Supplementary Materials and Methods

Reagents

All chemicals were from Sigma unless otherwise stated. PRR agonists were from Invivogen except RNA40 (iba Lifescience, normal and AF647-labeled) and CpG PTO 2006 (TIB Molbiol), see Table S2 and 3. iODNs used in this study with their respective sequences are listed in Table S3 and were from TIB Molbiol. Total human mRNA was isolated from HEK293T cells using the RNeasy kit on a QIAcube, both from QIAGEN. *S aureus* RNA as isolated as described [21]. Genomic human DNA was isolated from whole blood using QIAamp DNA Blood Mini Kit from Qiagen (51106) and phosphodiester DNA from TIB Molbiol. LL37 was from InvivoGen (see Table S2) and DOTAP from Roth, L787.2. LL37 was Atto488 (from Atto-Tec as a carboxy-reactive reagent)-labeled using standard procedures. For complex formation 5.8 µM RNA40 (approximately 34.4 µg/ml and equimolar to R848 used in this setting), 1 µM ssDNA (approximately 20 µg/ml and equimolar to CpG used in this setting, sequence see Table S3), genomic DNA (20 µg/ml) or bacterial RNA (20 µg/ml) was mixed together with 10 µg LL37 (see Table S2, Atto-488 where indicated) and left for one hour at RT. For the RNA-only or LL37-only conditions, the same amounts and volumes were used replacing one of the constituents by sterile, endotoxin-free H_2O. Ficoll was from Millipore. Antibodies used for flow cytometry and ImageStream analysis are listed in Table S4 as well as the recombinant cytokines used in this study. Constructs used for HEK293T transfection are listed in Table S5.

Study participants and sample acquisition

All patients and healthy blood donors included in this study provided their written informed consent before study participation. Approval for use of their biomaterials was obtained by the local ethics committee at the University of Tübingen, in accordance with the principles laid down in the Declaration of Helsinki as well as applicable laws and regulations. Whole blood from voluntary healthy donors was obtained at the University of Tübingen, Department of Immunology. Blood samples from psoriasis patients were obtained at the University Hospital Tübingen, Department of Dermatology. Psoriasis patients had a median age of 39.1 years (ranging from 23 to 59 years of age) with PASI scores >10 and did not receive any systemic treatments at the time of blood sampling. All samples obtained from psoriasis patients were processed simultaneously with samples from at least one healthy donor matched for age and sex.

Neutrophil isolation and stimulation

Whole blood (EDTA anticoagulant) was diluted in PBS (Thermo fisher, 14190-169) loaded on Ficoll (1.077 g/ml, Biocoll, Biochrom GmbH, ab211650) and centrifuged for 25 min at 366 x g and 21 °C without brake. All layers were discarded after density gradient separation except for the erythrocyte-

granulocyte pellet. Thereafter, erythrocyte lysis (using 1x ammonium chloride erythrocyte lysis buffer, see Table S6) was performed twice (for 20 and 10 min) at 4 °C on a roller shaker. The remaining cell pellet was carefully resuspended in culture medium (RPMI culture medium (Sigma Aldrich, R8758) + 10% FBS (heat inactivated, TH Geyer, 11682258) and 1.6 x 10^6 cells/ml were seeded (24 well plate). After resting for 30 min at 37°C, 5% CO_2, the cellsy were pre-treated with inhibitors (where indicated) for another 30 min and subsequently stimulated with the indicated agonists for 4 hours (for ELISA) or for 30 min to 2 h (for FACS analysis or microscopy).

PBMC isolation

Whole blood (EDTA anticoagulant) was diluted in PBS. After density gradient separation using Ficoll (described above), the PBMC layer was carefully transferred into a new reaction tube and diluted in PBS 1:1. The cell suspension was spun down at 1800 rpm for 8 min. The cells were then washed twice more in PBS, resuspended in culture medium (RPMI + 10% FBS (heat inactivated) + 1% L-glutamine), before counting and seeding.

