\chapter{Material and Methods}

\label{ch:Mat}

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\section{Ethical approval and recruitment of study participants}

Sample recruitment for psoriasis, PsA and the healthy volunteers were conducted under different ethics.

\subsection{Psoriasis patient recruitment}

Patient blood samples and skin biopsies were collected in collaboration with Professor Graham Ogg at the Weatherall Institute of Molecular Medicine, University of Oxford, and the Dermatology Department research nurses at the Churchill Hospital, Oxford University Hospitals NHS Trust. This was under approval from the Oxfordshire Research Ethics Committee (REC 14/SC/0106 and REC 14/NW/1153). After written informed consent, up to 60mL of blood from eligible psoriasis patients were collected into 10mL anticoagulant ethylenediaminetetraacetic acid (EDTA)-containing blood tubes (Vacutainer System, Becton Dickson).

Psoriasis patients were eligible for recruitment when age was over 18 years old, previously or newly diagnose fulfilling the Psoriasis Area and Severity Index (PASI) classification and in a flare. Recruited patients were required to present moderate to severe disease (PASI$>$5), not having taken antibiotics in the two weeks before sampling and na\"{i}ve for biological therapy. Availability of clinical information and written consent were also required. Detailed clinical information of the psoriasis cohort is included in Chapter \ref{ch:Results2} Table \ref{tab:Psoriasis\_cohort\_metadata}.

\subsection{PsA patient recruitment}

Sample recruitment was performed as part of the Immune Function in Inflammatory Arthritis (IFIA) study established in 2006 (REC/06/Q1606/139)in collaboration with Dr Hussein Al-Mossawi at the Botnar Research Centre and research nurses at the Nuffield Orthopaedic Centre, Oxford University Hospitals NHS Trust. Following informed written consent, approximately 30mL of both blood and synovial fluid aspirate (variable upon disease severity) were collected into 10mL anticoagulant sodium heparin coated tubes (Vacutainer System, Becton Dickson).

Eligibility of the PsA patients required age over 18 years old, previously or newly diagnosed according to the PsARC, including a physician global assessment questionnaire, with concomitant psoriasis and in a flare. Patients had to present an oligoarticular phenotype, not having taken antibiotics in the two weeks before sampling and be na\"{i}ve for biological therapy and preferably for any other treatment. Written consent and clinical data were also collected.

Further details about the cohort and clinical information can be found in Chapter \ref{ch:Results3} Table \ref{tab:PSA\_cohort\_metadata}.

\subsection{Healthy volunteer recruitment}

Recruitment of healthy volunteers was conducted as part of the Genetic Diversity and Gene Expression in White Blood Cells study with approval from the Oxford Research Ethics Committee (REC 06/Q1605/55). Up to 80mL of blood were collected into 10mL anticoagulant EDTA-containing blood tubes. Healthy individuals recruited in the study were required to be over 18 years old, preferably British or European, without family history of psoriasis, PsA, RA or SpA and not having suffered from an infectious process in the two weeks prior to sample recruitment. Written consent was required.

\section{Sample processing}

\label{sample\_processing}

Blood, synovial fluid and skin biopsies were processed straight after recruitment, following the appropriate protocols.

\subsection{PBMCs and synovial fluid cells isolation}

PBMCs were isolated from blood samples through density gradient separation using Ficoll-Paque with centriguation at 500g for 30 minutes at room temperature with minimum acceleration and no braking. Total synovial fluid (SF) mononuclear cells (SFMCs) were isolated by centrifugation at 500g for 5 min at room temperature in absence of density gradient. Samples were placed on ice, washed twice in ice cold Hank’s balanced salt solution (HBSS) without calcium or magnesium (Thermo Fisher Scientific) and resuspended in phosphate saline buffer (PBS, Gibco) supplemented with 0.5\% fetal calf serum (FCS, Invitrogen) and 2mM EDTA (Sigma), prior to separation of the different cell types. Cell numbers and viability were determined by manual counting using a haemocytometer with trypan blue (Sigma) for viability assessment.

