MATERIALS AND METHODS

Genetically Engineered Mouse Models (GEMMs)

S100A9 "floxed" mice were generated by Sergei Grivennikov lab by micro-injecting EUCOMM targeted C57Bl6 Agouti ES cells (S100a9$^{tm1a(EUCOMM)Wtsi}$, MGI:1338947, KO first allele, C57BL/6N-A/a JM8A3.N1; clone EPD0772_4_G10, obtained from EUCOMM/EuMMCR, IKMC project 85556) into C57BL/6-Albino blastocysts. Mice with a greater degree of potential chimerism were bred to C57Bl6 Albino strain (Jackson Laboratories) and germ line transmission was determined by coat color and confirmed by PCR. After germline transmission, the "conditional ready" S100a9^{tm1c} (floxed) allele, with loxP sites around exon 3, was generated by deletion of the FRT-lacz-neo cassette through crosses with pCAG-Flpe mice (MGI:2448985). Mice were routinely genotyped by PCR using the following primers: P2:s100a9-fw2 GGTGGGGTATGACTGCAAGA, P4:s100a9-rv2 ACAAATAGAAATGGAAACACCTTCT. The presence of wild-type S100a9 allele resulted in approximately 200 bp band and floxed allele - 370 bp.

S100A9 floxed mice were then crossed with DKO* mice with inducible double epidermal deletion of c-Jun and JunB [2] to generate inducible epidermal deletion of S100A9 (TKO*) in DKO* mice. Matings to generate experimental animals with TKO* and DKO* littermates were set up with breeders homozygote for the *c-jun* and *junb* floxed alleles and heterozygote for the *s100a9* floxed allele, with one parent hemizygous for the K5CreERT allele. Deletion of the floxed alleles in experimental cohorts was achieved by consecutive daily intraperitoneal injections of 1 mg tamoxifen dissolved in corn oil (Sigma). Mice were maintained in a mixed (C57BL/6×129S6/Sv) background and housed in Specific Pathogen-Free environment with free access to food and drink. All animal experiments were

conducted according to institutional policies and national and European guidelines.

Skin and joint inflammation scoring and assessment

Skin inflammation was scored as no phenotype, mild, moderate or severe, based on skin lesions/plaques in the ear region and ventral skin of the mouse (representative pictures shown in **online Supplemental Figure 1B**). The plot in Figure 1C was generated using a contingency table of all scored animals with 4 severity states and differences were evaluated using a Chi square test. Paw inflammation was only observed in mice with moderate/severe skin inflammation and was scored as absent or present, and similarly evaluated using a Chi square test. Both skin and joint inflammation was further analysed histologically and in the case of joints, by micro-CT and x-ray imaging. Nail lesions were assessed and quantified by histology in the hind paws, enthesitis was detected in the distal phalanges and defined as inflammation around the distal phalangeal bone. Bone marrow inflammation (osteitis) was detected in all bones associated with inflamed joints (epiphyses and diaphyses) and quantified based on histology.

Immunofluorescence and Immunohistochemistry

For immunofluorescence, paraffin embedded sections were processed for deparaffinization according to standard procedures and antigen retrieval was performed in a pressure cooker using citrate buffer pH6 for skin sections. and incubating with Carezyme I trypsin kit (1:1) from Biocare Medical for 30 minutes at RT for paw sections. Mouse tissue sections were stained overnight with primary antibodies specific for JunB (Cell Signaling, clone 37F9, ref # 3753, dilution 1:100), S100A9 (Santa Cruz, SC8115, dilution 1:100), Ly6B (BioRad, ref

MCA771GA, dilution 1:200). Human sections were stained with primary antibodies specific to S100A8 (Sigma, ref # HPA024372, dilution 1:750) and S100A9 (R&D ref # MAB5578, dilution 1:100). After overnight incubation with primary antibody, sections were washed 3 times with PBS and incubated with secondary antibodies conjugated to their corresponding fluorophores for 30 minutes at room temperature: Alexa Fluor 594 (Sigma, dilution 1:250), Alexa Fluor 488 (Sigma, dilution 1:250), Alexa Fluor 647 (Sigma, dilution 1:250). After secondary incubation, sections were washed 3 times with PBS, mounted and analysed using either fluorescent microscope or confocal.

Immunohistochemistry staining was performed by incubating the sections in 3% hydrogen peroxidase for 5 minutes after performing the antigen retrieval step, blocked in 10% donkey serum for 1 hr, and then incubated with primary antibody specific for pSTAT3 (Cell signaling, ref # 9131, dilution 1:100) overnight. Sections were then washed for 5 minutes in buffer, incubated for 30 minutes with diluted biotinylated secondary antibody, washed again and then incubated for 30 minutes with Vectastain Elite ABC Reagents. Finally, sections were washed, incubated in DAB peroxidase substrate solution and counterstain with hematoxylin.