Flow cytometry

After PMN isolation and stimulation, the purity and activation status of neutrophils were determined by flow cytometry. 200 µl of the cell suspension was transferred into a 96 well plate (U-shape) and spun down for 5 min at 322 x g, 4 °C. FcR block was performed using pooled human serum diluted 1:10 in FACS buffer (PBS, 1 mM EDTA, 2% FBS heat inactivated) for 15 min at 4 °C. After washing, the samples were stained for approximately 20-30 min at 4°C in the dark. Thereafter, fixation buffer (4% PFA in PBS) was added to the cell pellets for 10 min at RT in the dark. After an additional washing step, the cell pellets were resuspended in 150 µl FACS buffer. Measurements were performed on a FACS Canto II from BD Bioscience, Diva software. Analysis was performed using FlowJo V10 analysis software.

ELISA

Cytokines were determined in half-area plates (Greiner, Bio-one) using duplicates or triplicates and measuring with a standard plate reader. The assays were performed according the manufacturer's instructions (Biolegend, R&D systems), using appropriate dilutions of the supernatants. For LL37 determination a kit from HycultBiotech (HK321-02) was used following the manufacturer's instructions.

ImageStream analysis

ImageStream analysis was used to analyze internalization of RNA-LL37 complexes using spot-counts and tracking single cells. The cells were first seeded in a 96 well plate, 8 x 10^6 cells/ml, 125 µl per well. Subsequently, they were stimulated for 1 hour with RNA-AF647 (IBA technologies) and/or LL37-

Atto488 (kindly provided by Hubert Kalbacher, University of Tübingen). FcR block and surface staining (here CD15 PE) was performed as described above. After fixation, the cells were permeabilized with 0.05 % Saponin (Applichem, A4518,0100) for 15 min at RT in the dark. After washing, nuclei were stained with Hoechst 33342 (Sigma, B2261, 1 µg/ml) for 5 min at RT in the dark, washed and resuspended in 50 µl FACS buffer and transferred into a 1.5 ml Eppendorf tube. At least 10.000 cells were acquired for each sample with 40x magnification using an ImageStream X MKII with the INSPIRE instrument controller software (Merck-Millipore/Amnis). Data were analyzed with IDEAS Image analysis software. All samples were gated on single cells in focus.

Fluorescence Microscopy

The cells were seeded in a 96 well plate at 1.6 x 10^6 cells/ml, 125 µl per well. Subsequently they were stimulated with the complexes for 30 min and 1 hour using RNA-AF647 and/or LL37-Atto488. FcR block, staining, fixation and permeabilization was performed as for Flow cytometry. The cell pellets were resuspended in 50-100 µl FACS buffer. 40 µl of the cell suspension was pipetted on a Poly-L-Lysine coated coverslip (Corning, 734-1005) and the cells were left to attach for one hour in the dark. ProLong Diamond Antifade (Life technologies, P36965) was used to mount the coverslips on uncoated microscopy slides. The slides were left to dry overnight at RT in the dark and were then stored at 4 °C before microscopy. The measurements were conducted with a Nikon Ti2 eclipse (100x magnification) and the analysis was performed using Fiji analysis software.

Luminex analysis

All samples were stored at -70 °C until tested. The samples were thawed at room temperature, vortexed, spun at 18,000 x g for 1 minute to remove debris and the required sample volumes were removed for multiplex analysis according to the manufacturer's recommendations. The samples were successively incubated with the capture microspheres, a multiplexed cocktail of biotinylated, reporter antibodies, and a streptavidin-phycoerythrin (PE) solution. Analysis was performed on a Luminex 100/200 instrument and the resulting data stream was interpreted using proprietary data analysis software (Myriad RBM). Analyte concentrations were determined using 4 and 5 parameter, weighted and non-weighted curve fitting algorithms included in the data analysis package.

