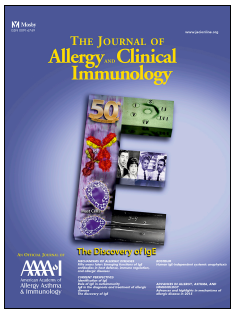
Enhancement of cutaneous immunity during ageing by blocking p38 MAPkinase induced inflammation

Accepted Manuscript



Enhancement of cutaneous immunity during ageing by blocking p38 MAPkinase induced inflammation

Milica Vukmanovic-Stejic, PhD, Emma S. Chambers, PhD, Mayte Suarez- Farinas, PhD, Daisy Sandhu, MD, Judilyn Fuentes-Duculan, MD, Neil Patel, MRCP, Elaine Agius, PhD, Katie E. Lacy, PhD, Carolin T. Turner, PhD, Anis Larbi, PhD, Veronique Birault, PhD, Mahdad Noursadeghi, PhD, Neil A. Mabbott, PhD, Malcolm H.A. Rustin, MD, James Krueger, MD PhD, Arne N. Akbar, PhD

PII: S0091-6749(17)31766-9

DOI: 10.1016/j.jaci.2017.10.032

Reference: YMAI 13129

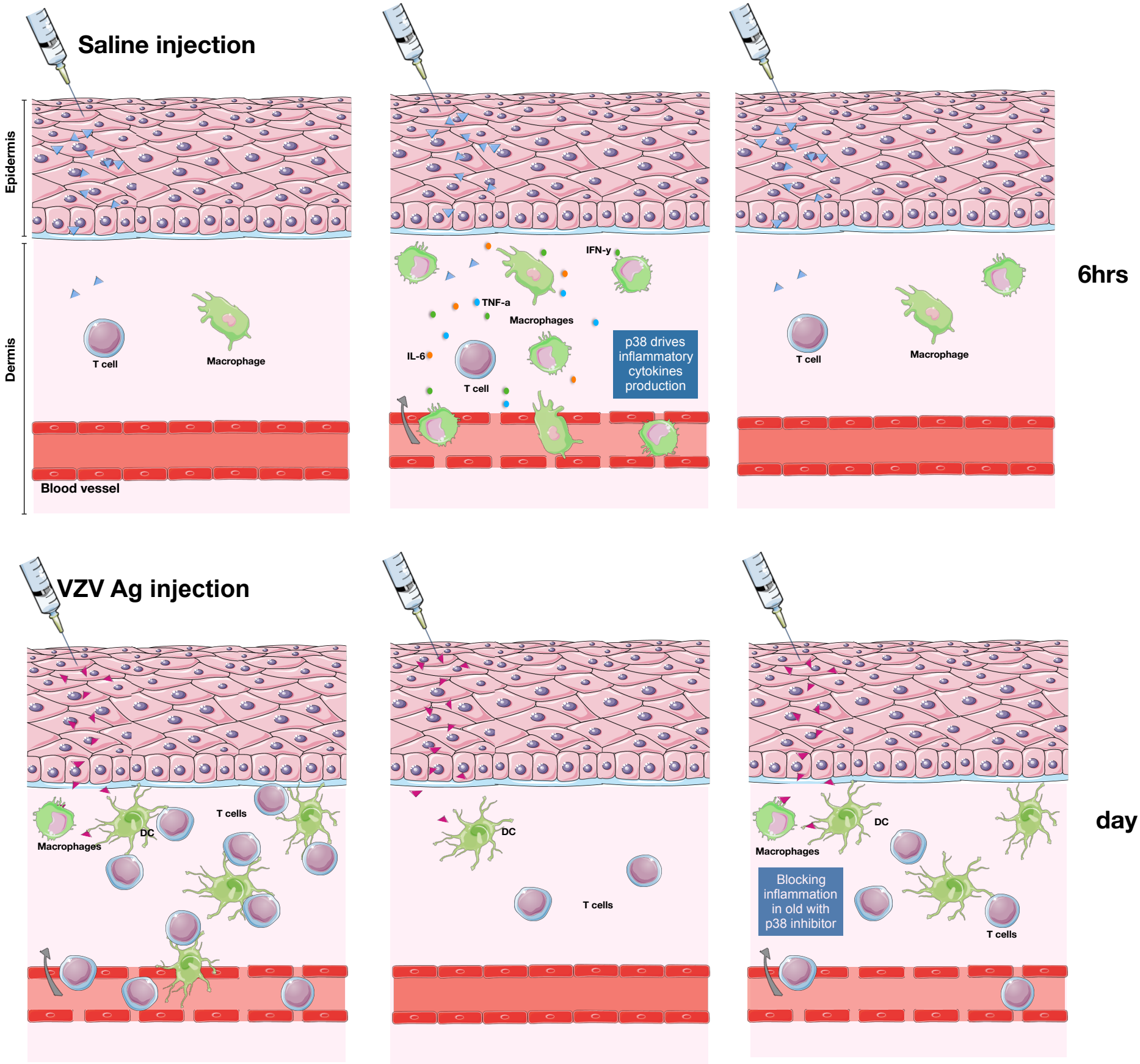
To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 8 August 2017

Revised Date: 13 October 2017

Accepted Date: 23 October 2017

Please cite this article as: Vukmanovic-Stejic M, Chambers ES, Farinas MS-, Sandhu D, Fuentes-Duculan J, Patel N, Agius E, Lacy KE, Turner CT, Larbi A, Birault V, Noursadeghi M, Mabbott NA, Rustin MHA, Krueger J, Akbar AN, Enhancement of cutaneous immunity during ageing by blocking p38 MAPkinase induced inflammation, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.10.032.


Saline injection
Epidermis
Dermis
T cell
Macrophage
Blood vessel
IFN-γ
TNF-a
Macrophages
IL-6
T cell
p38 drives inflammatory cytokines production
T cell
Macrophage
6hrs
VZV Ag injection
Macrophages
DC
T cells
DC
T cells
DC
Macrophages
Blocking inflammation in old with p38 inhibitor
T cells
day 7
Young
Old
Old
+
Losmapimod
(p38 inhibitor)

Enhancement of cutaneous immunity during ageing by blocking p38 MAPkinase induced inflammation

Milica Vukmanovic-Stejic, PhD[1*], Emma S. Chambers, PhD[1¶], Mayte Suarez- Farinas, PhD[2¶], Daisy Sandhu, MD[1,3], Judilyn Fuentes-Duculan, MD[2], Neil Patel, MRCP[1, 3], Elaine Agius, PhD[1, 3], Katie E. Lacy, PhD[1, 3, 4], Carolin T. Turner, PhD[1], Anis Larbi, PhD[5], Veronique Birault, PhD[6], Mahdad Noursadeghi, PhD[1], Neil A. Mabbott, PhD[7], Malcolm H.A. Rustin, MD[3], James Krueger, MD PhD[2], Arne N. Akbar PhD[1*]

1. Division of Infection and Immunity, University College London, London, WC1E 6EJ, England, UK

2. Laboratory for Investigative Dermatology, The Rockefeller University, New York, NY 10021, USA

3. Department of Dermatology, Royal Free Hospital, London, NW3 2QG, England, UK

4. NIHR Biomedical Research Centre at Guy's and St. Thomas's Hospitals and King's College London, Cutaneous Medicine and Immunotherapy, St. John's Institute of Dermatology, Division of Genetics and Molecular Medicine, King's College London School of Medicine, Guy's Hospital, King's College London, London, UK

5. Biomedical Sciences Institutes: Agency for Science, Technology and Research (A*STAR), Singapore 138648, Singapore.

6. The Francis Crick Institute, London NW1 1AT, UK

7. The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, UK

*Corresponding author:

Professor Arne N. Akbar or Dr Milica Vukmanovic-Stejic, The Rayne Building, 5 University Street, Division of Infection and Immunity, University College London, London, WC1E 6EJ. United Kingdom.

E-mail: a.akbar@ucl.ac.uk or m.vukmanovic-stejic@ucl.ac.uk

Tel: +44-203-108 2172

¶ M.S-F and E.S.C contributed equally to this work.

Funding: This work was funded by the Medical Research Council (MRC) Grand Challenge in Experimental Medicine (MICA) Grant (MR/M003833/1 to AA, MVS, VB, NM and MN), MRC New Investigator award (G0901102 to MVS), Dermatrust (to AA), British Skin Foundation (BSF5012 to AA) and National Institute for Health Research University College London Hospitals Biomedical Research Centre. MN was supported by the UK National Institute for Health Research Biomedical Research Centre award to UCLH.

Abstract

Background Immunity declines with age that leads to re-activation of varicella zoster virus (VZV). In humans, age associated immune changes are usually measured in blood leukocytes however this may not reflect alterations in tissue-specific immunity.

Objectives We used a VZV antigen challenge system in the skin to investigate changes in tissue specific mechanisms involved in the decreased response to this virus during ageing.

Methods We assessed cutaneous immunity by the extent of erythema and induration after intradermal VZV antigen injection. We also performed immune histology and transcriptomic analyses on skin biopsies taken from the site of challenge in young (<40 yrs) and old (>65 yrs) subjects.

Results Old humans exhibited decreased erythema and induration, $CD4^+$ and $CD8^+$ T cell infiltration and attenuated global gene activation at the site of cutaneous VZV antigen challenge compared to young subjects. This was associated with elevated sterile inflammation in the skin in the same subjects, related to p38 MAPK-related pro-inflammatory cytokine production ($p < 0.0007$). We inhibited systemic inflammation in old subjects by pre-treatment with an oral small molecule p38 MAP kinase inhibitor (Losmapimod), which reduced both serum C reactive protein (CRP) and peripheral blood monocyte secretion of IL-6 and TNF-α. In contrast, cutaneous responses to VZV antigen challenge was significantly increased in the same individuals ($p < 0.0006$).

Conclusion Excessive inflammation in the skin early after antigen challenge retards antigen-specific immunity. However this can be reversed by inhibition of inflammatory cytokine production that may be utilized to promote vaccine efficacy and the treatment of infections and malignancy during ageing.

Key Messages:

1) Cutaneous immunity to VZV decreases during ageing
2) Associated with excessive early skin inflammatory response
3) The inflammation is linked to p38 MAP kinase activation
4) An oral p38 inhibitor (Losmapimod) inhibits systemic inflammation
5) Short-term p38 treatment enhances the VZV skin response in old subjects

Capsule summary: Elevated cutaneous inflammation retards VZV-specific immunity. Inhibiting inflammatory cytokine production with p38 MAPkinase inhibitors enhances VZV-specific cutaneous immunity. Targeting inflammation may be used to promote vaccine efficacy and the treatment of malignancy during ageing.

Keywords: Ageing; p38 MAP kinase; VZV; inflammation

Abbreviations:

CBA – cytometric bead array

CRP - C reactive protein

DC – Dendritic cell

DEG - Differentially-expressed genes

DTH – Delayed type hypersensitivity

GSVA - Gene set variation analysis

IL – Interleukin

88 PV – perivascular infiltrate

89 TNF – Tumour necrosis factor

90 T_{RM} – Resident memory T cells

91 VZV – Varicella Zoster Virus

Introduction:

Older individuals have reduced immune function that predisposes them to an increased incidence of infection and malignancy(1, 2). In addition, vaccine efficacy against many pathogens is also reduced in these subjects(3). We developed a human experimental system to investigate antigen-specific immunity *in vivo* where healthy volunteers are challenged intradermally to induce antigen-specific delayed type hypersensitivity (DTH) responses. This enabled the investigation of the kinetics and the specificity of memory T cell expansion, and the interactions between different leukocytes after a single episode of immune stimulation *in situ*(4-6).

VZV is an alpha-herpes virus that causes chickenpox. After resolution of the initial infection VZV becomes latent within dorsal root ganglia but re-activates in older subjects causing herpes zoster (shingles)(7, 8). During both primary infection and latent virus reactivation the absence of T cell immunity results in VZV-induced pathology(9, 10). Therefore, decreased responsiveness to VZV challenge in aged skin is a good model for investigating immune decline during.

Old individuals exhibited reduced erythema and induration (clinical response) after injection of a VZV skin test antigen that was correlated with decreased T cell infiltration and proliferation in the skin. This was not due to defective macrophage activation(4) or reduced inherent function of skin resident memory T cells (T_{RM})(11). On the contrary, we identified a propensity of the skin of old but not young subjects to mount an over-exuberant pro-inflammatory response upon sterile challenge with a physiological saline solution. This was significantly inversely correlated with decreased VZV antigen responsiveness in the same individuals.

Previous studies demonstrated that systemic inflammation, indicated by elevated levels of serum IL-6, TNFα, and CRP, are strong predictors for frailty and mortality during ageing(12, 13). Ingenuity Pathway Analysis indicated a significant association between the inflammatory gene in the skin in old subjects with p38 MAP kinase pathway activation (p value of 1×10^{-18}). We tested the hypothesis that the magnitude of sterile pro-inflammatory response in the skin and reduced antigen-specific immunity in the same individuals was linked. To do this we treated old humans with the oral p38 MAP kinase inhibitor, Losmapimod, for 4 days to inhibit pro-inflammatory cytokine production. This resulted in a significant reduction of CRP and peripheral blood monocyte secretion of IL-6 and TNF-α after stimulation *in vitro* but significantly increased response to cutaneous response to VZV antigen challenge in the same individuals. Therefore, decreased VZV antigen challenge responsiveness in the skin of old subjects is related to excessive pro-inflammatory responses. Therefore, anti-inflammatory intervention may be a strategy for boosting cutaneous immunity during ageing.

Materials and Methods

Study design:

This work was approved by the Ethics Committee Queen's Square (London) and by institutional review board (UCL R&D). Healthy young individuals <40 years (n=97; median age, 29 years) and old individuals >65 years (n=78, median age, 75.5 years) were recruited (Supplementary table 1, Supplementary table 2). Exclusion criteria are described in the online methods section.. All volunteers provided written informed consent and study procedures were performed in accordance with the principles of the declaration of Helsinki.

Skin tests: VZV antigen (BIKEN, The Research Foundation for Microbial Diseases of Osaka University, Japan) was injected intradermally into sun unexposed skin of the medial proximal volar forearm as per manufacturer's instructions. Induration, palpability, and the change in erythema from baseline were measured and scored on day 3 as described previously(14). A clinical score (range 0-10) based on the summation of these parameters was then calculated(14). The injection site was sampled by skin biopsy at the different times after injection with VZV skin test antigen.

Losmapimod treatment: A sub group of 18 old volunteers (8 males, 10 females, age range 65-77: median age 69) were subjected to VZV antigen skin testing as described above. Approximately 2-3 months later volunteers received 15 mg Losmapimod (GW856553) BID for 4 days (provided by Glaxo-Smith-Klein under a Medical Research Council Industrial Collaboration Agreement). The Losmapimod 15 mg BID dose used in this study was chosen on the basis of the PK, PD and safety profiles of Losmapimod observed in GSK Phase I and II studies(15).

On day 4 of after Losmapimod treatment VZV skin test antigen was injected intradermally and clinical score recorded 48h later, as before. History of liver disease or elevated liver transaminases (>1.5 times the upper limit of normal) and abnormal ECG were additional

exclusion criteria for this part of the study. Serum CRP levels were measured using a high sensitivity assay (16). To assess compliance, *ex vivo* whole blood LPS-stimulation assays were performed before and 4 day after Losmapimod treatment(17). Briefly, peripheral blood was cultured with LPS (0-1 mg/ml) for 24 h (37^{o}C, 5% CO_2). Levels of TNF-α and IL-6 in plasma were assessed by cytometric bead array (CBA, BD).

Skin biopsies: Punch biopsies (5 mm diameter) from the site of antigen injection were obtained from young and old volunteers at various time-points (as indicated) post-VZV skin test antigen injection. Control skin punch biopsies from normal (un-injected) forearm skin were also obtained. Biopsies were frozen in OCT (optimal cutting temperature compound; Bright Instrument Company Ltd) as described(4, 11). 6µm sections were cut and left to dry overnight and then fixed in ethanol and acetone and stored at -80ºC.

Immunohistochemistry: Skin sections from normal, VZV skin test antigen or saline injected skin were stained with optimal dilutions of primary antibodies as described(4, 11) (Supplementary Table 3). The number of positively stained cells per mm^2 was counted manually using computer-assisted image analysis (NIH Image 6.1; http://rsb.info.nih.gov/nih-image). Cell numbers were expressed as the mean absolute cell number counted within the frame.

Immunofluorescence: Sections were stained with optimal dilutions of primary antibodies and followed by an appropriate secondary antibody conjugated to various fluorochromes as described(4, 11) (Supplementary Table 4). The number of cells in 5 of the largest perivascular infiltrates present in the upper and mid dermis were selected for analysis and an average was calculated(18). Macrophage images were imaged on the AxioScan Z1 slide scanner and Imaged on Zen Blue (Zeiss, Cambridge U.K.)

Skin Biopsy digestion for flow cytometric analysis: Skin biopsies (5 mm) were taken from normal and saline injected skin (6 h post-injection) and disaggregated by overnight incubation (37^{o}C; 5% CO_2) in 0.8 mg/ml collagenase IV (Sigma Aldrich) with 20% FCS. Single cell

suspensions were obtained by filtering the suspension through 100, 70 and 40 µm filters. Cells derived from skin biopsies were assessed by flow cytometric analysis on a BD Fortessa using FACSDIVA software (BD Biosciences), and subsequently analysed using FlowJo Version X (Treestar, Ashland, U.S.A). For details off antibodies used see Supplementary Table 5.

Cytometric Bead Array (CBA): IL-6, IL-8 and TNFα plasma concentrations were measured by CBA assay (BD), according to the manufacturer's protocol. The lower limit of detection for each analyte was 1.5 pg/ml.

Transcriptional analyses: 3 mm punch biopsies were collected from the injection site 6 or 72 h post injection with VZV antigen or normal saline, immediately frozen in RNAlater. Normal (un-injected) skin from the same site was collected as a control from each volunteer. Frozen tissue was homogenized and total RNA was extracted from bulk tissue homogenates using RNeasy Mini Kit (Qiagen). Details of gene expression analyses are in the online methods.

Where indicated, the human skin-punch microarray data were combined with a large collection of other primary cell gene-expression data sets (745 individual microarray data sets), available from the GEO database on the same Affymetrix Human Genome U133 Plus 2.0 expression array platform. The entire collection of primary cell expression data are available via the GEO accession number: GSE49910. Full details of each primary cell data set have been published(19). Upstream regulator analysis was performed with Ingenuity Pathway Analysis (Qiagen).

Statistics: Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, California, USA). Paired or unpaired t-test were used when data were normally distributed and non-parametric tests were utilised when data were not normally distributed. The Kruskall-Wallis test was used to compare three or more unpaired groups and a

202 2-tailed Mann-Whitney test was used when comparing only two unpaired groups. The Wilcoxon
203 matched pairs test was used when comparing two groups of matched data. Two way ANOVA
204 was used to compare the effects of Losmapimod and LPS.

