

fig. S1: Gating strategy for analysis of ILC2.

A. Isotype controls for ILC2 flow cytometry antibody stain. ILC2 are CD45⁺/CD3⁻/Lineage⁻/CRTH2⁺/IL7Rα⁺. **B – D** Isotype controls for CD1a expression on epidermal CD11c⁺ cells (**B.**), ILC2 (**C.**) and T cells (**D.**). **E.** Full gating strategy for isolation and analysis of ILC2. **F.** Isotype controls for strategy of isolation and analysis of ILC2.

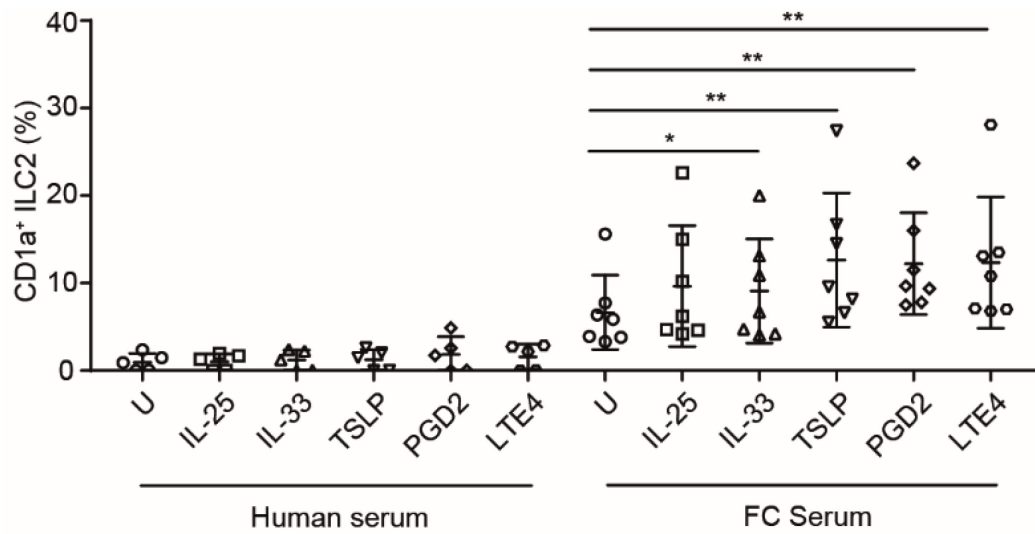


fig. S2: Stimulation of CD1a expression by blood derived ILC2.

ILC2 were cultured in media containing either human serum or fetal calf serum (FCS). Additional ILC2 activation stimuli, IL-25 (50 ng / ml), IL-33 (50 ng / ml), TSLP (50 ng / ml), PGD2 (100nM) or LTE4 (50 nM) (U = unstimulated), were added to the ILC2 culture for 72 hours. CD1a expression was determined by flow cytometry (n = 4 donors, t-test, and data represent 4 independent experiments).

Materials and Methods

Study design

The study was designed to test the hypothesis that ILC2 present antigen to CD1a-reactive lipid-specific T cells. Atopic dermatitis was diagnosed according to the UK refinements of the Hanifin and Rajka diagnostic criteria, and adult participants were only excluded if on systemic immunosuppression or topical calcineurin inhibitors. Clinic participants were recruited sequentially; blinding and randomization were not required as there was no intervention. Thus variation between the functional responses of different donors is expected as cells were isolated from individuals of different, age, gender, ethnicity and medical history, although broadly defined as healthy controls. Sample size was determined based on previous studies of CD1a-reactive T cell response frequencies in humans (1). All experiments were replicated as presented in the figure legends.

Production of human recombinant PLA2G4A.

PLA2G4A was produced in Sf9 insect cells using the baculovirus expression system. Briefly, the human PLA2G4A (Genbank no. BC114340) was inserted into the vector, pOPINE which adds a C-terminal his₆ tag (PMID: 17317681), and a recombinant baculovirus constructed as previously described (PMID: 25502201). Sf9 cells (1.0 L) were infected with the PLA2G4A baculovirus and harvested after 72 hours. Recombinant his-tagged PLA2G4A was purified from the cell lysate by a combination of immobilized metal affinity chromatography and gel filtration.

Antibodies and flow cytometry

For FACS surface staining the cells were labelled with the following anti-human antibodies purchased from BioLegend unless stated otherwise: CD3 (OKT3, Biolegend), CD19 (SJ25C1, BD Biosciences), CD123 (FAB301C, R&D Systems), CD11b (DCIS1/18), CD11c (BU15, Abcam), CD8

(RPA-T8), FcεRI (CRA-1), CD14 (MwP9, BD Biosciences), CD4 (OKT4), CD45 (2D1), CD56 (HCD56), CRTH2 (BM16, Miltenyi Biotec), IL-7Ra (A019D5), Live/Dead violet or Aqua (Invitrogen), CD1a (HI149). Intracellular cytokine staining was completed using the eBioscience FoxP3 Fix/Perm kit as per the manufacturer's instructions with Brefeldin-A (eBioscience) and anti-IL-13 (85BRD, eBioscience) antibody.

