3. Optimisation of methods for cytokine induced polarisation of primary human immune cells

3.1 Overview

Throughout this study, we aimed to characterise the response to cytokine induced polarisation in immune cells by analysing specific cell states generated *in vitro*. In this chapter, I describe the optimisation of our experimental setup for isolation, stimulation and polarisation of human naive $CD4^+$ T cells and monocyte derived macrophages. Finally, I summarise the results of a phenotypic characterisation of the cytokine polarisation cell states generated.

3.2 Differentiation and phenotyping of monocyte derived macrophages

To promote monocyte to macrophage differentiation, we cultured $CD14^+$ monocytes with M-CSF for 7 days and evaluated the efficiency of the differentiation compared with untreated monocytes. We observed that the expression of CD14, CD68, MerTK, and HLA-DR increased after seven days of differentiation (**Figure 3.1**), indicating that our cell culture conditions successfully induced monocyte to macrophage differentiation (153).

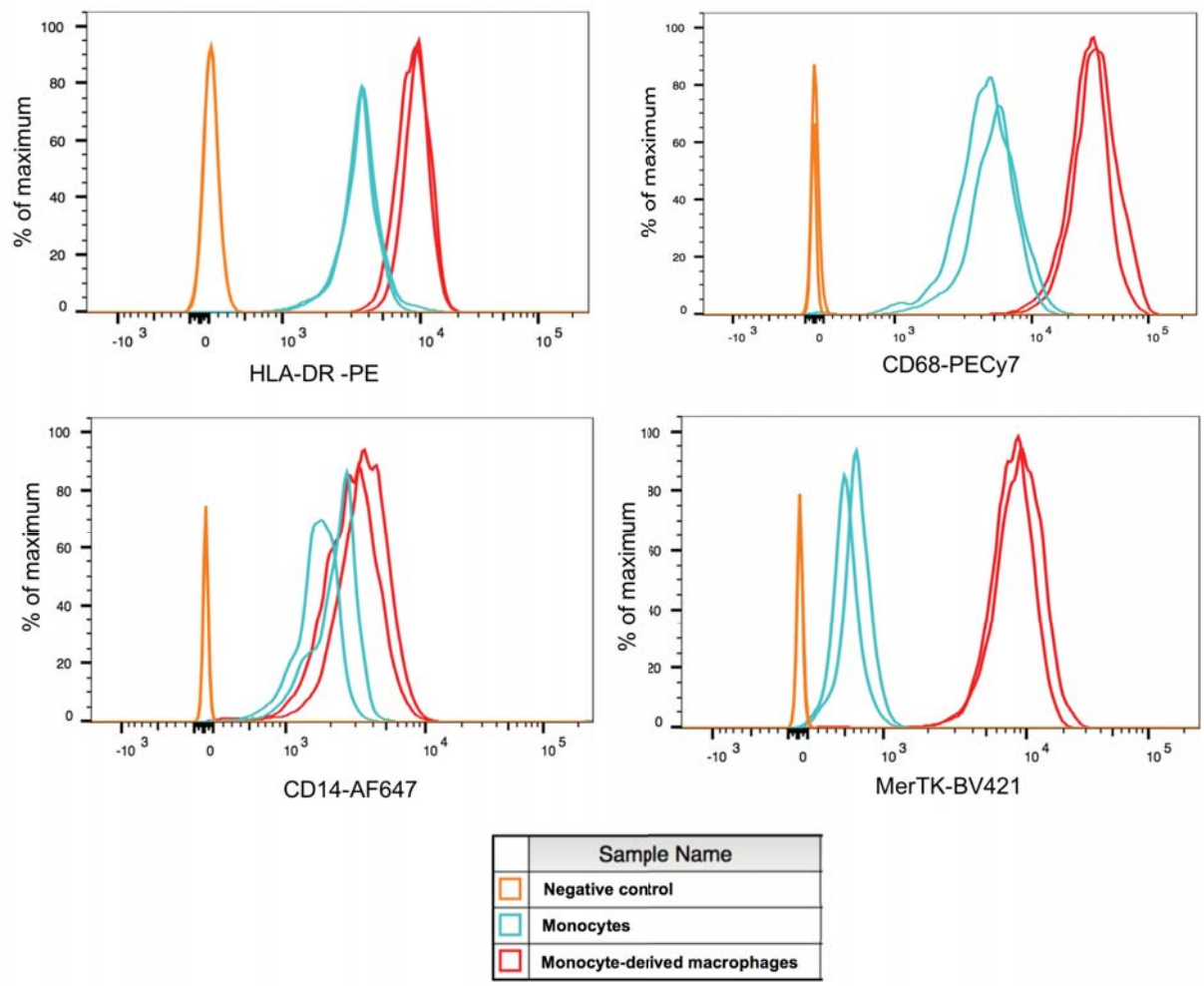

Figure 3.1 M-CSF promotes monocyte to macrophage differentiation. Following seven days of treatment with M-CSF, macrophages were stained for HLA-DR, CD68, CD14 and MErTK, and analysed via flow cytometry. Monocytes kept in culture without M-CSF for seven days as well as unstained cells were used as negative controls. Results are shown as percentage of maximum (normalised to mode measurements), with each color representing a different culture condition.

3.3 Isolation and stimulation of naïve CD4$^+$ T cells

Naive CD4$^+$ T cells were isolated from peripheral blood using magnetic selection followed by FACS. Isolated cells were of high purity, positive for CD45RA, and expressed low levels of CD25 and CD127 (**Figure 3.2**). Thus, we concluded that the population obtained was mostly composed of naive CD4$^+$ T cells and proceeded to use this method throughout our study.