\subsection{Skin biopsies processing and adherent assays}

KCs enrichment from skin biopsies was performed as described in Gutowska-Owsiak and colleagues \parencite{Gutowska‐Owsiak2012}. Skin biopsies (approximately 4mm) were washed with PBS, cut in 1mm width strips and incubated in 2U/mL of dispase II (Sigma) overnight at 4$^\circ$C. Following incubation, the epidermis was separated from the dermis. For RNA extraction, the epidermis was snap-frozen in liquid nitrogen. For chromatin accessibility assay, the epidermis was further digested in trypsin (Invitrogen) at 37$^\circ$C for 5 min to obtain a cell suspension that was filtered through a 70$\micro$m nylon strainer (BD) and washed with PBS. In some instances cells were manually counted and aliquoted in PBS prior to chromatin accessibility assay. In others, cells from each of the biopsies were resuspended in KGM-2 BulletKit (Lonza) supplemented with 0.06mM Ca$^2{+}$ and cultured in a collagen IV coated 96-well plate at 37{$^\circ$}C 5\% CO$\_2$ for 10 min or 3 hours, upon experimental requirements (see Chapter \ref{Results1}). After culture, cells in the 96-well plate were washed twice with 200$\micro$L of PBS and kept at 37{$^\circ$}C for downstream chromatin accessibility processing.

\subsection{Cryopreservation and cell culture}

Liquid nitrogen storage of 40-50x10$^6$ PBMCs was carried out using a modified version of the \parencite{Kent2009} protocol, where cells were pre-conditioned in RPMI 1640 complete medium (Lonza) supplemented with 2 mM L-glutamine (Sigma), 100U penicillin and streptomycin 100$\micro$g/mL (Sigma) and 50\% FCS for 30 minutes and, afterwards, diluted 1 in 2 in complete RPMI 1640 (supplemented as previously described) with 20\% dimethyl sulfoxide (DMSO, Sigma). PBMCs followed slow cryopreservation at -80{$^\circ$}C at -1{$^\circ$}C per minute and then transferred and stored for a minimum of two weeks in liquid nitrogen.

PBMCs were thawed quickly in a 37{$^\circ$}C water bath, resuspended in supplemented complete RPMI 1640 with 10\% FCS at a density of 10$^6$ cells/mL and rested for 30 min at 37{$^\circ$}C, 5\% CO$\_2$ in 25mL non-adherent polypropylene cell culture flasks (Greiner) followed by filtering through a 40$\micro$m cell strainer to obtain an homogenous cell suspension for FACS separation.

Cryopreserved normal human epidermal keratinocytes (NHEK, Lonza) in passage three were recovered and cultured at a cell density of 5x10$^6$ cells/mL in a 75 mL adherent cell culture flask (Greiner) in EpiLife basal medium (Gibco), following manufacturer's instructions. After recovery, NHEKs were trypsinised at room temperature for 8 minutes and trypsin was inactivated with EpiLife 10\% FCS. Cells were centrifuged at 180g for 10 min at room temperature and then manually counted with trypan blue as dead/live staining. NHEKs (16,000 cells) were seeded in a 96-well plate in 100uL of medium and cultured for 2 days at 37°C, 5\% CO$\_2$ to reach 90-100\% confluence (approximately 50,000 cells) before performing any ATAC protocol on the plate (further detailed in Chapter \ref{ch:Results1}). When used for Omni-ATAC, NHEKs after trypsinisation were processed through Ficoll density gradient (as previously explained for PBMCs isolation) to remove dead cells as recommended by \parencite{Corces2017}.

\subsection{Primary cell isolation using magnetic-activated cell sorting}

Primary cell subpopulations were separated using magnetic-activated cell sorting (MACS, Miltenyi) following the manufacturer's instructions. Consecutive positive selection was performed using Miltenyi beads for CD19$^+$, CD8$^+$, CD14$^+$ monocytes and CD4$^+$ cells (catalogue numbers 130-050-201, 130-045-101, 130-045-201 and 130-050-301) and AutoMACS Pro (Miltenyi) followed by a manual cell count with trypan blue. MACS separation was chosen over fluorescence-associated cell sorting (FACS) due to time and logistic constraints in the sample processing.

