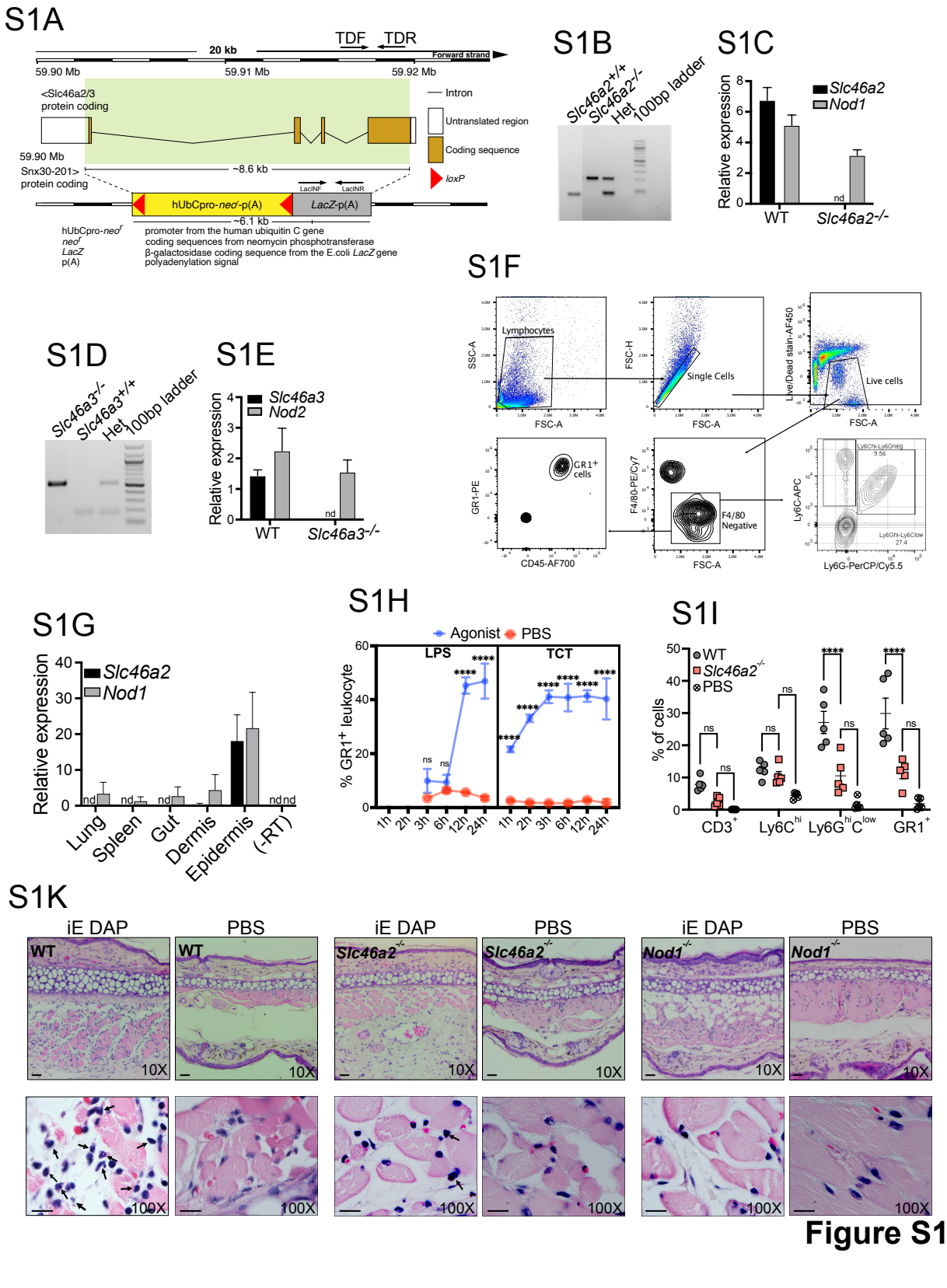
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TDF TDR
20 kb
Forward strand
59.90 Mb
59.91 Mb
59.92 Mb
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protein coding
Intron
Untranslated region
Coding sequence
loxP
59.90 Mb
Snx30-201>
protein coding
~8.6 kb
LacINF
LacINR
hUbCpro-neo'-p(A)
LacZ-p(A)
~6.1 kb
hUbCpro-neo^r
neo^r
LacZ
p(A)
promoter from the human ubiquitin C gene
coding sequences from neomycin phosphotransferase
β-galactosidase coding sequence from the E.coli LacZ gene
polyadenylation signal
S1B
Slc46a2+/+
Slc46a2-/-
Het
100bp ladder
S1C
Relative expression
Slc46a2
Nod1
nd
WT
Slc46a2-/-
S1D
Slc46a3-/-
Slc46a3+/+
Het
100bp ladder
S1E
Relative expression
Slc46a3
Nod2
nd
WT
Slc46a3-/-
S1F
Lymphocytes
SSC-A
FSC-A
FSC-H
Single Cells
Live/Dead stain-AF450
Live cells
GR1+ cells
GR1-PE
CD45-AF700
F4/80-PE/Cy7
F4/80 Negative
Ly6C-APC
Ly6G-PerCP/Cy5.5
S1G
Relative expression
Slc46a2
Nod1
nd
Lung
Spleen
Gut
Dermis
Epidermis
(-RT)
S1H
Agonist
PBS
LPS
TCT
% GR1+ leukocyte
ns
****
1h
2h
3h
6h
12h
24h
S1I
WT
Slc46a2-/-
PBS
% of cells
ns
****
CD3+
Ly6C^hi
Ly6G^hi C^low
GR1+
S1K
iE DAP
PBS
WT
Slc46a2-/-
Nod1-/-
10X
100X