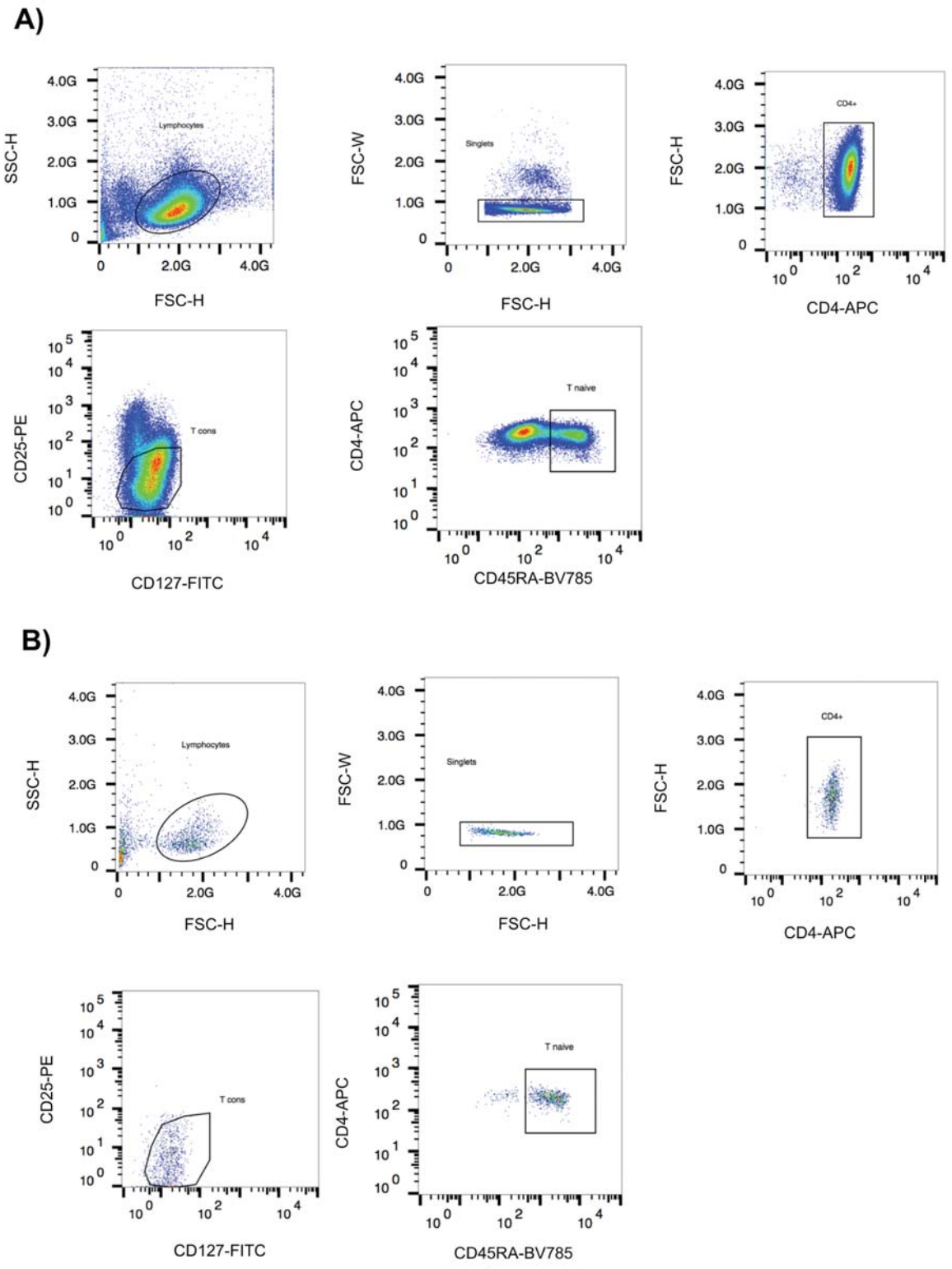

Figure 3.2 Naïve CD4$^+$ T cells are isolated using fluorescence activated cell sorting. **A)** CD4$^+$ cells were enriched from PBMCs, stained and separated via flow cytometry using the gating scheme in this figure. "Conventional T cells" (T_{cons}) were identified as CD25^{low} and naïve T cells as CD4$^+$ CD127^{high} CD25^{low} CD45RA$^+$. Example performed on PBMCs from one individual. **B)** After CD4$^+$ T cell isolation, purity was assessed by flow cytometry. On average, 90% of cells were CD4$^+$CD25^{low}CD127^{low}CD45RA$^+$. This is an example of such purity analysis performed on cells from one individual.

To assess the efficiency of T cell activation, following stimulation with anti-CD3/anti-CD28 antibodies we stained cells for the expression of the activation markers CD25 and CD69 (154). We observed that on average 85% of cells expressed CD69, and 80% increased CD25 expression after 16 hours of activation. A large $CD25^{+}CD69^{+}$ population emerged which was not present in the unstimulated samples (**Figure 3.3**). We thus concluded that stimulation was effective.