\subsection{Primary cell isolation using fluorescence-activated cell sorting}

Isolation of cell subpopulations from PBMCs and SFMCs to study the effect of cryopreservation in the chromatin landscape (Chapter \ref{ch:Results1}) (Chapter \ref{ch:Results3} was performed by FACS. PBMCs and SFMCs were resuspended in 1mM EDTA PBS (FACS buffer) at 10x10$^6$ cells/mL, stained with the appropriate antibody cocktail (Table \ref{tab:FACS\_antibodies}) for 30 min at 4{$^\circ$}C, washed with FACS buffer and centrifuged at 500g for 5 min at 4{$^\circ$}C. For the cell separation in the Chapter 3 samples, a modified FACS buffer supplemented with 3mM EDTA , 2\% FCS and 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Invitrogen) was used to avoid cell clumping after cryopreservation and short recovery in culture (as detailed previously). After removing the supernatant, cells were resuspended in FACS buffer prior to separation.

From the control samples of Chapter 3, CD14$^{+}$ monocytes and CD3$^+$ CD14$^{-}$ CD4$^{+}$ T cells were isolated using the SONY SH800 cell sorter. For the PsA samples, separation of CD19$^+$ B cells, memory T cells (CD3$^+$ CD14$^-$ CD4$^+$ CD45RA$^{-}$ and CD3$^+$ CD14$^-$ CD8$^+$ CD45RA$^-$), CD14$^+$ monocytes and CD56$^+$ NK from PBMCs and SFMCs was performed using FACS Aria (BD) cell sorter. Sorted cells were collected in 1.5mL tubes containing PBS 1\% FCS when used for ATAC-seq or only PBS when processed for scRNA-seq to avoid potential RNAse contamination. OneComp eBeads (eBioscience) were used for compensation of fluorescence spill over.

\begin{table}[htbp]

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\textbf{Surface} & \textbf{Fluorochrome} & \textbf{Manufacturer} & \textbf{Clone} & \textbf{Dilution} \\

\textbf{marker} & \textbf{PsA/Control} & \textbf{PsA/Control} & \textbf{PsA/Control} & \textbf{PsA/Control} \\

\midrule

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Viability & eFluor780 & - & eBioscience & 1:500/1:250\\

CD3 & FITC/AF700 & SK7/UCHT1 & BioLegend & 1:50/1:50\\

CD4 & APC & RPA-T4/RPA-T4 & BioLegend & 1:50/1:50\\

CD8a & PE & RPA-T8 & BioLegend & 1:100/-\\

CD45RA & BV421 & HI100 & BioLegend & 1:25/-\\

CD19 & PerCP-Cy5.5 & SJ25C1 & BioLegend & 1:50/-\\

CD14 & Pe-Cy7/FITC & M5E2/TUK4 & BioLegend/Miltenyi & 1:50/1:100\\

CD56 & BV510 & HCD56 & Biolegend & 1:25/- \\

\bottomrule

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\caption[Antibody panel used for FACS separation of primary cell populations in Chapter 3 controls and Chapter 5 PsA samples.]{\textbf{Antibody panel used for FACS separation of primary cell populations in Chapter 3 controls and Chapter 5 PsA samples.} Details regarding target molecule, fluorochrome, clone, supplier and dilution used for PBMCs and SFMCs staining are provided for each surface marker in the panel. For cell separation from Chapter 3, control PBMCs staining was only performed for CD3, CD4, CD14 and viability markers.}

\label{tab:FACS\_antibodies}

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\section{Experimental protocols}

\subsection{Chromatin Accessibility - ATAC}

Three different versions of the ATAC-seq protocol were progressively used in this thesis for assessment of chromatin accessibility in different primary cells, including CD14$^{+}$ monocytes, CD4$^+$ and CD8$^+$ T cells, CD19$^+$ B cells and CD56$^+$ NK cells, as well as in epidermal KCs isolated from skin biopsies. Fast-ATAC and Omni-ATAC were two subsequent versions published following the standard ATAC-seq protocol from \parencite{Buenrostro2013}, aiming to reduce the amount of mitochondrial DNA in the sequencing libraries and improve the signal-to-noise ratio of the original protocol. When using MACS separation, primary cells were manually counted, as specified above, and resuspended in PBS 1\% FCS.