205

206 **Results:**

207 ***Decreased response to VZV challenge in the skin during ageing***

208 We investigated the cutaneous response of young (<40 yrs) and old (>65 yrs) volunteers to VZV
209 antigen skin challenge. All volunteers had a prior history of chickenpox. At 72 hrs after VZV
210 injection, young subjects had obvious erythema and induration (clinical responses), whereas the
211 clinical response of old subjects was significantly lower and correlated inversely with increasing
212 age (Fig. 1A, $p<0.0001$, n=184, young n=97; old n=78, middle age 40-65 n=14, Supplementary
213 figure 1; for participant details see Supplementary Table 1, 2). There were no differences in the
214 response of male and female donors in young and old age groups ($p = 0.5$, Supplementary
215 table 2). The decrease in clinical score was associated with decreased $CD4^+$ T cell
216 accumulation at the site of VZV challenge in the skin at all time points investigated (Fig 1B,C,D).
217 There was a highly significant correlation between the clinical score (measured at 72hrs) and
218 the extent of $CD4^+$ T cell accumulation and old subjects (measured at 7 days; Fig. 1E). We
219 stratified old individuals into those who had a low skin response to VZV (clinical score of <4;
220 88% of old volunteers) and those who showed similar responses to young subjects with a
221 clinical score >4 (86% of young volunteers).

222 The decreased cutaneous response to VZV in old donors was not related to differences in the
223 number of resident memory $CD4^+$ or $CD8^+$ T cells defined by expression of CD69 alone or the
224 combination of CD69 and CD103(20, 21) in the skin of young and old individuals(11)
225 (Supplementary Fig. 2).

226

227 ***Reduced Dendritic cell/ T cell interaction and T cell proliferation after VZV challenge in***
228 ***old individuals***

Immune clusters containing both antigen presenting cells and T cells (referred to as skin associated lymphoid tissue) are generated in the skin during cutaneous immune responses(22). A highly significant increase in the number of $CD11c^+$ DCs was observed in the skin of young but not old individuals at different times after VZV antigen challenge (Fig. 2A,B). Dendritic cells accumulate around blood vessels and form large perivascular clusters with $CD4^+$ T cells (Fig. 2C) and to a lesser extent $CD8^+$ T cells (not shown). By 3 days after VZV antigen challenge the majority of the DCs within these infiltrates in the skin of young individuals were CD1c-negative and expressed DC-LAMP, a marker of mature inflammatory DCs. In the skin of old individuals after VZV antigen challenge, DC infiltration was significantly reduced compared to the young group (Fig. 2A,B).

In young individuals, proliferating ($Ki67^+$) $CD4^+$ T cells were undetectable in normal skin but a significant increase was observed from 3 days post-VZV antigen challenge compared to baseline (Supplementary Fig 3A, B). Proliferation of $CD8^+$ T cells was also observed but to a lesser extent than in $CD4^+$ T cells (Supplementary Fig. 3 C, D). In contrast, in the skin of old individuals $CD4^+$ and $CD8^+$ T cell proliferation was extremely low even after 7 days of VZV antigen challenge (Supplementary Fig. 3). This indicates that in young subjects, the increased accumulation of T cells in the skin after VZV antigen challenge occurs in part from their proliferation at the site of injection.

We investigated whether decreased endothelial cell activation contributed to the reduced T cell infiltration in the skin of old subjects. At 6 h after VZV antigen injection, 20% of the $CD31^+$ capillary loops in young and old skin expressed both E-selectin and VCAM-1, that was significantly higher than the expression observed in unchallenged (Day 0) skin from either age group (Supplementary Fig. 4). This suggests that endothelial cells in old individuals are not

defective and can be activated to the same extent as young subjects early in the response. At later time points, capillary loops in young individuals showed significantly increased expression of E-selectin when compared to old individuals (Supplementary Fig. 4). Therefore, reduced response to VZV antigen challenge was not due to defects in the initiation of the response, but was related to either active inhibition and/or lack of immune amplification at later stages.

Global decrease in the magnitude of gene expression in the skin following VZV challenge in old individuals

We next performed global gene expression analyses to identify genes that may be associated with the decreased response to VZV antigen challenge in old subjects. In each young or old donor, skin punch biopsies were taken from the site of either VZV antigen challenge (6h and 72h post-injection) or saline injection (6h and 72h post-injection; control) in the contralateral arm of the same individual (resulting in a total of 4 biopsies per individual). The gene expression was compared to the signature in biopsies taken for normal, un-injected skin from 6 young and 9 old donors (Fig. 3). In a previous study we showed that there was no evidence for inflammatory responses in either group at steady state(11).

Six hours post-VZV antigen challenge there were 935 significantly up-regulated and 1042 down-regulated genes in the skin of young individuals and 820 up-regulated and 550 down-regulated. genes in the old group (Supplementary Fig. 5A). Although similar pathways were activated in the skin of young and old individuals at 6 hours after VZV antigen challenge, the magnitude of their expression was reduced in the old group (Supplementary Fig. 5B, 5C). At 72h after VZV antigen challenge, young individuals exhibited a strong transcriptional response that was considerably reduced in the old group (>5000 differentially-expressed genes (DEG) in young, 666 DEG in the old, Fig. 3A,B). The top 30 genes that are significantly differentially expressed are shown in Fig. 4B, indicating that the same genes are upregulated in the both groups but that

the expression was reduced in the old subjects, indicating attenuated immune amplification. Genes associated with T cell and DC activation including *ITGAX* (which encodes CD11c), *CD2, CD28*, *CD69*, *CD83, CD86, EOMES, ICOS*, and *STAT1* were more highly expressed in the skin at 72 hrs of VZV challenge in young compared to the old group (Supplementary Table 6). There was activation of signalling pathways associated with immune responses, inflammation, and immune response to viruses, and pathways induced by type I IFN and IFN-γ signalling in young but not old individuals (Supplementary Fig. 5).

Saline injection induces an inflammatory response in old donors which inversely correlates with cutaneous VZV response

Sterile saline solution was a physiological control that was injected into to the contralateral arm of the same individuals who received VZV skin antigen challenge (Fig 4A). In young individuals, saline injection had a negligible effect on gene expression compared to normal skin (Fig. 4A, B; 30 DEG at 6 hrs post injection). However, in old subjects, saline injection induced significant early expression of numerous inflammatory genes (Fig. 4A, B; 856 DEG, FDR<0.05; FCH>2, Supplementary Table 7) including *IL6, CXCL8* and *PTX3* and also genes indicative of myeloid cell activation including *CXCL2, IL1B, ICAM1* and *FCGR3A* (Fig. 4B; Supplementary Table 7).

Using Ingenuity Pathway Analysis we found a significant association with predicted p38 MAP kinase pathway activation (p value of 1×10^{-18}) when the genes that were upregulated after saline injection (6 hours) in old skin were compared to unchallenged control old skin. The majority of the top 30 genes activated after 6 hours saline injection (Fig, 4C; indicated by asterisk) are induced by p38 MAP kinase signalling or are regulators of p38 MAP kinase activation. Pathway analysis further suggested that the response to saline in the skin of old

subjects involved the activation of inflammatory pathways relating to Type I IFN production, TNF-α signalling, MAP kinase activation, IFN-γ responses and IL-17 production (Fig. 4D). Principal component analysis demonstrated that gene expression in response to saline injection in young and old individuals was distinct (Supplementary Fig. 6B) and that a large number of the genes which were upregulated by 6 h after saline injection in old skin were also induced by VZV antigen challenge at the same time point (Fig. 4C, Supplementary Fig. 6). These data indicate that the early transcriptional response to VZV antigen challenge in the skin of old subjects includes an inflammatory component that may not be specific for the antigen itself.

We investigated if the propensity to exhibit sterile inflammatory responses at 6 hours after nonspecific (saline-induced) inflammation in ageing skin was associated with decreased clinical response to VZV challenge in the contralateral arm of the same individuals at 48 hours. To address this, the expression levels of 384 genes designated as positive regulators of inflammatory response (Supplementary Table 8) were compared in the skin of young and old individuals (n=10 old, n=6 young) at 6 h after saline injection. Using GSVA analysis each individual was assigned a numerical score (denoted as inflammatory index) based on the variation in expression of all these inflammatory genes. A highly significant inverse correlation was observed when the inflammatory index value to saline injection for each individual was plotted against the clinical response to intradermal VZV antigen challenge in the contralateral arm (Fig. 4E). A similar, significant inverse correlation was observed between the expression level of IL-1β, IL-6, IL-12p35 and IL-12p40 as determined by qRT-PCR and an individual's clinical response to VZV antigen challenge were compared (Supplementary Fig 7). This indicates an association between propensity to exhibit early sterile inflammation and reduced responses to VZV antigen challenge in the skin of the same old subjects *in vivo*.

Non-specific inflammation induced by saline injection is associated with mononuclear phagocytes

In order to identify the cell type which may contribute to the elevated pro-inflammatory response to saline injection in ageing skin, the DEG identified at 6 h after saline-injection (FDR<0.05; FCH>2, Table S2) were imported into BioLayout Express^{3D} (see methods; Fig. 5A). Comparison of the mean cellular expression profiles of the gene clusters derived from this network graph suggested that many of the genes within them were strongly associated with cells of the monocyte/macrophage lineage (Fig. 5A, Supplementary Table 9). Furthermore, immunohistological analysis also demonstrated a significant increase in the number of CD163^{+} mononuculear phagocytes in the skin of old subjects within 6h of saline injection (Fig. 5B,C). This was confirmed by multi-parameter flow cytometry where we identified significantly increased proportions of HLA-DR^{+}CD14^{+} mononuclear phagocytes in old compared to young skin biopsies 6h after saline injection (Fig. 5D and Supplementary Fig 8). This rapid increase in the frequency of CD14 expressing mononuclear phagocytes was probably a result of recruitment from the blood as these cells were not in cycle (not shown)(23). The increase in "inflammatory" monocytes in old subjects was transient and coincided with the transient sterile inflammatory response that was only observed at 6 h but not 24 h after saline injection. A similar significant transient increase in mononuclear phagocytes is observed when old individuals were injected with VZV (Supplementary Fig 4E).

Short-term p38 MAP kinase-blockade improves clinical response to VZV skin challenge in older individuals

We tested the hypothesis that excessive pro-inflammatory cytokine secretion that is driven by p38 MAP kinase signalling in mononuclear phagocytes early in the skin response. To do this we treated 18 healthy old volunteers who had a low previous skin response to VZV challenge

353 (clinical score <4) with Losmapimod, a potent and selective oral p38 MAP kinase inhibitor,
354 before VZV antigen re-challenge in the skin(16, 24) (Fig 6A). These individuals were
355 investigated 2-3 months after the first skin test and were pre-treated with the drug for 4 days
356 before re-challenge with VZV antigen in the skin. In parallel studies we showed that the re-
357 challenge of old volunteers with VZV skin test antigens did not significantly boost their original
358 clinical score (n=14, p=0.58, Supplementary Fig. 9).

359

360 CRP production in the liver is upregulated by p38 MAP kinase-dependent cytokines such as IL-
361 1 and IL-6(25, 26). Serum CRP was significantly reduced after Losmapimod pre-treatment (Fig.
362 6B, p=0.04, n=18). In addition, TNFα, IL-6 and IL-8 production by LPS-stimulated PBMCs from
363 the same donors was also significantly reduced after Losmapimod pre-treatment (Fig. 6C,
364 Supplementary Fig 10). In contrast, Losmapimod pre-treatment significantly enhanced the
365 clinical response to VZV antigen challenge in the skin of 13 of 18 old subjects (Fig. 6D, n=18,
366 p=0.0006). This increase in clinical score to VZV challenge correlated with the decrease in CRP
367 in the serum in the same individuals (Fig 6E). Losmapimod pre-treatment had no effect on $CD4^+$
368 or $CD8^+$ T cell function in response to CD3 and IL-2 stimulation in the same donors as defined
369 by cytokine expression (IFNγ, IL-2 and TNFα) or proliferation as defined by Ki67 expression
370 (Supplementary Fig 10). Histological assessment of biopsies collected from 4 old subjects who
371 showed an increased clinical response after Losmapimod treatment showed that there was a
372 significant increase in the number of $CD11c^+$ DCs in the perivascular infiltrates (p=0.04) that
373 were associated with increased numbers of $CD4^+$ T cells in immune clusters (representative
374 experiment shown in Fig. 6F, top and bottom right panels). These clusters were not found in 4 of
375 the individuals who did not respond to Losmapimod treatment (Fig. 6F top and bottom left
376 panels). These clusters resembled those found after VZV challenge of skin in young subjects
377 (see Fig. 2). This shows that p38 MAP kinase inhibition significantly reduced pro-inflammatory

responses and that this was associated with enhancement of antigen-specific immune responses in the skin of old individuals *in vivo*.

Discussion

The cutaneous recall response to intradermal antigen challenge is a manifestation of immune memory and this reaction decreases with age(4, 27-29). We have investigated reasons for this decrease in order to explain the increased incidence of cutaneous infection and malignancy during ageing(2, 30). Early inflammation (erythema and induration, 48 hours) is required to initiate the cascade of events leading to optimal cellular infiltration in the skin that occurs later (peak at 7 days)(14). An unexpected observation therefore was that excessive inflammation during the early phase after VZV challenge hinders the amplification of the response in old subjects. The response to VZV in older individuals is not inhibited from the outset since the endothelium of old and young subjects are activated equally at 6 h and gene expression at this time is similar. Furthermore, the decreased response after VZV antigen challenge in these individuals was not due to intrinsic changes in the functionality of cutaneous T_{RM} cells or macrophages since these cells from both age groups were equally responsive when isolated from skin and activated *in vitro*(4, 11).

In other studies, elevated systemic inflammation has been shown to have a negative impact on the cutaneous recall response to candida antigens (31), however this study did not investigate the response of old subjects or events that occur in the skin itself. The reduced efficacy of vaccination has also been linked to excessive inflammation for influenza(32), yellow fever(33), tuberculosis(34) and Hepatitis B vaccines(35). Furthermore, inflammatory macrophages in patients with chronic artery disease suppress T cell activation and expansion *in vitro* and this is associated with defective VZV-specific T cell immunity in the peripheral blood of these patients(36). The proposed mechanism for this inhibition involves the upregulation of the inhibitory receptor ligand PD-L1 on the inflammatory macrophages that inhibit function of PD1 expressing T cells(36). This suggests that the infiltration of inflammatory monocytes that

express PD-L1 during sterile inflammation may block early activation of cutaneous resident memory T cells (T_{RM}) since this latter population expresses significantly higher levels of PD-1 during ageing(11).

Type I IFN has been shown to interfere with antigen-specific T cell responses and excessive levels of these mediators impair the clearance of both viral and mycobacterial infections in mice *in vivo*(37, 38). In the present study we also found a strong type I IFN signature in the skin of old subjects after saline injection although other inflammatory pathways were also upregulated. The impact of excessive inflammation on the inhibition of antigen-specific T cell function is particularly important for ageing since older individuals have widespread low grade systemic inflammation termed “inflammageing”(12) that is linked to expression of inflammasome gene modules that may underpin clinical frailty and immune dysfunction(13).

It is not clear why saline injection induces an early but transient inflammation in the skin of old individuals (observed at 6 h but not at 24 h after injection). However, NaCl itself is pro-inflammatory and has been shown to induce Th17 cells whilst conversely inhibiting $Foxp3^+$ Treg function(39, 40) and to also activate inflammatory cascades in monocytes and bone-marrow derived macrophages *in vitro*(41, 42). The saline control that we used in the current study contained 0.9% NaCl, which is similar to the concentration used in the diluent of the VZV skin test antigen (0.68% NaCl). Therefore a component of the transcriptional response of old subjects to VZV antigen would also include a response to NaCl in the diluent that may hinder the induction of antigen-specific immunity in old subjects. This response is not observed in young subjects. We identified mononuclear phagocytes as the source of the saline-induced cutaneous inflammation.

Many of the inflammatory mediators induced by saline injection in older subjects were linked directly or indirectly to the activation of the p38 MAP kinase pathway. Many pharmaceutical companies have generated small molecule p38 MAP kinase inhibitors in humans *in vivo* in phase I,II and III trials to block inflammatory diseases/disorders(43). Although most trials with p38 MAP kinase inhibitors were discontinued because of hepatotoxicity after long term treatment (>3 months) and adaptation of cell-signalling pathways leading to reduced drug efficacy(44), these inhibitors do not show evidence of toxicity in the short-term (weeks) in humans *in vivo.* We therefore treated old subjects who were not responsive to cutaneous VZV antigen challenge with Losmapimod (GW856553), a selective, reversible, competitive inhibitor of p38 MAP kinase, to test the hypothesis that reducing inflammation in the skin would enhance antigen-specific cutaneous immunity. The key observation was that Losmapimod pre-treatment significantly enhanced the cutaneous response to VZV in older subjects. Although our previous studies have shown that that p38 inhibition can enhances T cell proliferation *in vitro*(45-47), in the current study Losmapimod treatment did not affect peripheral blood T proliferation or cytokine production after stimulation CD3/IL-2. Thus the enhancement of cutaneous immunity is likely to be due to the anti-inflammatory effects of the drug.

This raises the question of whether the short-term inhibition of p38 MAP kinase signalling and/or inhibition of inflammation would also enhance immunity in other tissues. An interesting possibility is that this would be a strategy to improve vaccine efficacy that is decreased in ageing individuals(3, 48). Another point to consider is that increasing the strength of adjuvants to enhance vaccine responses during ageing may be counter-productive if they further increase inflammatory responses and it may be important to stratify old vaccinees on the basis of their baseline inflammatory responses in the future(13). Our study may appear to challenge the

concept that antigen-specific immunization is more successful in the presence of an adjuvant that is designed to increase inflammatory responses. However while adjuvants may enhance the induction of immunity in draining lymph nodes, excessive inflammation that is present at the site of the effector phase of a response may inhibit T cell responsiveness. This may be a mechanism to protect against pathology induced by excessive immune stimulation in the tissues. Furthermore excessive inflammation is detrimental for cancer progression(49) and the temporary inhibition of inflammation in this situation may be a strategy for boosting immunotherapy in these patients. While the current challenge is to identify the optimal way to reduce excessive inflammation and to enhance immunity in ageing humans, it is serendipitous that some drugs that may do this have already been developed and may therefore be repurposed.

Acknowledgements.