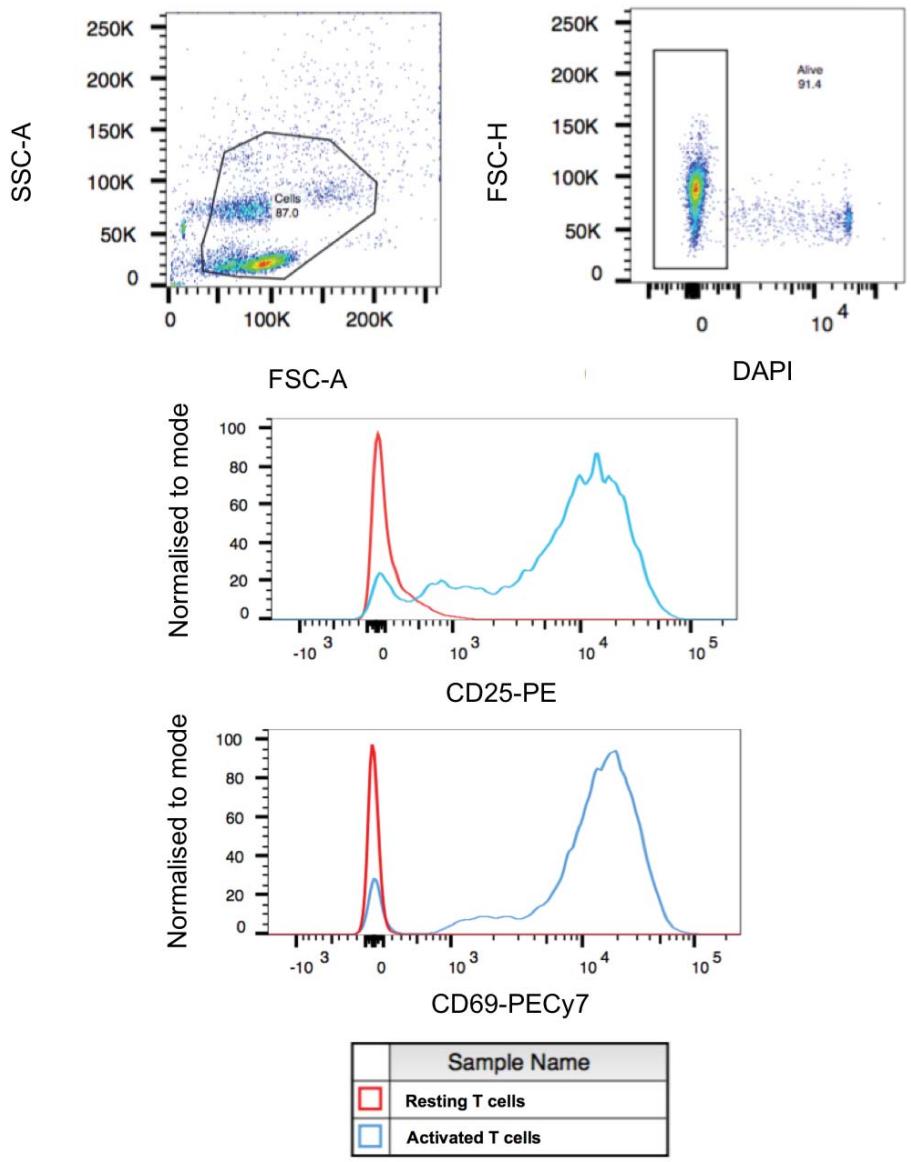

Figure 3.3 T cells express CD25 and CD69 following activation. After 16 hours of activation, the viability and surface marker expression of $CD4^{+}$ T cells were analysed by flow cytometry. Only $DAPI^{-}$ cells were used to determine the percentage of CD69 and CD25 expressing cells.

3.4 Determination of optimal T cell activation conditions

Given that activation beads tightly interact with T cells, their mechanical removal inherently results in cell loss. Thus, we proceeded to estimate the ratio of beads to cells that achieved the maximum efficiency of activation with the minimal cell loss upon beads removal. To do this, we stimulated naïve $CD4^+$ T cells with a decreasing beads-to-cells ratio and assessed the activation efficiency through the expression of CD69 at 16 hours. The data from these experiments was used to build a titration curve (**Figure 3.4A**). We found that both a 1:1 and a 1:2 beads to cells ratio achieved on average over 90% CD69 expressing cells (**Figure 3.4A**). Since the difference in activation was minimal, we decided to measure the cell loss observed with a 1:2 beads to cells ratio. To estimate the percentage of cells lost due to beads removal, we stimulated naive $CD4^+$ T cells and magnetically removed the beads after 16 hours of culture. The cells were then counted via flow cytometry using counting beads. The total counts were compared with a sample from the same individual in which no removal of beads was performed. On average, magnetic removal of activation beads at the beads-to-cells ratio of 1:2 caused 30% cell loss (**Figure 3.4**). We considered this number acceptable to use throughout the rest of the study.

We wanted to ensure that the presence of cytokines would not affect the efficiency of T cell activation. To assess this, we stimulated naïve $CD4^+$ T cells for 16 hours as previously described in the presence or absence of polarising cytokines. Following cell culture, the expression of CD69 and CD25 was measured by flow cytometry and compared to unstimulated $CD4^+$ T cells cultured for 16 hours. We observed a clear upregulation of both CD69 and CD25 in $CD4^+$ T cells upon activation which was independent from the polarising condition (**Figure 3.5**). More than 90% cells expressed CD69 regardless of the cytokines they were polarised with, and on average 75% expressed CD25. Therefore, these results implicated that cytokines did not affect the percentages of activated T cells.