Cytometric bead array

A cytometric bead array was performed using the "Human inflammatory cytokine kit" from BD Bioscience (551811) and following the manufacturer's instruction. 25 µl of samples and standards were added to 25 µl of the capturing bead mixture. Additionally, 25 µl of PE detection reagent was added all tubes and incubated for 3 h at RT in the dark. Thereafter, 1 ml of wash buffer was added to each tube and centrifuges at 200 x g for 5 minutes. The supernatant was carefully removed and the pellet was resuspended in 300 µl wash buffer. Measurements were performed with the FACS Canto II

from BD Bioscience operated using Diva software. Analysis was performed with Soft Flow FCAP Array v3 analysis software from BD Bioscience.

Transwell experiments

In the inserts 100 µl of PBMC suspension was added to the inserts (0.8 x 10^6 cells/insert). PBMCs and Neutrophils were used from the same donor in each experiment. In the lower chamber, either PMNs were seeded as described above (same plate size, same volume, same cell concentration) using polycarbonate, 24 well plates, 3 µm pores, Corning, 734-1570) and stimulated for 4 h with stimuli as indicated. Alternatively, media containing the stimuli only (i.e. no PMNs) or media containing only MIP-1β (30 and 150 pg /ml), IL-16 (300 and 1500 pg/ml) or SDF-1α (control, 100 ng/ml) and no PMNs were added. After 4 h, the lower compartment was harvested and FACS staining was performed as described above. The total number of migrated cells was acquired using counting beads (Biolegend, 424902) on a FACS Canto II (BD Bioscience) with Diva software. Analysis was performed using FlowJo V10 analysis software.

Transient transfection of HEK293T cells

HEK293T cells were transiently transfected using the $CaPO_4$ method as described [22]. Cells were seeded in 24-well plates at a density of 14 x 10^5 cells/ml 2-3 h prior to transfection. For the transfection of one well, 310 ng of plasmid DNA (100 ng TLR plasmid, 100 ng firefly luciferase NF-κB reporter, 10 ng *Renilla* luciferase control reporter, and 100 ng EGFP plasmid) was mixed with 1.2 µl of a 2 M $CaCl_2$ solution and filled up with sterile endotoxin-free H_20 to obtain a total reaction volume of 10 µl. After the addition of 10 µl of 2X HBS solution (50 mM HEPES (pH 7.05), 10 mM KCl, 12 mM Glucose, 1.5 mM Na_2HPO_4), the mixture was then added to the cell suspension. As negative controls, TLR coding plasmids were replaced by empty vectors carrying the appropriate backbone of the TLR plasmids. After the addition of the transfection complexes, the cells were incubated either for 24 h followed by stimulation, or kept for 48 h without stimulation (MyD88 expression). For stimulation, the media was aspirated and replaced by fresh growth medium in which TNFα or the different TLR ligands (R848, CpG, RNA40) with or without IRS were diluted to appropriate concentrations. TLR8 activation with RNA40 was facilitated by complexation of the RNA with DOTAP (L787.1, Roth). RNA40 and DOTAP were first diluted in 1X HBS separately. Next, RNA40/HBS was diluted 1:3 in DOTAP/HBS. The solution was carefully mixed by pipetting up and down. After 15 minutes of incubation at RT, the mixture was 1:6.7 diluted in growth medium (with or without IRS) and finally dispensed (500 µl/well) into the wells containing transfected HEK293T cells. Each tested condition was measured in triplicates. The cells were stimulated and inhibited for 24 h at 37°C.

