Functional genomics of psoriasis

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A thesis submitted in partial
fulfilment of the requirements for the degree of
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Abstract

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This is my abstract...

Acknowledgements

Thank you, thank you, thank you.

Declarations

I declare that unless otherwise stated, all work presented in this thesis is my own. Several aspects of each project relied upon collaboration where part of the work was conducted by others.

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Abbreviations

Abbreviation	Definition
ATAC-seq	
ChIPm	
Fast-ATAC	
Omni-ATAC	
PI	Protein inhibitor
PsA	
PSO	
QC	
qPCR	quantitative polymerase chain reaction
RA	Rheumatoid arthritis
SDS	Sodium dodecyl sulfate
SF	Synovial fluid

Chapter 1

Material and Methods

1.1 Ethical approval and recruitment of study participants

Sample recruitment for the two different phenotypes and the healthy volunteers were conducted under different ethics.

1.1.1 PSO patient recruitment

Patient blood samples and normal or psoriatic skin biopsies were collected in collaboration with the Dermatology Department research nurses at the Churchill Hospital, Oxford University Hospitals NHS Trust and Professor Graham Ogg at the Weatherall Institute of Molecular Medicine, University of Oxford under approval from the Oxfordshire Research Ethics Committee (REC 09/H0606/71 and 08/H0604/129). After written informed consent, up to 60 mL of blood from eligible PSO patients were collected into 10 mL anticoagulant EDTA-containing blood tubes (Vacutainer System, Becton Dickson).

PSO patients were eligible for recruitment when meeting the following criteria:

- over 18 years old
- previously or newly diagnose, in a flare and going into biologic therapy for the first time

- fulfillment of the clinically accepted Psoriasis Area and Severity Index (PASI) classification for PSO diagnosis (**Fredriksson1978**)
- moderate to severe disease (PASI¿5)
- less than 2 weeks without antibiotics unless used for prophylaxis
- available clinical information and written consent

Detailed clinical information of the PSO cohort is included in (Chapter **??**)(Table **??**).

1.1.2 PsA patient recruitment

Sample recruitment was performed as part of the Immune Function in Inflammatory Arthritis (IFIA) study established in 2013 (REC/06/Q1606/139)in collaboration with research nurses at the Nuffield Orthopaedic Centre, Oxford University Hospitals NHS Trust and Dr Hussein Al-Mossawi at the Botnar Research Centre. Following informed written consent, blood (30 mL) and synovial fluid aspirate (variable upon disease severity) were recruited into 10 mL anticoagulant sodium heparin coated tubes (Vacutainer System, Becton Dickson).

Eligibility of the PsA patients was upon fulfillment of the following criteria:

- over 18 years old
- previously or newly diagnose, with concomitant PSO, in a flare and going into biologic therapy for the first time
- fulfillment of the clinically accepted PsA Response Criteria (PSARC) including a physician global assessment questionnaire (**Philipp2011**; **Clegg1996**)
- oligoarticular phenotype and naïve for any treatment
- less than 2 weeks without antibiotics unless used for prophylaxis

- available clinical information and written consent

Further details about the cohort and clinical information can be found in (Chapter **??**)(Table **??**).

1.1.3 Healthy volunteer recruitment

Recruitment of healthy volunteers was conducted as part of the study Genetic Diversity and Gene Expression in White Blood Cells with approval from the Oxford Research Ethics Committee (REC 06/Q1605/55). Up to 80 mL of blood were collected into 10 mL anticoagulant EDTA-containing blood tubes, similarly to the PSO sample recruitment.

The criteria for healthy individuals to participate in the study was:

- over 18 years old and preferably British or European
- no family history of PSO, PsA, RA or SpA
- matched sex and age with the PSO cohort
- less than 2 weeks since last infectious process
- available clinical information and written consent

1.2 Sample processing

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

1.2.1 PBMC and synovial fluid cells isolation

PBMC were isolated from blood samples through density gradient separation using Ficoll-Paque. Total synovial fluid (SF) cells (SFC) were isolated by centrifugation at 500g for 5 min. Both were washed twice in Hanks balanced

salt solution 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 ethylenediaminetetraacetic acid (EDTA, Sigma)prior to cell types separation. Cell numbers and viability were determined by manual count using a haemocytometer and trypan blue (Sigma).

