Cell Stem Cell

Supplemental Information

dsRNA Released by Tissue Damage Activates TLR3 to Drive Skin Regeneration

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Supplemental Figure Legends

Supp. Fig 1: Related to Figure 1; Microarray analysis at wound closure but prior to regeneration indicates TLR3 signaling signature.
A) Microarray analysis was performed on healed scars at the earliest time of wound closure and re-epithelialization, prior to morphogenesis (~WD12) on Low Regenerating (LR) and High Regenerating (HR) strains of mice.
B) 25 Genes of overlap from Fig **1B** between top 200 genes in HR mice and top 200 genes in dsRNA treated keratinocytes from Karim et al. Fold changes and p-values are from mouse array. Genes in bold are associated with dsRNA recognition or induced by interferon, known TLR3 effects.
C) Wound closure and healing were monitored daily in strain-matched control and TLR3 null mice and average day of scab detachment (SD) as an indication of epithelialization; n = 6 mice.
D) Representative image of non-wounded murine skin after control or poly (I:C) injection (500ng/mouse) during telogen showing no change in hair cycle.

Supp. Fig. 2: Related to Figure 2; Gene expression analysis and qRT-PCR verification of gene expression changes on late stage microarrays.
A) Microarray analysis was performed on healed scars at WD16 on three LR and HR mouse scars as indicated.
B) Signaling pathways enriched and selected changed genes in samples with high regeneration.
C) Top 5 genes associated with enriched signaling pathways in **1B**.
D) Selected significantly changed interleukins, chemokines, and cytokines in HR.
E) Top gene ontology "functions" enriched in HR.
F) qRT-PCR verification of microarray gene expression of selected genes: Interleukin 6 (IL-6); interleukin 10 (IL-10), forkhead box protein P3 (Foxp3). Data represent the Mean ± SE of the fold change in gene expression; n = 5-10; *p < 0.05
G) Mean fold change in IL-6 mRNA with RelA-specific or scrambled control siRNA in the presence of poly (I:C) (20μg/mL) in keratinocytes as determined by qRT-PCR and normalized to housekeeping gene, RPLP0.

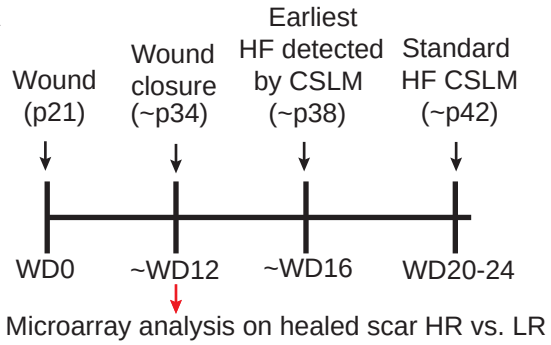
Supp. Fig 3: Related to Figure 3; P-STAT3 and IL-6 protein expression during wound healing after Poly IC or rmIL-6 treatment.
P-Stat3 and IL-6 immunohistochemistry in healing murine wounds after poly (I:C) treatment at WD3 or rmIL-6 treatment at WD7; Wounds were harvested 24hrs after treatment and representative images are shown. IL-6 and P-Stat3 positivity is increased during early wound healing (WD4) compared to late wound healing (WD8). Scale bar = 50 μm; original magnification: 40X.

Supp. Fig 4: Related to Figure 4; Increased stratification and altered keratinocyte morphology after IL-6 and TLR3 activation in keratinocytes.
A) Cleaved caspase 3 and Ki-67 immunohistochemistry on healed murine wounds after rmIL-6 treatment; representative images are shown. Scale bar = 100μm; original magnification: 20X.
B) Mean fold change in TLR3 mRNA with TLR3-specific siRNA or siCON (control siRNA) 24 hours after poly (I:C) treatment of NHEKs for 24 hours as determined by qRT-PCR and normalized to housekeeping gene, RPLP0. N=3, * p < 0.05.
C) Keratinocyte morphology 72 hours after 24 hours of poly (I:C) (20μg/mL) or control treatment to NHEK as determined immunofluorescence staining with phalloidin (green), pan-cadherin (red) and DAPI (blue). Magnification = 60X.