Figure S1

Figure S1, related to Figure 1: *Slc46a2* expression in the epidermis is lost in knockout animals, while DAP-muropeptide triggers rapid neutrophil recruitment to the skin

(**A**) Design of *Slc46a2* and *Slc46a3* null alleles. The gene region was replaced with ZEN-UB1 targeting cassette by homologous recombination. Primers used for genotyping are indicated. Adapted from velocigene (www.velocigene.com) and Ensembl genome browser.

(**B**) Agarose gel of PCR genotyping for validation of *Slc46a2* knockout mice.

(**C**) Quantitative RT-PCR from WT and *Slc46a2*$^{-/-}$ mouse epidermis for *Slc46a2* and *Nod1* expression. *Slc46a2*$^{-/-}$ epidermis showed no *Scl46a2* expression.

(**D**) Agarose gel of PCR genotyping for validation of *Slc46a3* knockout mice.

(**E**) Quantitative RT-PCR from WT and *Slc46a3*$^{-/-}$ mouse epidermis for *Slc46a3* and *Nod2* expression. *Slc46a3*$^{-/-}$ epidermis showed no *Scl46a3* expression.

(**F**) Gating strategy for scoring GR1^{+} and Ly6C&G leukocytes by flow cytometry.

(**G**) Expression analysis of *Slc46a2* and *Nod1* in indicated mouse organs. Maximum expression of both *Slc46a2* and *Nod1* was observed in the epidermis. nd; not detected.

(**H**) Neutrophil recruitment to pinnae were measured at indicated time points after intradermal injection of10 µl of 10 µg/ml LPS or 8 µM TCT in one ear compared to a similar volume of PBS injection, as a control, in the contralateral ear. TCT triggered robust and rapid neutrophil recruitment in the skin while the response to LPS is slower.

(**I**) Recruitment of immune cells stained with CD3, Ly6C and Ly6G, or Gr-1 3hrs after 30µM iE-DAP or PBS challenge in pinnae. Both Ly6G^{hi} & LyC^{low} and Gr-1^{+} staining showed a similar pattern of neutrophil recruitment in WT and *Slc46a2* mutant mice, with the mutant showing a dramatic reduction in neutrophil recruitment with either assay. Neither Ly6C^{hi} nor CD3^{+} cells were significantly recruited compared to WT pinnae at this time point.

(**K**) Representative H&E stained histological sections from the mouse ear 3 hr after intradermal injection of 10µl of 30µM iE-DAP or equal volume of PBS in WT, *Slc46a2*$^{-/-}$, and *Nod1*$^{-/-}$ mice. Images in inset show an area at higher magnification. iE-DAP recruited inflammatory cells in WT but not in *Slc46a2*$^{-/-}$, and *Nod1*$^{-/-}$ mice skin, whereas PBS injection did not induce inflammatory reaction; multi-lobed neutrophils indicated with arrows.

In panels C, E, and G each condition shown as a mean with SEM from three independent experiments. For panel H, each data point is mean with SEM from one trial with 5 mice at each time point and uses two-way ANOVA and Tukey's multiple comparisons tests. Panel I, each dot represents an individual animal, data pooled from two to four separate trials and analyzed by two-way ANOVA and Tukey's multiple comparisons tests. Panels B, D, and K are representative images of at least three independent experiments. **** $P < 0.0001$; ** $P < 0.01$; ns, not significant. The scale bar is 100µM.

