Supplementary Materials and Methods

***PL Tong* et al. 2014**

The Skin Immune Atlas: Three-Dimensional Analysis of Cutaneous Leukocyte Subsets by Multiphoton Microscopy

Tissue processing for histology and staining

Ear pinnae, dorsal back, footpad and tail skin of albino C57BL/6 mice were fixed in 10% neutral buffered formalin before embedding in paraffin. Five μm sections were cut and then stained with haematoxylin & eosin (H&E), toluidine blue or with Milligan's trichrome stain.

Tissue processing for confocal microscopy

Ears of C57BL/6 mice were de-haired and treated with dispase I (BD), 5 U/mL in PBS for 1.5 h followed by 24 h fixation in 10% formalin. After fixation, tissues were embedded in paraffin, sectioned at 2 μm thickness, counterstained with DAPI (Invitrogen) and imaged using the Leica TCS SP5 confocal microscope.

Tissue processing for flow cytometric analysis

Preparation of epidermal and dermal single cell suspensions has been performed previously (Tschachler *et al*., 2004; Roediger *et al.*, 2013). Cell numbers were determined by adding 20,000 AccuCheck Counting Beads (Spherotech) to a 100 μl aliquot taken from a 5 mL cell suspension per ear. Cell suspensions were stained with fluorochrome-conjugated antibodies diluted in "FACS buffer" (PBS containing 5% FCS, 2 mM EDTA, and 0.02% sodium azide). Fluorochrome-conjugated antibodies to the following cell surface molecules were used: CD2 (RM-5); CD3 (145-2C11); CD11b (M1/70); CD45 (30-F11); CD64ab (X54-5/7.1); CD90.2 (53-2.1); TCR γδ (GL3; all from BD Biosciences); CD326 (G8.8; from eBioscience); I-A/I-E (M5/114.15.2; from BioLegend). After staining, cell suspensions were resuspended in FACS buffer containing 0.5 μg/mL DAPI (Invitrogen) for dead cell exclusion. Samples were acquired

using a LSR-II 5 laser or LSR Fortessa flow cytometer (BD Biosciences). Flow cytometric data were analyzed using FlowJo 9.4.11 software (TreeStar Inc. Ashland, OR).

Calculation of nearest distance from cells to the vasculature by automated image analysis

Images were processed, visualized and automatically analyzed using Imaris (Bitplane) integrated with custom code written in MATLAB (MathWorks). Each cell was assigned a Spot object and the vasculature demarcated by a Surface object. Spot positions were manually adjusted if necessary (23.5% on average). A custom coded but fully integrated Immune Atlas analysis module was then called from within Imaris. This code transferred the precise 3-dimensional coordinates of every object into MATLAB and calculated the nearest Euclidean distance from every cell to the vasculature (in µm). The custom module then exported all numerical values into Comma Separated Values (CSV) spreadsheets, and further analyzed statistically using R and MATLAB. The custom-developed module is freely available for academic use at http://www.matebiro.com/software/immuneatlas.

Passive cutaneous anaphylaxis

Female mice from all groups were injected intra-dermally (*i.d.)* with 20 µL of IgE anti-DNP mAb (Liu *et al*., 1980) at a concentration of 5 µg/mL (*i.e.*, 100 ng dose) diluted in HMEM-Pipes (Sigma-Aldrich) in the right ear and equal volume of vehicle in the left ear. For back skin, mice were injected (*i.d.*) with either IgE anti-DNP mAb or vehicle (HMEM-Pipes). 16 h later, mice were injected *i.v.* (tail vein) with DNP-HSA (200 µg in 100 µl, in 0.9% saline and containing 1% Evans blue (Gurr-Searle Diagnostic)). Mice were euthanized 30 min later and whole ears or 6 mm punch biopsies of dorsal back skin were obtained and weighed. To extract the Evans blue dye, the tissues were diced into pieces approximately 1-2 mm^2 and incubated in 0.3 mL formamide at 55°C overnight. After incubation, tissue pieces were separated from the formamide solution by

centrifugation at 13,000 rpm for 5 min and the extraction of Evans blue was quantified by absorption at 610 nm and data normalized to the weight of the sample.

Experimental design and statistical analysis

For animal experiments, sex-matched adult mice (8-12 weeks of age, unless otherwise indicated) were used. In ageing experiments, the same ear skin was subsequently subjected to flow cytometric analysis and MPM counts adjusted accordingly. Statistical analyses were performed using Prism 6 (GraphPad Software, Inc) and where relevant, data are presented as mean ± S.E.M. For comparisons of two groups, an unpaired Student's *t*-test or Mann-Whitney *U* test was used. For multiple comparisons, an unpaired one-way analysis of variance (ANOVA) was used followed by a Tukey or Dunnett's multiple-comparison test where appropriate. A difference between groups was considered significant if $P < 0.05$.

Reference

Liu FT, Bohn JW, Ferry EL, *et al*. (1980) Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. *J. Immunol*. 124(6); 2728–2737.

Roediger B, Kyle R, Yip KH, *et al*. (2013) Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat Immunol.* 14(6): 564-73.

Tschachler E, Reinisch CM, Mayer C, *et al.* (2004) Sheet preparations expose the dermal nerve plexus of human skin and render the dermal nerve end organ accessible to extensive analysis. *J Invest Dermatol.* 122(1):177-82.