Supplementary Materials for

Targeting human Plasmacytoid Dendritic cells through BDCA2 prevents skin inflammation and fibrosis in a novel xenotransplant mouse model of Scleroderma

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Materials and methods

Isolation of plasmacytoid dendritic cells (pDC)

For healthy pDC, commercial cryo-human pDC (Stemcell Technology) from healthy subjects, matched for gender and age, as well as pDC isolated from PBMC isolated from healthy cone donations, using Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec) following manufacturer protocol, were used. Firstly, the non-pDC are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against lineage-specific antigens and Anti-Biotin MicroBeads. Followed by depletion of non-pDC, using an LD MACS® column and magnetic field MACS Separator (Miltenyi Biotec), the pre-enriched pDC were labeled with pDC-specific CD304 (BDCA-4/Neuropilin-1) Diamond MicroBeads and isolated by positive selection over a MS MACS Column and magnetic field MACS Separator (Miltenyi Biotec). Purified pDC were then counted and tested for purity by FACS staining with mouse anti-human antibodies directed against lineage markers (VioBlue-CD3, CD14, CD19, CD56 and CD11c), APC-Vio770 HLA-DR, PerCPVio770-CD123 (IL-3R) and PE- CD304 (BDCA4) Abs. The purity of pDC was >98%.

RNA sequencing of healthy pDC

Total RNA was harvested from commercial healthy human pDC. Firstly, cells were thawed from 4 donors according to manufacturer protocol and cultured as above in RPMI1640+10% FBS+1% PS (unstimulated), 1 µM ODN2216 with and without 10ug/ml CBS004. RNA was extracted from cells using RNeasy minikit (Qiagen) according to manufacturer's protocol. Ovation® RNA-Seq System V2 (NuGEN) was used to amplify total RNA from all samples. Briefly, first-strand cDNA was made and used to generate double-stranded cDNA followed by a SPIA® amplification. cDNA were quantified by using Qubit dsDNA BR Assay kit (Thermo Fisher Scientific) and the quality was checked by using D1000 screen tape on a Tapestation (Agilent). Covaris S2 sonicator (Woburn) was used to fragment all the cDNA at a size of 200bp. 50 ng cDNA was used to make libraries by using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (Ipswich) without any size selection. The size distribution of the final libraries were checked using the Tapestation and quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific). All the libraries were pooled at a concentration of 10 ng and was sequenced on a HiSeq 3000 instrument (Illumina). Pooled sequence data were demultiplexed using

Illumina bcl2fastq software, allowing no mismatches in the read index sequences. Raw paired-end sequence data in Fastq format were quality-checked using FastQC software.[1] Cutadapt software[2] was used to trim poor quality bases (Phred quality score < 20) and contaminating adapter sequences from raw reads. Reads trimmed to fewer than 30 nucleotides and orphaned mate-pair reads were discarded. Reads were aligned to human hg38 analysis set reference sequences, obtained from UCSC database[3] using the splicing-aware STAR aligner.[4] STAR aligner was run in 2-pass mode, with known splice junctions supplied in GTF file format, obtained from hg38 RefSeq gene annotation table from UCSC database using Table Browser tool.[5] The resulting alignments in BAM file format were checked for quality using QualiMap software [6] and Picard tools,[7] with the latter being used to also mark PCR/Optical duplicate alignments. BAM files were sorted and indexed using Samtools software[8] and visualised using IGV browser.[9] Bioconductor R package RSubread[10] was used to extract raw sequenced fragment counts per transcript using RefSeq hg38 transcript annotation set. Paired-end reads were counted as a single fragment and multi-mapping read pairs were counted as a fraction of all equivalent alignments. Raw count data were normalised for library size differences using median ratio method,[11] as implemented in DESeq2 R Bioconductor package.[12] DESeq2 was also used to perform additional data QC steps and differential expression analyses. False Discovery Rate (FDR) was calculated using Benjamini-Hochberg multiple testing correction. Genes below 5% FDR threshold were considered differentially expressed. Differentially expressed gene expression was visualised as clustered heatmaps using the Pheatmap R package,[13] using log-transformed normalised gene expression values as input. Principal Component Analysis (PCA) was carried out using the 'prcomp' R function, using the expression of 1000 most variable genes as input. Gene enrichment analyses and annotation were performed using R Bioconductor packages clusterProfiler[14] and ReactomePA.[15] Additionally, KEGG[16] pathways were visualised using Pathview package.[17]