Toluidine blue and H&E of paw sections were performed using standard protocols (CNIO histopathology unit). Quantification of H&E and immunohistochemistry images were performed using ImageJ software 1.38X. Each slide was measured in 3-5 different fields, and the average was calculated for each sample. Toluidine blue analysis was carried out in digital slide imaging performed by DotSlide scan to quantify cartilage areas in joints with the programme ZEN 3.1. Each slide was measured in 3-10 different fields, and the average was calculated for each

sample. For IF quantification, whole ear skin and paw samples were imaged with a TCS-SP5 confocal microscope (Leica Microsystems) equipped with AOBS and HCX PLAN APO 20×/0.7N.A. dry objective. Image processing, measurements, assembly, and editing were performed using Image J. Absolute numbers of neutrophils were quantified from whole ear sections, whereas quantification of neutrophils in synovial spaces and bone marrow of paws was calculated per selected joint area. Each slide was measured in 3-5 different joint regions, and the average was calculated for each paw.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from frozen tissue and homogenized in 1 mL of TRIZOL reagent (Life Technologies) using a Precellys tissue homogenizer followed by a chloroform extraction. The extracted solution was incubated with an equal volume of isopropanol (Sigma-Aldrich) and 1 µL glycoblue (Life Technologies) at room temperature for ten minutes. The solution was then centrifuged, and the precipitate was washed once with 75% ethanol. The solution was centrifuged again and the supernatant was removed from the sample and resuspended in DEPC treated water. Total RNA yield and purity were analysed using a NanoDrop 2000 Spectrophotometer (Sigma Aldrich) and adjusted to a standard concentration prior to cDNA synthesis. To quantify mRNA expression, 2 µg of total RNA was reverse transcribed into cDNA using a high capacity first strand cDNA synthesis kit (Promega) as per manufacturer's instructions. qRT-PCR was performed using 2 µL cDNA, 5 µL SYBR Green (Promega), 7 µL DEPC treated water, and 0.4 µM of forward and reverse primers. *Rpl4* was used as a housekeeping gene.

Flow cytometry

Ear skin epidermis was separated from the dermis by dispase digestion (0.12mg/mL) for 30 min at 37ºC. After mechanical dissociation, the cell suspension was filtered through a 70 µm cell strainer and centrifuged 5 min at 1500 rpm. Pellet was re-suspended in staining buffer (PBS with 1% BSA and EDTA), counted and labelled for 30-45 min at 4ºC with the following conjugated surface markers: CD45-APC-Cy7 (Biolegend, clone 30-F11, dilution 1:400), CD49f-PE-Cy7 (Biolegend, 1:400), CD11b-FITC (Biolegend, 1:200), Ly6G-PE (Biolegend, 1:400). After labelling, cells were washed with staining buffer, stained with DAPI to exclude dead cells and analyzed and sorted by FACS ARIA (BD, San Jose CA). At least 25,000 alive single events were collected. All data are analyzed using FlowJo 7.6.5 (Treestar, Oregon). Cells were filtered through a 40 µm cell strainer onto 5 mL polypropylene sterile tubes. Sorting procedures were done excluding dead cells and doublets. Neutrophils and epidermal cells were purified into Trizol LS (Thermo Fisher Scientific) for RNA isolation and 500 ng-1 µg mRNA per sample was used for cDNA synthesis and qPCRs.

MicroCT and radiographic imaging

Paws were scanned using a µCT 40 (Scanco Medical AG, Brüttisellen, Switzerland) at 55kV and 145 µA. Integration time was set to 250 ms and an isometric voxel size of 6 µm was used. The articulating joint between intermediate and proximal phalangeal bone of the third digit of the right back-paw was scanned and evaluated. For mid-shaft evaluation BV/TV (bone volume to tissue volume), Ct.Th (cortical thickness) and Mdens (density of the bone) were evaluated. Additionally, BS/√BV was calculated to quantify the bone surface roughness. The

volume of interest (VOI) for mid-shaft analysis was set to the region ranging from 31% (distal) of the length of the bone to 65% (proximal) of the length of the bone. Assessment of joint bone morphology and mineralization were carried out over a stack of 300 images with the center of the VOI located in the joint space. Joint bone volume (JBV) and density (JBVdens) were evaluated. To quantify the remodeling process in the joint region, the volume of high-density bone (HDJBV) was quantified as well as the density of the full mineralized area (DFJBV) according to two thresholds (high density: 345-1000 1/cm, low density 295-1000 1/cm, both linear attenuation coefficient). 3D joint distance was evaluated measuring the distance of the two joint surfaces with an orthogonal line from the proximal surface towards the distal surface. Additionally, subchondral bone thickness was evaluated in a 2D projection of the 3D joint dataset in a lateral view. Thickness was measured orthogonal to the distal subchondral bone surface in the 2D projection at the mid of the distal joint.