Author contribution: MV-S designed the histological experiments and sample collection; performed histological experiments and data analysis and interpretation; and contributed to the writing of the manuscript. **MS-F** performed all analysis of microarray data. **ESC** performed IF staining, mononuclear phagocyte assessment by flow cytometry and in vitro LPS assays and data interpretation. **DS**, **NP**, and **AE** performed histological analysis (immunofluorescent and immunohistochemical staining) and data analysis. **JF-D** performed RNA extraction and QPCR analysis and advised on immunohistochemical staining and counting. **KEL**, **DS** and **NP** performed clinical procedures and sample collection. **VB** and **MN** contributed to study design and organized ethical and regulatory permission for the Losmapimod study. **AL** helped with the design with flow panels and provided access to A* core Flow-Cytometry facility. **NAM** performed modelling analysis of transcriptional data including Biolayout express. **MHR** was the clinical lead for the study and was involved with scientific discussions. **JK** and **MS-F** provided expert advice on transcriptional analysis of human skin. **AA** was involved in the overall design of the study, initiated and coordinated the collaborative interaction between the different research groups, interpreted the data, contributed writing and edited the manuscript.

We thank Dr. Iain Laws, Dr. Ruchira Glaser and Dr. Robert Henderson at GSK for support in developing this project. We are also grateful to Glaxo-Smith Kline for providing the drug Losmapimod (MICA agreement with the MRC) and to the Losmapimod team at GSK for organizing the dispatch of the clinical supply of the drug in this Investigator Led study. We thank Professor David Lomas for advice on this project. We are grateful to Mr. Hugh Trahir for technical assistance with the immunohistology experiments. We would especially like to thank the blood and skin donors who volunteered for this study and to our Research Nurses Ms Megan Harries and Ms. Michelle Berkley for their outstanding work

492 **Competing interests:** The authors declare that they have no competing interests related to this
493 work.

References:

1. Gavazzi G, Krause KH. Ageing and infection. Lancet Infect.Dis. 2002; 2:659-66.
2. Diffey BL, Langtry JA. Skin cancer incidence and the ageing population. Br.J.Dermatol. 2005; 153:679-80.
3. Bouree P. Immunity and immunization in elderly. Pathol.Biol.(Paris) 2003; 51:581-5.
4. Agius E, Lacy KE, Vukmanovic-Stejic M, Jagger AL, Papageorgiou AP, Hall S, et al. Decreased TNF-{alpha} synthesis by macrophages restricts cutaneous immunosurveillance by memory CD4+ T cells during aging. The Journal of Experimental Medicine 2009; 206:1929-40.
5. Reed JR, Vukmanovic-Stejic M, Fletcher JM, Soares MV, Cook JE, Orteu CH, et al. Telomere erosion in memory T cells induced by telomerase inhibition at the site of antigenic challenge in vivo. J.Exp.Med. 2004; 199:1433-43.
6. Vukmanovic-Stejic M, Sandhu D, Sobande TO, Agius E, Lacy KE, Riddell N, et al. Varicella Zoster-Specific CD4+Foxp3+ T Cells Accumulate after Cutaneous Antigen Challenge in Humans. J Immunol 2013; 190:977-86.
7. Oxman MN. Herpes Zoster Pathogenesis and Cell-Mediated Immunity and Immunosenescence. JAOA: Journal of the American Osteopathic Association 2009; 109:S13-S7.
8. Levin MJ. Immune senescence and vaccines to prevent herpes zoster in older persons. Curr Opin Immunol 2012; 24:494-500.
9. Abendroth A, Arvin AM. Immune evasion as a pathogenic mechanism of varicella zoster virus. Semin Immunol 2001; 13:27-39.
10. Vossen MT, Biezeveld MH, de Jong MD, Gent MR, Baars PA, von Rosenstiel IA, et al. Absence of circulating natural killer and primed CD8+ cells in life-threatening varicella. J Infect Dis 2005; 191:198-206.
11. Vukmanovic-Stejic M, Sandhu D, Seidel JA, Patel N, Sobande TO, Agius E, et al. The Characterization of Varicella Zoster Virus-Specific T Cells in Skin and Blood during Aging. J Invest Dermatol 2015; 135:1752-62.
12. Franceschi C, Campisi J. Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 2014; 69:S4-S9.

13. Furman D, Chang J, Lartigue L, Bolen CR, Haddad F, Gaudilliere B, et al. Expression of specific inflammasome gene modules stratifies older individuals into two extreme clinical and immunological states. Nat Med 2017; 23:174-84.
14. Akbar AN, Reed JR, Lacy KE, Jackson SE, Vukmanovic-Stejic M, Rustin MH. Investigation of the cutaneous response to recall antigen in humans in vivo. Clin Exp Immunol 2013; 173:163-72.
15. Watz H, Barnacle H, Hartley BF, Chan R. Efficacy and safety of the p38 MAPK inhibitor losmapimod for patients with chronic obstructive pulmonary disease: a randomised, double-blind, placebo-controlled trial. The Lancet Respiratory Medicine; 2:63-72.
16. Lomas DA, Lipson DA, Miller BE, Willits L, Keene O, Barnacle H, et al. An oral inhibitor of p38 MAP kinase reduces plasma fibrinogen in patients with chronic obstructive pulmonary disease. J Clin Pharmacol 2012; 52:416-24.
17. Fehr S, Unger A, Schaeffeler E, Herrmann S, Laufer S, Schwab M, et al. Impact of p38 MAP Kinase Inhibitors on LPS-Induced Release of TNF-α in Whole Blood and Primary Cells from Different Species. Cellular Physiology and Biochemistry 2015; 36:2237-49.
18. Vukmanovic-Stejic M, Agius E, Booth N, Dunne PJ, Lacy KE, Reed JR, et al. The kinetics of CD4Foxp3 T cell accumulation during a human cutaneous antigen-specific memory response in vivo. J Clin.Invest 2008; 118:3639-50.
19. Mabbott NA, Baillie JK, Brown H, Freeman TC, Hume DA. An expression atlas of human primary cells: inference of gene function from coexpression networks. BMC Genomics 2013; 14:632.
20. Watanabe R, Gehad A, Yang C, Scott LL, Teague JE, Schlapbach C, et al. Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. Sci Transl Med 2015; 7:279ra39.
21. Thome JJ, Farber DL. Emerging concepts in tissue-resident T cells: lessons from humans. Trends Immunol 2015; 36:428-35.
22. Natsuaki Y, Egawa G, Nakamizo S, Ono S, Hanakawa S, Okada T, et al. Perivascular leukocyte clusters are essential for efficient activation of effector T cells in the skin. Nat Immunol 2014; 15:1064-9.
23. McGovern N, Schlitzer A, Gunawan M, Jardine L, Shin A, Poyner E, et al. Human dermal CD14(+) cells are a transient population of monocyte-derived macrophages. Immunity 2014; 41:465-77.

24. Watz H, Barnacle H, Hartley BF, Chan R. Efficacy and safety of the p38 MAPK inhibitor losmapimod for patients with chronic obstructive pulmonary disease: a randomised, double-blind, placebo-controlled trial. Lancet Respir Med 2014; 2:63-72.
25. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994; 372:739-46.
26. Kumar S, Boehm J, Lee JC. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat Rev Drug Discov 2003; 2:717-26.
27. Moesgaard F, Lykkegaard NM, Norgaard LP, Christophersen S, Mosbech H. Cell-mediated immunity assessed by skin testing (Multitest). I. Normal values in healthy Danish adults. Allergy 1987; 42:591-6.
28. Marrie TJ, Johnson S, Durant H. Cell-mediated immunity of healthy adult Nova Scotians in various age groups compared with nursing home and hospitalized senior citizens. J Allergy Clin.Immunol 1988; 81:836-43.
29. Sadaoka K, Okamoto S, Gomi Y, Tanimoto T, Ishikawa T, Yoshikawa T, et al. Measurement of varicella-zoster virus (VZV)-specific cell-mediated immunity: comparison between VZV skin test and interferon-gamma enzyme-linked immunospot assay. J Infect Dis 2008; 198:1327-33.
30. Laube S. Skin infections and ageing. Ageing Res.Rev. 2004; 3:69-89.
31. Pence BD, Lowder TW, Keylock KT, Vieira Potter VJ, Cook MD, McAuley E, et al. Relationship between Systemic Inflammation and Delayed-Type Hypersensitivity Response to Candida Antigen in Older Adults. PLoS ONE 2012; 7:e36403.
32. Parmigiani A, Alcaide ML, Freguja R, Pallikkuth S, Frasca D, Fischl MA, et al. Impaired antibody response to influenza vaccine in HIV-infected and uninfected aging women is associated with immune activation and inflammation. PLoS One 2013; 8:e79816.
33. Muyanja E, Ssemaganda A, Ngauv P, Cubas R, Perrin H, Srinivasan D, et al. Immune activation alters cellular and humoral responses to yellow fever 17D vaccine. J Clin Invest 2014; 124:1.
34. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 2010; 466:973-7.
35. Fourati S, Cristescu R, Loboda A, Talla A, Filali A, Railkar R, et al. Pre-vaccination inflammation and B-cell signalling predict age-related hyporesponse to hepatitis B vaccination. Nat Commun 2016; 7:10369.

36. Watanabe R, Shirai T, Namkoong H, Zhang H, Berry GJ, Wallis BB, et al. Pyruvate controls the checkpoint inhibitor PD-L1 and suppresses T cell immunity. The Journal of Clinical Investigation 2017; 127:2725-38.
37. Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, et al. Blockade of chronic type I interferon signaling to control persistent LCMV infection. Science 2013; 340:202-7.
38. Teijaro JR, Ng C, Lee AM, Sullivan BM, Sheehan KC, Welch M, et al. Persistent LCMV infection is controlled by blockade of type I interferon signaling. Science 2013; 340:207-11.
39. Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, et al. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. Nature 2013; 496:513-7.
40. Hernandez AL, Kitz A, Wu C, Lowther DE, Rodriguez DM, Vudattu N, et al. Sodium chloride inhibits the suppressive function of FOXP3+ regulatory T cells. J Clin Invest 2015; 125:4212-22.
41. Zhang WC, Zheng XJ, Du LJ, Sun JY, Shen ZX, Shi C, et al. High salt primes a specific activation state of macrophages, M(Na). Cell Res 2015; 25:893-910.
42. Min B, Fairchild RL. Over-salting ruins the balance of the immune menu. J Clin Invest 2015; 125:4002-4.
43. Patterson H, Nibbs R, McInnes I, Siebert S. Protein kinase inhibitors in the treatment of inflammatory and autoimmune diseases. Clinical & Experimental Immunology 2014; 176:1-10.
44. Sweeney SE. Therapy: The as-yet unfulfilled promise of p38 MAPK inhibitors. Nat Rev Rheumatol 2009; 5:475-7.
45. Di Mitri D, Azevedo RI, Henson SM, Libri V, Riddell NE, Macaulay R, et al. Reversible senescence in human CD4+CD45RA+. J.Immunol. 2011; 187:2093-100.
46. Lanna A, Henson SM, Escors D. The kinase p38 activated by the metabolic regulator AMPK and scaffold TAB1 drives the senescence of human T cells. Nat Immunol 2014.
47. Lanna A, Gomes DC, Muller-Durovic B, McDonnell T, Escors D, Gilroy DW, et al. A sestrin-dependent Erk-Jnk-p38 MAPK activation complex inhibits immunity during aging. Nat Immunol 2017; 18:354-63.
48. Dominguez A, Godoy P, Torner N. The Effectiveness of Influenza Vaccination in Different Groups. Expert Rev Vaccines 2016:1-14.
49. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008; 454:436-44.

50. Suarez-Farinas M, Haider A, Wittkowski KM. "Harshlighting" small blemishes on microarrays. BMC Bioinformatics 2005; 6:65.
51. Wu Z, Irizarry RA. Preprocessing of oligonucleotide array data. Nat Biotechnol 2004; 22:656-8; author reply 8.
52. Hanzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. BMC Bioinformatics 2013; 14:7.
53. Freeman TC, Goldovsky L, Brosch M, van Dongen S, Maziere P, Grocock RJ, et al. Construction, visualisation, and clustering of transcription networks from microarray expression data. PLoS Comput Biol 2007; 3:2032-42.

1. Gavazzi G, Krause KH. Ageing and infection. Lancet InfectDis. 2002;2(11):659-66.
2. Diffey BL, Langtry JA. Skin cancer incidence and the ageing population. BrJDermatol. 2005;153(3):679-80.
3. Bouree P. Immunity and immunization in elderly. PatholBiol(Paris). 2003;51(10):581-5.
4. Agius E, Lacy KE, Vukmanovic-Stejic M, Jagger AL, Papageorgiou AP, Hall S, et al. Decreased TNF-{alpha} synthesis by macrophages restricts cutaneous immunosurveillance by memory CD4+ T cells during aging. The Journal of Experimental Medicine. 2009;206(9):1929-40.
5. Reed JR, Vukmanovic-Stejic M, Fletcher JM, Soares MV, Cook JE, Orteu CH, et al. Telomere erosion in memory T cells induced by telomerase inhibition at the site of antigenic challenge in vivo. JExpMed. 2004;199(10):1433-43.
6. Vukmanovic-Stejic M, Sandhu D, Sobande TO, Agius E, Lacy KE, Riddell N, et al. Varicella Zoster-Specific CD4+Foxp3+ T Cells Accumulate after Cutaneous Antigen Challenge in Humans. J Immunol. 2013;190(3):977-86.
7. Oxman MN. Herpes Zoster Pathogenesis and Cell-Mediated Immunity and Immunosenescence. JAOA: Journal of the American Osteopathic Association. 2009;109(6 suppl 2):S13-S7.
8. Levin MJ. Immune senescence and vaccines to prevent herpes zoster in older persons. Curr Opin Immunol. 2012;24(4):494-500.
9. Abendroth A, Arvin AM. Immune evasion as a pathogenic mechanism of varicella zoster virus. Semin Immunol. 2001;13(1):27-39.
10. Vossen MT, Biezeveld MH, de Jong MD, Gent MR, Baars PA, von Rosenstiel IA, et al. Absence of circulating natural killer and primed CD8+ cells in life-threatening varicella. J Infect Dis. 2005;191(2):198-206.

11. Vukmanovic-Stejic M, Sandhu D, Seidel JA, Patel N, Sobande TO, Agius E, et al. The Characterization of Varicella Zoster Virus-Specific T Cells in Skin and Blood during Aging. J Invest Dermatol. 2015;135(7):1752-62.

12. Franceschi C, Campisi J. Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 2014;69(Suppl 1):S4-S9.

13. Furman D, Chang J, Lartigue L, Bolen CR, Haddad F, Gaudilliere B, et al. Expression of specific inflammasome gene modules stratifies older individuals into two extreme clinical and immunological states. Nat Med. 2017;23(2):174-84.

14. Akbar AN, Reed JR, Lacy KE, Jackson SE, Vukmanovic-Stejic M, Rustin MH. Investigation of the cutaneous response to recall antigen in humans in vivo. Clin Exp Immunol. 2013;173(2):163-72.

15. Watz H, Barnacle H, Hartley BF, Chan R. Efficacy and safety of the p38 MAPK inhibitor losmapimod for patients with chronic obstructive pulmonary disease: a randomised, double-blind, placebo-controlled trial. The Lancet Respiratory Medicine.2(1):63-72.

16. Lomas DA, Lipson DA, Miller BE, Willits L, Keene O, Barnacle H, et al. An oral inhibitor of p38 MAP kinase reduces plasma fibrinogen in patients with chronic obstructive pulmonary disease. Journal of clinical pharmacology. 2012;52(3):416-24.

17. Fehr S, Unger A, Schaeffeler E, Herrmann S, Laufer S, Schwab M, et al. Impact of p38 MAP Kinase Inhibitors on LPS-Induced Release of TNF-α in Whole Blood and Primary Cells from Different Species. Cellular Physiology and Biochemistry. 2015;36(6):2237-49.

18. Vukmanovic-Stejic M, Agius E, Booth N, Dunne PJ, Lacy KE, Reed JR, et al. The kinetics of CD4Foxp3 T cell accumulation during a human cutaneous antigen-specific memory response in vivo. J ClinInvest. 2008;118(11):3639-50.

19. Mabbott NA, Baillie JK, Brown H, Freeman TC, Hume DA. An expression atlas of human primary cells: inference of gene function from coexpression networks. BMC Genomics. 2013;14:632.

20. Watanabe R, Gehad A, Yang C, Scott LL, Teague JE, Schlapbach C, et al. Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. Sci Transl Med. 2015;7(279):279ra39.

21. Thome JJ, Farber DL. Emerging concepts in tissue-resident T cells: lessons from humans. Trends Immunol. 2015;36(7):428-35.

22. Natsuaki Y, Egawa G, Nakamizo S, Ono S, Hanakawa S, Okada T, et al. Perivascular leukocyte clusters are essential for efficient activation of effector T cells in the skin. Nature immunology. 2014;15(11):1064-9.

23. McGovern N, Schlitzer A, Gunawan M, Jardine L, Shin A, Poyner E, et al. Human dermal CD14(+) cells are a transient population of monocyte-derived macrophages. Immunity. 2014;41(3):465-77.

24. Watz H, Barnacle H, Hartley BF, Chan R. Efficacy and safety of the p38 MAPK inhibitor losmapimod for patients with chronic obstructive pulmonary disease: a randomised, double-blind, placebo-controlled trial. The Lancet Respiratory medicine. 2014;2(1):63-72.

25. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature. 1994;372(6508):739-46.

26. Kumar S, Boehm J, Lee JC. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat Rev Drug Discov. 2003;2(9):717-26.

27. Moesgaard F, Lykkegaard NM, Norgaard LP, Christophersen S, Mosbech H. Cell-mediated immunity assessed by skin testing (Multitest). I. Normal values in healthy Danish adults. Allergy. 1987;42(8):591-6.

28. Marrie TJ, Johnson S, Durant H. Cell-mediated immunity of healthy adult Nova Scotians in various age groups compared with nursing home and hospitalized senior citizens. J Allergy ClinImmunol. 1988;81(5 Pt 1):836-43.

29. Sadaoka K, Okamoto S, Gomi Y, Tanimoto T, Ishikawa T, Yoshikawa T, et al. Measurement of varicella-zoster virus (VZV)-specific cell-mediated immunity: comparison between VZV skin test and interferon-gamma enzyme-linked immunospot assay. J Infect Dis. 2008;198(9):1327-33.

30. Laube S. Skin infections and ageing. Ageing ResRev. 2004;3(1):69-89.

31. Pence BD, Lowder TW, Keylock KT, Vieira Potter VJ, Cook MD, McAuley E, et al. Relationship between Systemic Inflammation and Delayed-Type Hypersensitivity Response to Candida Antigen in Older Adults. PloS one. 2012;7(5):e36403.

32. Parmigiani A, Alcaide ML, Freguja R, Pallikkuth S, Frasca D, Fischl MA, et al. Impaired antibody response to influenza vaccine in HIV-infected and uninfected aging women is associated with immune activation and inflammation. PloS one. 2013;8(11):e79816.

33. Muyanja E, Ssemaganda A, Ngauv P, Cubas R, Perrin H, Srinivasan D, et al. Immune activation alters cellular and humoral responses to yellow fever 17D vaccine. J Clin Invest. 2014;124(10):1.

34. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010;466(7309):973-7.

35. Fourati S, Cristescu R, Loboda A, Talla A, Filali A, Railkar R, et al. Pre-vaccination inflammation and B-cell signalling predict age-related hyporesponse to hepatitis B vaccination. Nature communications. 2016;7:10369.

36. Watanabe R, Shirai T, Namkoong H, Zhang H, Berry GJ, Wallis BB, et al. Pyruvate controls the checkpoint inhibitor PD-L1 and suppresses T cell immunity. The Journal of Clinical Investigation. 2017;127(7):2725-38.

37. Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, et al. Blockade of chronic type I interferon signaling to control persistent LCMV infection. Science. 2013;340(6129):202-7.

38. Teijaro JR, Ng C, Lee AM, Sullivan BM, Sheehan KC, Welch M, et al. Persistent LCMV infection is controlled by blockade of type I interferon signaling. Science. 2013;340(6129):207-11.

39. Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, et al. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. Nature. 2013;496(7446):513-7.

40. Hernandez AL, Kitz A, Wu C, Lowther DE, Rodriguez DM, Vudattu N, et al. Sodium chloride inhibits the suppressive function of FOXP3+ regulatory T cells. J Clin Invest. 2015;125(11):4212-22.

41. Zhang WC, Zheng XJ, Du LJ, Sun JY, Shen ZX, Shi C, et al. High salt primes a specific activation state of macrophages, M(Na). Cell Res. 2015;25(8):893-910.

42. Min B, Fairchild RL. Over-salting ruins the balance of the immune menu. J Clin Invest. 2015;125(11):4002-4.

43. Patterson H, Nibbs R, McInnes I, Siebert S. Protein kinase inhibitors in the treatment of inflammatory and autoimmune diseases. Clinical & Experimental Immunology. 2014;176(1):1-10.

44. Sweeney SE. Therapy: The as-yet unfulfilled promise of p38 MAPK inhibitors. Nat Rev Rheumatol. 2009;5(9):475-7.

45. Di Mitri D, Azevedo RI, Henson SM, Libri V, Riddell NE, Macaulay R, et al. Reversible senescence in human CD4+CD45RA+. JImmunol. 2011;187(5):2093-100.

46. Lanna A, Henson SM, Escors D. The kinase p38 activated by the metabolic regulator AMPK and scaffold TAB1 drives the senescence of human T cells. Nature immunology. 2014.

47. Lanna A, Gomes DC, Muller-Durovic B, McDonnell T, Escors D, Gilroy DW, et al. A sestrin-dependent Erk-Jnk-p38 MAPK activation complex inhibits immunity during aging. Nature immunology. 2017;18(3):354-63.

48. Dominguez A, Godoy P, Torner N. The Effectiveness of Influenza Vaccination in Different Groups. Expert Rev Vaccines. 2016:1-14.

49. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454(7203):436-44.

Figure Legends

Figure 1. Cutaneous response to Varicella Zoster Virus (VZV) antigen is reduced in old individuals

Healthy young and old volunteers were injected VZV skin antigen test (female =circles and male = diamonds). A clinical score at day 3 in response to VZV, was calculated based on induration, palpability and redness. (A) Clinical score versus participant's age. (B) Haematoxylin and eosin staining (x10), PV, perivascular infiltrates 5mm punch biopsies were performed on days 0, 1, 3 or 7 post injection (with 4-7 volunteers per timepoint. (C) Representative skin sections stained for CD4 (green) (original magnification: x400). (D) Collated data of T cell numbers at different times after VZV injection in young and old volunteers. Each symbol represents the average number of $CD4^+$ T cells within perivascular infiltrates for each individual (n=4-7 per time point; Mann Whitney test, horizontal bar represent the mean). (E) Clinical score at 48 h (peak clinical response) correlated with the number of $CD4^+$ T cells in the perivascular infiltrate at the peak of cellular response on day 7 (n=10 young [black circles] and 22 old [white circles]). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Figure 2. Perivascular cluster formation is reduced in the skin of old individuals.

5 mm punch biopsies were performed on days 0, 1, 3 or 7 post- VZV injection (with 3-6 volunteers per time point). (A) Representative staining of skin sections immunostained for CD11c (original magnification x10). (B) Cumulative data showing mean $CD11c^+$ cell number per field (young - filled bars, old- open bars). Data shown as mean ± SEM. * = $p<0.05$, ** = $p<0.01$. (C) Representative staining showing $CD11c^+$ DC (red) and $CD4^+$ T cells (green) in a perivascular cluster (representative young donor, day 3 after VZV injection, x400).

Figure 3. Transcriptomic analysis of young and old skin after VZV antigen challenge.

3mm punch biopsies were collected from old (n=10) and young individuals (n=6) at 6 and 72 h post-VZV injection. Normal skin punch biopsies were collected from and additional group of young (n=9) and old individuals (n=6). Total skin RNA was isolated, amplified and hybridized to Affymetrix Human Genome U133 2.0 plus arrays. (A) Heatmap showing the relative expression of differentially expressed genes between VZV injected and normal skin in young (left panel) and old (right panel) at FCH>2 and FDR>0.05 in normal/unmanipulated skin, 6 hours post-VZV and 72 hours post-VZV challenged skin in each grouup. For each gene, only the probeset with the largest average expression is shown. Unsupervised clustering was carried out using Pearson correlation distance with Mcquitty agglomeration scheme of DEG at 6 h following VZV. (B) Table shows top 30 up-regulated genes at 72h in young and old subjects compared to normal skin in each group.

Figure 4. Comparison of global gene expression between normal, saline-injected and VZV antigen-injected skin.

(A) Schematic representation of biopsy collection for transcriptional analysis. (B). Heatmap showing the relative expression of DEG (FCH>2 and FDR>0.05) between normal skin and saline-injected skin at 6 hours after treatment in young (left panel) and old (right panel) individuals. (C) Table showing the top 20 upregulated genes at 6 hours in the saline-injected old and young skin compared to normal skin. Genes not reaching statistical significance are indicated in blue. Asterisk indicates genes related to p38 MAP kinase signalling. (D) Bubble plot shows expression of pathways in saline-injected skin versus normal skin. KEGG and GO collection, as well as a curated skin-related collection were interrogated and the most relevant pathways amongst them with FDR<0.05 are presented. (E) Inflammatory index was calculated

for each individual (see methods) and plotted against VZV clinical score at 72 hours (young n=6 and old n=10).

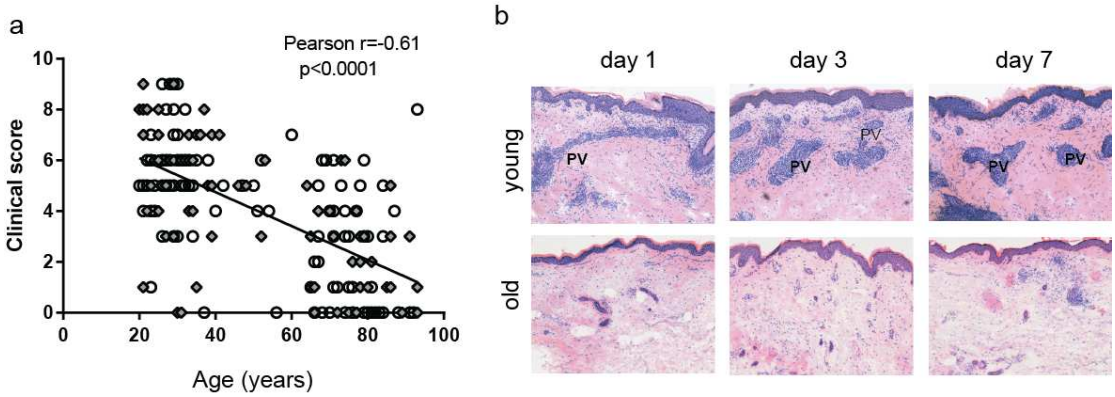
Figure 5 Identification of a monocyte/macrophage-related gene expression signature in saline-injected aged skin.

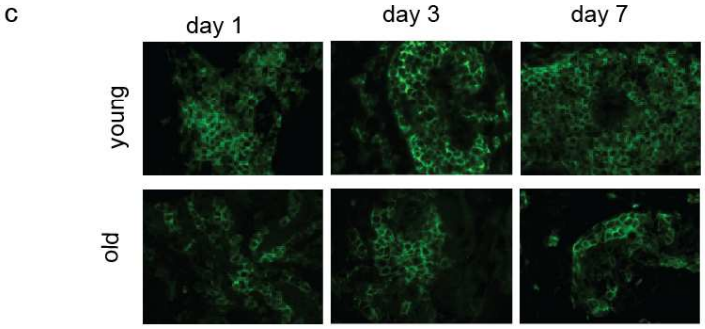
(A) Transcriptomic analysis using the tool Biolayout Express^{3D} of the genes upregulated in the skin of elderly humans 6 hours after saline treatment which clusters together in a large network of monocyte/macrophage-related genes (C_, cluster no.; nodes represent individual genes; edges represent Pearson correlations >0.7). (B) representative images of CD163 stained saline-injected skin from young and old and (C) cumulative data of $CD163^+$ cells in paired analysis from normal and saline injected skin at 6 hours individuals (n=4-5 per age group). (D) The frequency of mononuclear phagocytes determined as being $CD45^+$ Lineage- ($CD3^-$, $CD19^-$, $CD20^-$ and $CD56^-$) and $HLA\text{-}DR^+$ and either $CD14^+$ and/or $CD16^+$, expressed as a % of $CD45^+$ lineage negative, in young (white) and old (black) pre- and post-saline as assessed by flow cytometry. Data assessed by paired t-test.

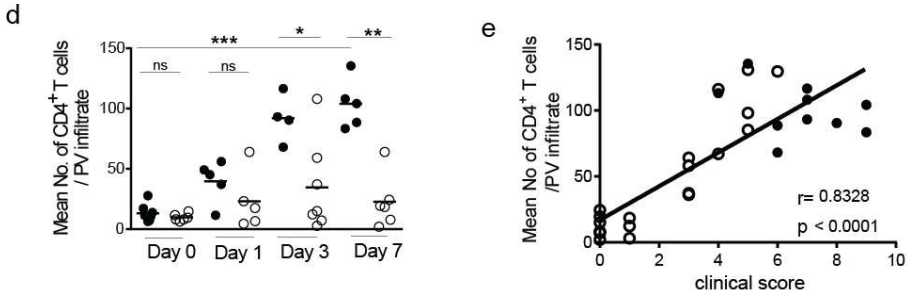
Figure 6. Effects of Losmapimod treatment on VZV response in the skin.

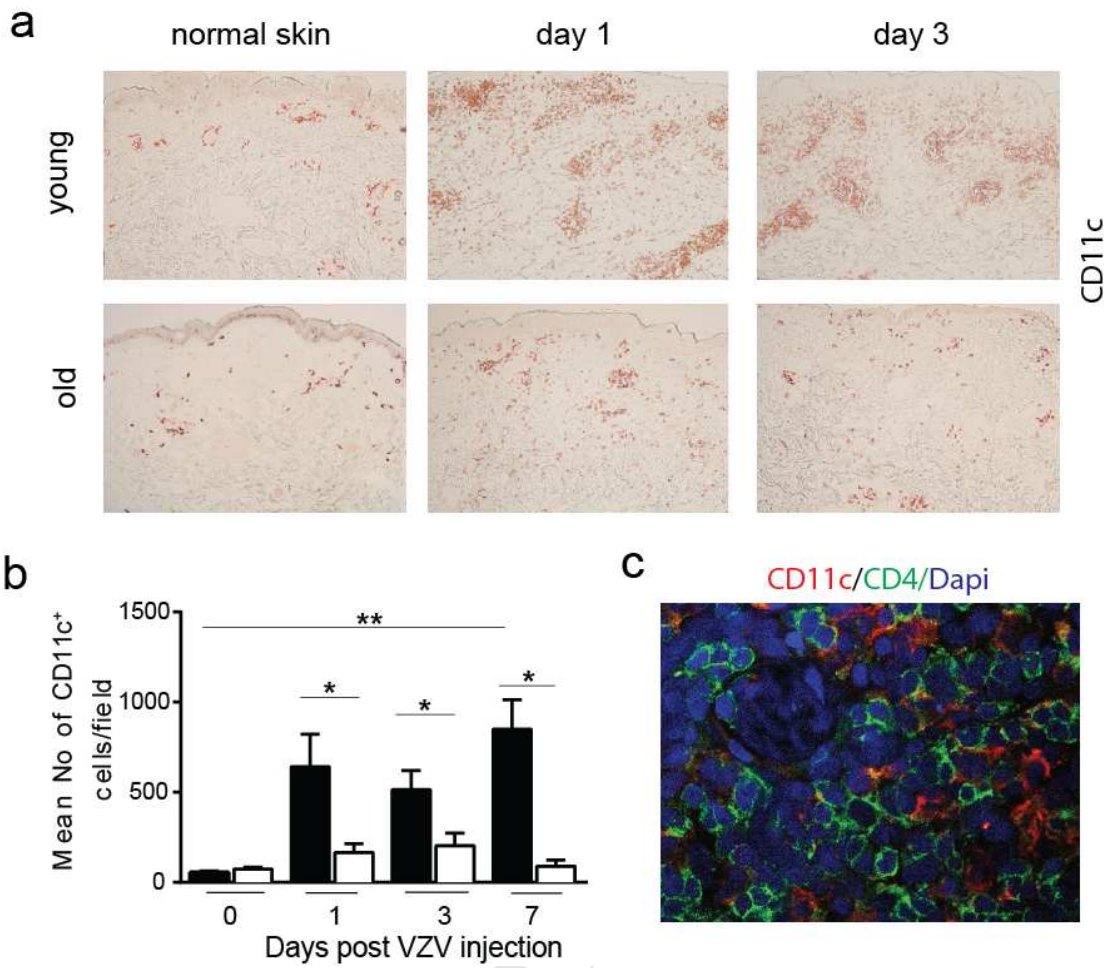
(A). Responses to VZV skin challenge were investigated in old individuals (n=18, 8M, 10F) pre- and post-Losmapimod treatment (15 mg twice daily for 4 days). (B) Serum CRP levels before pre- and post-Losmapimod treatment (n=18, p=0.04, Wilcoxon paired test). (C) Whole blood LPS stimulation was performed pre- and post-Losmapimod treatment, and TNF-α production measured by CBA (LPS p<0.0001, Losmapimod p<0.0001, Two way ANOVA n=18). (D) Clinical score was measured at 48 hrs after VZV antigen challenge pre and post Losmapimod (p=0.0006, Wilcoxon paired test, red symbol indicates the mean). (E) Correlation between change in serum CRP and change in clinical score after Losmapimod treatment (Pearson correlation). (F)

Representative images of skin sections collected 7 days post-VZV injection, stained for CD4 (red) and CD11c (pale blue) pre- and post-Losmapimod treatment in one of the individuals who showed an increased clinical score in response to VZV improved after Losmapimod treatment (top and bottom right panels) and one of individuals whose clinical score remained low following Losmapimod treatment (top and bottom left panels). White arrows indicate a dendritic cell interacting with surrounding T cells.

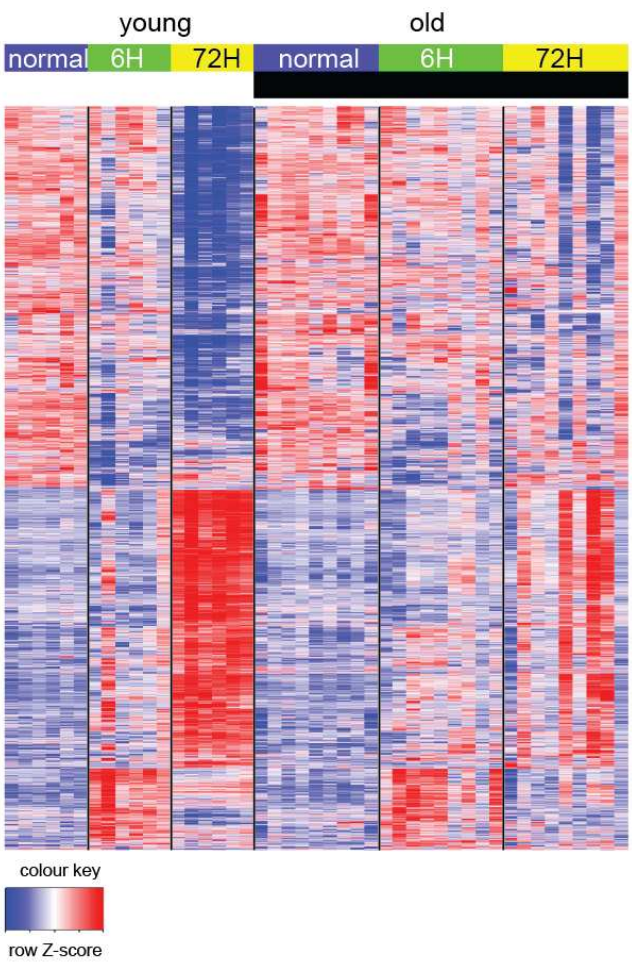

a
Pearson r=-0.61
p<0.0001
Clinical score
Age (years)
b
day 1
day 3
day 7
young
old
PV


c
day 1
day 3
day 7
young
old


d
Mean No. of CD4+ T cells / PV infiltrate
ns
ns
***
*
**
Day 0
Day 1
Day 3
Day 7
e
Mean No of CD4+ T cells /PV infiltrate
r= 0.8328
p < 0.0001
clinical score


a
normal skin
day 1
day 3
young
old
CD11c
b
Mean No of CD11c+ cells/field
1500
1000
500
0
**
*
*
*
0
1
3
7
Days post VZV injection
c
CD11c/CD4/Dapi