1.2.2 Primary cell isolation using magnetic-activated cell sorting

For the work related to PSO and healthy volunteers, primary cell subpopulations were separated using magnetic-activated cell sorting (MACS, Miltenyi). Positive selection was performed for consecutive isolation of $CD19^+$ B cells, $CD8^+$ T cells, $CD14^+$ monocytes and $CD4^+$ T cells with AutoMACS Pro (Miltenyi) and cells were manually counted as previously described. MACS separation was chosen over Fluorescence-associated cell sorting (FACS) due to time and logistic constrains in the sample processing and therefore cell numbers in down stream application may not be as exact.

1.2.3 Primary cell isolation using fluorescence-activated cell sorting

Primary cell subpopulations from controls to study the effect of cryopreservation in chromatin states (Chapter 3) or PsA blood and SF samples were isolated by FACS. PBMC and SFC were resuspended in PBS 1mM EDTA (FACS buffer) at $10x10^6$ cells/mL, stained with the appropriate antibody cocktail (Table 1.1) for 30 min at 4°C, washed with FACS buffer and centrifuged at 500g for 5 min at 4°C. For the samples used in Chapter 3, a modified FACS buffer supplemented with 3 mM EDTA , 2% FCS and 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Invitrogen) was used

to avoid cell clumping after cryopreservation and short recovery.After removing the supernatant, cells were resuspended in FACS buffer prior to separation.

In the controls samples of Chapter 3 only $CD14^+$ monocytes and $CD3^+$ $CD14^-$ $CD4^+$ T cells were isolated in 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 was performed using FACS Aria (BD) cell sorter from both PBMC and SFC. Bulk sorted cells were collected in 1.5mL tubes in PBS 1% FCS, whilst single cell and small bulk sorting was performed in 96-well plates in the appropriate buffer (See RNA-seq section). Different nozzle sizes were chosen for bulk and single-cell sorting and OneComp eBeads (eBioscience) were used for compensation of fluorescence spill over.

Surface marker	Fluorochrome PsA/CTL	Manufacturer PsA/CTL	Clone PsA/CTL	Dilution PsA/CTL
Viability	eFluor780	-	eBioscience	1:250
CD3	FITC/AF700	SK7/UCHT1	BioLegend	xxx/1:50
CD4	APC	RPA-T4/RPA-T4	BioLegend	1:50/1:50
CD8a	PE	RPA-T8	BioLegend	xxx
CD45RA	BV421	HI100	BioLegend	xxx
CD19	PerCP-Cy5.5	SJ25C1	BioLegend	xxx
CD14	Pe-Cy7/FITC	M5E2/TUK4	BioLegend/Miltenyi	xxx/1:100
CD56	BV510	NCAM16.2	BD	xxx

Table 1.1: Details regarding target molecule, fluorochrome, clone, supplier and dilution used for PBMC and SFC staining are provided for each of the antibodies in the panel. In controls only CD3, CD4 and CD4 markers were used.

1.2.4 Skin biopsies processing and adherent assay

KC enrichment from skin biopsies was performed as described in Gutowska-Owsiak and colleagues (GutowskaOwsiak and Schaupp 2012). 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°C. The epidermis

was separated from the dermis and either snap-frozen in liquid nitrogen (for RNA extraction) or further digested in trypsin (Invitrogen) at 37°C for 5 min, when used for chromatin accessibility assay. After digestion the resulting cell suspension was filtered through a 70µm nylon strainer (BD) and washed with PBS. In some instances cells were manually counted and aliquoted for ATAC-seq processing. In others, cell 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 4°C for 10 min or 3 hours, upon experimental requirements (see Chapter X). After culturing, cells were washed twice with 200µL of PBS and kept at 37°C for downstream processing.

1.3 Experimental protocols

1.3.1 Cryopreservation and cell culture

For the controls samples in Chapter 3, 40-50x10^6 of PBMC were freeze-thawing using a modified version of the (Kent 2009) protocol, where cells were pre-conditioned in RPMI 1640 (brand) complete medium supplemented with2 mM L-glutamine, 100U penicillin and strep 100µg/mL 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). PBMC underwent slow cryopreservation at -80°CC in isopropanol at -1°C per minute and stored for a minimum of two weeks in liquid nitrogen. PBMC were thaw, resuspended in supplemented complete RPMI 1640 with 10% FCS at a density of 10^6 cells/mL and rested for 30 min at 37C, 5% CO2 in 25mL non-adherent polypropylene cell culture flasks followed by filtering through a 40µm to obtain an homogenous cell suspension undergoing FACS separation. Frozen Normal human epidermal keratinocytes (NHEK) in passage 3 were recovered and cultured at a cell density of 5x10^6 cells/mL in a 75 mL adherent cell