Affinity analysis of humanized mAbs by BIAcore

A BIAcore T200 was used with BIAcore run buffer (HBS-EP) at pH7.4. 692RU of huBDCA2-Fc was immobilized to a CM5 chip (CFJB156) utilising 5 µg/ml of huBDCA2-Fc with the BIAcore EDC/NHS kit according to the manufacturer's instructions. Two-fold dilutions of the anti-human CD303 BDCA2 antibody (AC144) (Miltenyi Biotec), chimeric CBS004 (Capella Bioscience) or humanized CBS004 mAbs (LONZA, Cambridge) were injected starting at 200 nM down to 3.1 nM with a contact time of 60s at a flow of 30 or 60 µl/min at 25C followed by an off-rate wash for 5 minutes with BIAcore buffer. Regeneration of the chip was achieved with two injections of 10 µl of 10 mM NaOH/1M NaCl between samples. The BIAcore T200 software was used to calculate Ka (1/Ms), Kd (1/s) and the KD (nM).

Competition ELISA assay

Wells were coated with 0.5 µg/ml of human (hu) BDCA2-Fc in 1x PBS pH 7.4 O/N at 4°C, added at 100 µl per well. Blocking was achieved with 4 % Skimmed Milk (Marvel) in PBS for 2 h at RT. To the well, 50µof huBDCA-2-Fc protein was added in a dilution range [1-0.001 µg/ml] in 1 % Skimmed Milk in 1x PBS (buffer) plus 50 µl of CBS004, or AC144 in buffer at [0.04 µg/ml] was added for 1 h at RT.

Bound CBS004 and AC144 were detected by secondary antibodies: Mouse Anti-Human IgG (anti-CH1-HRP) (BD Pharmigen) at 1:1000 or Peroxidase conjugated Affinipure Donkey anti-mouse IgG (anti-mouse IgG-HRP) at 1:5000 (Jackson ImmunoResearch), respectively, in buffer. Control 2nd antibody: anti-CH1-HRP and anti-mouse IgG-HRP in 1 % Skimmed Milk / 1x PBS; 100 µl/well. Development achieved by TMB (ThermoFisher) and Stop reaction by H_2SO_4 and the absorbance read at 450nm.

Peripheral blood mononuclear cells handling

Blood samples from 15 SSc patients enrolled at the Scleroderma clinic within the Leeds Institute of Rheumatic and Musculoskeletal Medicine (UK) and from 15 healthy subjects, matched for gender and age, were analyzed in this study. All SSc patients enrolled fulfilled the 2013 EULAR/ACR classification criteria for SSc. All subjects signed informed consent to participate in the study and all related procedures. Peripheral blood mononuclear cells (PBMC) were isolated from EDTA anti-coagulated blood by density gradient separation using prefilled Leucosep™ tubes (Greiner Bio-One Ltd, UK). PBMC were cultured in RPMI1640 containing 10% FBS and 1% Penicillin Streptomycin (PS) (Gibco Laboratories, Grand Island, NY).