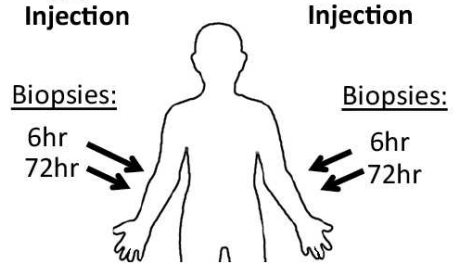
a



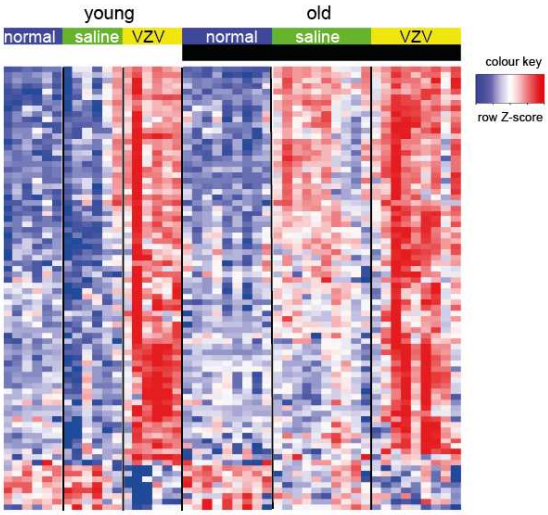
b

SYMBOL	FCH_Y.VZV72vsNormal	FCH_O.VZV72vsNormal
IDO1	501.15	23.16
GNLY	239.05	13.84
FCGR1B	172.34	27.93
KLRC2	161.18	6.94
GZMB	153.6	27.28
SLAMF7	143.7	9.4
FCGR1A	142.94	36.36
PLAC8	126.06	16.55
FPR1	122.26	21.67
BATF2	107.99	8.29
AIM2	105.88	9.16
IL18RAP	104.85	5.79
KLHDC7B	104.71	15.22
NKG7	92.32	7.54
IL7R	90.77	5.89
GBP5	89.3	13.43
FAIM3	75.55	7.56
CXCL9	75.49	35.84
SLAMF6	73.51	6.38
SLAMF1	73.03	9.3
CTLA4	70.87	6.79
OASL	68.86	10.51
CLEC4E	68.5	12.19
XCL1	65.46	8.69
LCK	64.86	8.09
SH2D1A	62.29	7.27
EPSTI1	62.18	15
CD247	61.49	5.58
IRF1	60.96	10.22
CCL5	60.73	11.64

a



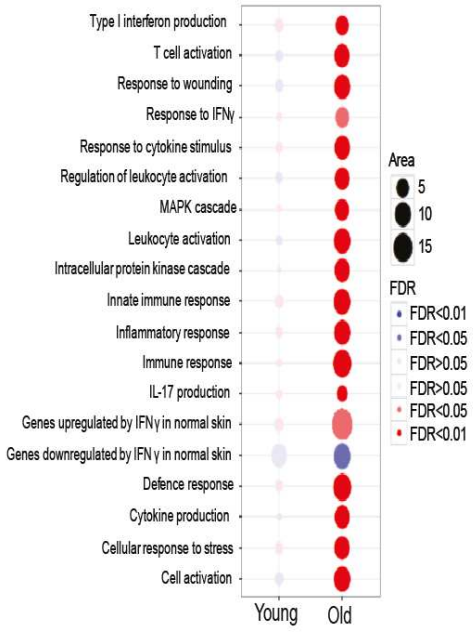
b



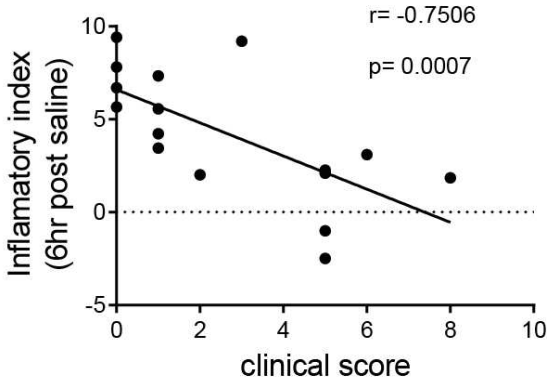
c

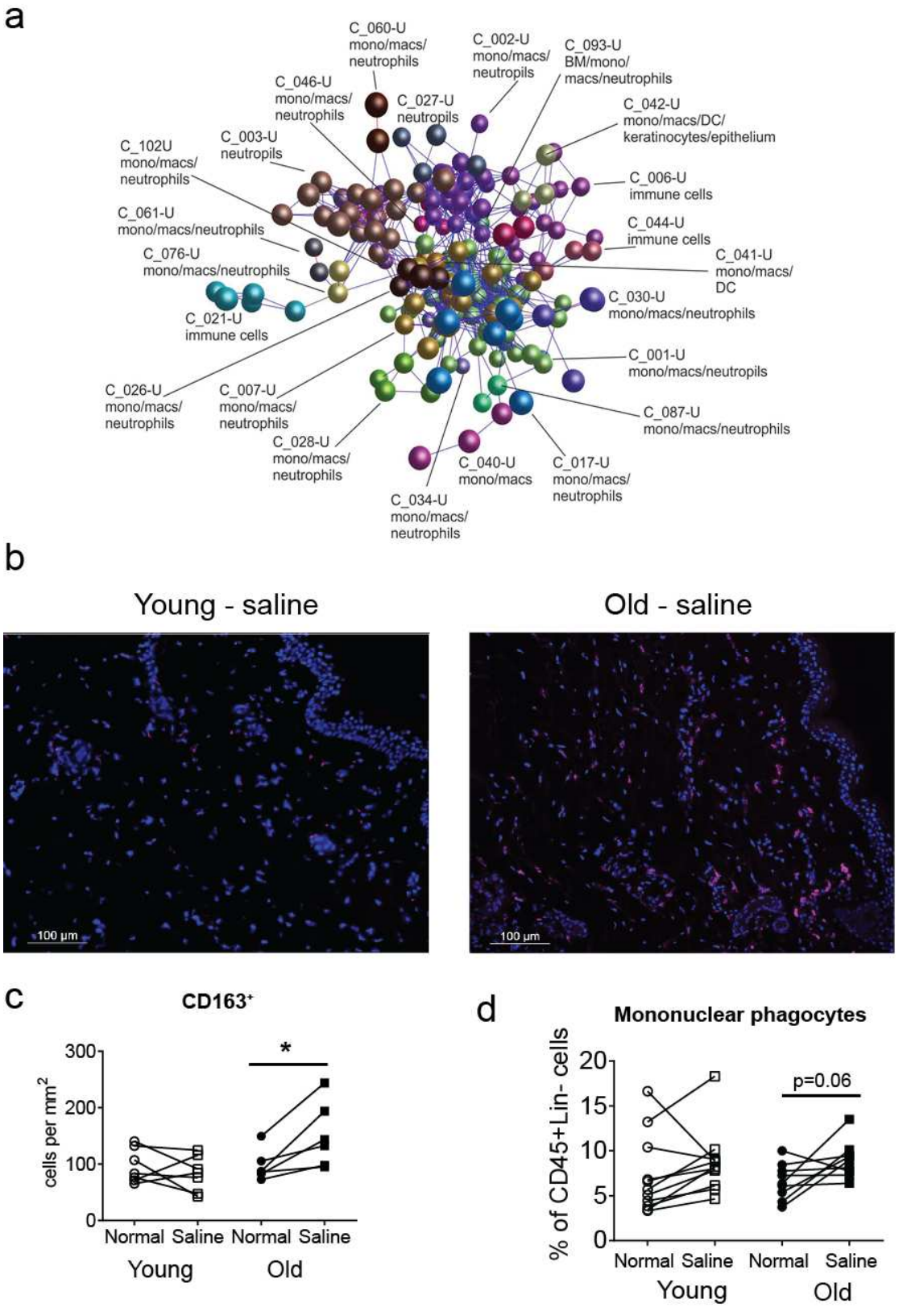
	SYMBOL	FCH_Old Saline 6hrs vs Normal	FCH_Young Saline 6hrs vs Normal
*	FOSL1	51.5	21.63
*	FPR1	39.86	8.29
	PTX3	33.34	7.49
*	SERPINA1	24.27	1.04
*	IL6	23.67	4.03
	FCGR1A	23.29	5.47
*	MMP1	21.34	8.28
*	SELE	20.69	2.33
	FCGR1B	18.96	5.84
*	MMP3	18.63	22.16
*	CXCL1	18.01	4.51
*	CXCL8	17.67	4.09
*	IL1RL1	16.88	2.3
*	PROK2	15.75	5.61
	BCL2A1	14.06	1.88
*	CXCL2	13.86	2.49
*	IL1B	12.96	2.68
*	HAS3	11.94	18.49
*	S100A9	11.24	7.39
*	SELL	11.06	1.49
*	FCGR3B	10.9	2.44
*	NAMPT	10.74	2.56
*	CXCL10	9.85	-2.63
*	TNFAIP6	9.51	3.02
*	PTGS2	9.47	6.01
*	CH25H	9.44	2.36
	CYP27B1	8.81	6.12
*	CCL2	8.79	1.68
	ADAMTS8	8.42	1.66
*	THBS1	8.23	2.95

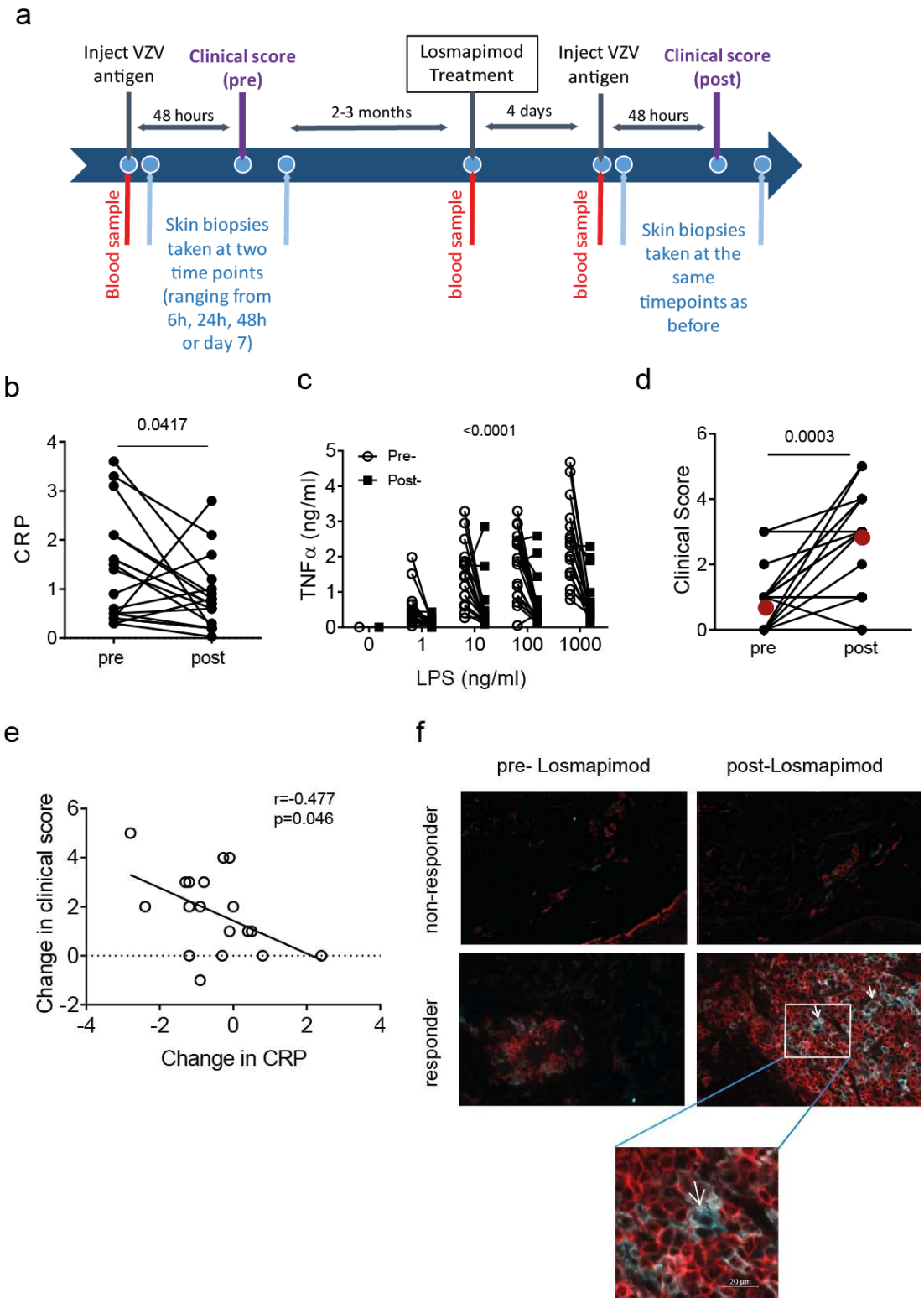
d



e




a
C_060-U mono/macs/ neutrophils
C_002-U mono/macs/ neutropils
C_093-U BM/mono/ macs/neutrophils
C_046-U mono/macs/ neutrophils
C_027-U neutropils
C_042-U mono/macs/DC/ keratinocytes/epithelium
C_003-U neutropils
C_102U mono/macs/ neutrophils
C_006-U immune cells
C_061-U mono/macs/neutrophils
C_044-U immune cells
C_076-U mono/macs/neutrophils
C_041-U mono/macs/ DC
C_030-U mono/macs/neutrophils
C_021-U immune cells
C_001-U mono/macs/neutropils
C_026-U mono/macs/ neutrophils
C_007-U mono/macs/ neutrophils
C_087-U mono/macs/neutrophils
C_028-U mono/macs/ neutrophils
C_040-U mono/macs
C_017-U mono/macs/ neutrophils
C_034-U mono/macs/ neutrophils
b
Young - saline
Old - saline
100 µm
100 µm
c
CD163+
cells per mm2
300
200
100
0
*
Normal Saline Normal Saline
Young
Old
d
Mononuclear phagocytes
% of CD45+Lin- cells
20
15
10
5
0
p=0.06
Normal Saline Normal Saline
Young
Old


a
Inject VZV antigen
Clinical score (pre)
48 hours
2-3 months
Losmapimod Treatment
4 days
Inject VZV antigen
48 hours
Clinical score (post)
Blood sample
Skin biopsies taken at two time points (ranging from 6h, 24h, 48h or day 7)
blood sample
blood sample
Skin biopsies taken at the same timepoints as before
b
0.0417
CRP
pre
post
c
<0.0001
Pre-
Post-
TNFα (ng/ml)
LPS (ng/ml)
d
0.0003
Clinical Score
pre
post
e
r=-0.477
p=0.046
Change in clinical score
Change in CRP
f
pre- Losmapimod
post-Losmapimod
non-responder
responder

List of Supplementary material:

Tables:

Figures:

Supplementary Figure 6. Overlap of differentially expressed genes (DEG) in the skin of young and old individuals in the skin after injection of saline or VZV antigen.

Supplementary Figure 7. Inflammatory response induced by saline injection inversely correlates with the response to VZV antigen challenge

Supplementary Figure 8. Saline injection increases the frequency of HLA-DR+ cells and mononuclear phagocytes in old but not young individuals.

Supplementary figure 9. Repeat skin testing with VZV skin antigen does not affect the clinical response in old individuals.

Supplementary Figure 10. Effect of Losmapimod treatment on immune function

Supplementary text:

Methods:

Participant exclusion criteria: Individuals with history of neoplasia, immunosuppressive disorders or inflammatory skin disorders were excluded from this investigation. Furthermore, we excluded individuals with co-morbidities that are associated with significant internal organ or immune dysfunction including heart failure, severe COPD, diabetes mellitus and rheumatoid arthritis and individuals on immunosuppressive regimes for the treatment of autoimmune or chronic inflammatory diseases (e.g. oral glucocorticoids, methotrexate, azathioprine and cyclosporin). We did not exclude volunteers with a history of uncomplicated hypertension or hypercholesterolaemia as this would have prevented the majority of ageing volunteers from participating in this study. The blood pressure and cholesterol level were not specifically measured for each volunteer, but those volunteers taking medication for a previously confirmed diagnosis of hypertension or hypercholesterolaemia were identified.

PBMC stimulation: PBMCs were isolated as standard and then subsequently stored at -80oC. The PBMCs were defrosted, counted and then cultured overnight at 5x105 cells/ml with plate bound anti-CD3 (1µg/ml) and IL-2 (50IU/ml) for eighteen hours at 37oC with 5% CO2. Brefeldin A (5µg/ml) was added two hours into the incubation. The cells were removed and cell surface stained with the following antibodies CD3, CD4, CD8 (clones UCHT1, SK3, SK9 respectively; BD) and Live/Dead after two washed the cells were fixed and permeabilised in Foxp3 staining buffers (as per the manufacturer's instructions; eBiosciences) and intracellularly stained with the following antibodies: IFNγ, IL-2, TNFα and Ki67 (clones 4S.B3, MQ1-17H12, Mab11 and Ki-67 respectively; Biolegend. Samples were acquired on the BD Symphony (BD Biosciences) and were subsequently analysed using FLowJo Version X (Treestar).

Transcriptional analysis of skin biopsies: Target amplification and labelling was performed according to standard protocols using Nugen Ovation WB Kit. RNA was hybridized to Affymetrix Human Genome U133 2.0 plus arrays. Affymetrix gene chips were scanned for spatial artefacts using the Hirshlight package[50]. Gene expression measures were obtained using the GCRMA algorithm[51] and was modelled using mixed-models in R's limma framework. Differences between groups were estimated from this model and its significance assessed using the moderated (paired/unpaired) t-test. Resulting P-values were adjusted for multiple hypotheses using the Benjamini-Hochberg procedure. Gene set variation analysis (GSVA)[52] was used to obtained the per-pathway scores for each patient and sample; using a collection of skin-specific pathways curated by the Krueger lab.

Network analysis of the genes expressed within skin biopsies was performed as described[19]. Briefly, normalized, nonlog-transformed, annotated, gene-expression data were imported into BioLayout Express[3D] (www.biolayout.org), a tool designed specifically for the visualization of large gene-expression network graphs[53]. Network graphs were then created using a Pearson correlation coefficient cut-off threshold of

r = 0.95. Each network graph was then clustered into groups of genes sharing similar profiles using the Markov clustering algorithm. The graphs were then explored to understand the biological significance of the gene clusters, identify those expressed by the young and old skin samples and their functional relationships to the other cell populations represented.

Figure legends:

Supplementary Figure 1. Effect of age on clinical response to intradermal injection of VZV antigen. Healthy young and old volunteers were injected with 0.02 ml VZV skin test antigen and clinical score based on a combination of extent of induration, palpability and redness at the injection site was measured at day 3 post challenge. Volunteers were split into age groups and mean, median and range f clinical scores calculated (A). Graph shows mean ± SEM for each age group. Mann-Whitney test was used to compare changes between age groups

.**Supplementary Figure 2. Phenotype of CD4 and CD8 T cells resident in normal skin of young and old individuals**. Sections of normal skin were immunostained to detect CD4, CD8, CD69, CD103 using an indirect immunofluorescence method. (A). Representative image of normal skin immunostained for CD4 (green), CD69 (red) and CD103 (white). (B) The proportion of $CD4^+$ cells expressing CD69 in young and old skin (n=11). (C) The proportion of $CD4^+CD69^+$ cells expressing CD103 in young and old skin (n=10). (D) The proportion of $CD8^+$ cells expressing CD69 in young and old skin (n=11). (E) The proportion of $CD8^+CD69^+$ cells expressing CD103 in young and old skin (n=10). For B-E the line indicates the mean.