Suction blister technique

Suction blister cups were applied to the skin of the forearm of adult patients with atopic dermatitis (AD) and healthy individuals, at a vacuum pressure of 250 mmHg. Blisters were generated over the site of a house dust mite intradermal injection or over unchallenged skin. Blisters were formed within 30 – 90 min of suction application. Blister fluid was aspirated 24 hours later using a 30 - gauge needle. Fluids were then centrifuged at 1500 rpm for 5 min at 4°C and the concentration of TSLP was measured by multiplex array (multiplex bead array) and in separate studies the cells were stained with cell surface antibodies for flow cytometric isolation of ILC2 and T cells utilized for RNA Sequencing analysis.

RNA Sequencing

Suction blister fluid and blood derived PBMCs were centrifuged at 1500 rpm for 5 minutes at 4°C to pellet the blister-infiltrating cells, which were re-suspended in PBS. Blister and blood cell populations were isolated by flow cytometry and collected directly into TRIzol LS; T cells (CD3⁺ CRTH2⁺) and ILC2 (Lin⁻ CD45⁺ CD3⁻ IL-7Rα⁺ CRTH2⁺). The manufacturer's protocol was followed for TRIzol LS mRNA extraction as far as "Phase Separation". The RNA containing phase was then processed using Qiagen RNeasy mini kit and contaminating DNA was removed using Ambion Turbo DNase. The total purified RNA was then processed using a NuGEN Ovation RNA-seq system V2 (Ultralow DR multiplex kit). Samples were sequenced on an Illumina HiSeq 2000. Following QC analysis with the fastQC package (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>), reads were aligned using STAR (2)

against the human genome assembly (NCBI build37 (hg19) UCSC transcripts). Non-uniquely mapped reads and reads that were identified as PCR duplicates using Samtools (3) were discarded. Gene expression levels were quantified as read counts using the featureCounts function (4) from the Subread package (5) with default parameters. RPKM values were generated using the edgeR package (6).

Cell sorting and culture

PBMCs were isolated from healthy adult donors under local ethics approval (National Research Ethics Service Committee South Central, Oxford C, 09/ H0606/71). ILC2 were isolated and cultured as previously described (7). Briefly, the lineage (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, CD123, and Fc ϵ RI)⁻, CD45⁺, IL7R α ⁺, CRTH2⁺ ILC2 population was sorted into 96-well plates at 100 cells per well and resuspended in MLR of gamma-irradiated PBMCs from three healthy volunteers (2x10⁶ cells / ml) coupled with 100 IU / ml IL-2 and PHA. After 4 – 6 weeks the growing cells were tested by flow cytometry staining to ensure a pure population of lineage⁻ CRTH2⁺IL-7R α ⁺ ILC2 was obtained (fig. S1E and S1F). Autologous T cells were isolated from donor PBMCs prior to isolation of ILC2 using magnetic-activated cell sorting (CD3 Microbeads Miltenyi Biotech.).

Cytokines and TLR agonists

For stimulation studies ILC2 were incubated in culture with 50 ng / ml of TSLP (Peprotech), or 10 μ g / ml PamCSK (Invivogen), 1 μ g / ml LPS (Invivogen), 10⁸ cells / ml HKSA (Invivogen) for 6 - 72 hours at 37°C as noted in the figure legend. Cells were then centrifuged at 1500 rpm for 5 minutes at 4°C and supernatant saved for enzyme activity and cells used for RT-PCR analysis.

Quantitative RT-PCR

mRNA extraction was performed using a TurboCapture 96 mRNA kit (Qiagen, 72251) following the manufacturer's instructions. cDNA was prepared from the mRNA using M-MLV reverse transcriptase (Invitrogen). Taqman probes for GAPDH (Hs02786624_g1), PLA2G4A (Hs00996912_m1), PLA2G4C (Hs01003754_m1) were used to analyze gene expression of ILC2 cultures on a QuantStudio7 Flex real time PCR machine.

Analysis of immune cells within human skin biopsies

To analyze CD1a⁺ populations in the skin, samples of human skin were processed as follows. Samples were taken under GCP guidance with ethical approval of the NRES Committee South Central. Subcutaneous fat was removed using scissors and residual fat cells were scraped from the underside of the skin and hairs from the upper side using a sharp scalpel. To analyze the cells of whole thickness skin samples, the explants were cut in to < 0.5mm pieces using scalpel and scissors in petri dishes and incubated in collagenase P (1 mg / ml Roche) containing media overnight at 37°C in the petri dish. After overnight digestion, the remaining tissue was homogenized with a Pasteur pipette and endonuclease deoxyribonuclease I (DNase I) 200 Kunitz unit/ml (Roche 10104159001) was added for 15 minutes at room temperature then passed through a 70 µm strainer (VWR) and washed with cold 10 mM EDTA solution. After centrifugation, the pellet was resuspended in cold RPMI and passed through a 40 µm strainer ready for further analyses.