\subsubsection{Standard ATAC-seq}

Standard ATAC-seq was used to generate data from NHEKs and skin biopsies (Chapter \ref{Results1}), healthy volunteers to test the effect of cryopreservation in the chromatin landscape (Chapter \ref{Results1}) and cohort 1A primary immune cells isolated from blood of psoriasis and control samples (Chapter \ref{Results2}). ATAC-seq was performed using an estimated number of 50,000 cells as described in Buenrostro \textit{et al.}, 2013 with minor modifications. Cells lysis was carried out for 10 min, the isolated nuclei were transposed for 40 min at 37{$^\circ$}C using the Nextera Tn5 transposase (Illumina) and DNA was purified with the PCR MinElute kit (Qiagen), following the manufacturer's instructions. Additional modifications of the protocol for 96-well plates were implemented when processing KCs isolated from skin biopsies following \parencite{Bao2015} (as later detailed in Chapter \ref{ch:Results1}). All transposed samples were simultaneously amplified and singled indexed for 11 PCR cycles using modified Nextera primers from Buenrostro \textit{et al.},2013, after appropriate assessment of the approximate required number of qPCR cycles. The resulting DNA libraries were purified using the MinElute PCR purification kit (Qiagen) and a 1.8X (v/v) of Agencourt AMPure XP Magentic Beads (Beckman Coulter) to remove the remaining adaptors and primer dimers.

\subsubsection{Fast-ATAC}

An improved ATAC-seq protocol was published in Nature Methods Corces et al., 2016, called Fast-ATAC. Optimised for hematopoietic cells it combined cell lysis and transposition into a single step. In this thesis, Fast-ATAC was performed in skin biopsies (Chapter \ref{Results1}), cohort 1A primary immune cells isolated from blood of psoriasis and control samples (Chapter \ref{Results2}) and primary immune cells isolated from blood and SF of PsA patients (Chapter \ref{Results3}). Fast-ATAC was conducted as described by Corces et al., 2016 with minor modifications. Following the advice from Corces and colleagues, approximately 20,000 cells (MACS or FACS sorted) were washed with 200$\micro$L of PBS, centrifuged at 500g for 5 min at 4$^\circ$C and incubated in the lysis/transposition buffer containing digitonin (Roche) for 30 min at 37{$^\circ$}C and agitation at 400rpm, as specified in \parencite{Corces2016}. Following transposition DNA was prepared and purified as per ATAC-seq except with 13 cycles of PCR amplification after appropriate cell cycle determination in a pilot set of samples.

\subsubsection{Omni-ATAC}

Omni-ATAC, a third generation of ATAC-seq was published by \parencite{Corces2017}. Omni-ATAC is a universal protocol optimised for any cell type. It includes independent steps for cell lysis and transposition reactions, similarly to the standard ATAC-seq protocol. In this protocol, a gentle cell lysis (using NP40, Tween-20 (Sigma) and digitonin) is followed by a washing step in a buffer containing Tween-20 to remove mitochondrial DNA and other cell debris prior to the transposition step, maximising signal and minimising off-target DNA contamination. NHEK transposed DNA was simultaneously amplified and indexed, as detailed in the ATAC-seq standard protocol, for 8 PCR cycles and purified using Qiagen PCR purification columns. Omni-ATAC was performed in 50,000 NHEKS that were manually counted (Chapter \ref{Results1}).