FACS analysis of pDC

PBMC were labelled for FACS staining with mouse anti-human antibodies directed against antibodiy mix containing lineage markers (Vioblue-CD3, CD14, CD19, CD56 and CD11c) (BD Biosciences), APC-Vio770 HLA-DR, PerCPVio770-CD123 (IL-3R) and PE- CD304 (BDCA4) FITC-CD303 (BDCA2) (Miltenyi Biotec). The plate was then incubated at 4°C for 30 minutes followed by addition of 200 µl of FACS buffer and centrifugation at 300g for 10 minutes at 4°C. Supernatants were decanted, and cell wash repeated using 200 µl of ice-cold Dulbecco's PBS. Cell pellets were re-suspended in 200ul of Fixation/permeabilization buffer (e-Biosciences) and the plate incubated at 4°C for 30 minutes. Following centrifugation, cells were washed once in 200 µl of perm/wash buffer (e-Biosciences). Finally, the cells were re-suspended in FACS buffer for flow cytometry analysis. The data acquisition was performed on LSRII 4 laser flow cytometer (BD Biosciences), and the analysis was conducted using FACS DIVA software (BD Biosciences). pDC gating strategy excluded dead cells using Aminoactinomycin D (7-AAD) (BD Biosciences) and lineage-, and HLA-DR+, with sequential gating for human CD45+CD123+CD304+. For competition assay between CBS004 and AC144, both antibodies were incubated with fixed PBMC on ice for 30 minutes. For intracellular detection of IFN, prior to resuspending in FACS buffer, cells were labelled with APC IFN-I (Miltenyi Biotec) at 4°C 30 minutes. For pDC subtyping, PBMC were counted and plated in 96 well plate and stimulated for 18 hours using 1 µM ODN 2216. FACS staining was performed using antibody mix detailed above, plus BV650-PD-L1(CD274) and APC-R-700 CD80 (BD Biosciences). Cells then resuspended and analysed using FACS analysis for gating on pDC using Lineage- DR+ CD123+CD304+ and for sub-typing of the three activated pDC populations based on PD-L1 and CD80 expression: P1, PD-$L1^+CD80^-$; P2, PD-$L1^+CD80^+$; P3, PD-$L1^-CD80^+$.

Organotypic 3D skin rafts (OSR)

Primary normal human dermal fibroblasts and keratinocytes (from caucasian female breast tissue) (Promocell) were used to generate a skin-like 3D culture. These cells were routinely cultured in DMEM+10%FBS+1%PS and complete Keratinocyte Growth Medium 2+1%PS (Promocell), respectively, and handled according to user guidelines. Firstly, fibroblast-collagen cultures were prepared in Falcon cell culture inserts and placed into Falcon 6 Well Deep Well TC-Treated Polystyrene Plates (BD Biosciences). These cultures were prepared on ice by adding PureCol bovine type 1 collagen (Advanced Matrix), followed by 10x HBSS (ThermoFisher Scientific) (bringing to the correct pH using NaOH single droplets until media turned pink) and then $2x10^5$ fibroblasts in FBS, following the composition ratio of 8:1:1. Using chilled stripettes, 2.5 ml of the mixture was added carefully to each well. Cultures were left at 37°C for 2 hours without CO_2. Complete KGM™ Keratinocyte Growth Medium BulletKit™ (Lonza) was then added into the well (12.5 ml), and on top of the set collagen culture (2.5 ml) and left overnight at 37°C with 5% CO_2. Media was carefully removed from the gel and $2x10^6$ keratinocytes were seeded in 2 ml of media/well and left overnight. Cultures were finally placed into Air-Liquid Interphase (ALI) by carefully removing all media and adding 10 ml of ALI media into the bottom of the wells. ALI contained Complete KGM Lonza media without BPE supplement, with the addition of 50 µg/ml of ascorbic acid, 1 mg/ml BSA, 10 µg/ml Transferrin, and 1.1 mM of $CaCl_2$ (Promocell). Cultures were media changed every 2-3 days and left for 5 days. On day 5, ALI was supplemented with supernatants from pDC treated as above (CTR; no ODN stimulation, ODN; ODN stimulation, ODN+CBS004; ODN stimulation plus 10 µg/ml CBS004) to produce a final concentration of 6000 pg/ml of IFN in the ODN experiment (determined via ELISA, approx. dilution of supernatants 1:20). Cultures were left for 48 h. 3 mm punch biopsies were taken and harvested for histology analysis. The remaining culture was collected into 1 ml of TRIzol™ and processed for RNA extraction as described below.