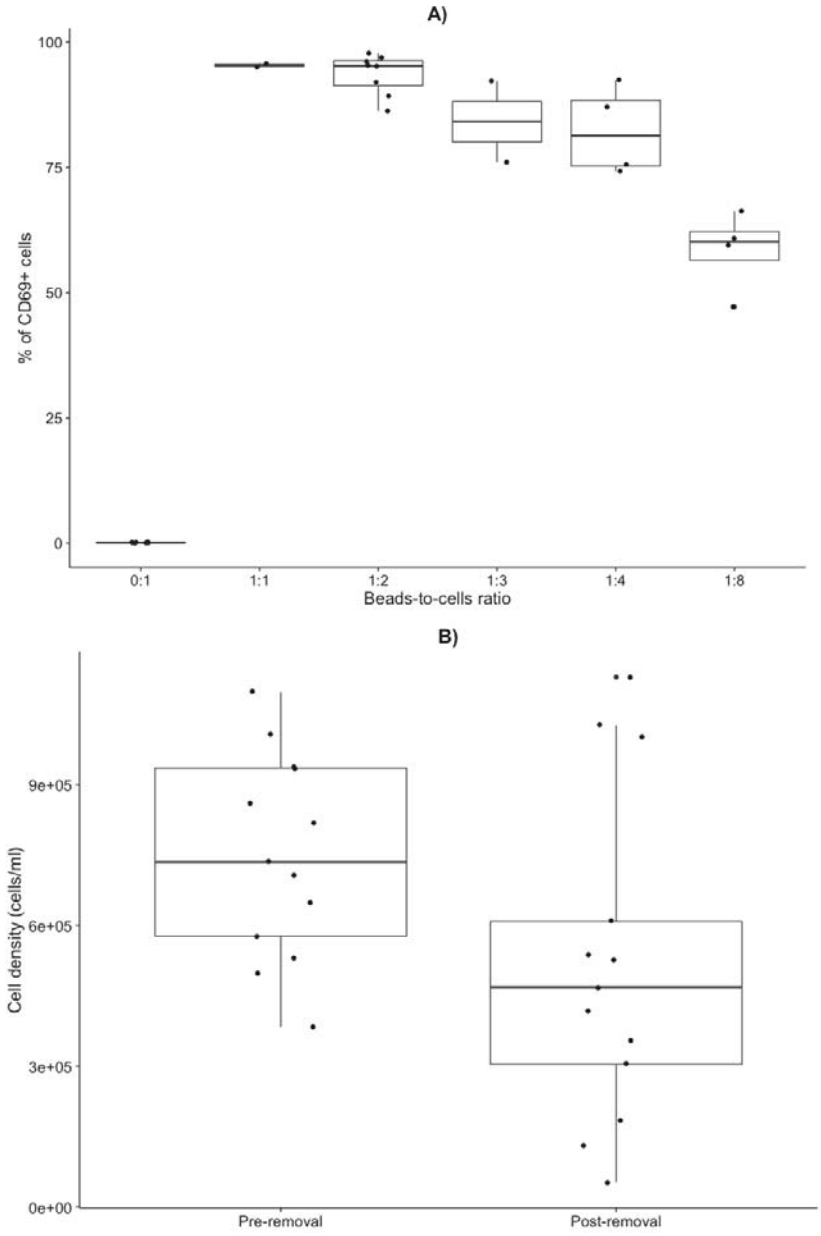

Figure 3.4 Determination of the optimal ratio of T cells to activation beads. Naive $CD4^+$ T cells were incubated for 16 hours with activation beads. Next, beads were removed and cells counted using flow cytometry. Boxplots represent data from nine independent experiments. **A)** Titration curve showing the percentage of CD69 expressing cells at different cells-to-beads ratios. **B)** Number of cells per millilitre before and after magnetic removal of activation beads at a 1:2 ratio. A 30% cell loss was observed.