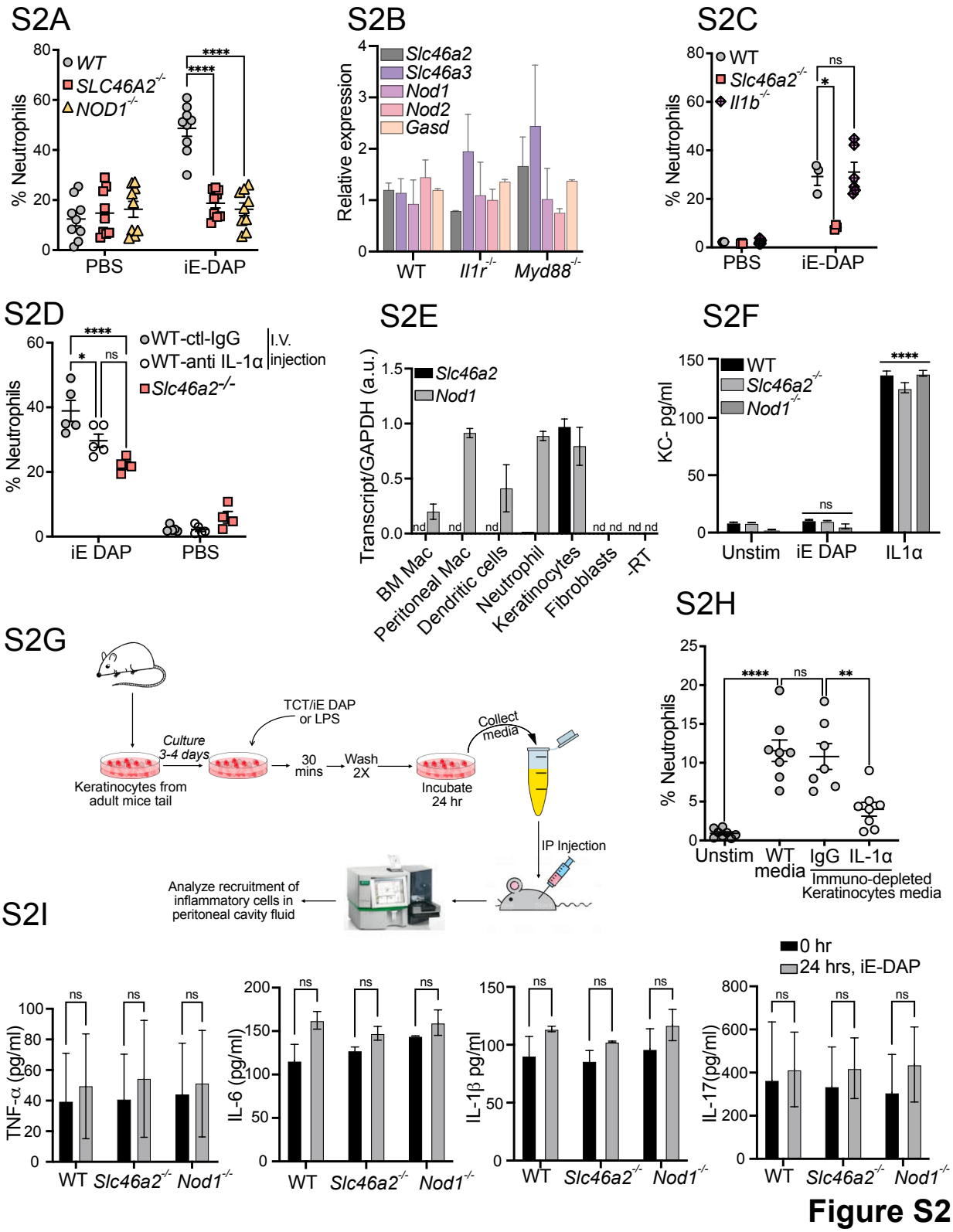

S2A
% Neutrophils
WT
SLC46A2-/-
NOD1-/-
PBS
iE-DAP
S2B
Relative expression
Slc46a2
Slc46a3
Nod1
Nod2
Gasd
WT
Il1r-/-
Myd88-/-
S2C
% Neutrophils
WT
Slc46a2-/-
Il1b-/-
PBS
iE-DAP
S2D
% Neutrophils
WT-ctl-IgG
WT-anti IL-1α
Slc46a2-/-
I.V. injection
iE DAP
PBS
S2E
Transcript/GAPDH (a.u.)
Slc46a2
Nod1
BM Mac
Peritoneal Mac
Dendritic cells
Neutrophil
Keratinocytes
Fibroblasts
-RT
S2F
KC- pg/ml
WT
Slc46a2-/-
Nod1-/-
Unstim
iE DAP
IL1α
S2G
Keratinocytes from adult mice tail
Culture 3-4 days
TCT/iE DAP or LPS
30 mins
Wash 2X
Incubate 24 hr
Collect media
IP Injection
Analyze recruitment of inflammatory cells in peritoneal cavity fluid
S2H
% Neutrophils
Unstim
WT media
IgG
IL-1α
Immuno-depleted Keratinocytes media
S2I
0 hr
24 hrs, iE-DAP
TNF-α (pg/ml)
IL-6 (pg/ml)
IL-1β pg/ml
IL-17(pg/ml)
WT
Slc46a2-/-
Nod1-/-

Figure S2

Figure S2, related to Figure 1: *Slc46a2*-dependent response to topical DAP-muropeptides recruits neutrophils and induces IL-1a

(**A**) Neutrophil recruitment was measured in WT, *Slc46a2*$^{-/-}$ or *Nod1*$^{-/-}$ mice pinnae 3 h after topical application of 10 µl of 30µM iE-DAP to tape stripped mouse skin. WT skin responded to the iE-DAP challenge unlike *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ skin.

(**B**) RT-qPCR analysis of *Slc46a2, Slc46a3, Nod1*, and *Nod2* expression in keratinocytes isolated from WT, *Il1r*, and *Myd88* mutant mice. None of these genes were significantly changed in any of these genotypes.

(**C**) Neutrophil recruitment was measured in WT, *Il1b*$^{-/-}$ and *Slc46a2*$^{-/-}$ mice pinnae in response to intradermal challenge with 10 µl of 30µM iE-DAP. No significant difference was observed in cell recruitment in *IL1b*$^{-/-}$ mice compared to WT.

