observed that TNF-α seems to have no effect on $CD4^+$ T cells but significant effects on macrophages, where it promotes secretion of proinflammatory

cytokines, apoptosis and necroptosis. Polarisation of macrophages with IL-26 shared some of these features, possibly regulated by NFκB. Conversely, there seems to be no effect in macrophages stimulated with IL-23 or in T cells stimulated with IL-21, IL-27, and IL-10.

Finally, we assessed the reproducibility of LC-MS/MS coupled to TMT-labelling for quantitative proteomic analysis in $CD4^+$ T cells. The results of this methodology appear to be reproducible and correlate well with observations at the RNA level.

5.2 Future perspectives

5.2.1 Characterisation of heterogeneity and plasticity in cytokine induced cell polarisation

Despite our success in identifying pathways and possibly involved in cytokine induced cell polarisation, the results from this study suggest that cells in culture respond differently to the same cytokine stimulus. For example, we observed that TGF-β only induces FoxP3 expression in 65% of cells, and the same is true for other conditions and TFs. Thus, it is unclear if the remaining cells do not respond to the stimulus, or if several types of response coexist, generating a heterogeneous population. Furthermore, polarised cells are plastic and can interconvert from one phenotype to another (3, 65). Single-cell RNA-sequencing (scRNA-seq) is a high-resolution technique which can successfully identify subsets of cells that display characteristic gene expression profiles within a population (205, 206). Thus, performing a similar study using scRNA-seq instead of bulk RNA-seq would inform on the existence of subpopulations of cells upon cytokine induced polarisation. According to our results, this would be particularly useful for studying Th2, Th17 and iTreg cells stimulated for five days. Furthermore, scRNA-seq can also characterise changes in gene expression throughout time with higher resolution, accounting for differences in response time between individual cells (207).

5.2.2 Identification of novel drug targets

Some of the results of this study are particularly relevant for drug development. For example, TNF-α blockade is a major therapeutic strategy for treating conditions such as RA (2, 119), however the role of TNF-α in disease is still unclear and some patients are unresponsive to treatment. Here we found that the effects of this cytokine on macrophages are very similar to those of IL-26. An immediate question is if the combination of IL-26 and TNF-α blockade

would improve the current treatment. To evaluate this, we would need to recapitulate the results in a more powered study and to characterise the mechanism behind this observation. For example, the effect of autocrine TNF-α secretion would need to be discarded. Furthermore, characterising the response to cytokines like IL-1β, IL-23, and IL-27 on different cell types might help identifying more specific strategies for cytokine blockade which could be used as therapies.

5.2.3 Surface marker validation and characterisation of cytokine induced cell states *in vivo*

In this study, we identified potential novel markers to isolate $CD4^+$ T cell subset directly from blood. However, these markers were only based on mRNA levels and need further validation, specially given our power limitations. In the short term these markers should be used for staining cells with antibodies specific for them, and further isolating cell subsets enriched in the expression of these markers from peripheral blood using flow cytometry. The cytokine secretion pattern of these cells could then be characterised using flow cytometry, Luminex and ELISA. Direct isolation of cytokine induced cell states from peripheral blood would significantly improve our understanding of cell subsets in the human immune system, reducing the need of *in vitro* models.

In the long term, developing a proteomics based approach to identify surface markers would be useful. Based on the high correlation of our LC-MS/MS and RNA-seq data, we hypothesise that LC-MS/MS would be an appropriate methodology. To achieve this, the next step would be to quantify the full proteome of specific cell types using TMT LC-MS/MS. This study has already been started in our group for the cell states here described.

5.2.4 Intersection with GWAS variants and prioritisation of cell states relevant to autoimmunity

Improving our understanding of diseases like MS, RA and IBD is a major motivation for genetic studies. Genome wide association studies (GWAS) have identified hundreds of variants linked to autoimmunity (208-210). Most of these localise to non-coding regions (211) and are difficult to interpret. The study of their contribution to disease is complicated by the multiplicity of cell types and cell states in the immune system (44) and it remains unclear in which cell states these variants have a functional implication.

To assess the implication of risk variants in each cell state, gene expression data from this study could be intersected with catalogues of GWAS SNPs linked to immune disease (212). If variants are highly enriched within genes differentially expressed in a particular cell state, this cell might be prioritised for the studied disease (212). In the short term, this is attainable with our. Moreover, a similar study with larger sample size and sequencing depth is already being carried out by our research group.

However, most risk variants are non-coding and will not be unequivocally assigned to a target gene based solely on genomic proximity. Hence, in the longer term a characterisation of the epigenetic context and chromatin landscape upon cytokine induced polarisation is needed. To this end, ChIP-seq (213, 214) can be performed on the cytokine induced cell states described here. Next, specific chromatin modifications which mark active enhancers, active promoters or inactive chromatin (213) can be identified. These annotations can then be used to prioritise relevant cell states by intersecting the chromatin marks with GWAS SNPs (215). This approach is expected to achieve a better resolution than enrichment analyses which use only RNA-seq data. ChIP-seq is already being performed in our research group for the cell states here described.

5.2.5 Epigenetic characterisation of cytokine induced cell states

Throughout this study we failed to identify substantial differences in gene expression after 16 hours of cytokine polarisation in $CD4^+$ T cells. The next step would be to repeat the analysis for selected conditions using a more powered design. However, it is also possible that only epigenetic changes are apparent at this time point. To assess this, we would need to characterise the chromatin landscape, as well as the activity of specific TFs in these cell states. This could be done using the assay of transposase accessible chromatin followed by sequencing (ATAC-seq) (216), which has been successful in mapping chromatin accessibility and TFBS occupancy (217). This approach would help us assess hypotheses generated by our results, for example whether Sox4 and Msx2 really coordinate part of the Th17 and iTreg differentiation programs.