\subsubsection{Quality control and sequencing}

Indexed and amplified ATAC samples were assessed for tagmentation profile on an Agilent 2200 or 4200 Tapesation with the D1000 high sensitivity DNA tape (Agilent) as part of the quality control. Quantification of the library concentration was performed by qPCR using the Kapa assay from Roche, following the manufacturer's instructions. Pools of 12 to 16 libraries were sequenced on up to 3 lanes of the HiSeq4000 Illumina platform by the Oxford Genomics Centre, at the Wellcome Centre for Human Genetics (WCHG), aiming for 50 million paired-end reads.

\subsection{Chromatin immunoprecipitation with sequencing library preparation by Tn5 transposase}

For chromatin immunoprecipitation (ChIP) a low cell input protocol known as ChIPmentation (ChIPm) was used \parencite{Schmidl2015}. The H3K27ac histone mark (active enhancer and promoter marker) was assayed in four cells types (CD14$^+$ monocytes, total CD4$^+$, total CD8$^+$ and CD19$^+$). For each ChIPm reaction, 100,000 cells were MACS sorted, as described in \ref{sample\_processing}, fixed with 1\% formaldehyde (Sigma) and snap frozen in dry ice and ethanol prior to storage at -80{$^\circ$}C. Fixed cells were resuspended in SDS lysis buffer, sonicated for 8 min using Covaris M220(Covaris) with a duty factor of 5\% and processed downstream for ChIPm as in Schmidl \textit{et al.}, 2015. Immunoprecipitation was carried out with 1$\micro$g of the Diagenode Ab (C15410196). For each sample, an aliquot of chromatin was processed in parallel without incubation with the anti-H3K27ac Ab (control input). Tagmentation of the control input was performed using 1ng of DNA.

Amplification by qPCR was performed in each of the samples and control inputs to determine the appropriate number of full cycles required to reach one-third of the final fluorescence to minimise the presence of PCR replicates upon NGS. Libraries were then amplified for the number of determined cycles minus one and simultaneously dual indexed using the primers optimised by \parencite{Buenrostro2015}.

\begin{table}[htbp]

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\textbf{Buffer} & \textbf{SDS} & \textbf{EDTA} & \textbf{Tris-HCl} & \textbf{NaCl} & \textbf{Triton} & \textbf{PI} & \textbf{NP-40} \\

\textbf{name} & & & \textbf{pH8} & & \textbf{-X100} & & \\

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\textbf{SDS lysis} & 0.25\% & 1mM & 10mM & - & - & 1X & - \\

&&&&&&&\\

\textbf{ChIP} & - & 1mM & 10mM & 233mM & 1.66\% & 1X & - \\

\textbf{equilibration} & & & & & & \\

&&&&&&&\\

\textbf{Beads} & 0.1\% & 50mM & 10mM & 150mM & - & 1X & 1\% \\

\textbf{washing} & & & & & & & \\

&&&&&&&\\

\textbf{ChIP} & 0.1\% & 1mM & 10mM & 140mM & 1\% & 1X & - \\

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\caption[ChIPm buffers modified from Schmidl \textit{et. al}, 2015]{\textbf{Composition of the three modified buffers in house for the ChIPm protocol: SDS lysis buffer, ChIP equilibration buffer, beads washing buffer and ChIP buffer. For each of the buffers the final concentration for each reagent is indicated. The final volume prepared for each buffer was adjusted with Ambion water depending on the number of samples processed at the time. Sodium dodecyl sulfate (SDS); PI (proteinase inhibitor). Suppliers: SDS Sigma, EDTA X, Tris-HCl pH8 X, Triton-X100 X, NP-40 Sigma, NaCl X, PI Roche.}}