(**D**) Mice were intravenously injected with IL-1a blocking antibody (1 µg/mouse), or isotype-matched IgG control, 1 hour prior to intradermal injection of 10 µl of 30 µM iE-DAP, and 3 h later neutrophil recruitment was measured. Neutralization of IL-1a significantly reduced the recruitment of leukocytes in WT mice.

(**E**) Expression analysis of *Slc46a2* and *Nod1* in specific cell types isolated from mice, by qRT-PCR. The highest expression of *Slc46a2* was observed in keratinocytes. n.d; not detected.

(**F**) Primary dermal fibroblasts from WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ mice were challenged with iE-DAP (30 µM) or IL-1a (10 ng/ml) for 24hrs and CXCL1 (KC) was measured by ELISA from culture media. KC was induced in fibroblasts treated with IL-1α, regardless of their *Slc46a2* or *Nod1* genotype, but fibroblasts were unresponsive to iE-DAP.

(G) Schematic representation of experimental design for preparation of and bioassay analysis of conditioned media from primary mouse keratinocyte cultures.

(**H**) Anti-IL-1a antibody was used to deplete this cytokine from keratinocyte conditioned media prior to IP injection. IL-1a depleted media showed reduced neutrophil recruitment compared to control IgG antibody-treated media.

(**I**) TNFα, IL-6, IL-1β and IL-17 cytokines measured by ELISA from WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ keratinocyte media before and after challenge with 30 µM iE-DAP for 24hrs. No significant induction in any of these cytokines was detected.

For panels A, C, D, and H each dot represents an individual animal, data pooled from two to four separate trials, and analyzed by two-way ANOVA and Tukey's multiple comparisons tests. In panels B, E, F, and I, data is shown as a mean of three independent experiments with SEM. B, F and I use two-way ANOVA and Tukey's multiple comparisons test to determine significance. **** $P < 0.0001$; ** $P < 0.01$; ns, not significant.