culture flask (brand) in EpiLife basal medium (Gibco) following manufacturer's instructions. After recovery NHEK were trypsinised at room temperature for 8 minutes followed by trypsin inactivation with EpiLife 10% FCS, centrifugation at 180g for 10 min at room temperature and manual counting with trypan blue. NHEK were seeded in a 96-well plate in 100uL of medium at a cell density of 160 cells/µL. NHEK were cultured for 2 days to a 90-100% confluence before being used downstream.

1.3.2 ATAC-seq, Fast-ATAC and Omni-ATAC

Improved versions of the ATAC-seq protocol were progressively used in the 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. The subsequent version aimed to reduce the amount of mitochondrial DNA and improve the ratio of signal to noise for this technique.

After MACS separation, primary cells were manually counted as above specified and they were resuspended in PBS with 1% FCS. As previously stated, due to reduced accuracy of manual cell counting compared to FACS sorting, in my experiments ATAC-seq was performed using an estimated number of cells between 50,000 to 100,000. ATAC-seq was performed as described in Buenrostro *et al.*, 2013 with minor modifications. Cells were centrifuged at 500g for 5 min at 4°C. After removing the supernatant cells were lysed for 10 min, the nuclei were transposed using the Nextera Tn5 transposase (Illumina) for 40 min at 37°C and DNA was purified using the PCR MinElute kit (Qigen). Additional modifications and performance in 96-well plates were implemented for KC and they will be described in

After appropriate determination of the amount of DNA amplification using qPCR, samples were amplified and singled indexed for 11 PCR cycles using modified Nextera primers from Buenrostro *et al.*,2013 (Table1.2). The

resulting DNA libraries were purified using the MinElute kit and additional Agencourt AMPure XP Magentic Beads (Beckman Coulter), according to the manual specifications, to remove the remaining adaptors and primer dimers.

Primer name	Full sequence
Ad1.noMX	AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG
Ad2.1	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT
Ad2.2	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
Ad2.3	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
Ad2.4	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
Ad2.5	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGT
Ad2.6	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT
Ad2.7	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGT
Ad2.8	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGT
Ad2.9	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGT
Ad2.10	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
Ad2.11	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
Ad2.12	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT
Ad2.13	CAAGCAGAAGACGGCATACGAGATATCACGACGTCTCGTGGGCTCGGAGATGT
Ad2.14	CAAGCAGAAGACGGCATACGAGATACAGTGGTGTCTCGTGGGCTCGGAGATGT
Ad2.15	CAAGCAGAAGACGGCATACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGT
Ad2.16	CAAGCAGAAGACGGCATACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGT
Ad2.22	CAAGCAGAAGACGGCATACGAGATTGTGACCAGTCTCGTGGGCTCGGAGATGT
Ad2.23	CAAGCAGAAGACGGCATACGAGATAGGGTCAAGTCTCGTGGGCTCGGAGATGT

Table 1.2: Name and full sequence of the PCR primers used for amplification, indexing and pooling of the ATAC-seq and ChIPm samples in this thesis. These primers were designed by Buenrostro *et al.*, 2013 and they are an modified version of the Nextera Illumina primers optimised for larger molecular weight DNA fragments from low input samples. All samples were indexed with the universal primer Ad1.noMx and one of the additional 18 primers. The indexing sequence of each of the primers is in blue text.

Following the Nature Methods publication of Corces et al., 2016, the initial ATAC-seq protocol was replaced by a modified version named Fast-ATAC. It was specifically optimised for hematopoietic cells and combined cell lysis and transposition in a single step. Fast-ATAC was performed as described by Corces et al., 2016 with minor modifications. Since 5,000 cells was considered the lower limit to generate good quality data in Fast-ATAC, in my experiments I used 20,000 MACS or FACS sorted cells, to account for inaccurate manual cell counting as well as possible cell loss over centrifugation steps. The Fast-ATAC reaction was performed for 30 min at 37°C and agitation at 400rpm. DNA was purified as in ATAC-seq and libraries were generated following 13 cycles of PCR amplification, following appropriate cell cycle determination. Purification following PCR were performed using Agencourt AMPure XP Magentic Beads only.