D) Quantitation of length to width ratio of keratinocyte morphology as in 4C.
E) Vimentin and keratin 5 immunofluorescence staining in NHEK after poly (I:C) or control as in 4C.
F) Mean fold change in VIM mRNA after poly (I:C) (20μg/mL) addition to NHEK for 24 hours at indicated time points as determined by qRT-PCR and normalized to housekeeping gene, RPLP0.
G) Quantification of vimentin expression via flow cytometry in NHEK after poly (I:C) or control as in 4F or normal fibroblasts.
H) Non-directional keratinocyte migration (μm/hr) after vehicle control or poly (I:C) (20μg/mL) continuous exposure for 7 days.
*$p < 0.05$ by Student's T-test or Single Factor ANOVA.

Supp. Fig. 5: Related to Figure 5; TLR3 KO mice have fewer γδT-cells and WIHN is not impacted in NSG mice.
A) Mean percentage of TCRγδ cells in newly healed wounds in wild type and TLR3 KO mice. Representative FlowJo vX dot plots are shown. N= 3-5 mice per genotype; *$p = 0.001$.
B) WIHN is not affected in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice lacking T and B cell lineages. Cross-sectional H&E histology through the middle of healed scar at WD22 in NSG mice, average number of hair follicles =13; n = 5 mice as measured by CSLM. Regenerated hair follicles are marked with arrows. Scale bar = 100 μm. Original objective: 20X.