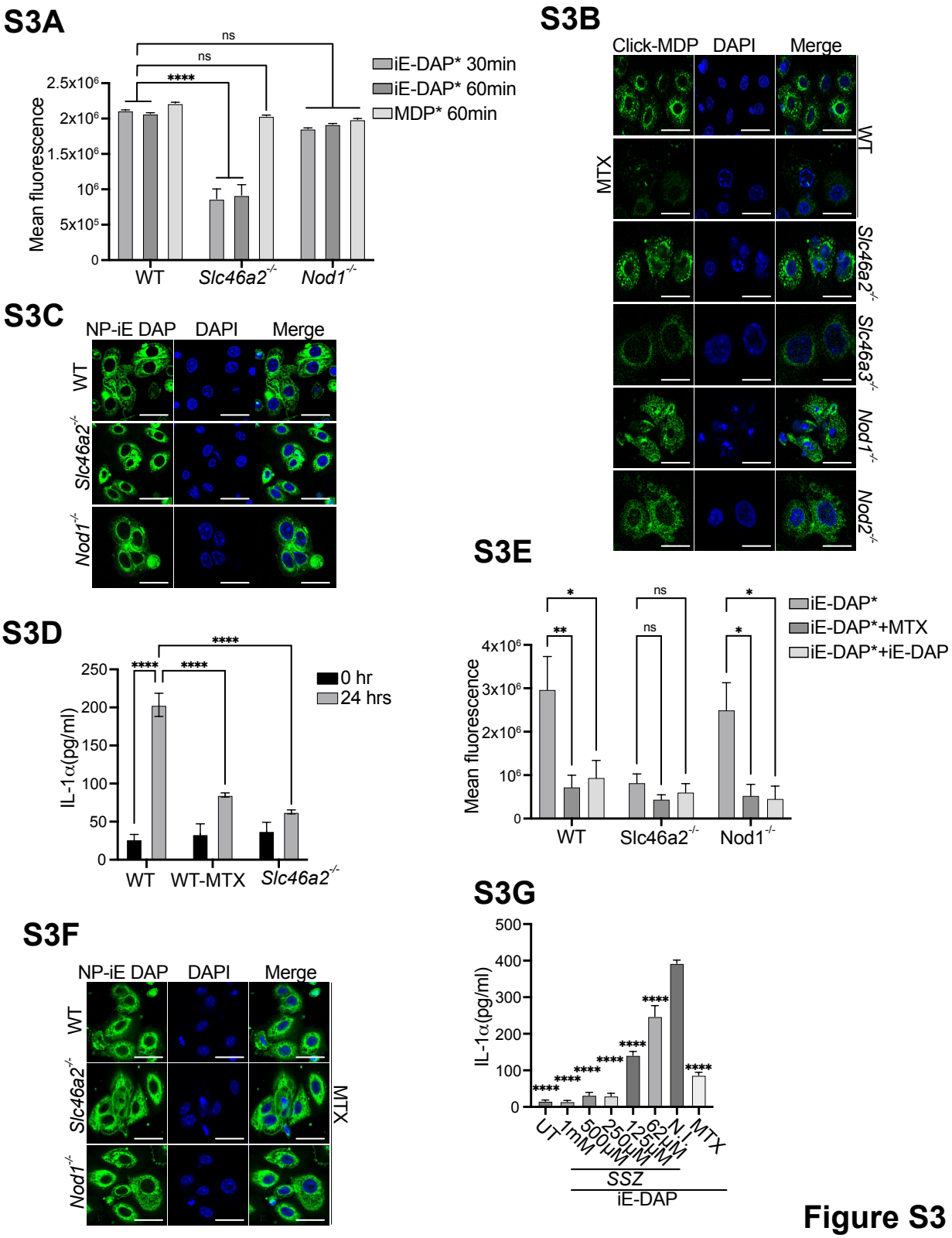

S3A
ns
ns
****
iE-DAP* 30min
iE-DAP* 60min
MDP* 60min
Mean fluorescence
2.5x10⁶
2x10⁶
1.5x10⁶
10⁶
5x10⁵
0
WT
Slc46a2⁻/⁻
Nod1⁻/⁻
S3B
Click-MDP
DAPI
Merge
MTX
WT
Slc46a2⁻/⁻
Slc46a3⁻/⁻
Nod1⁻/⁻
Nod2⁻/⁻
S3C
NP-iE DAP
DAPI
Merge
WT
Slc46a2⁻/⁻
Nod1⁻/⁻
S3D
****
****
****
0 hr
24 hrs
IL-1α(pg/ml)
250
200
150
100
50
0
WT
WT-MTX
Slc46a2⁻/⁻
S3E
*
ns
*
**
ns
*
iE-DAP*
iE-DAP*+MTX
iE-DAP*+iE-DAP
Mean fluorescence
4x10⁶
3x10⁶
2x10⁶
10⁶
0
WT
Slc46a2⁻/⁻
Nod1⁻/⁻
S3F
NP-iE DAP
DAPI
Merge
WT
Slc46a2⁻/⁻
Nod1⁻/⁻
MTX
S3G
IL-1α(pg/ml)
500
400
300
200
100
0
****
UT
1mM
500μM
250μM
125μM
62μM
N.I.
MTX
SSZ
iE-DAP

Figure S3

Figure S3, related to Figure 3: MTX blocks the transport of DAP muropeptides through SLC46A2 and MDP through SLC46A3

(**A**) WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ keratinocytes were challenged with 30 μM click-iE DAP or 10μM click-MDP for 30 minutes and 60 minutes, as labeled, washed extensively, and then lysed. Muropeptides were then detected in these lysates with click-reacted CalFluor 488 Azide and fluorescence quantified. With click-iE-DAP, *Slc46a2*$^{-/-}$ keratinocytes showed significantly reduced fluorescence intensity compared to WT or *Nod1*$^{-/-}$ keratinocytes, while no change in fluorescence intensity was observed with click-MDP in all genotypes.