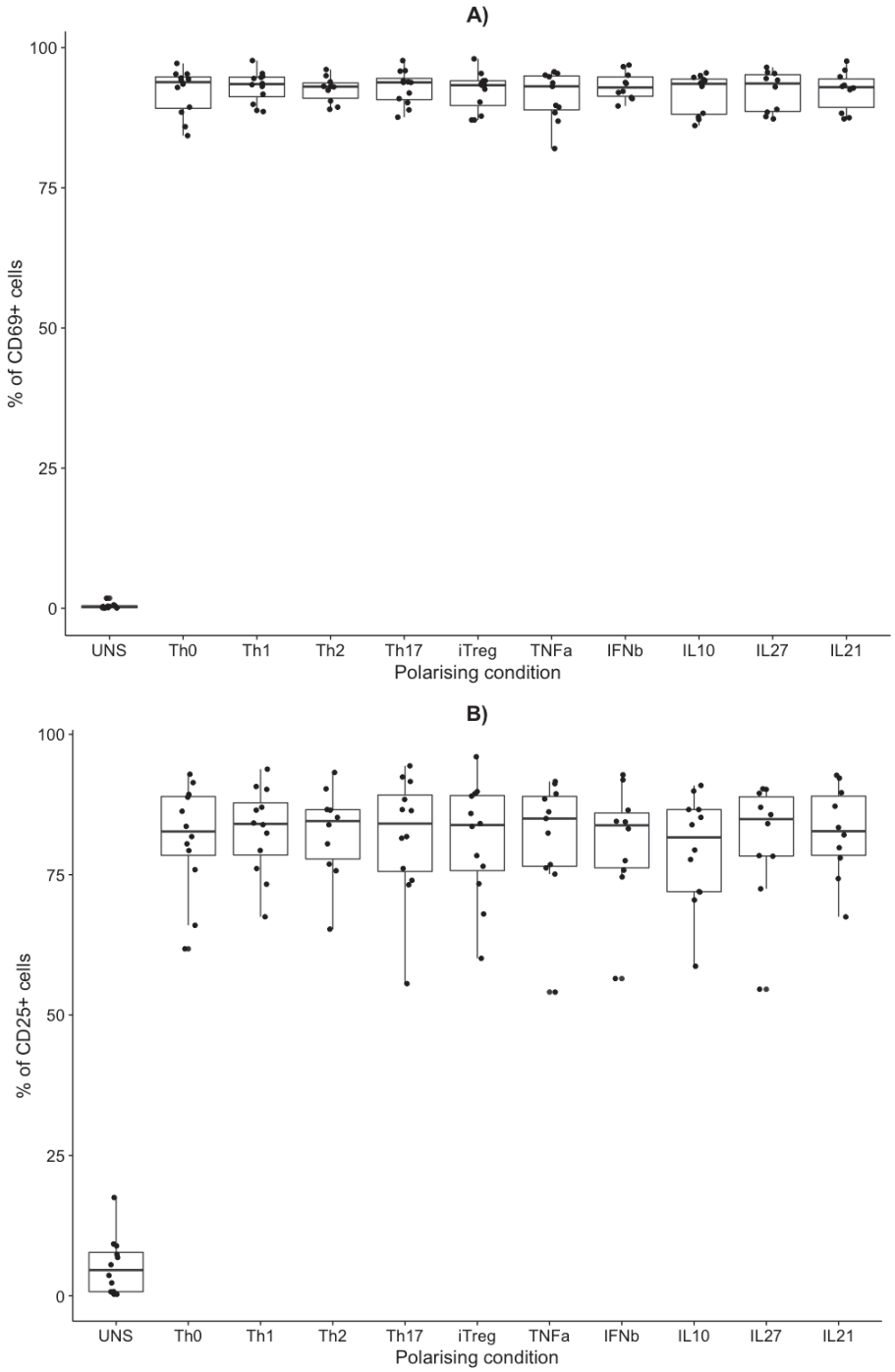

Figure 3.5 Cytokine polarising conditions do not modify activation efficiency. Naive $CD4^+$ T cells were incubated with polarising cytokine cocktails and activation beads. Percentage of live cells expressing **A)** CD69 and **B)** CD25 expression was assessed by flow cytometry after 16 hours. Box plots represent 12 independent biological replicates.

3.5 Cytokine polarisation of naive CD4$^+$ T cells

Next, we sought to investigate whether cytokines polarised CD4$^+$ T cells to known cell states. In order to assess the expression of Tbet, Gata3, and FoxP3, we stimulated and polarised naïve CD4$^+$ T cells to Th1, Th2, Th17 and iTreg lineages for 5 days. Cells were then fixed, permeabilised, and stained with antibodies against these proteins. The percentage of TF-expressing cells was estimated by flow cytometry. We observed increased expression of GATA-3 and FoxP3 in Th2 and iTreg lineages, respectively. Approximately 75% of cells stimulated with IL-4 expressed GATA-3, and on average 65% of TGF-β stimulated cells were FoxP3$^+$ (**Figure 3.6**). Therefore, we concluded that our Th2- and iTreg-polarising cocktails efficiently triggered the respective differentiation programs. These results also confirmed that the concentration of cytokines used was appropriate. Since Th17 and iTreg polarisation both need TGF-β, and yet Th17 cells did not express FoxP3 (**Figure 3.6A**), these results indirectly suggested a good efficiency of Th17-polarisation.

Since T-bet was expressed at variable levels across all lineages, reporting percentages of Tbet-expressing cells was not as informative as for the other TFs. However, we observed that the expression level of T-bet, measured as MFI, was higher in Th1 cells as compared to other polarised cell fates (**Figure 3.6C**). Therefore, we concluded that our cocktail successfully polarised CD4$^+$ T cells to the Th1 phenotype.