Omni-ATAC, a third generation of ATAC-seq was published by Corces et al., 2017. It consisted in an universal protocol with individual cell lysis and transposition reactions intercalated with a washing step, to remove mitochondrial DNA and other cell debri Omni-ATAC was performed as described by Corces and colleagues (**Corces2017**) using 50,000 cells.

Following either of the three protocols, DNA tagmentation profiles were assessed with the D1000 high sensitivity DNA tape (Agilent) as part of the quality control and quantified using the Kapa kit (Roche), following the manufacturer's instructions. Pools of 12 to 16 libraries were sequenced in one to 3 lanes of the HiSeq4000 Illumina platform by the Oxford Genomics Centre at the Wellcome Trust Centre for Human Genetics.

1.3.3 Chromatin Immunoprecipitation with sequencing library preparation by Tn5 transposase

Assessment of histone marks modification in the chromatin of PSO patients from Cohort 1B and four age matched healthy volunteers was performed

using a low cell input Chromatin Immunoprecipitation (ChIP) method know as ChIPmentation (ChIPm). For each individual and cell type three histone marks, including H3K27ac, H3K4me1 and X were tested in 100,000 cells and compared to an input control processed in parallel. Samples were processed following the protocol published by Schmidl and colleagues (**Schmidl2015**) with some modifications. Aliquots of 600,000 cells of MACS sorted cell types, as described in 1.2, were fixed with 1% formaldehyde (Sigma) and snap frozen in dry ice and ethanol prior to storage at -80°C. Chromatin sonications of the different individuals and cell types were performed in one batch using Covaris M220(Covaris). Each of the aliquots was resuspended in 130µL of SDS lysis buffer (Table1.3), sonicated for 8 min using a duty factor of 5% and aliquoted for single ChIPm reactions prior to long term storage at -80°C.

Sonicated chromatin aliquots were thawed and resuspended in appropriate volume of ChIP equilibration buffer (Table1.3), in order to achieve the appropriate concentration of NaCl and Triton-X100. For the immunoprecipitation step, samples were incubated with the appropriate amount of antibody (Table 1.4) overnight in rotation at 4°C. Protein-A Dynabeads (Invitrogen) were also washed three times with Beads wash buffer (Table1.3) and blocked with yeast tRNA (supplier) overnight in rotation at 4°C. Beads were washed twice with the aforementioned washing buffer and resuspended in SDS lysis buffer before being added to the sample-antibody mix. One of the advantages of this protocol is the tagmentation of the chromatin when still bound to the beads prior to protein decross-linking, which allows preventing overtagmentation of the DNA.

qPCR was performed in each of the purified ChIPm DNA samples to identify the number of full cycles required to reach one-third of the final fluorescence. Libraries were amplified for the number of cycles minus one determined with this strategy, allowing to minimise the total number of PCR replicates. The primers used for amplification and indexing are the ones

optimised by Buensotro and colleagues (Table1.2). The number of amplification cycles for each of the samples is recorded in

Reagent	Final concentration	Supplier
SDS lysis buffer		
SDS	0.25%	Sigma
EDTA	1mM	X
Tris-HCl pH 8	10mM	Sigma
PI	1X	Roche
Water	-	Ambion
ChIP equilibration buffer		
Triton-X100	1.66%	Sigma
EDTA	1mM	X
NaCl	233mM	X
Tris-HCl pH 8	10mM	Sigma
PI	1X	Roche
Water	-	Ambion
Beads washing buffer		
SDS	0.1%	Sigma
EDTA	50mM	X
NaCl	150mM	X
NP-40	1%	Sigma
Tris-HCl pH 8	10mM	Sigma
PI	1X	Roche
Water	-	Ambion

Table 1.3: Composition of the three modified buffers in house for the ChIPm protocol: SDS lysis buffer, ChIP equilibration buffer and beads washing buffer. For each of the buffers the reagents, composition and supplier are indicated.The final volume prepared for each buffer was adjusted depending on the number of samples processed at the time. Sodium dodecyl sulfate (SDS), PI (proteinase inhibitor).

Histone mark	Feature	µL per sample	Manufacturer and catalo
H3K427ac	Promoter	X	
H3K4me1	Active enhancer	X	
X	X	X	

Table 1.4: Details regarding the histone marks, the the most likely chromatin state delineated, the amount of antibody required per reaction and the supplier and catalogue num of the antibodies.

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