(**B**) Fluorescent confocal microscopy of WT, *Slc46a2*$^{-/-}$, *Slc46a3*$^{-/-}$ and *Nod1*$^{-/-}$ keratinocytes, challenged with 10μM "click-MDP" for 1h. WT, *Slc46a2*$^{-/-}$, and *Nod1*$^{-/-}$ keratinocytes show a similar import of click-MDP whereas *Slc46a3*$^{-/-}$ keratinocytes displayed reduced click-MDP import. Also, MTX-treated WT keratinocytes showed diminished transport of click-MDP.

(**C**) Fluorescent confocal microscopy of WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ primary keratinocytes treated with NPs loaded with iE-DAP and immunofluorescent dye. Localization of dye inside the keratinocytes shows the successful delivery NP delivery of cargo. No change in fluorescence intensity was observed in all genotypes.

(**D**) IL-1α in culture media of WT and *Slc46a2*$^{-/-}$ keratinocytes after stimulating with 30 μM iE DAP for 24 hours, measured by ELISA. WT keratinocytes were also treated with 250 μM MTX along with iE-DAP. Like *Slc46a2* deficiency, MTX prevented IL-1α release in iE-DAP-treated WT keratinocytes.

(**E**) Similar to (A), MTX (250μM) or unlabeled iE-DAP (30μM) interfered with the cellular uptake of click-iE-DAP in WT or *Nod1*$^{-/-}$, keratinocytes, while in *Slc46a2*$^{-/-}$ cells import was low and unchanged.

(**F**) Immunofluorescent images of WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ keratinocytes pretreated with 250 μM MTX and then iE-DAP delivered by dye-loaded NP. Addition of MTX did not affect the NP-mediated dye delivery in any genotype.

(**G**) IL-1α ELISA of supernatant from WT keratinocytes treated with increasing concentrations of SSZ. SSZ potently inhibits the IL-1α release from keratinocytes. UT is untreated cells and N.I. is no inhibitor.

In panels A, D, E, and G data is shown as a mean and SEM for at least three independent experiments and analyzed by two-way ANOVA and Tukey's multiple comparisons. Panels B, C, and F are representative images of at least three independent experimental results. **** $P < 0.0001$; ** $P < 0.01$; ns, not significant. The scale bar is 10μM.