Mass Spectrometry

Proteins from the ears of wildtype control (n=3), DKO*(n=5), TKO* (n=4) were extracted using the RIPA buffer protocol (Millipore, Billerica, MA). Twenty micrograms of total protein from each sample were digested with Trypsin/Lys C mix (Promega, Madison, WI) following the manufacturer's instruction. Resulting peptide mixtures were chromatographically separated on a reverse-phase C18 column (10cm x 75µm, 3 µm, 120 Å) and analyzed on a Velos Pro Dual-Pressure Linear Ion Trap mass spectrometer (Thermo Fisher Scientific) as described previously[3].

Peptide spectral matching and protein identification were achieved by database search using Sequest HT algorithms in a Proteome Discoverer 1.4 (Thermo

Fisher Scientific). Raw spectrum data were searched against the UniProtKB/Swiss-Prot protein database for mouse (May 25, 2019). Main search parameters included: trypsin, maximum missed cleavage site of two, precursor mass tolerance of 1.5 Da, fragment mass tolerance of 0.8 Da, and variable modification of oxidation/hydroxylation of methionine, proline, and lysine (+15.995Da). A decoy database search was performed to calculate a false discovery rate (FDR). Proteins containing one or more peptides with FDR≤0.05 were considered positively identified and reported. For all proteins, the total number of peptide spectral matches (PSMs) reported by the Protein Discoverer 1.4 was used for quantification. Statistical significance of differences between the mean values for DKO* and TKO* compared to WT was determined by t-test, with statistical significance between two independent groups considered for $p<0.05$. Volcano plots were generated using ggplot2 R package (version 3.3.5). Upregulated and downregulated peptides were displayed in red and blue respectively, while peptides with p-value > 0.05 were considered non-significant and displayed in gray. Venn diagram was generated using Venn Diagram R package (version 1.7.1) using only peptides with a p-value < 0.05 and a positive log2 fold change for each of the comparisons. Up-regulated peptides shared or exclusive to one or more comparison were next analyzed for Gene Ontology (GO) Biological Processes (Enrichr, http://amp.pharm.mssm.edu/Enrichr/) and the top 10 most significant biological pathways presented in the Alluvial plot. Only peptides with an indexed gene reference were used for GO analysis and represented in the subsequent graphs and very few genes were left out, mostly immunoglobulin chains isoforms (UniProtKB accession numbers: P01864, P01756, P01631, P01635, P01638, P01796, P01644, P01630, P01843, P04945,

P06330, Q91V76, P01636, P01643, P01674, P01878, P01668, P01654, P01660, P01675, P01786, P01896, P03987-2, Q3U4G0-2, Q3UJC8-2, Q8BWQ6-3, Q9CYI0, Q9D5Q8-3, Q9D5Y0, P10404, P18527). Heatmap was generated using pheatmap R package (version 1.0.12). Alluvial plot was generated using ggalluvial R package (version 0.12.3). Peptides represented in the heatmap and alluvial plot were enriched in the referenced processes, obtained in the gene ontology analysis performed in EnrichR as previously described. The scale of the heatmap is log2 fold change of each comparison, only significant peptides are represented (p-value < 0.05). when a peptide was not significant genes in one condition it was assigned a log2 fold change of 0, and depicted in white.

The connectivity network was based on publicly available sources of protein-protein interaction information to visualize physical and functional interactions. Nodes are connected by 735 edges, a significant increase in interactions above the 237 edges that one would expect from a random set of proteins of same size and degree distribution, indicating that the proteins are biologically connected. Nodes associated with Gene Ontology (GO) terms are highlighted according to neutrophil granulation (76 nodes; red), innate immunity (22 nodes; purple), and TNF-alpha response (16 nodes; green). Line weight of edges or connections indicates the combined score of the probabilities from evidence channels, corrected for the probability of randomly observed interaction, heavy lines indicating stronger evidence of interaction [4].