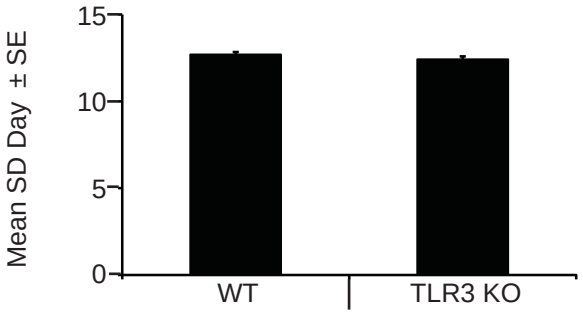
A



B

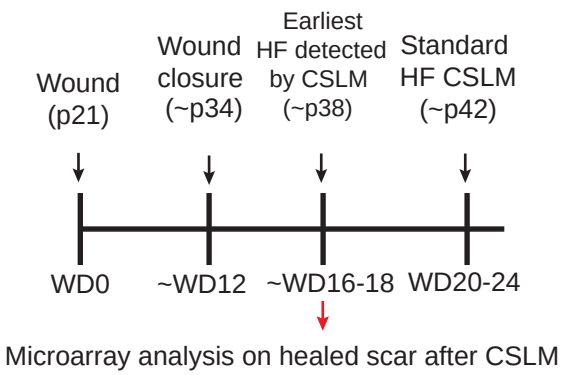
Gene Symbol	Gene Name	p-value	Fold Δ	Gene Function
IFIT1	Interferon-Induced Protein With Tetratricopeptide Repeats 1	3.12E-05	3.9194	Interferon induced; anti-viral RNA binding protein
LCE3	Late Cornified Envelope 3	1.27E-02	3.6914	Precursor of cornified envelope
IFI44	Interferon-Induced 44kDa protein	1.81E-05	3.6779	Interferon induced; microtubular structure
OAS1	2',5' Oligosynthetase 1	1.35E-04	2.7335	Innate immune response to viral infection
ISG15	Interferon Stimulated Gene 15	8.24E-05	2.6225	Activated by IFNs; Ubiquitin-like protein; targets RIG-I
IRF7	Interferon Regulatory Factor 7	2.54E-06	2.2975	Innate immune response against DNA/RNA virus
OAS2	2',5' Oligosynthetase 2	1.73E-03	2.2851	Innate immune response to viral infection
CXCL11	Chemokine (C-X-C Motif) Ligand 11	1.36E-04	2.1059	Induced by IFNs, skin immune response
OAS3	2',5' Oligosynthetase 3	1.69E-04	2.0748	Innate immune response to viral infection
RSAD2	Radical S-Adenosyl Methionine Domain Containing 2	1.78E-04	2.0589	Interferon-inducible; anti-viral activity
OASL	2',5' Oligosynthetase-Like	4.49E-04	1.9994	Innate immune response to viral infection
BST2	Bone Marrow Stromal Antigen	7.70E-05	1.9656	IFN-induced anti-viral factor
DHX58	DEXH (Asp-Glu-X-His) Box Polypeptide 58	4.28E-04	1.9042	Regulates DDX58; directly binds dsRNA
DDX58/RIG-I	Retinoic Acid-Inducible Gene 1 Protein	7.15E-04	1.8025	Recognizes dsRNA; innate immune response
PARP14	Poly (ADP-Ribose) Polymerase Family, Member 14	2.36E-04	1.7923	Post-translational modification of histones after DNA damage
XAF1	XIAP Associated Factor 1	3.41E-04	1.7069	Negative regulator of "inhibitor of apoptosis" (XIAP) proteins
HERC6/HERC5	HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase	2.20E-04	1.6458	Ubiquitin ligase
IFI35	Interferon-Induced 35kDa protein	3.86E-05	1.6281	Interferon induced
CXCL9	Chemokine (C-X-C Motif) Ligand 9	7.13E-02	1.6075	T-cell trafficking; immune and inflammatory response
MMP9	Matrix Metalloproteinase 9 (Gelatinase)	4.07E-02	1.6037	Tissue remodeling
CMPK2	Cytidine Monophosphate (UMP-CMP) Kinase 2	4.71E-04	1.5895	Nucleotide synthesis salvage pathway
GBP4	Guanylate Binding Protein 4	2.75E-02	1.3757	Induced by IFNs; hydrolyzes GTP
DEFB103B	Beta-Defensin 3	8.88E-02	1.2254	Antimicrobrial peptide
HELZ2	Helicase With Zinc Finger	3.28E-01	1.1411	Helicase
TRIM5	Tripartite Motif Containing 5	2.75E-04	1.1368	Blocks viral replication

C



D

A



B

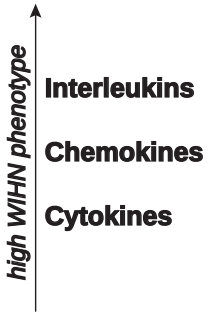
Signaling Pathway	P-value
JAK/STAT	***0.0000276***
Corticotropin Releasing Hormone	0.000154
IL-6 Signaling	***0.000212***
IL-10 Signaling	0.000626
Acute Phase Response	***0.000758***

C

Gene Symbol	Fold Δ	P-value
IL-6	3.348	*0.0253*
TNFAIP6	2.518	0.0498
SOCS3	2.183	0.0317
SERPINE1	2.175	0.0141
Fos	2.02	0.000291

D

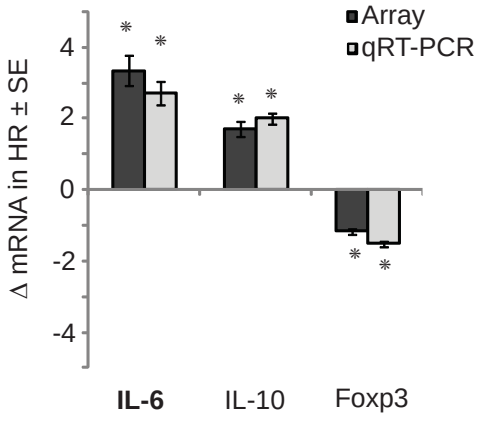
Gene Symbol	Fold Δ	P-value
Wdr92	6.26	0.0150
Ccl24	***4.23***	***0.0065***
Ptgs2	3.72	0.0042
Il-6	***3.35***	***0.0253***
Nr4a1	2.92	0.0012
Cxcl2	***2.52***	***0.0467***
Cxcl1	***2.10***	***0.0025***
Il-1b	***1.93***	***0.0175***
Ccl3	***1.79***	***0.0186***
Il-10	***1.71***	***0.0341***