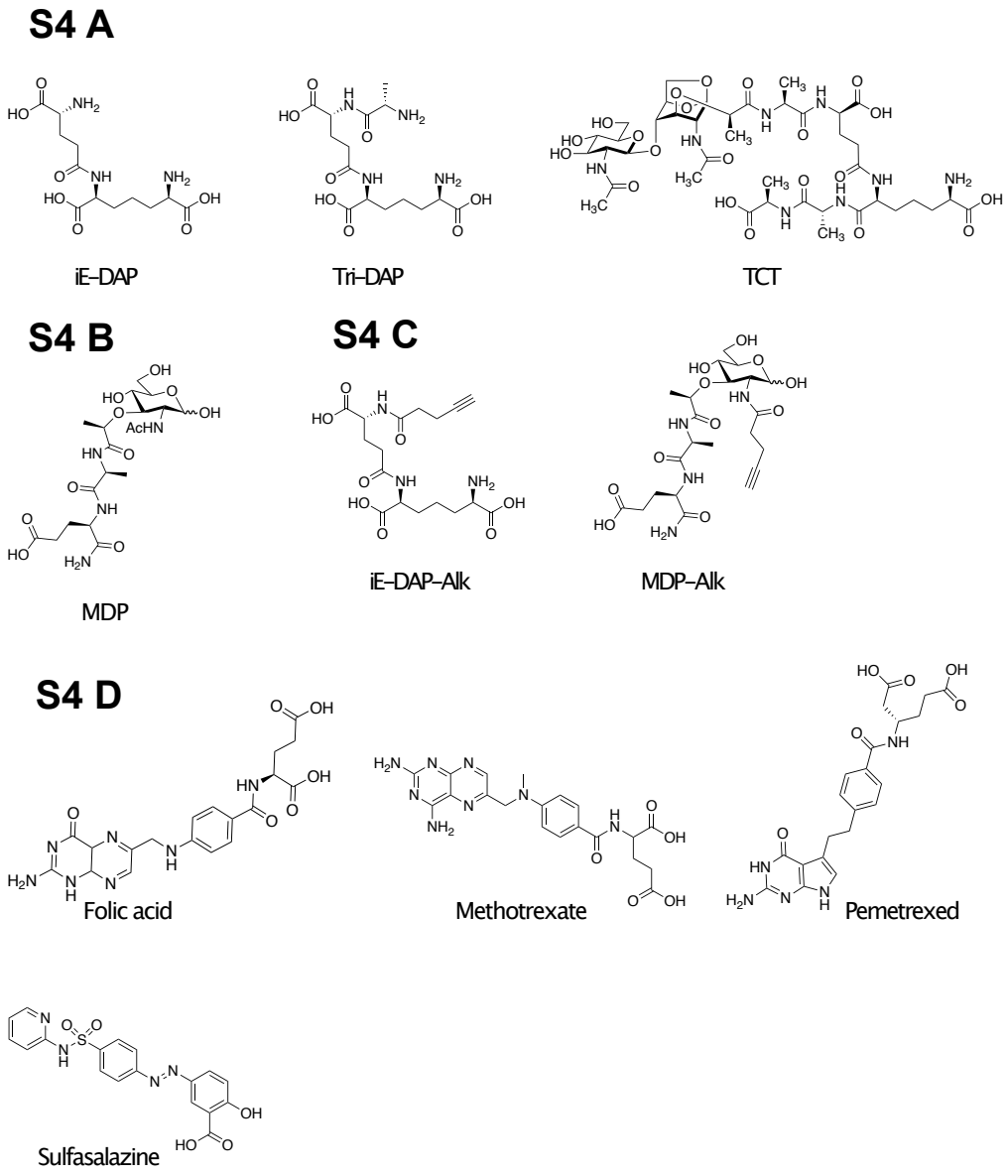


Figure S4

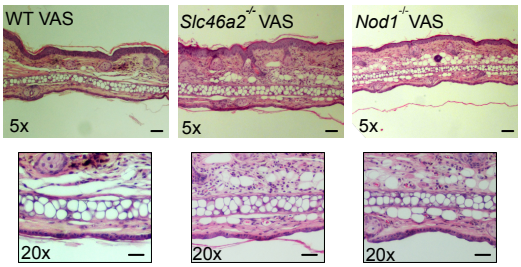
Figure S4, related to Figure 4. Structure of different chemical compounds used in this study.

(**A**) and (**B**) Derivatives of DAP type PGN and MDP respectively.

(**C**) Alkyne-modified iE-DAP and MDP used in click chemistry assays.

(**D**) Chemical structure of different inhibitors used in this study.

S5A



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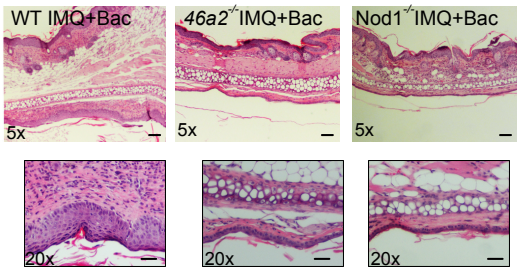

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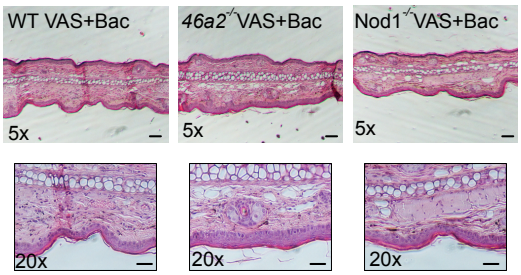

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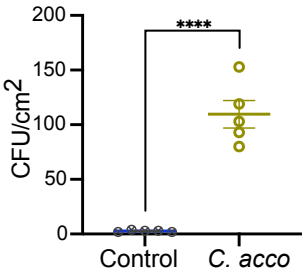

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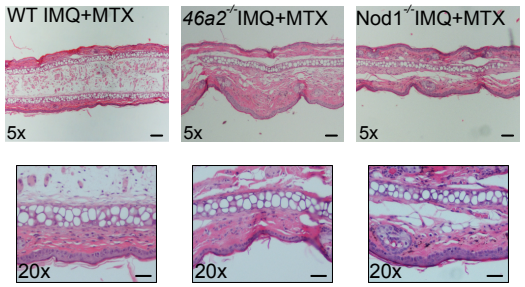