\subsection{RNA extraction and gene expression quantification}

\subsubsection{RNA extraction}

Following MACS isolation of the different cell types between 2-3x10$^6$ cells were resuspended in 350$\micro$L of RNAProtect (Qiagen) or RLT buffer (Qiagen) supplemented with 0.1\% of beta-mercaptoethanol (BM, Sigma) and snap frozen in dry ice before storage at -80{$^\circ$}C. Cells isolated from Cohort 1A psoriasis and control samples (Chapter \ref{ch:Results2} Table \ref{tab:Psoriasis\_cohort\_metadata} and \ref{tab:Control\_cohort\_metadata}) were preserved in RNAProtect, which stops any biochemical reaction and transcriptional activity whilst maintaining cell integrity. At early stages of the project, the time frame to process the acquired samples was uncertain and RNAProtect was chosen as the most appropriate strategy to preserve cells for future RNA extraction to guarantee high quality incase storage exceeded 6 months. In the psoriasis and control samples from Cohort 1B (Chapter \ref{ch:Results2} Table \ref{tab:Psoriasis\_cohort\_metadata} and \ref{tab:Control\_cohort\_metadata}) and PsA samples (Chapter \ref{ch:Results3}), cells were resuspended in 0.1\% BM supplemented RLT buffer, which lysis cells and prevents RNA degradation. When starting from RNAProtect preserved material, cells were centrifuged at 300g for 10 min at room temperature, the supernatant were removed and the pellets were resuspended in 350$\micro$L of RLT 0.1\% BM buffer. All cell lysates were homogonised using the QIAshredder (Qiagen) prior to RNA extraction.

Total RNA was extracted using the AllPrep DNA/mRNA/microRNA Universal kit (Qiagen) following the manufacturer's instructions. RNA extractions were performed in batches of 12 samples, including all cell types from each individual processed and a balanced numbers of psoriasis and control samples, to minimise batch effect correlating with phenotype. Basic quantification was performed with NanoDrop (Thermo Scientific) before storage at -80{$^\circ$}C.

\subsubsection{RNA-seq}

RNA-seq quality control (QC), quantification, library preparation and sequencing were carried out by Oxford Genomics Centre at the WCHG in two independent batches of samples, each including Cohort 1A or Cohort 1B, respectively. Processing of samples in two batches was due to logistics of patient recruitment in the project. RNA quality control and quantification were assessed with the Bioanalyzer (Agilent). Samples were depleted from ribosomal RNA using Ribo-Zero rRNA Removal kit (Illumina) prior to cDNA synthesis and library preparation using TruSeq Stranded Total RNA (Illumina). This method allowed to preserve non-polyadenylated transcripts including nascent pre-mRNA (unspliced) and functionally relevant lncRNAs. For each of the cohorts, all libraries were pooled together and sequenced over several lanes of HiSeq4000 aiming a depth of approximately 50 million total reads per sample to maintain an appropriate level of sensitivity for subsequent expression analysis.

\subsubsection{Gene expression quantification by qPCR array}

Expression of immune-relevant genes was profiled by qPCR using the RT2 Profiler PCR Array (PAHS-3803Z, Qiagen) in collaboration with UCB. This platform included primers to test expression for 370 key genes involved in immune response during autoimmunity and inflammation, as well as appropriate house-keeping genes for normalisation. In brief, RNA was extracted, as detailed previously, from CD14$^+$ monocytes, mCD4$^+$ and mCD8$^+$ cells. Reverse-transcription for cDNA synthesis and qPCR gene expression quantification was performed by UCB following the PCR array's manufacturer’s instructions.

\subsubsection{Single-cell RNA-seq}

\label{scRNA\_processing}

scRNA-seq data was generated using 10X Genomics technology Chromium single cell 3' expression library preparation kit (PN-120267) by the Oxford Genomics Centre at the WCHG. Briefly, PBMCs and SFMCs were made into a cell suspension. Approximately 3,000 cells from the PBMCs or SFMCs suspensions were partitioned into single-cell gel beads in emulsion (GEMs) using the 10X Chromium controller system. Reverse-transcription for cDNA synthesis was performed within the GEMs, which included a 16bp 10x barcode, a 10bp unique molecular identifier (UMI) and poly-dT primers. The cDNA was released from the GEMs, followed by PCR amplification, enzymatic fragmentation and size selection. Afterward, appropriate sequencing Illumina indexes were incorporated into the samples through library preparation. Sequencing was performed using PE HiSeq4000 with 26bp for read 1 and 98bp for read 2 at a depth of approximately 50,000 reads per cell, following standard 10X library sequencing requirements.