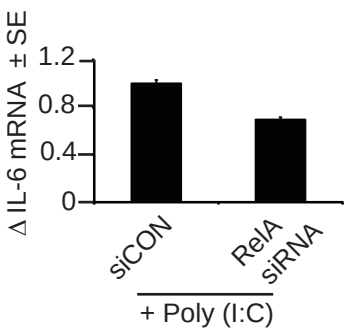
E

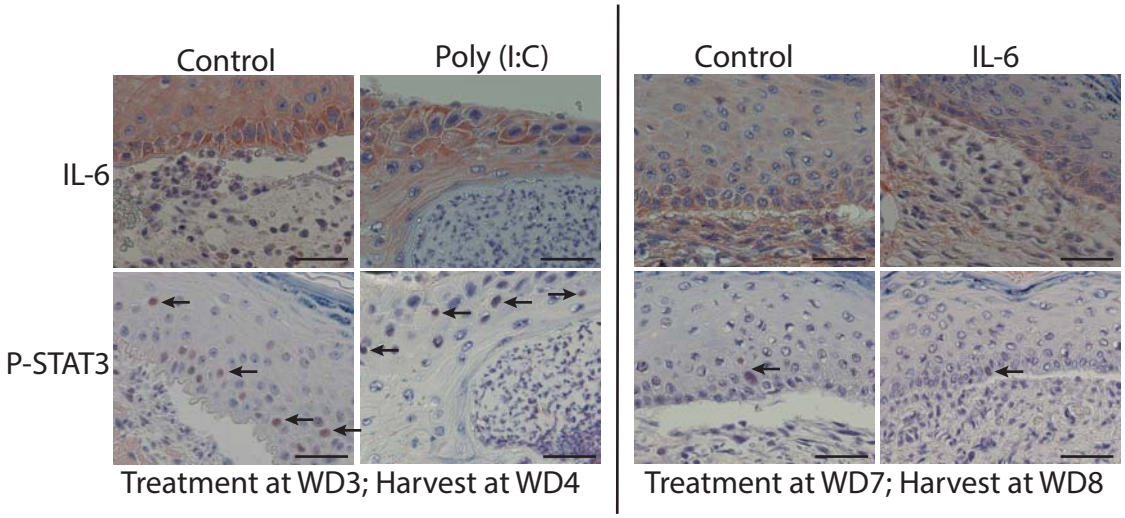
GO Bio Function Term	# of genes	P-value
Hematological Function	195	0.00001 < P < 0.01
Inflammatory response	110	0.00001 < P < 0.01
Infection mechanism	105	0.0001 < P < 0.01
Cell-mediated immunity	93	0.00001 < P < 0.01
Organism development	77	0.0001 < P < 0.01