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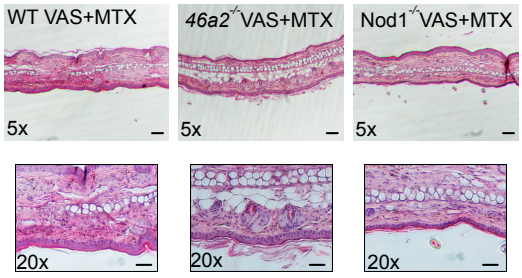
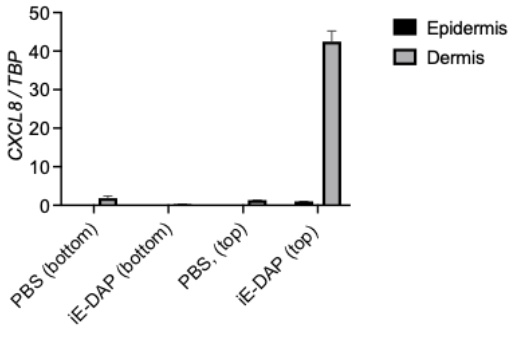


S5G



S5H

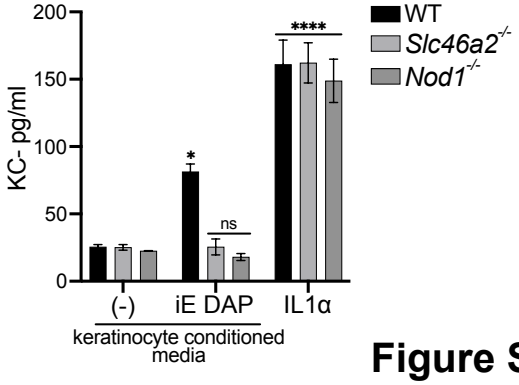


Figure S5

Figure S5, related to Figure 5: MTX blocks the psoriatic inflammation in skin

(**A**) Representative H&E stained histology of ear sections from VAS (Vaseline, as a vehicle) applied to skin from WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ mice. VAS applied to skin did not show signs of inflammation in any genotype.

(**B**) H&E stained histology sections from IMQ (3 days) and *C. accolens* (3 days, Bac) applied skin. WT skin shows hyper inflammation compared to *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ mice skin.

(**C**) Representative H&E stained histology of ear sections from VAS (Vaseline, as a vehicle, 3 days) plus *C. accolens* (3 days, Bac) applied to skin from WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ mice. VAS plus *C. accolens* applied skin did not show signs of inflammation in any genotype.

(**D**) Enumeration of CFUs from the ear pinnae of mice 24 h after topical application of *C. accolens* (10^7 CFU). Control ear pinnae left unassociated. Each dot represents an individual mouse.

(**E**) H&E stained histology sections from IMQ and methotrexate (MTX) treated skin from WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ mice. After the application of MTX, WT skin was less inflamed compared to IMQ-treatment in Fig. 5B, whereas MTX had minimal effect on *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ mice skin,.

(**F**) Representative H&E stained histology of ear sections from VAS (Vaseline, as a vehicle) and methotrexate (MTX) applied to skin from WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ mice. VAS-applied skin did not show signs of inflammation in any genotype.

(**G**) CXCL8 expression was analyzed in skin organoids after iE-DAP challenge to the top (epidermal) or bottom (dermal) layer. CXCL8 response was only observed in dermal fibroblast when an iE-DAP challenge was delivered to the top epidermal layer.

(**H**) WT mouse primary dermal fibroblasts were cultured for 24 h in conditioned media from WT, *Slc46a2*$^{-/-}$ or *Nod1*$^{-/-}$ mouse keratinocytes, that were stimulated or not with 30 μM iE-DAP for 24 h. As a control WT, *Slc46a2*$^{-/-}$ and *Nod1*$^{-/-}$ fibroblasts were treated with IL1-α (10ng/ml). KC was induced in fibroblasts cultured in condition media from WT keratinocytes treated with iE-DAP, but not the mutant cells, while fibroblasts treated with IL1-α produced KC in all genotypes.

Panels A, B, C, E, and F are representative images of at least three independent experimental trials. In panel D, each dot represents a separate trail and was analyzed by unpaired t-test. Panel G is representative of three independent trials, error bars display technical variation. In panel H, data is shown as a mean for at least three independent experiments with SEM and analyzed by two-way ANOVA and Tukey's multiple comparisons test. **** $P < 0.0001$; ** $P < 0.01$; ns, not significant. The scale bar is 100μM.