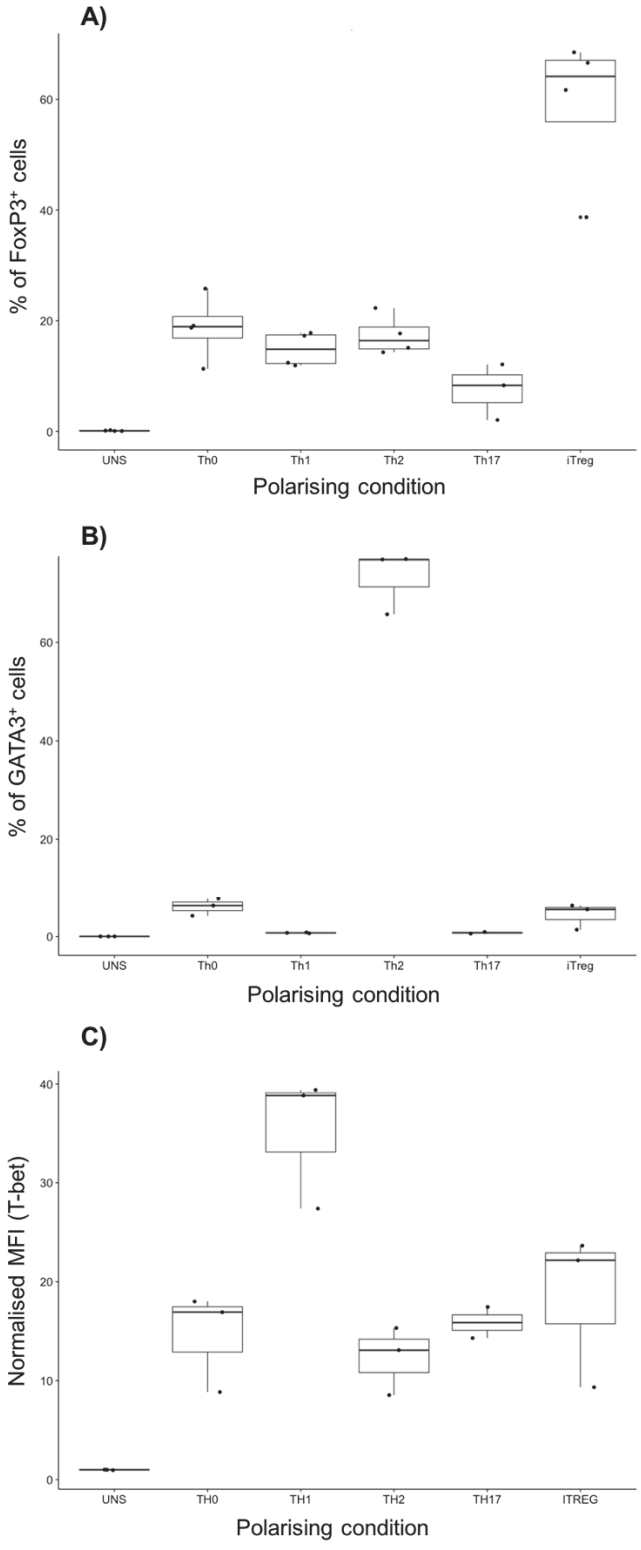

Figure 3.6 Cytokines polarise CD4$^+$ T cells towards Th2 and iTreg lineages. Polarisation of CD4$^+$ T cells to Th1, Th2 and iTreg was assessed by flow cytometry after five days. **A)** Percentages of cells expressing Gata3 and **B)** FoxP3 were calculated. **C)** T-bet expression was represented as MFIs normalised to the negative control. Box plots correspond to observations from three independent biological replicates.