ELISA assays

PBMC (1-2*10^6 cells) and pDC (1-4*10^4) were maintained for 16 h in RPMI1640+10% FBS+ 1% (PS) (Gibco Laboratories) in 96 well round bottom plates. Cells were cultured with or without 1 µM of TLR9 (ODN2216) (Milteny Biotec) or 4 µM of Imiquimod (InvivoGen) in presence or absence of increasing concentration of CBS004 or control human IgG1 (Crown Biosciences) [0.0005-10 µg/ml]. Cell-free supernatant was harvested after 16 h by centrifugation at 300g for 10 minutes and IFN alpha levels were evaluated using commercially available PBL-Elisa kit (PBL Assay Science), according to manufacturer recommendations. This kit detects 14 out of 15 identified human IFN-α subtypes. They are: IFN-αA, IFN-α2, IFN-αD, IFN- αB2, IFN-αC, IFN-αG, IFN-αH, IFN-αI, IFN-αJ1, IFN-αK, IFN-α1, IFN-α4a, IFN-α4b, and IFN-αWA.

Xenotransplant mouse models of human pDC activation (XenoSCID)

All mice used were severe combined immunodeficient (CB17/Icr-Prkdcscid/IcrIcoCrl, Charles River) between 4 to 8 weeks of age, housed in accordance with local and Home Office regulations. For the

Aldara model, mice were shaved on the back and received topical Aldara application (5% Imiquimod; Meda Health Sales). After 12h, a second application of Aldara and an intraperitoneal (i.p.) injection of CBS004 mAb (5mg/kg) or control human IgG was administrated. 12 h later the mice received an intravenous (i.v.) tail injection of $2.5x10^5$ human pDC. Mice were then euthanized after a further 12h and skin harvested using a punch biopsy and processed for gene expression and FACS analysis. For the bleomycin-induced fibrosis model, 100 µl Bleomycin (Sigma) at 200 µg/ml in PBS was injected subcutaneously into a single location on the shaved back of mice once every other day for 3 weeks. Additionally, some mice received 2.5 $x10^5$ human pDC i.v. on day 0, 7 and 14 following first BLM injection. CBS004 or human IgG (5mg/Kg) were injected i.p. every 5 days starting 24 hours prior to first bleomycin injection. Different pDC donors were used for the different timepoints, however all conditions were subjected to the same donor at the same time. ODN-induced IFN response was confirmed *in vitro* as outlined above.

FACS analysis of XenoSCID samples

Skin samples from mice were enzymatically digested to release cells using 1 mg/ml collagenase D (Roche), 0.5 mg/ml dispase (Roche) and 0.1 mg/ml DNase-I (Invitrogen, Carlsbad, CA, USA) in Hanks' balanced salt media (Sigma-Aldrich Corp). For FACS analysis, the released cells were stained with antibodies against human CD45, CD123, CD304 (Miltenyi Biotec). Gating strategy excluded dead cells using Aminoactinomycin D (7-AAD) (BD Biosciences) and sequential gating for human CD45+CD123+ CD304+ as mentioned above.

ISG response analysis of XenoSCID and organotypic 3D models

RNA was extracted using TRIzol™ Plus RNA Purification Kit (Thermo Fisher Scientific) as per the manufacturer's instruction. Briefly, skin was homogenized in TRIzol using two 7 mm metal beads and a TissueLyser LT (Qiagen). Homogenates were centrifuged to separate an RNA containing aqueous phase, after which it was further purified by PureLink columns and genomic DNA removed by DNase (Life Technologies, Carlsbad, CA, USA). Eluted RNA was converted to cDNA using RT2 First Strand Kit (Qiagen). Next, the cDNA was mixed with an appropriate RT2 SYBR Green Mastermix (Qiagen). Mouse and Human Type I Interferon Response RT2 Profiler PCR Arrays (Qiagen) were performed and relative expression determined using the ΔΔCT method and normalized for 5 housekeeping genes according to manufacturer's guidance. For the generation of the composite ISG score in the XenoSCID model, qRT-PCR was also performed for mouse mRNA targets *Mx1* (FWD 5'-tcagtcttccttggcagcag-3' REV 5'-tagaggactggtctgcagct-3', *Isg15* (FWD 5'-cgcagactgtagacacgctta-3' REV 5'-ctcgaagctcagccagaact-3'), *Viperin* (FWD 5'-tgaagcgtggcggaaagtat-3' REV -5'-tccttcccatctcagcctca-3'), *Ifit1* (FWD 5'-ttgcaccacactagcttgca-3' REV 5'-gggatggaagcactcacagt-3'), and *Cxcl10* (FWD 5'-gctcaagtggctgggatg-3' REV 5'-gaggacaaggagggtgtgg-3'), all of which were shown to be pDC-stimulated ISGs from the preliminary array data (increased >2-fold within the skin of mice between CTR and Aldara+pDC conditions). *Gapdh* (FWD 5'-cagcaaggacactgagcaag-3' REV 5'-tattatgggggtctgggatg-3') was used as housekeeping gene control and used to calculate relative expression determined using the ΔΔCT method. Composite ISG score is an average of the fold