%\subsection{Single-cell analysis of the V(D)J T cell receptor repertoire}

%Single-cell sequencing of the V(D)J segments from TCR transcripts was performed simultaneously with the 10X Genomics technology Chromium 5' expression library kit (PN-1000014) by the Oxford Genomics Centre at the WCHG. In short, full-length cDNA was amplified by PCR with primers against the 5’ and 3’ ends of the barcode sequences inherent to the 10X Genomics bead technology. The amplified material was divided for use in 5' total scRNA-seq (as specified previously in \ref{scRNA\_processing}) and also for enrichment of the TCR by PCR amplification with specific primers. TCR enriched cDNA was followed by enzymatic fragmentation and size selection in order to obtain variable length fragments spanning the V(D)J segments. Library prep and indexing was followed by Illumina HiSeq4000 xxxx bp single-end sequencing at a depth of XXX reads per cell.

\subsection{DNA extraction and rs4672405 genotyping}

DNA isolation was performed using the AllPrep DNA/mRNA/microRNA Universal kit (Qiagen) following the manufacturer's instructions. Quantification was performed using NanoDrop (Thermo Scientific) and samples were kept at -80{$^\circ$}C for long term storage. The extracted DNA was amplified by PCR using forward (5'-CACTGTGGAGGGAGGAACAA-3') and reverse (5'-CGTGTTGGCCAGGATAGTCT-3') primers annealing up and down stream the SNP rs4672505, respectively. An aliquot of the sample was run on a 1% agarose gel to check for amplification of a 390bp PCR fragment. The remaining was purified using MinElute PCR purification kit, quantified by dsDNA Qbit kit (Invitrogen) according to the manufacturer's instructions and prepared for Sanger sequencing using the Mix2Seq kit and service (Eurofins). The forward and reverse sequences were analysed with BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

\subsection{Mass cytometry using cytometry by time of flight (CyTOF)}

Mass cytometry assay was performed by Dr Nicole Yager in collaboration with UCB following their in-house protocol for the CyTOF instrument. Briefly, an aliquot of whole blood and SF were fixed for 5 min with 1.6\% paraformaldehyde (PFA) within 30 min of venipuncture/aspiration, respectively. These samples were defined as time 0h. In addition, another aliquot of whole blood and SF were incubated at 37{$^\circ$}C for 6h in the presence of the protein transport inhibitors 1X BD GolgiStop (BD) and 1X BD GolgiPlug (BD), containing monesin and brefeldin A, respectively. Treatment with monensin and brefeldin A prevents the extracellular transport of cytokines from the cells and allowed measuring the intrinsic cytokine production rate in basal conditions. After 5h 45 mins the samples were treated with cisplatin to facilitate discrimination of dead cells, and then fixed 5 min with 1.6\% PFA. These samples were defined as time 6h. After fixation of time 0h or 6h samples, red blood cells were lysed and cell suspensions were washed with PBS and stained with Abs against the cell surface markers of the intra-cellular staining (ICS) panel (Table \ref{tab:CyTOF}). The samples were further permeabilised and stained with Abs of the ICS panel against the intracellular targets (Table \ref{tab:CyTOF}).

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\textbf{Markers from the ICS CyTOF panel} \\

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CD248, CD19, GP38, FAP, CD8a, IL8, CD16, CD25, CD123, CD-11b \\

IL-17F, IL-17A, IL-10, CD11c, CD14, IL6, IFN-$\gamma$, GM-CSF, CD45\\

CD45RO, CD56, HLA-DR, IL-13, CD117, CD4, IL4, IL-2, TNF$\alpha$,\\

IL-21, FceR, CD3, CD161\\

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\caption[Molecules targeted by the mass cytometry ICS staining panel in whole blood and SF.]{\textbf{Molecules targeted by the mass cytometry ICS staining panel in whole blood and SF.} The molecules targeted by the Abs used in the ICS staining panel are listed. Note the panel also included Abs recognising surface markers to identify the cell populations of interest for further analysis of the intracellular cytokine production.}

\label{tab:CyTOF}

\end{table}

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