3.6 Discussion

In this study we optimised a system for assessing cytokine polarisation of human immune cells *in vitro*. We demonstrated that in our experimental set up human monocytes expressed high levels of CD14, a well known marker of the monocyte/macrophage lineage (72), as well as medium levels of HLA-DR, CD68 and MerTK. *In viv*o, human monocytes differentiate into DCs or macrophages upon migration to the tissues (73-75). Each of these differentiation programs is triggered by the presence of specific stimuli. Whereas GM-CSF with IL-4 induces differentiation into DCs, M-CSF induces differentiation into macrophages (155). We used M-CSF to induce the monocyte to macrophage differentiation program (94). When comparing the phenotype of monocyte derived macrophages with monocytes, we saw a sharp upregulation of CD68, MerTK and HLA-DR. MHC-II molecules, including HLA-DR, are known to be upregulated in both DCs and macrophages as compared to monocytes, and are thus a general marker of differentiation (153). On the other hand, MerTK is a known marker of mature tissue macrophages not expressed by DCs (153), informing on the type of differentiation. Thus, this indicated that our protocols for $CD14^+$ cell isolation followed by M-CSF treatment yielded high purity monocyte derived macrophages.

Naïve $CD4^+$ T cells can be identified based on the expression of the splice variant of the CD45 molecule ($CD45RA^+$ cells) as opposed to the effector and memory cells that express the CD45RO isoform (156). In our study, cells were selected based on low expression of CD25, and high CD127 and CD45RA expression. Flow cytometry analysis consistently showed high purity of this population. *In viv*o, naïve $CD4^+$ T cells egress from the thymus and circulate through peripheral blood before homing to secondary lymphoid organs such as lymph nodes in response to a chemokine gradient (157). In the lymph nodes, naive $CD4^+$ T cells are stimulated by DCs, who provide two signals necessary to successfully drive activation (48-53). In order to mimic this process *in vitro,* we stimulated T cells with beads coated with anti-CD3 and anti-CD28 antibodies. This stimulation induced an increase in CD25 and CD69 expression. This is expected, as CD25 allows the cell to respond to IL-2, a cytokine essential for T cell proliferation in response to activation signals (158). This upregulation was observed even at 16 hours, generally thought of as an early time point. Furthermore, expression of CD69, a transmembrane lectin protein upregulated shortly after T cell activation (154, 158), was also seen at 16 hours and further confirmed that cells responded to activation. These results suggested that our T cell activation method was efficient. Furthermore, they also validated the choice of 16 hours as a relevant time point for transcriptional analysis. This time point was chosen so as to characterise the transcriptome

before the activation of cell cycle genes. At five days, on the other hand, we expect to find more defined lineage specific signatures.

Magnetic removal of activation beads is an important cause of cell loss. For this reason, we decided to reduce by half the number of activation beads in the cell culture. Our results showed almost no difference in activation efficiency between the two bead ratios. Thus, this reduction maintained the efficiency of activation in our cell population, while cell loss was reduced to approximately 30%.

Next, we proved experimentally that cytokine stimuli did not affect the percentage of stimulated cells, even when regulatory (anti-inflammatory) cytokines such as IL-10 were used. Then, we verified that each T cell subset expressed its master regulatory TF (35, 36, 159, 160). We also found that T cell polarisation to Th1, Th2, Th17 and iTreg occurred at a later time point of T cell activation, since lineage specifying TFs were not expressed at 16 hours but rather appeared at high levels after five days. We observed that cytokines seemed to act in addition to T cell activation, triggering further specialisation. These results are in agreement with the two-step model of T cell polarisation proposed in the literature (161-163).

Our results suggest that T cell activation itself is enough to induce expression of T-bet regardless of cytokines in the microenvironment. The addition of IL-12, however, caused significant upregulation of this TF. These results reflect the fact that T cell lineages sometimes share the expression of master regulators, and are not clearly defined cell types, but rather functional states with remarkable plasticity (29, 43, 64, 67, 164-166). This is in agreement with previous observations of interconversion between T cell polarisation states upon stimulation with cytokines (65). However, the effect of autocrine cytokines cannot be ruled out and might explain the induction of T-bet even in Th0 cells. To test this, we would need to perform a new experiment, adding anti-IFNg antibody to the culture media. Furthermore, strong TCR stimulation can also induce Th1 polarisation (33).

In summary, we have optimised a methodology for studying cytokine induced polarisation of human primary immune cells *in vitro*. The populations of human monocytes and naïve $CD4^+$ T cells obtained with this methodology appeared to be highly pure and could respond efficiently to monocyte to macrophage differentiation and T cell activation. Polarised cells expressed phenotypes previously described in the literature, implicating that our experimental setup provides a valid model for studying human cytokine biology *in vitro*.