difference in relative expression between test group and CTR for each mRNA. Test and CTR group were linked with mouse litter and experimental timepoints.

Histology

3 mm punch biopsies from mice and patients were formalin-fixed and embedded in paraffin. Sections were cut at 5 μM and subjected to haematoxylin and eosin (H&E) staining. Masson trichrome was used to dye collagen blue and muscle red to identify the extent of fibrosis in the skin samples. Briefly, 3 dyes are used sequentially; Weigerts iron haematoxylin for nuclei, ponceau fuchsin for muscle, cytoplasm and erythrocytes, and methyl blue for collagen. In between stains, slides are washed in water. Prior to adding the final methyl blue dye, two Phoshotungstic acid incubations were performed. Slides were mounted post water rinse, dehydration in alcohol and Xylene. Antigen retrieval was performed using 10 mM pH 6.0 sodium citrate and sections were stained with anti-MX1 antibody (Abcam) and pSTAT1 Tyr701 (Cell signaling) followed followed by ImmPRESS™ (Peroxidase) Polymer Anti-Rabbit IgG Reagent (Vector Laboratories), and visualised with 3, 3-diaminobenzidine (DAB) (Vector Laboratories). Mouse spleen, healthy skin and negative staining were performed for controls. Microscopic analysis was performed using an Olympus BX50 with MicroFire (Optronics) and images captured using Stereo Investigator software at 20X magnification. For epidermal and dermal measurements, each condition was performed in triplicate mice. For each mice, 1 20X H&E representative image was used to take 10 measurements. Epidermal measurement was taken from the top of the skin section to the basement membrane, while the dermal measurement also included up until the top of the muscle layer.

Western blotting

Total protein was extracted from skin biopsies in M-PER mammalian protein extraction reagent and resolved by SDS-PAGE (10-15% Tris-Glycine), transferred onto Hybond nitrocellulose membrane (Amersham biosciences) and probed with antibodies specific for MX1. Immunoblots were visualized with species-specific HRP conjugated secondary antibodies (Sigma) and ECL (Thermo/Pierce) on a Biorad ChemiDoc imaging system.

Measurement of collagen in XenoSCID skin samples

Soluble collagen was quantified using the Sircol soluble collagen assay (Biocolor). Punch biopsy skin samples were obtained from XenoSCID and protein extracted and homogenised using M-PER mammalian protein extraction reagent (Thermo Scientific) and two 7mm metal beads. The samples were then further extracted using acetic acid–pepsin solution according to the manufacturer's protocol. Briefly, 100 μl of sample was added to 1 ml of the colorimetric reagent and agitated for 30 min followed by centrifugation at 10,000*g* for 10 min. The SR dye was released from the pellet with alkali reagent and absorbance measured at 555 nm using a microplate reader. Collagen concentration was calculated using the standard curve generated from collagen reference standards. Concentrations were normalised for total protein concentrations calculated by Pierce™ BCA Protein Assay Kit (Thermo Scientific).

Statistical analysis

GraphPad Prism 7 software (GraphPad 50 Software) was used for statistical analysis. Pearson's correlation was used to analyze the association between all studied parameters. One-way analysis of variance combined with Mann-Whitney test or unpaired and paired two tailed t-test were used to evaluate statistically significant differences between groups. Data were expressed as the mean ± standard error (SE). Significance was considered with a P value less than 0.05.

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