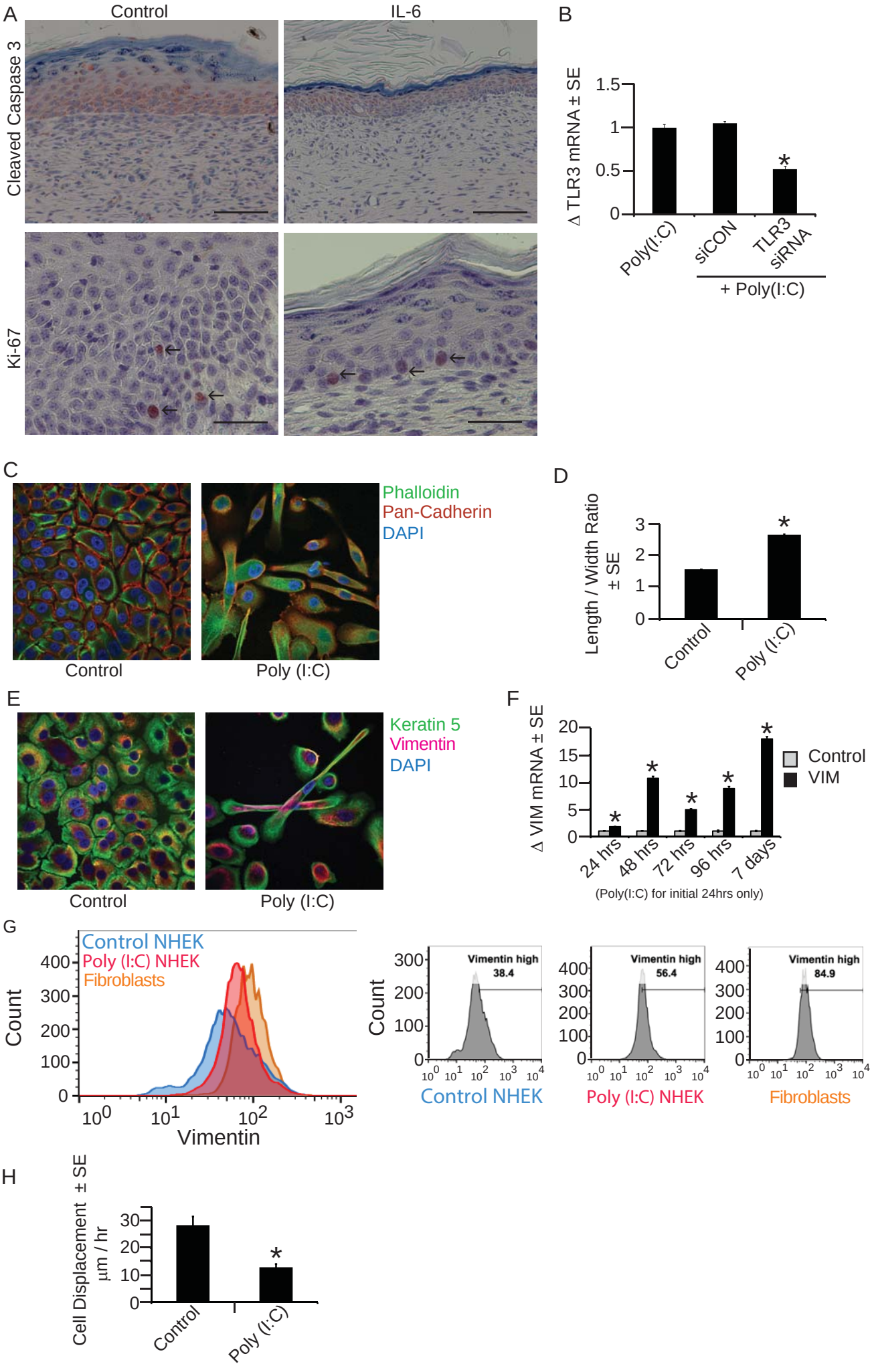
F



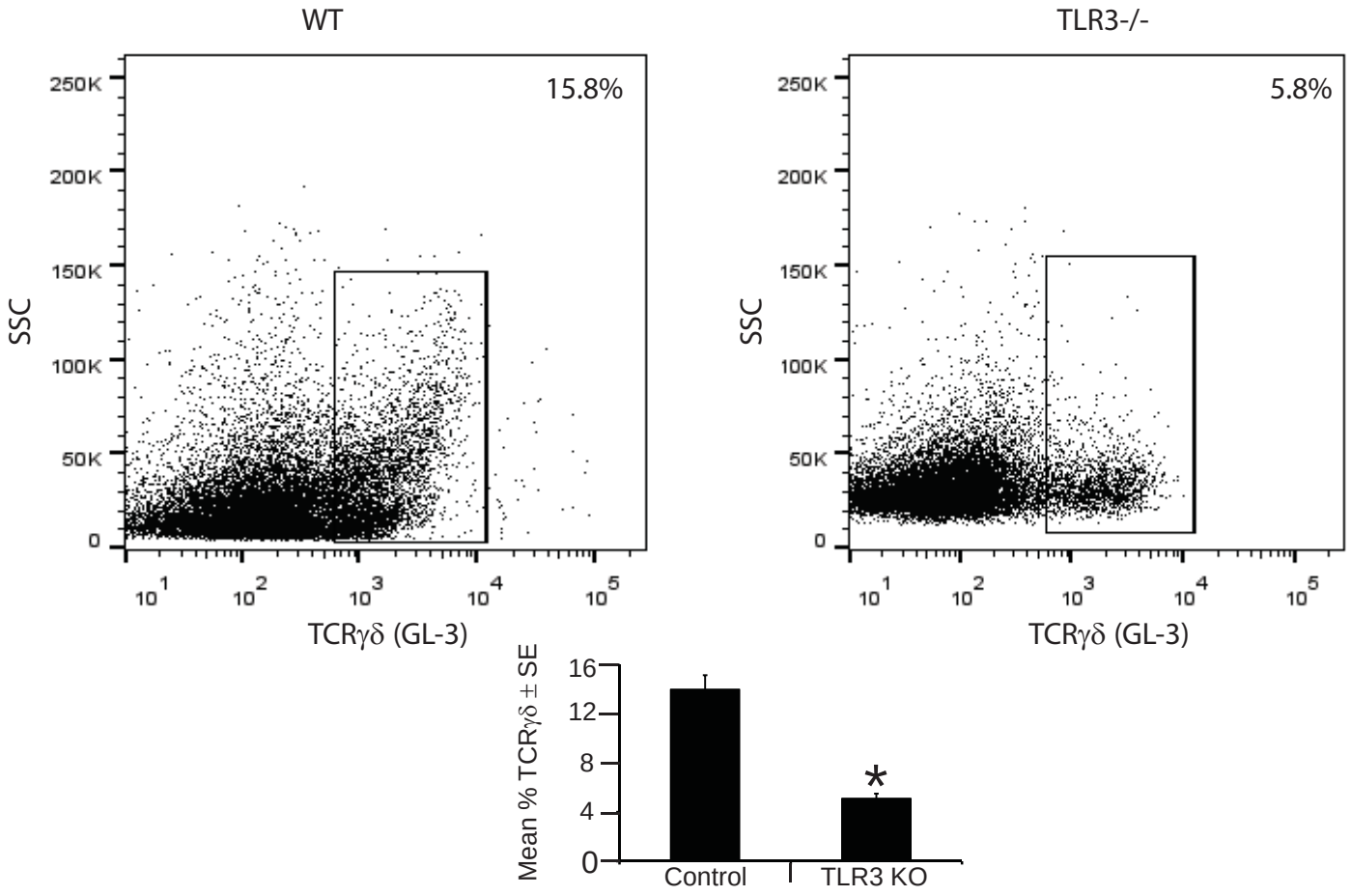
G




Control
Poly (I:C)
Control
IL-6
IL-6
P-STAT3
Treatment at WD3; Harvest at WD4
Treatment at WD7; Harvest at WD8


A
Control
IL-6
Cleaved Caspase 3
Ki-67
B
Δ TLR3 mRNA ± SE
1.5
1
0.5
0
*
Poly(I:C)
siCON
TLR3 siRNA
+ Poly(I:C)
C
Phalloidin
Pan-Cadherin
DAPI
Control
Poly (I:C)
D
Length / Width Ratio ± SE
3
2
1
0
*
Control
Poly (I:C)
E
Keratin 5
Vimentin
DAPI
Control
Poly (I:C)
F
Δ VIM mRNA ± SE
20
15
10
5
0
*
Control
VIM
24 hrs
48 hrs
72 hrs
96 hrs
7 days
(Poly(I:C) for initial 24hrs only)
G
Control NHEK
Poly (I:C) NHEK
Fibroblasts
Count
400
300
200
100
0
10^0 10^1 10^2 10^3
Vimentin
Vimentin high
38.4
56.4
84.9
10^0 10^1 10^2 10^3 10^4
Control NHEK
Poly (I:C) NHEK
Fibroblasts
H
Cell Displacement ± SE
μm / hr
30
20
10
0
*
Control
Poly (I:C)