Supplementary Figure 3. Proliferation of T cells following VZV antigen challenge is reduced in the old. (A) Representative immunostaining of ki67 expression in skin afterday 3 and 7 post-VZV injection (Ki67 green; CD4 red). (B)

Cumulative data showing the frequency of $CD4^+$ cells expressing Ki67 per perivascular infiltrate (young - filled bars, old- open bars). (C) Representative immunostaining on days 3 and 7 post-injection: Ki67 (green) and CD8 (red). (D) Cumulative data of the percentage of $CD8^+$ cells expressing Ki67 per perivascular infiltrate in each donor. Data shown as mean ± SEM. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Supplementary Figure 4. Activation of dermal endothelium at site of VZV challenge Immunofluorescence staining for CD31 and E-selectin or VCAm1 expression was performed on skin sections from biopsies taken from normal skin and 6 h, day 1 and day 3 after cutaneous challenge with VZV antigen from young and old volunteers (n=4-5 per age group at each time point). The number of double positive staining vessels expressed as a proportion of the total number of vessels in the superficial and mid-dermis of each section was used for analysis for each individual. (A) Representative images are shown CD31 (green) and E-selectin (red). (B) cumulative data (p values indicated, Mann Whitney test). (C) Expression of VCAM1 on $CD31^+$ capillary loops 6hrs after VZV injection. (D) Healthy young and old volunteers (n=5) were injected with 0.02 ml VZV skin antigen test and 5 mm punch biopsies were performed 6hrs post injection. Skin sections were immunostained with CD11c, CD4 and neutrophil elastase and the number of positive cells was counted per field. For B-D data shown as mean ± SEM. (E) Normal and VZV (6 hours post-injection) paired skin biopsies were assessed for mononuclear phagocyte numbers by immunofluorescence staining utilising HLA-DR, CD14 and CD16. Any cell that was HLA-DR+ and CD14+ and/or CD16+ was defined as being a mononuclear phagocyte, analysis was performed in young (black, n=5) and old (white, n=5). For E data was assessed by a paired t-test * = $p<0.05$..

Supplementary Figure 5. Pathway analysis of gene expression in young and old skin at 6 and 72 h post VZV antigen challenge . Differentially expressed genes

between VZV injected and normal skin in young or old at FCH>2 and FDR>0.05. Unsupervised clustering was carried out using Pearson correlation distance with Mcquitty agglomeration scheme. (A) Venn diagrams show numbers of DEG at 6 h following VZV antigen injection compared to normal skin. Up-regulated genes are shown in red, down-regulated genes in blue. (B) Table shows top 30 up-regulated genes at 6 h in young, genes not significantly up-regulated in old skin are indicated in bold italics. (C)**.** Bubble plot representing the overall representation of relative gene expression in VZV-injected skin versus normal skin. KEGG and GO collection, as well as a curated skin-related collection were interrogated and the most relevant pathways amongst them with FDR<0.05 are presented. The area of each circle is proportional to the differences in the GSVA-derived pathway scores between VZV-injected and normal skin in each group. Colours indicate the direction of dysregulation red (up) and blue (down). Colour intensity represents the strength of the dysregulation determined by FDR.

Supplementary Figure 6. Overlap of differentially expressed of genes (**DEG) in the skin of young and old individuals in the skin after injection of saline or VZV antigen.** A selection of differentially expressed genes of interest are indicated for each (red, up-regulated and blue, down-regulated). (B) Principal component analysis of global gene expression in normal skin and 6 h after injection with saline or VZV antigen. (C) Table shows top 30 up-regulated genes at 6 h in saline and VZV injected skin.

Supplementary Figure 7. Inflammatory response induced by saline injection inversely correlates with the response to VZV antigen challenge. The expression of individual inflammatory genes in the skin 6 h after saline was compared by qRT-PCR analysis and plotted against the clinical score following VZV antigen injection at 48 hours.

Supplementary Figure 8. Frequency of HLA-DR$^+$ cells and mononuclear phagocytes following saline injection 5mm punch biopsies were collected from normal or injected skin and digested overnight to provide single cell suspension. (A) Gating strategy employed to identify mononuclear phagocytes and dendritic cells in human skin; CD45+ lineage cocktail negative single cells were identified, subsequently, HLA$^-$DR$^+$ CD14$^+$ and/or CD16$^+$ were mononuclear phagocytes and HLA-DR+CD14-CD16- were dendritic cells (DCs) either CD141$^+$ or CD11c$^+$ DCs. (B) cumulative data of mononuclear phagocyte populations 24 hours post-saline injection. (C) Phenotype of mononuclear phagocytes in the young and old donors (CD14$^+$CD16$^-$ grey, CD14$^+$CD16$^+$ white, CD16$^+$CD14$^-$ black). * = $p<0.05$

Supplementary figure 9. Repeat skin testing with VZV skin antigen does not affect the clinical response in old individuals. 14 individuals with clinical score below 4 were re-challenged with VZV in the skin 2-5 months after the original skin test. Clinical score for both skin tests are shown in the table ($p>0.5$).

Supplementary figure 10. Effect of Losmapimod treatment on immune functionA). Whole blood LPS stimulation was performed pre- and post-Losmapimod treatment in the same donors, and IL-6 and IL-8 was production measured by CBA (LPS $p<0.0001$, Losmapimod $p<0.0001$, Two way ANOVA n=18). PBMCs were stimulated overnight with CD3 and IL-2 were assessed by flow cytometric analysis in CD4+ T cells (B) for intracellular cytokine expression and (C) Ki67 expression and additionally in CD8 T cells (D) for intracellular cytokine expression and (E) Ki67 expression pre- and post-losmapimod treatment (white circles and black squares respectively). Figure B-E were assessed by a paired t-test and no significant difference was observed.

	Old female	Old male	Young female	Young male
number	47	31	56	41
Age range	65-93	65-93	20-39	20-39
Average age	75.7	77.5	28.3	29.6
Median age	74	77	29	29
Score range	0-8	0-6	0-9	0-9
Mean score	2.3	1.9	5.5	5.5
Median score	2	1	5	6

Supplementary Table 2:

	young	middle	old
number	97	14	78
Age range	20-39	41-64	65-93
Median age	29	52	75.5
Gender	56F/41M	8F/6M	47F/31M
Score range	0-9	0-7	0-8
Mean score	5.5	4.5	2.18
Median score	6	5	2

Supplementary Table 3:

Antibody name	**Clone**	**Company**
CD11c	B-ly6	BD Bioscience
CD4	SK3	BD Bioscience
CD163	5C6-FAT	Acris
DC-LAMP	104.G4	Beckman Coulter
Neutrophil elastase	NP75	Dako

Antibody name	Clone	Company
CD4	SK3 or YNB46.1.8	BD Bioscience
CD8	RPA-T8	BD Bioscience
Ki67 - FITC	B56	BD Bioscience
CD31 - FITC	WM59	BD Bioscience
CD11c	B-ly6	BD Bioscience
Ki67 - FITC	B56	BD Bioscience
CD69	FN50	Biolegend
CD103	2G5.1	Thermofisher
Foxp3 - Biotin	PCH101	eBioscience
PD-1	NAT105	Abcam

Supplementary Table 3:

E-selectin	ENA1	Abcam
CD163	RM3/1	Abcam

Antibody name	Clone	Company
CD11c	3.9	Biolegend
CD14	HCD14	Biolegend
CD16	3G8	Biolegend
CD19	HIB19	Biolegend
CD20	2H7	Biolegend
CD56	HCD56	Biolegend
CD163	GHI/61	Biolegend
HLA-DR	104.G4	BD Biosciences
CD3	UCHT1	BD Biosciences
CD45	2D1	BD Biosciences

Supplementary table 6

	lgFCH_Y.VZ	FCH_Y.VZV	pvals_Y.VZ	fdrs_Y.VZV	StatusFCH2	lgFCH_Y.VZ	FCH_Y.VZV	pvals_Y.VZ
117_at	0.93	1.91	0.0275	0.0616	0	0.35	1.28	0.399
1294_at	2.01	4.02	6.27E-07	7.08E-06	1	-0.85	-1.8	0.0233
1316_at	-0.89	-1.86	0.00755	0.021	0	-1.21	-2.31	0.000401
1405_i_at	5.92	60.73	1.67E-07	2.30E-06	1	0.18	1.13	0.861
1438_at	-1.83	-3.56	3.25E-07	4.05E-06	-1	-0.55	-1.46	0.0963
1552256_a	-1.41	-2.66	8.26E-07	8.95E-06	-1	-0.4	-1.32	0.134
1552263_a	1.43	2.7	7.78E-05	0.000416	1	0.16	1.11	0.65
1552264_a	1.18	2.26	1.79E-05	0.000118	1	0.47	1.39	0.0678
1552283_s	-1.35	-2.54	0.0093	0.025	-1	-0.69	-1.62	0.173
1552286_a	-1.24	-2.36	2.20E-06	2.03E-05	-1	-0.76	-1.69	0.00243
1552302_a	2.4	5.28	2.08E-09	6.28E-08	1	0.74	1.67	0.0371
1552303_a	2.32	4.98	1.36E-12	2.37E-10	1	0.43	1.35	0.115
1552316_a	2.98	7.89	4.59E-09	1.18E-07	1	-0.32	-1.25	0.479
1552318_a	2.33	5.03	4.84E-06	3.94E-05	1	-0.94	-1.92	0.0483
1552320_a	4.5	22.67	7.06E-11	4.61E-09	1	0.47	1.38	0.427
1552323_s	1.04	2.05	0.00937	0.0252	1	-1.4	-2.64	0.000579
1552327_a	-2.1	-4.29	8.29E-09	1.94E-07	-1	-1.83	-3.55	2.67E-07
1552343_s	1.99	3.98	8.90E-06	6.59E-05	1	-0.54	-1.46	0.197
1552344_s	1.29	2.45	1.39E-06	1.39E-05	1	1.07	2.1	4.08E-05
1552365_a	-1.53	-2.88	6.04E-05	0.000334	-1	-0.29	-1.22	0.422
1552367_a	-1.43	-2.69	0.000795	0.00307	-1	-0.22	-1.17	0.588
1552398_a	2.57	5.93	0.00971	0.0259	1	0.36	1.29	0.707
1552400_a	-0.36	-1.28	0.421	0.545	0	-1.8	-3.49	0.00011
1552474_a	-1.01	-2.02	0.000301	0.00133	-1	-0.72	-1.65	0.00848
1552480_s	4.8	27.87	8.41E-10	3.11E-08	1	0.66	1.58	0.335
1552482_a	-1.18	-2.27	0.00039	0.00166	-1	-0.47	-1.38	0.144
1552485_a	1.33	2.52	0.000143	0.000702	1	1	2	0.00345
1552486_s	1.61	3.05	1.08E-06	1.12E-05	1	1.16	2.23	0.000271
1552487_a	-0.47	-1.39	0.162	0.261	0	1.44	2.71	5.33E-05
1552491_a	0.01	1.01	0.975	0.984	0	1.33	2.51	9.99E-06
1552496_a	-1.43	-2.69	3.85E-07	4.67E-06	-1	-0.35	-1.27	0.176
1552497_a	6.2	73.51	3.24E-11	2.55E-09	1	0.26	1.2	0.743
1552502_s	-1.19	-2.29	0.00142	0.00503	-1	0.01	1.01	0.981
1552509_a	-2.25	-4.77	7.50E-05	0.000402	-1	-0.88	-1.85	0.103
1552532_a	-1.15	-2.23	3.82E-07	4.65E-06	-1	-0.12	-1.09	0.562
1552553_a	2.85	7.19	7.98E-09	1.88E-07	1	0.95	1.93	0.0321
1552562_a	0.33	1.26	0.148	0.243	0	1.01	2.01	3.72E-05
1552566_a	-1.54	-2.9	0.00148	0.00522	-1	0.52	1.43	0.267
1552575_a	-3.54	-11.64	7.78E-12	8.67E-10	-1	-0.93	-1.9	0.0354
1552584_a	4.63	24.75	3.45E-10	1.57E-08	1	0.53	1.44	0.408
1552612_a	2.69	6.45	2.08E-07	2.78E-06	1	0.16	1.11	0.74
1552613_s	2.44	5.42	9.23E-10	3.32E-08	1	0.27	1.21	0.436
1552618_a	1.04	2.06	0.0146	0.0365	1	0.66	1.58	0.115
1552619_a	-1.2	-2.29	0.0103	0.0271	-1	-1.38	-2.6	0.00332
1552626_a	1.41	2.66	1.89E-06	1.79E-05	1	0.48	1.4	0.0809
1552633_a	2.14	4.41	2.50E-10	1.24E-08	1	0.13	1.09	0.655

Supplementary table 7

	lgFCH_Y.Sa	FCH_Y.Salir	pvals_Y.Sal	fdrs_Y.Salir	StatusFCH2	lgFCH_Y.Sa	FCH_Y.Salir	pvals_Y.Sal
117_at	-0.34	-1.26	0.417	1	0	-0.14	-1.1	0.734
1552487_a	-0.01	-1.01	0.975	1	0	0.91	1.88	0.00797
1552491_a	-0.16	-1.12	0.56	1	0	0.7	1.62	0.0147
1552641_s	-0.25	-1.19	0.556	1	0	0.31	1.24	0.465
1553749_a	-0.05	-1.04	0.818	1	0	0.47	1.38	0.0366
1553787_a	-0.24	-1.18	0.557	1	0	-0.96	-1.95	0.0201
1553789_a	-0.26	-1.2	0.455	1	0	-0.9	-1.87	0.0106
1553861_a	0.17	1.12	0.702	1	0	0.38	1.3	0.382
1554008_a	0.22	1.16	0.543	1	0	1.42	2.69	0.000145
1554283_a	0.04	1.03	0.902	1	0	0.8	1.74	0.0133
1554406_a	0.47	1.38	0.55	1	0	0.21	1.16	0.789
1554704_a	-0.45	-1.36	0.192	1	0	-1.6	-3.04	1.09E-05
1554748_a	0.06	1.04	0.914	1	0	-0.08	-1.06	0.882
1554834_a	0.42	1.34	0.458	1	0	0.85	1.81	0.136
1555131_a	0.25	1.19	0.499	1	0	-1.23	-2.34	0.00163
1555167_s	-0.41	-1.33	0.488	1	0	1.36	2.56	0.0252
1555318_a	-0.35	-1.28	0.608	1	0	-1.83	-3.56	0.00906
1555427_s	0.06	1.04	0.881	1	0	0.7	1.63	0.0637
1555600_s	0.64	1.55	0.294	1	0	1	1.99	0.103
1555638_a	0.17	1.12	0.85	1	0	0.1	1.07	0.915
1555756_a	0.45	1.37	0.555	1	0	0.3	1.23	0.692
1555760_a	-0.43	-1.34	0.354	1	0	0.14	1.11	0.752
1555870_a	0.36	1.28	0.394	1	0	-0.63	-1.55	0.134
1556069_s	-0.39	-1.31	0.537	1	0	-0.23	-1.17	0.717
1556081_a	-0.54	-1.45	0.0793	1	0	0.46	1.37	0.136
1556185_a	-0.34	-1.27	0.356	1	0	0.14	1.1	0.715
1556210_a	-0.36	-1.29	0.387	1	0	-1.42	-2.67	0.00116
1556211_a	-0.18	-1.13	0.639	1	0	-1.02	-2.02	0.00843
1556212_x	-0.03	-1.02	0.944	1	0	-0.9	-1.87	0.0204
1556253_s	0.52	1.43	0.264	1	0	-0.07	-1.05	0.876
1556300_s	-1.38	-2.61	0.0126	1	0	0.21	1.16	0.7
1556321_a	-0.35	-1.28	0.313	1	0	0.67	1.59	0.059
1556361_s	-0.26	-1.2	0.541	1	0	0.81	1.75	0.0592
1556385_a	0.07	1.05	0.911	1	0	1.2	2.3	0.0465
1556402_a	-0.45	-1.37	0.26	1	0	-0.61	-1.52	0.13
1556579_s	0.3	1.23	0.512	1	0	-0.03	-1.02	0.939
1556589_a	-0.15	-1.11	0.74	1	0	-1.08	-2.12	0.0185
1556590_s	-0.06	-1.04	0.91	1	0	-0.82	-1.76	0.115
1556758_a	-0.43	-1.34	0.186	1	0	0.16	1.12	0.617
1556770_a	-0.31	-1.24	0.537	1	0	-1.07	-2.09	0.0363
1556867_a	-0.04	-1.03	0.931	1	0	1.55	2.93	0.00148
1556989_a	-0.83	-1.78	0.168	1	0	0.35	1.28	0.554
1557155_a	1.22	2.33	0.00354	1	0	1.23	2.34	0.0033
1557383_a	-0.26	-1.2	0.578	1	0	-1.42	-2.68	0.00346
1557458_s	0.2	1.15	0.57	1	0	0.7	1.63	0.0491
1557553_a	0.18	1.13	0.779	1	0	-0.18	-1.14	0.767

Supplementary table 8

PositiveRegulators
ABCB9
ADAM10
ADAM17
ADAM8
ADK
ADORA2B
AIF1
AKT1
AP3B1
AP3D1
AQP3
ATF1
ATF2
AXL
BAD
BCAR1
BCL10
BCL2
BDKRB1
BIRC2
BIRC3
BLM
BLOC1S3
BMI1
BTK
C1QA
C1QB
C1QC
C1R
C1RL
C1S
C2
C3
C3AR1
C4BPB
C6
C7
CACNB3
CADM1
CAMK1D
CARD11
CARD9
CASP8
CAV1
CCL19
CCL21