ELISA

The following ELISA kits from R&D Systems were used following manufacturer's protocols: mouse (ref# DY2065) and human (ref# DY5578) S100A9 DuoSet, mouse (ref# DY8596-05) and human (ref# DY-8226-05) S100A8/S100A9 Heterodimer DuoSet (ref# DY8596-05), mouse (ref# DY3059) and human (ref# DY4570-05) S100A8 DuoSet, mouse (ref# M6000B) and human (ref# D6050) IL-6 Quantikine, mouse and human (ref# DTA00D) TNF-alpha Quantikine (ref# MTA00B), mouse (ref# M1700) and human (ref# D1700) IL-17A Quantikine ELISA.

Human samples and analyses

Serum samples of 24 active psoriasis (Ps) patients, 24 active psoriatic arthritis (PsA) patients (according to the Classification Criteria for Psoriatic Arthritis (CASPAR) [1]) and 24 healthy controls (HC; mean ± SD age: 40.5 ± 7.7 years; 13 males/11 females) were analysed in the study. Ps patients (14 males/10 females) had a mean±SD age of 42.9±92.1 years and a mean± SD skin disease activity of 9.5 ± 2.5 units according to the psoriasis area and severity index (PASI). 13 Ps patients received topical treatment only, 8 patients received methotrexate and 3 patients received fumaric acid. PsA patients (12 males/12 females) had a mean ± SD age of 45.2±8.3 years, a mean ± SD joint disease activity of 25.6 ± 6.0 units according to Disease Activity in Psoriatic Arthritis (DAPSA) score and a mean±SD skin disease activity of 6.4 ± 3.2 units according to PASI. 8 PsA patients received only topical treatment plus non-steroidal anti-inflammatory drugs, 14 patients received methotrexate and 2 received leflunomide. In all patient serum analyses for S100 proteins and cytokines were

carried out. In 8 from the 24 PsA patients, additional synovial fluid (derived from knee joint puncture) analyses for S100 proteins and cytokines were carried out.

Human skin sections (n=5, 2 males/3 females with mean ± SD: 5 healthy, 40.6 ± 4.04 years; 5 psoriatic 41.8 ± 6.30 years; PASI score 8.2 ± 2.4 units, DAPSA score 25.7 ± 1.6 units) were briefly washed in deionized water and blocked for 1 hour in PBS supplemented with 5% BSA and 2% horse serum. S100A8 and S100A9 polyclonal rabbit primary antibodies (provided by J Roth/T Vogel, Münster) were incubated overnight at 4 °C. Sections were washed in PBS and incubated with the 1 µg/ml polyclonal donkey anti-rabbit Alexa Fluor 555 secondary antibody (abcam, ab150062) and 0.1 *µ*g/ml DAPI for 2 hours at ambient temperature. For imaging, the slides were mounted in aqueous fluorescence mounting medium (Dako) and sealed with commercial nail polish. Images were acquired on a Nikon Eclipse N*i*-U microscope and processed using the ImageJ distribution Fiji. From each patient sample 2 images were taken, and for each image fluorescence intensity was measured in 3 regions of interest (average 120x120 µm) within the epidermis.

Statistical analyses

Statistical analyses were performed using Prism (version 9.2.0, GraphPad Software). Bar graphs are shown in the form of mean ± SEM. Comparisons between 2 groups were evaluated using unpaired (Figures 5N and 6A) and paired (6B and S6A) t-test. Differences between 3 or more groups were determined using an ordinary one-way ANOVA with Tukey post-hoc test when Gaussian Distribution could be assumed (Figures 1B,D,E,F,G,H,I,J; 2C,D,F; 3B,C,E,F; 6C; S1C,D,E; S2A,D,E,F,G,H,I; S4C,D; S5C,D; S6C). In cases where Gaussian Distribution could not be assumed due to small sample sizes or expected unequal

variances between groups (i.e. gene knockout), Kruskal Wallace nonparametric tests were performed (Fig 5A-L). A Chi square test was performed on the contingency tables generated to compare categorical data between two groups (Fig 1C and 2A). Statistical significance in all cases was considered as *p>0.05, **p>0.01 and ***p>0.001.

SUPPLEMENTARY REFERENCES

1. Taylor, W., et al., *Classification criteria for psoriatic arthritis: development of new criteria from a large international study.* Arthritis Rheum, 2006. **54**(8): p. 2665-73.
2. Zenz, R., et al., *Psoriasis-like skin disease and arthritis caused by inducible epidermal deletion of Jun proteins.* Nature, 2005. **437**(7057): p. 369-75.
3. Pu, X. and J.T. Oxford, *Proteomic Analysis of Engineered Cartilage.* Methods Mol Biol, 2015. **1340**: p. 263-78.
4. Szklarczyk, D., et al., *STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets.* Nucleic Acids Res, 2019. **47**(D1): p. D607-D613.