Dual Luciferase Reporter Assay

After checking transfection efficiency via EGFP fluorescence microscopy, HEK293T supernatants were aspirated and 60 µl of 1X passive lysis buffer (E194A, Promega) added per well. The plate was then incubated for 15 minutes at RT on the plate shaker and subsequently stored at -80 °C for at least 15 minutes to facilitate complete cell lysis. After thawing, 60 µl were transferred into a V-bottom 96-well plate and centrifuged for 10 minutes at 2500 rpm and 4 °C to pellet cell debris. 10 µl supernatant were then transferred into a white microplate and each condition was measured in triplicates using the FLUOstar OPTIMA device (BMG Labtech). Firefly and Renilla luciferase activity were determined using the Promega Dual luciferase kit. Both enzyme activities were measured for 12.5 s with 24 intervals of 0.5 s, respectively. The data was analyzed by calculating the ratio of the two measured signals, thereby normalizing each firefly luciferase signal to its corresponding Renilla luciferase signal. The ratios were represented as the relative light units (RLU) of NF-κB activation.

Statistics

Experimental data was analyzed using Excel 2010 (Microsoft) and/or GraphPad Prism 6 or 7 (GraphPad Software, Inc.), microscopy data with ImageJ/Fiji, flow cytometry data using FlowJo software version 10. When extreme values occurred, outliers were statistically identified using the ROUT test at high (0.5%) stringency and normality tested using the Shapiro-Wilk test for the subsequent choice of a parametric or non-parametric tests. p-values (α=0.05, β=0.8) were then calculated and multiple testing was corrected for in Prism as indicated in figure legends. Values < 0.05 generally considered statistically significant. These were denoted by * throughout, even if the calculated p-values were considerably lower than 0.05. Comparisons made to unstimulated control unless indicated otherwise by brackets.

Supplementary Figure Legends

Supplementary figure S1

Aqua Live-dead flow cytometric viability analysis of unstimulated PMNs after 4 hours in culture (A, n=9 combined from several experiments) and including chloroquine (CQ) 30 min pre-incubation (B, n=5-9). (C) as in B but including CpG stimulation and ELISA analysis as indicated (n=7). A-C represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). * $p<0.05$ according to Wilcoxon signed rank sum (B) and one-way ANOVA with Sidak correction (C).

Supplementary figure S2

Luminex multiplex cytokine analysis of PMN supernatants (screening analysis). Mean values of TNF-α, IL-1β, IL-6, IL-16 and MIP-1β for n=2 donors shown.

Supplementary figure S3

ELISA of IL-8 (A) or MIP-1β (B) secreted from psoriasis PMNs (n=3) or PMNs from sex-and age-matched healthy donors (n=3-10) in response to LPS treatment. Combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). * $p<0.05$ according to Kruskall-Wallis test with Dunn's correction (A, B).

Supplementary figure S4

Flow cytometric cell count of migrated CD4 T cells (A), CD8 T cells (B) and $CD14^{+}HLA\text{-}DR^{+}$ monocytes (C) quantified in transwell assays with total PBMCs in the upper and SDF-1α (positive control) in the lower compartment (A-C, n=6-7, $p>0.05$ for treatments vs media according to Friedman or one-way ANOVA test, respectively). A-C represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). * $p<0.05$ according to Friedmann test with Dunn's correction (A, B), or one-way ANOVA with Dunnett's correction for multiple testing (C).

Supplementary figure S5

NF-κB dual luciferase reporter assay in HEK293 cells, transfected with NF-κB firefly luciferase reporter, *Renilla* control reporter and plasmids for either TLR9 (A), empty vector (B) or MyD88 (C), subsequently stimulated with CpG ODN (A), recombinant TNF (B) or left unstimulated (C), without (arrow) or with IRS661, IRS954, IRS869 and IRS546 (n=2 each). (D) Aqua Live-dead flow cytometric viability analysis of PMNs treated with IRS661 and IRS954 only (4 h, n=5-9). (E) IL-8 (release from PMNs stimulated with LPS (E, n=4-6) with or without IRS661 (1 nM), IRS954 (50 nM) pre-incubation (30 min) quantified by ELISA. (E) as in D but stimulation with RNA-LL37 and pre-incubation with chloroquine and measuring MIP-1β release (CQ, 10 µM, n=6). D-F represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). In A-C one representative

of ‘n’ replicates is shown (mean+SD). * $p<0.05$ according to two-way ANOVA (A), one-way ANOVA with Dunnett’s correction (only TNF or MyD88 group, respectively, B, C), Kruskall-Wallis test with Dunn’s correction (D) or one-way ANOVA with Sidak correction (E, F).