To isolate the epidermis 1 cm² sections of skin were placed epidermis down in a petri dish containing 5 U / ml dispase at 4°C overnight. The epidermal layer was then peeled from the dermis with forceps and chopped up and placed in an Eppendorf of 0.5 % Trypsin 0.02 % EDTA at 37°C for 15 minutes. The samples were homogenized with a Pasteur pipette and strained through a 40 µm filter and diluted and washed in FCS containing media. The single cell suspension was then stained with cell surface marker antibodies for flow cytometry.

ELISpot functional assays

CD1a reactivity was assessed by IFN- γ and IL-22 ELISpot (Mabtech AB). ELISpot plates (Millipore Corp., MA) were coated with anti-cytokine capture antibody overnight at 4°C (Mabtech AB). ILC2 were pulsed with HDM (7 μ g / ml), PLA2G4A (1 μ g / ml) (with or without 1 μ M inhibitor MAFP (methyl arachidonyl fluorophosphonate)) or heat-killed *S. aureus* (10^8 cells / ml) overnight, and were then washed and resuspended in FCS ILC2 media (RPMI supplemented with 2 mM L-glutamine, 100I U / mL penicillin, and 100 μ g / mL streptomycin, Sodium Pyruvate, non-essential amino acids, BME, hepes, plus 10% FCS). The plates were washed six times with RPMI-hepes and blocked for 1 h with ILC2 FCS media. A total of 50 000 T cells were plated per well to which 50 000 ILC2 were added for co-culture with 10 μ g / ml anti-HLA A,B,C (W6/32 eBioscience) and HLA-DR (L243, eBioscience) antibodies to inhibit ILC2-MHC-TCR interaction.

In some wells 10 μ g / mL anti-CD1a blocking antibody (OKT-6), 10 μ g / mL IgG1 isotype control, cPLA2 inhibitor MAFP (10 μ M) or anti-TLR2 (pab-hstlr2, Invivogen) and anti-TLR4 (pab-hstlr4, Invivogen) antibodies (10 μ g / ml) were added to ILC2 before addition of T cells. Wells were set-up in duplicate or triplicate. Phorbol myristate acetate 10 ng / mL and Ionomycin 500 ng / mL stimulation was included as a positive controls for T cells and ILC2, and T cells alone in the absence of ILC2 and ILC2 alone were included as a negative controls. After overnight incubation at 37°C and 5% CO₂, culture supernatants were recovered, and plates were washed x 6 in PBS-Tween 0.05% and incubated with 1 μ g / mL of biotin-linked anti-IFN- γ or anti-IL22 monoclonal antibody (Mabtech AB) for 2 h. After washing 6 times in PBS-Tween 0.05%, the plates were incubated for 1 h with streptavidin-alkaline phosphatase (Mabtech AB). Spots were visualized using an alkaline phosphatase conjugate substrate kit (Biorad, Hercules) and enumerated using an automated ELISpot reader (Autimmun Diagnostika gmbh ELISpot Reader Classic). Number of IFN γ or IL-22 producing T cells per 50 000 cells presented on result graphs was calculated as the total number of spots enumerated minus the number of IFN γ or IL-22 spots in the T cell alone negative control wells.

To assess the functional role of CD1a expressed by ILC2 we relied on the use of CD1a blocking antibody clone OKT6 and calculating the CD1a dependent response by analysing the differential signal of unblocked versus anti-CD1a-mAb-blocked conditions. The variability of human data and of the efficacy/completeness of neutralising antibody experiments was accounted for by careful replication and analysis of a number of donors with experiments completed on different occasions. Concentration of OKT6 used for blocking has been previously optimized (8). Prior incubation of ILC2 with anti-CD1a preceding antigen pulsing was undertaken to aid CD1a blocking.

PLA₂ biochemical activity assays

Cytosolic and secreted PLA₂ activity of recombinant PLA₂G4A, heat-killed *S. aureus* preparation and ILC2 culture supernatant were measured using either a cytosolic PLA₂ kit (Cayman Chemicals) or a secretory PLA₂ kit (Cayman Chemicals) according to the manufacturer's protocols. Arachidonoyl thio-PC is the substrate for cPLA₂ and hydrolysis of the arachidonoyl thioester bond at the sn-2 position by cPLA₂ releases a free thiol which can be detected by DTNB (5,5'-dithiobis[2-nitrobenzoic acid]) producing a colored precipitate. For the sPLA₂ kit in the presence of PLA₂, cleavage of the substrate (diheptanoyl thio-PC) at the sn-2 position results in release of the thiol group. In both assays absorbance was measured with a spectrophotometer (Clariostar) (wavelength 414 nm) to give a measure of PLA₂ activity.

Statistical analysis

The one-way ANOVA tests with Tukey's multiple comparison tests (alpha 0.05), and paired and unpaired two-tailed T-tests were performed using GraphPad Prism version 6.00 (GraphPad Software), as indicated in the figure legends. Error bars represent standard deviation of the mean. The number of biological replicates and number of times the experiments were independently performed are recorded in the figure legends. Exact p values are given in the supplementary source data file (Table S1).

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