SUPPLEMENTARY FIGURES LEGENDS

Supplementary Figure 1: A. GEMMs generated for this study and experimental time course. **B.** Disease severity scored as no phenotype, mild, moderate or severe based on ear and ventral skin inflammation **C.** Weight loss associated with disease progression in in mice with inducible dual epidermal deletion of c-Jun and JunB (DKO*) (n=75). Serum S100A9 **(D)** (n=85) and interleukin (IL)-17 **(F)** (n=29) according to disease severity in DKO* mice.

Supplementary Figure 2: A. Representative pictures of Psoriatic arthritis (PsA) phenotype observed in wildtype (WT) mice and in mice with inducible dual epidermal deletion of c-Jun and JunB (DKO*) and triple epidermal deletion of c-Jun, JunB and S100A9 (TKO*) with quantification of digit width/length (control n=4; DKO* n=7; TKO* n=7). **B.** Micro-CT image of the right hind limb showing the

two different areas of the third distal phalanx that were analysed (red). **C.** Micro-CT scans of the hind paws showing finger deformation (red arrows) and skin inflammation (red arrows) in DKO* and TKO* mice, as well as micro-CT images showing a cross-sectional view through the joint, 3D- reconstruction of the diaphysis and the third distal interphalangeal joint in WT, DKO* and TKO* mice. **D-I.** Bone parameters measured by micro-CT in the third hind paw digit of WT, DKO* and TKO* mice: **D.** Subchondral bone volume . **E.** Total periarticular bone density stated in linear attenuation (1/cm). **F.** Periarticular -bone volume of highly mineralized bone tissue measured. **G.** Total periarticular bone utilizing a lower threshold of 295 1/cm. **H.** Cortical bone volume at the mid-shaft of the distal phalanx **I.** Density of highly mineralized bone volumes in the joint-bone area.

Supplementary Figure 3: A. Mosaic composite of confocal images from whole ear slides with (original) and without (edited) Munro-like microabscessess of wild-type (WT) mice and mice with inducible dual epidermal deletion of c-Jun and JunB (DKO*) and triple epidermal deletion of c-Jun, JunB and S100A9 (TKO*) (green=Ly6b; red=S100A9; purple=DAPI). (Scale bar=250μm; * Munro-like microabscessess in *Stratum corneum*; ** autofluorescence in cartilage). **B.** Confocal scans of paws stained with Ly6b (green) and S100A9 (red). Scale: bar=1000μm, bone marrow close up: 100μm . **C-D.** Confocal microscopy-based quantification of the percentage of neutrophils (Ly6B-positive) **(C)** and the percentage of S100A9-positive neutrophils **(D)** per total bone marrow area in WT, DKO* and TKO* mice (n = 6-8 mice).

Supplementary Figure 4: A-B. Volcano plots showing significantly upregulated (red) and downregulated (blue) proteins in mice with inducible dual epidermal deletion of c-Jun and JunB (DKO*) compared to wild-type (WT) mice (A) and in

mice with triple epidermal deletion of c-Jun, JunB and S100A9 (TKO*) compared to WT mice in proteomic analyses. **C.** Connectivity network diagram of proteins related to neutrophil degradation (red nodes), innate immunity (purple) and TNFα mediated signalling (green). Line weight of connections indicates the combined score of the probabilities from evidence channels, corrected for the probability of randomly observed interaction, heavy lines indicating stronger evidence of interaction.

Supplementary Figure 5: A-B. FACS cell sorting strategy to isolate keratinocytes and neutrophils for gene expression analyses. **C.** Percent of CD45+ population. **D.** Percent of neutrophils in CD45+ population (n=4-7).

Supplementary Figure 6: A. Serum and synovial fluid concentration of vascular endothelial growth factor (VEGF) and lipocalin-2 (LCN2) in patients with psoriatic arthritis (PsA) (n=8, ns= non-significant). **B**. Correlation between psoriatic skin disease activity (psoriasis area and severity index, PASI) and serum calprotectin (S100A8/A9, upper row) or serum S100A9 (lower row) in psoriasis (Ps, left panels) and PsA patients (middle panels). Correlation between joint disease activity (Disease Activity in Psoriatic Arthritis score, DAPSA) and serum calprotectin (S100A8/A9, upper row) or serum S100A9 (lower row) in PsA patients (right panels). Serum values are given in ng/mL (r= Spearmans correlation coefficient). **C.** Serum levels of VEGF and LCN2 in healthy controls (HC) and patients with either Ps or PsA (each group n=24).