ACCEPTED MANUSCRIPT

Supplementary table 9

Gene symb	Symbol	Descriptior	ID	EntrezID	Chrom	ChromLoc	PathwayID	PathDesc
BID;211725	BID	BH3 intera	211725_s_	637	22	18216905	04115, 042	p53 signalir
C1orf38;20	C1orf38	chromoson	207571_x_	9473	1	28199054	-	-
C1orf38;21	C1orf38	chromoson	210785_s_	9473	1	28199054	-	-
C5AR1;220	C5AR1	complemer	220088_at	728	19	47813103	04080, 046	Neuroactiv
CCR1;2050	CCR1	chemokine	205098_at	1230	3	46243199	04060, 040	Cytokine-cy
CCR1;2050	CCR1	chemokine	205099_s_	1230	3	46243199	04060, 040	Cytokine-cy
CD300A;20	CD300A	CD300a mc	209933_s_	11314	17	72462521	-	-
CHST11;22	CHST11	carbohydra	226368_at	50515	12	1.05E+08	00532, 009	Chondroitir
CHST11;22	CHST11	carbohydra	226372_at	50515	12	1.05E+08	00532, 009	Chondroitir
CLEC4E;22	CLEC4E	C-type lecti	222934_s_	26253	12	8685900	-	-
CLEC7A;15	CLEC7A	C-type lecti	1554406_a	64581	12	10269375;	-	-
CLEC7A;15	CLEC7A	C-type lecti	1555756_a	64581	12	10269375;	-	-
CLEC7A;22	CLEC7A	C-type lecti	221698_s_	64581	12	10269375;	-	-
CSF2RB;20	CSF2RB	colony stim	205159_at	1439	22	37309674	04060, 042	Cytokine-cy
CTSS;2029(	CTSS	cathepsin S	202902_s_	1520	1	1.51E+08	04142, 046	Lysosome,
CYBB;2039	CYBB	cytochrom	203923_s_	1536	X	37639269	4670	Leukocyte t
CYTH4;219	CYTH4	cytohesin 4	219183_s_	27128	22	37678494	-	-
DOK3;2235	DOK3	docking pr	223553_s_	79930	5	176928914	-	-
EFHD2;222	EFHD2	EF-hand do	222483_at	79180	1	15736390	-	-
EMR2;207(	EMR2	egf-like mo	207610_s_	30817	19	14843204	-	-
FCGR2A;20	FCGR2A	Fc fragmen	203561_at	2212	1	1.61E+08	04666, 053	Fc gamma I
FGR;20843	FGR	Gardner-Ra	208438_s_	2268	1	27938802	4062	Chemokine
HCK;20801	HCK	hemopoiet	208018_s_	3055	20	30639990	04062, 046	Chemokine
IGSF6;2064	IGSF6	immunoglo	206420_at	10261	16	21652605	-	-
ITGAX;210	ITGAX	integrin, al	210184_at	3687	16	31366508	4810	Regulation
LILRA2;207	LILRA2	leukocyte i	207857_at	11027	19	55085256	-	-
LILRA2;211	LILRA2	leukocyte i	211100_x_	11027	19	55085256	-	-
LILRA2;211	LILRA2	leukocyte i	211101_x_	11027	19	55085256	-	-
LILRB1;211	LILRB1	leukocyte i	211336_x_	10859	19	55128628;	-	-
LILRB1;229	LILRB1	leukocyte i	229937_x_	10859	19	55128628;	-	-
LILRB2;207	LILRB2	leukocyte i	207697_x_	10288	19	54777675	-	-
LILRB2;210	LILRB2	leukocyte i	210146_x_	10288	19	54777675	-	-
LILRB3;211	LILRB3	leukocyte i	211135_x_	11025	19	54720146	4662	B cell recep
LYN;20262	LYN	v-yes-1 Yan	202625_at	4067	8	56792385	04062, 046	Chemokine
LYN;20262(	LYN	v-yes-1 Yan	202626_s_	4067	8	56792385	04062, 046	Chemokine
LYN;21075	LYN	v-yes-1 Yan	210754_s_	4067	8	56792385	04062, 046	Chemokine
LYZ;155574	LYZ	lysozyme (r	1555745_a	4069	12	69742133	-	-
NA;204961	NA	NA	204961_s_	NA	-	-	-	-
NA;210225	NA	NA	210225_x_	NA	-	-	-	-
NA;210784	NA	NA	210784_x_	NA	-	-	-	-
NA;211133	NA	NA	211133_x_	NA	-	-	-	-
NA;227184	NA	NA	227184_at	NA	-	-	-	-
NA;228685	NA	NA	228685_at	NA	-	-	-	-
NADK;2136	NADK	NAD kinase	213607_x_	65220	1	1682677	00760, 011	Nicotinate
PILRA;2197	PILRA	paired imm	219788_at	29992	7	99971067	-	-
PILRA;2222	PILRA	paired imm	222218_s_	29992	7	99971067	-	-
PTAFR;206	PTAFR	platelet-act	206278_at	5724	1	28473677	04020, 040	Calcium sig
PTAFR;211	PTAFR	platelet-act	211661_x_	5724	1	28473677	04020, 040	Calcium sig
PTPRE;221	PTPRE	protein tyr	221840_at	5791	10	129705324	-	-

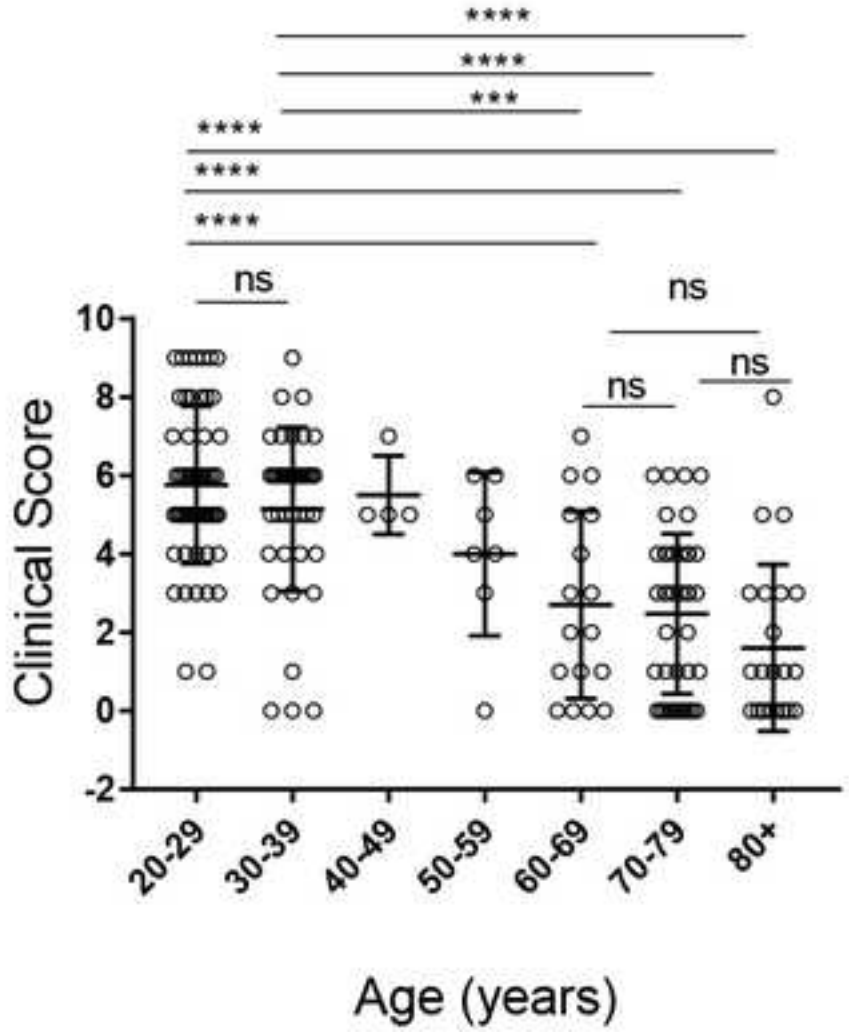
Supplementary Figure No.1

Click here to download high resolution image

A

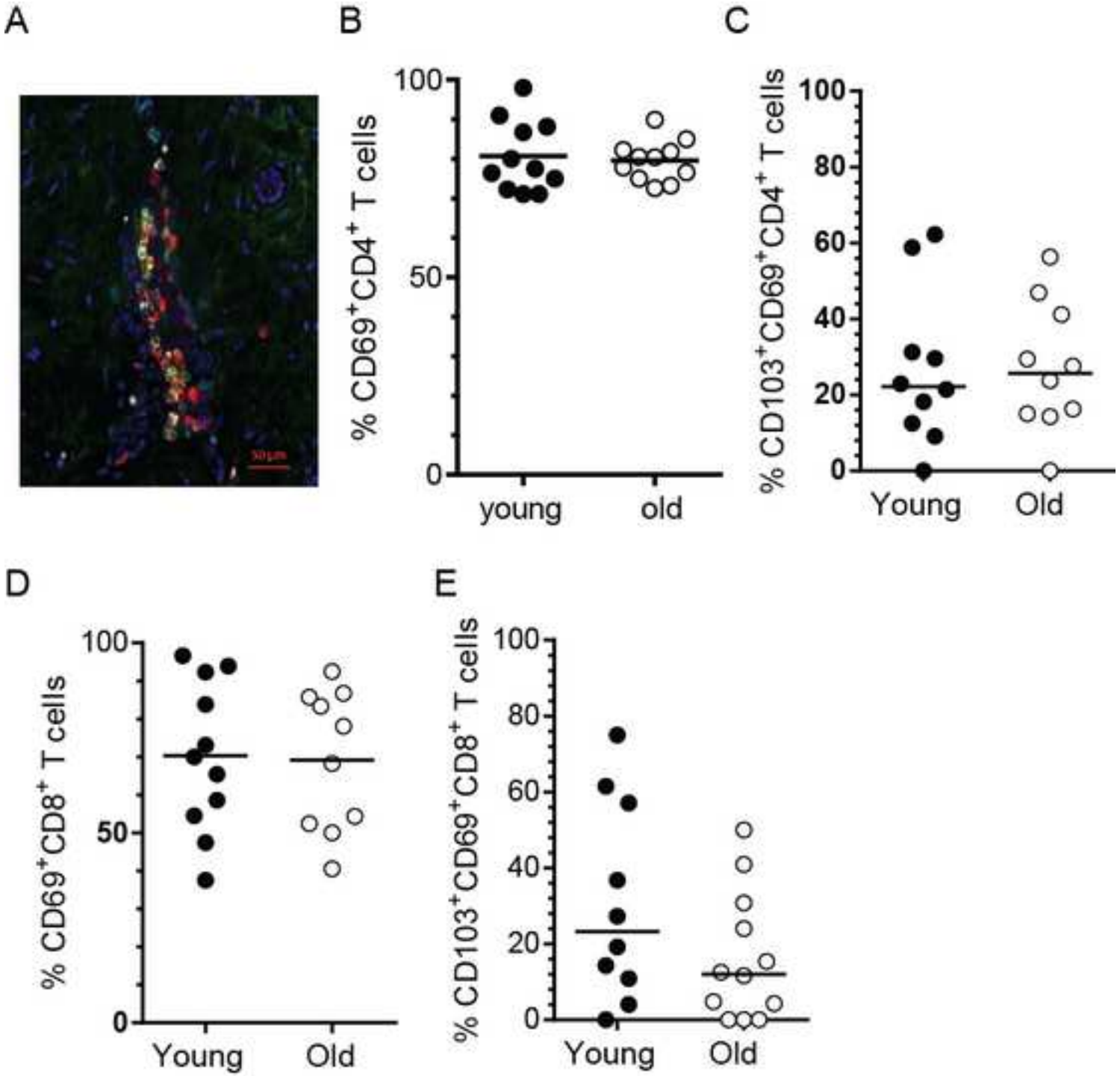
	20-29	30-39	40-49	50-59	60-69	70-79	80+
number	55	41	4	7	17	40	23
Score range	1-9	0-9	5-7	0-6	0-7	0-6	0-8
Mean score	5.76	5.15	5.5	4	2.71	2.48	1.61
Median score	6	6	5	4	2	3	1

B



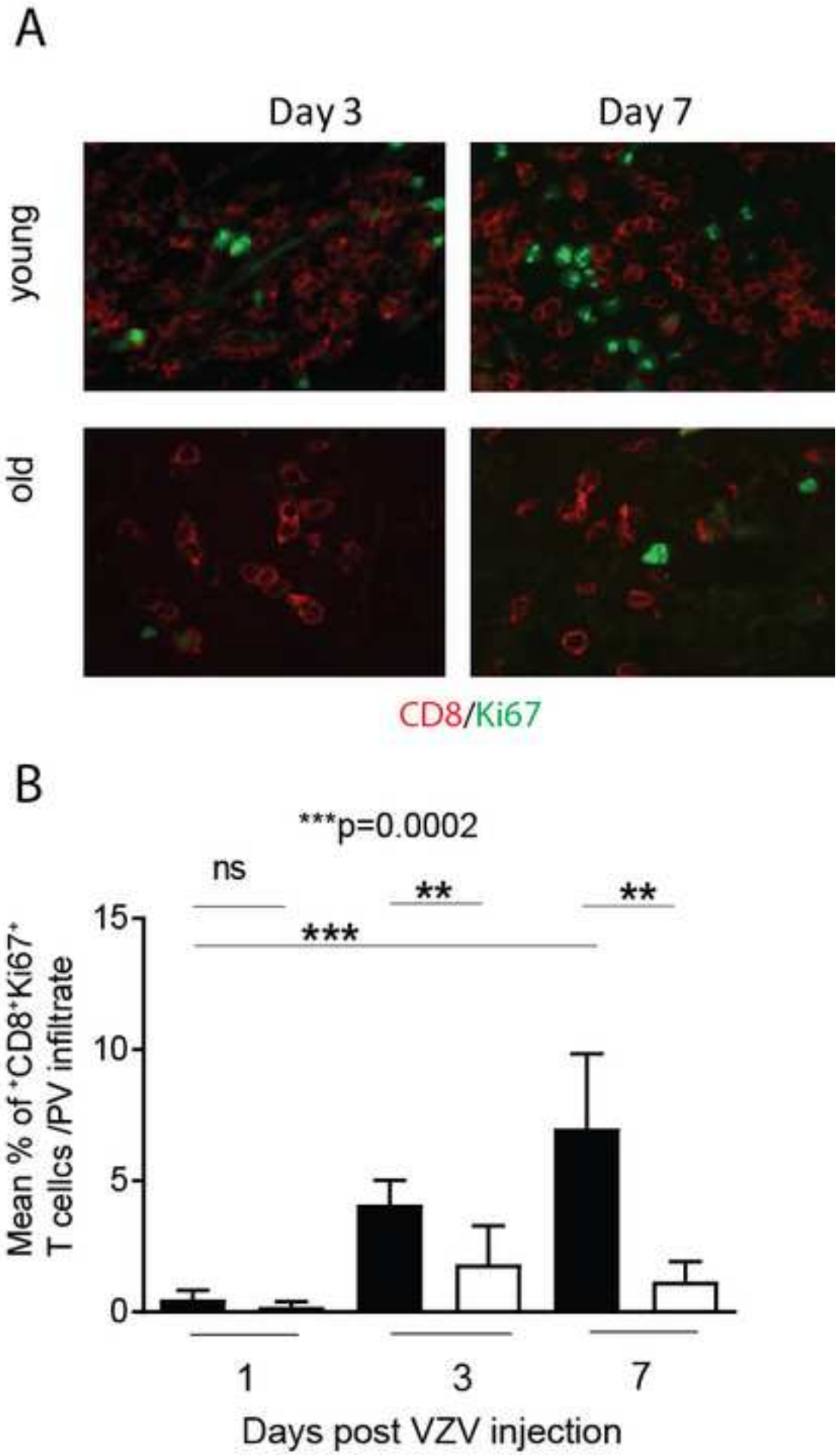
Supplementary Figure No.2

Click here to download high resolution image



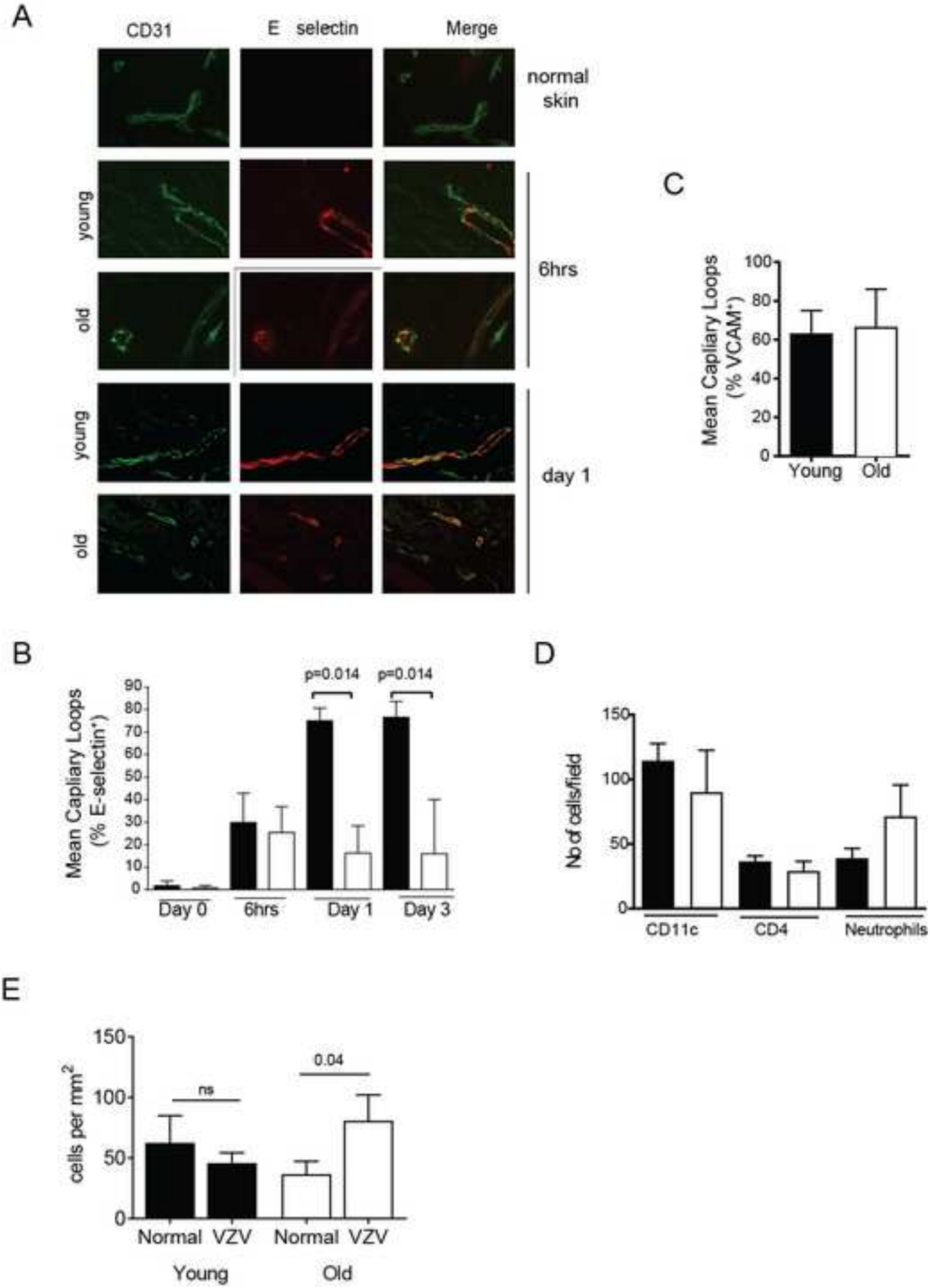
Supplementary Figure No.3

Click here to download high resolution image



Supplementary Figure No.4

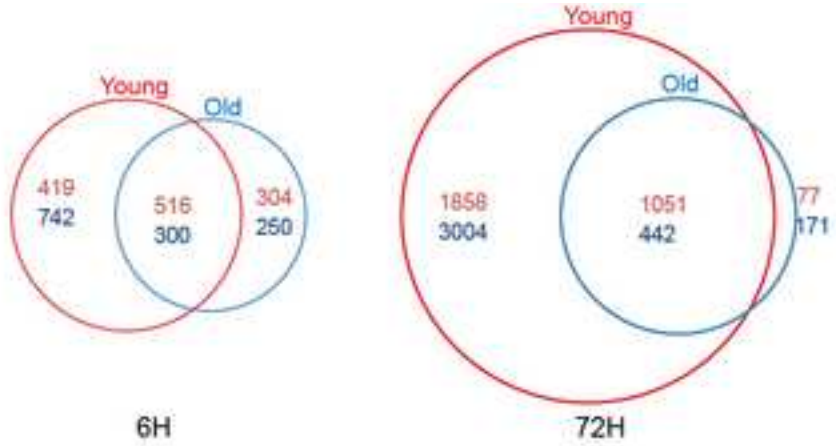
Click here to download high resolution image