A



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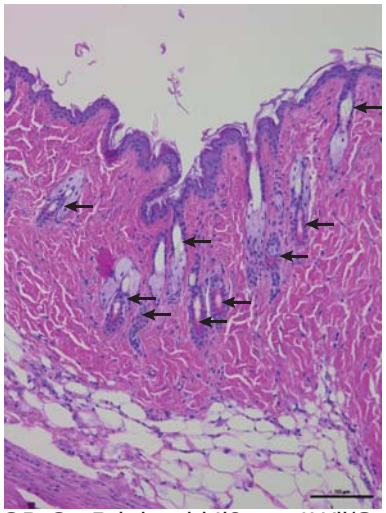

NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ

Expanded Experimental Methods

Antibodies

Rabbit polyclonal antibodies to phosphorylated Stat 3, Stat3, Ki-67, cleaved caspase 3, β-actin and secondary anti-rabbit HRP were obtained from Cell Signaling Technology (Danvers, MA). Anti-cytokeratin 1 (Krt1), anti- IL-6, anti- KRT15 (LHK15), anti-Wnt7b (ab94915) and anti-pan-cadherin antibodies was obtained from Abcam (Cambridge, MA). Mouse monoclonal β-catenin antibody (14) and vimentin (RV202) were obtained from BD Biosciences (San Jose, CA) and rabbit polyclonal keratin 5 antibody was purchased from Covance (Princeton, NJ). FITC-Phalloidin was obtained from Sigma (St Louis, MO). Mouse and rabbit IgG isotype controls were purchased from Invitrogen (Camarillo, CA).

Treatments during WIHN assay

Recombinant mouse IL-6 protein (R&D Systems, Minneapolis, MN) and poly (I:C) (High Molecular Weight; InVivogen, San Diego, CA) was diluted in sterile PBS immediately prior to injection. Cucurbitacin I (Tocris Biosciences/R&D Systems, Minneapolis MN) was dissolved in 10% EtOH/PBS to a final concentration of 1mg/mL prior to injection. RNase III enzyme (Life Technologies, Grand Island NY) was diluted in supplied reaction buffer

Keratinocyte Migration Assay

Keratinocytes were seeded onto LabTek II Chambered Coverglass 8-well slides (Thermo, cat. 155409) which had been coated with rat tail collagen I (Gibco, cat: A10483-01). Poly (I:C) was first added (20 ug/ml) 2 days after plating cells (~30% confluent). Subsequently, fresh culture medium (KGM-Gold) and poly (I:C) was applied every other day. At the time of imaging, cells were exposed to poly (I:C) for 7 days. Extended time-lapse imaging was done at the JHU Microscope Facility (acknowledgements: Barbara Smith and Dr. Scot Kuo) for 24 hours inside a humidified, 5% CO2 chamber. Cell migration rate was quantified using SlideBook Reader 6 software (Intelligent Imaging Innovations) by measuring overall cell displacement over the course of one hour (18 cells per video, 3 videos per condition).

ChIP

The amplicon for Gli2 promoter site 4 encompasses TTCCAGGAA on chr2: 121621123-121621782 in the Encode database.
It was amplified with the primer sequences: a) forward: 5'CACAGATAAGCTGAGTCACAGGA3'; b) reverse: 5'TCCTGTTCACATTGACGCC3'. The amplicon for β-catenin promoter site 4 encompasses TTCCTGGAA on chr3: 41264037-41264357 in the Encode database.
This was amplified with the primer sequences: a) forward: 5'TGCCTTTGCATCAACAACAAGG3'; b) reverse: 5'TCAGAAACCAACTGGTCATGTCT3'.