Supplementary Figure No.5

Click here to download high resolution image

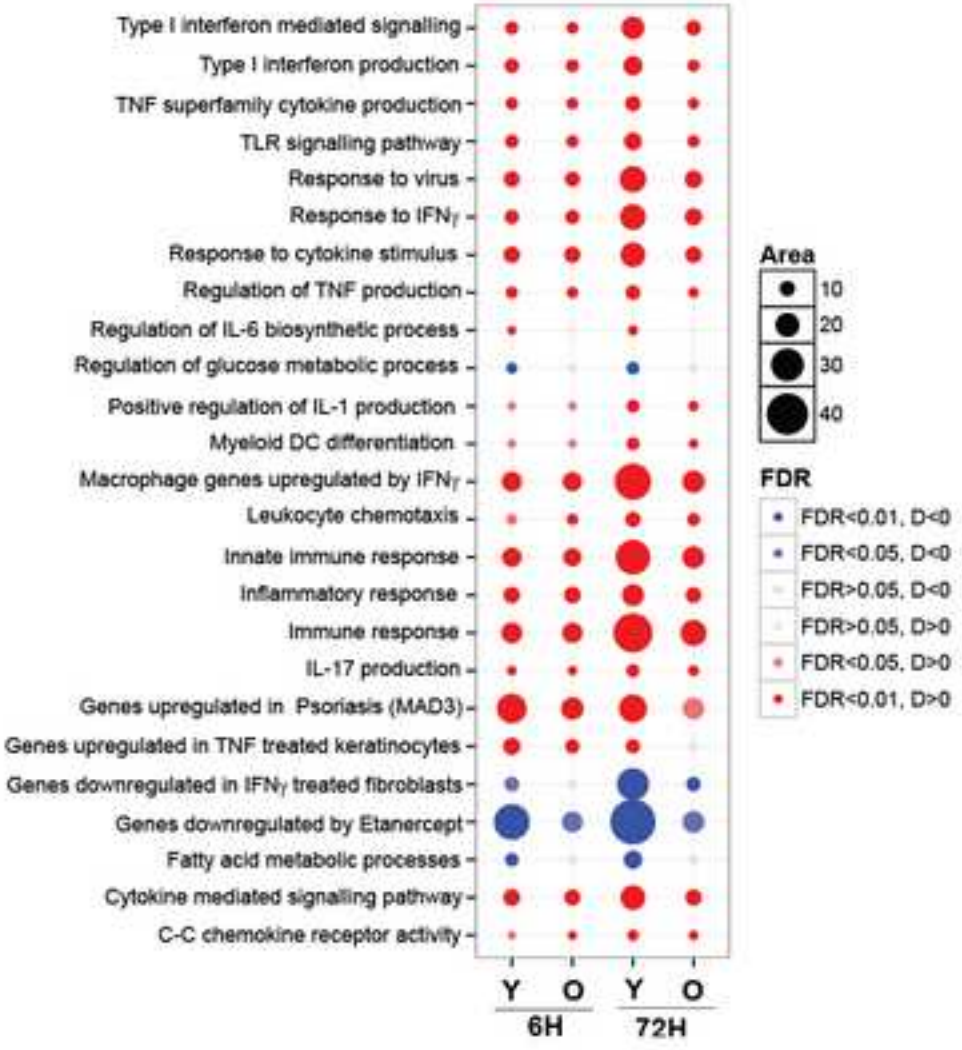
A



B

SYMBOL	FCH_Y:VZV6vsNormal	FCH_O:VZV6vsNormal
FOSL1	160.28	37.58
MMP3	125	12.16
MMP1	70.59	14.24
IL6	62.35	46.84
PTX3	61.67	50.09
HAS3	59.81	8.84
IL1B	50.15	30.45
S100A7A	***49.06***	***3.59***
CCL8	43.61	13.15
OASL	41.32	20.38
IL1B	37.77	21.85
FCGR1B	35.01	44.73
CXCL1	34.28	20.41
S100A9	33.83	8.69
FPR1	29.69	44.21
FCGR1A	28.99	49.84
DEFB4A	***28.32***	***-1.54***
PTGS2	26.68	9.52
ICAM1	24.56	15.67
CYP27B1	23.16	8.13
CXCL2	23	14.85
CD274	21.13	9.94
TNFAIP6	19.6	24.5
IRF1	19.32	10.22
CCL20	***15.22***	***1.28***
CXCL8	15.03	10.76
CCL3	14.49	9.04
RSAD2	14.37	5.38
RGS16	14.31	8.94
MMP12	14.28	8.55

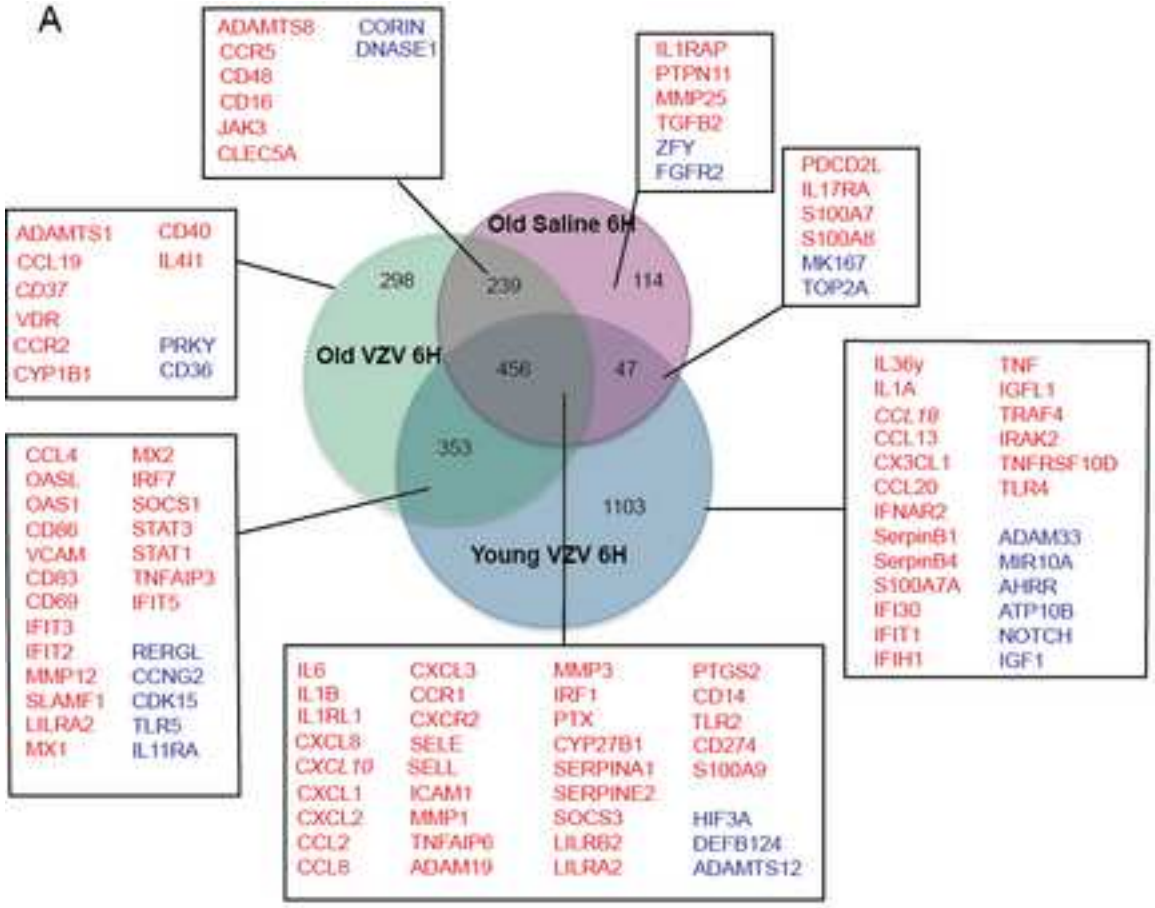
C



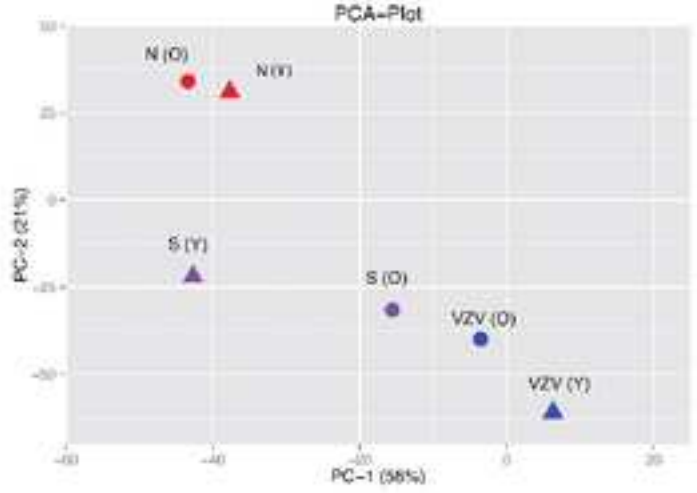
Supplementary Figure No.6

Click here to download high resolution image

A



B

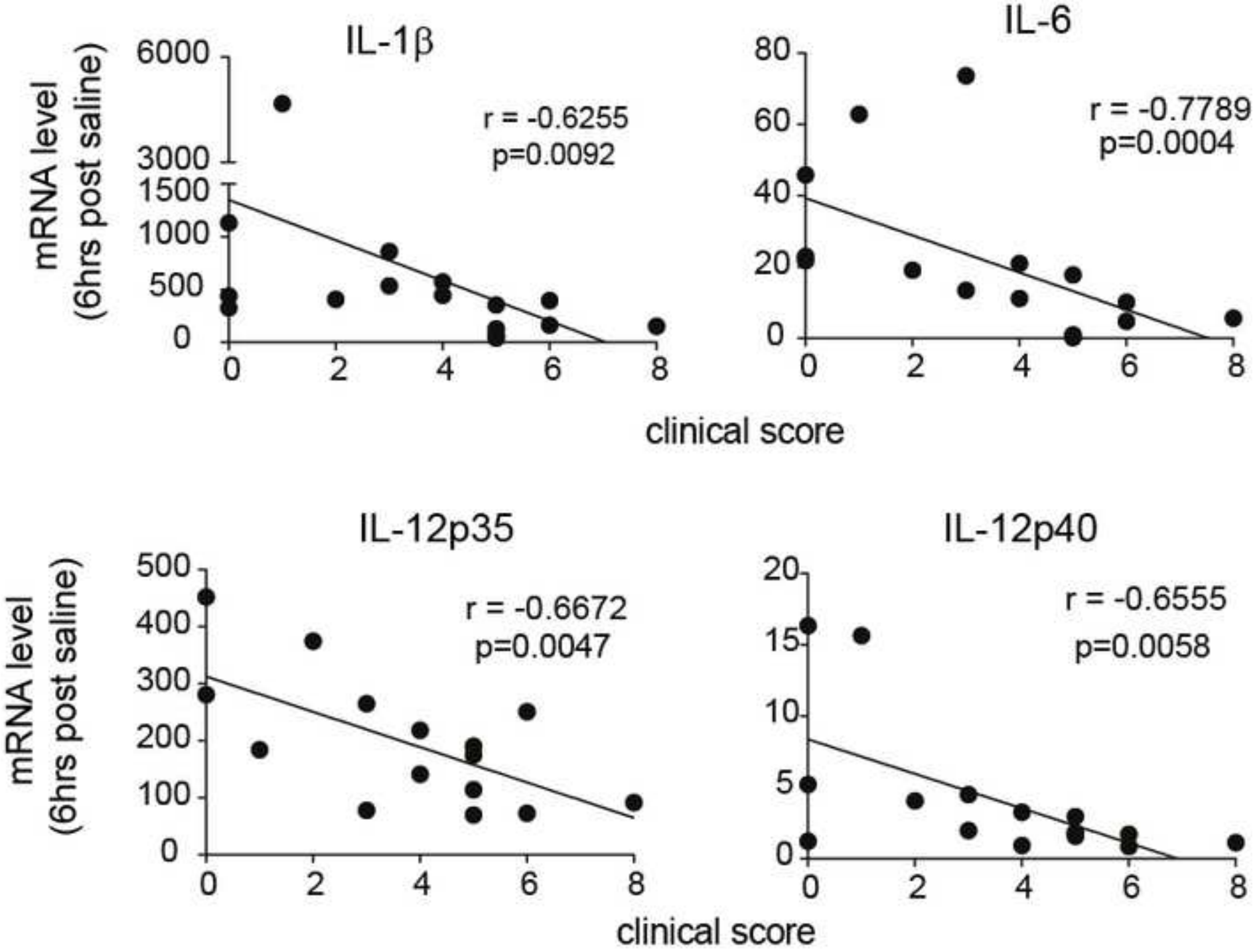


C

SYMBOL	FCH_Old Saline 6hrs vs Normal	FCH_Old VZV 6 hrs vs Normal
FOSL1	51.5	37.58
FPR1	39.86	44.21
PTX3	33.34	50.09
SERPINA1	24.27	28.06
IL6	23.67	46.84
FCGR1A	23.29	49.84
MMP1	21.34	14.24
SELE	20.69	28.23
FCGR1B	18.96	44.73
MMP3	18.63	12.16
CXCL1	18.01	20.41
CXCL8	17.67	10.76
IL1RL1	16.88	13.08
PROK2	15.75	7.82
BCL2A1	14.06	19.3
CXCL2	13.86	14.86
IL1B	12.96	30.45
HAS3	11.94	8.84
S100A9	11.24	8.69
SELL	11.06	9.11
FCGR3B	10.9	7.74
NAMPT	10.74	15.94
CXCL10	9.85	29.78
TNFAIP6	9.51	24.5
PTGS2	9.47	9.52
CH25H	9.44	11.49
CYP27B1	8.81	8.13
CCL2	8.79	11.63
ADAMTS8	8.42	12.22
THBS1	8.23	8.14

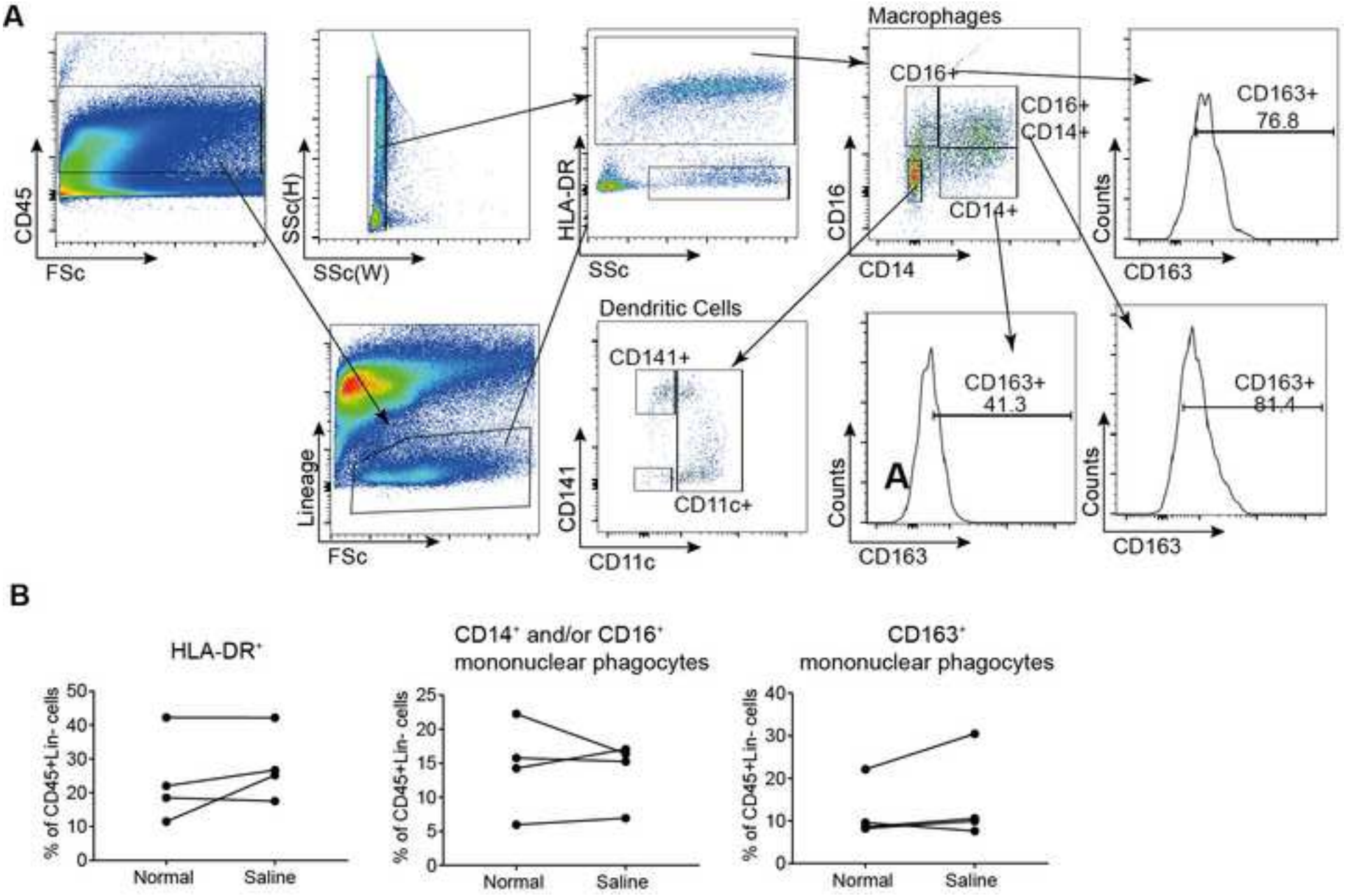
Supplementary Figure No.7

Click here to download high resolution image



Supplementary Figure No.8

Click here to download high resolution image



Supplementary Figure No.9

Click here to download high resolution image

	1st	2nd
donor 1	1	0
donor 2	2	3
donor 3	0	3
donor 4	4	4
donor 5	0	0
donor 6	4	4
donor 7	1	1
donor 8	0	0
donor 9	1	1
donor 10	0	0
donor 11	0	0
donor 12	4	4
donor 13	4	3
donor 14	4	4

Supplementary Figure No.10

Click here to download high resolution image