Supplementary Tables

Table S1: Summary of primary PMN responses across experiments.

Analyte	LPS	PMA	R848	RNA	Pam2	Pam3	CpG DNA	genomic DNA/ssDNA	RNA + LL37	genomic DNA/ssDNA + LL37
IL-8	++	+++	++	+/-	+++	++	+++	-	++	-
CD62L	+++	+++	-	-	+++	++	+++	-	+	-
ROS	+/-	+++	-	-	-	-	-	-	-	-

Table S2: Commercial TLR ligands and inhibitors

Component	**company**	**Product**
LL37	InvivoGen	tlrl-l37
LPS-EK (ultrapure)	InvivoGen	tlrl-peklps
R848 (Resiquimod)	InvivoGen	tlrl-r848-5
Chloroquine	InvivoGen	tlrl-chq
PMA	InvivoGen	tlrl-pma
Pam2CSK4	InvivoGen	tlrl-pm2s-1
Pam3CSK4	InvivoGen	tlrl-pms

Table S3: RNA/DNA and inhibitors

Component	Sequence	company
CpG2006	5'TsCsGsTsCsGsTsTsTsTsGsTsCsGsTsTsTsTsGsTsCsGsTsT3'	TIB
RNA40	5'GsCsCsCsGsUsCsUsGsUsUsUsGsUsGsUsGsAsCsUsC3'	iba
ssDNA60	5'AC$(AC)_{28}$AC3'	TIB
IRS546	5'TsCsCsTsGsCsAsGsGsTsTsAsAsGsT3'	TIB
IRS661	5'TsGsCsTsTsGsCsAsAsGsCsTsTsGsCsAsAsGsCsA3'	TIB
IRS869	5'TsCsCsTsGsGsAsGsGsGsGsTsTsGsT3'	TIB
IRS954	5'TsGsCsTsCsCsTsGsGsAsGsGsGsGsTsTsGsT3'	TIB

ssRNA40 was obtained from IBA. The backbone is phosphorothioate.

Table S4: Antibodies and recombinant proteins

Item	fluorophore	species	isotype	company	Product
Isotype control	PE	mouse	IgG1 kappa	eBioscience	12471442
Isotype control	FITC	mouse	IgM	BioLegend	401605
Isotype control	APC	mouse	IgG1 kappa	BD	550854
Isotype control	BV421	mouse	IgG1 kappa	BioLegend	400157
Isotype control	FITC	mouse	IgG2b	BioLegend	402207
Anti-hCD15	PE	mouse	IgG1 kappa	BioLegend	323006
Anti-hCD66b	FITC	mouse	IgG1 kappa	BioLegend	305103
Anti-hCD62L	BV421	mouse	IgG1 kappa	BioLegend	30482
Anti-hCD14	PE	mouse	IgG1 kappa	ImmunoTool	21620144
Anti-hCD4	PE	mouse	IgG1 kappa	BioLegend	300508
Anti-hCD8	APC	mouse	IgG1 kappa	ImmunoTool	21810086
Anti-hHLA-DR	FITC	mouse	IgG2b	BioLegend	327006
Recombinant hMIP-1β	-	-	-	ImmunoTool	11343223
Recombinant hIL-16	-	-	-	ImmunoTool	11340163
Recombinant hSDF-1α	-	-	-	ImmunoTool	11343363

Table S5: Plamids used for HEK293T transfection

Plasmid name Insert	Vector backbone	Insert
EGFP	pC1-EGFP	EGFP
NF-κB reporter	pGL3	6x NFKB response element
Renilla	pRL-TK	Renilla
hTLR7	pcDNA3.1 (+)	hTLR7
hTLR8	pcDNA3.1 (+)	hTLR8
hTLR9	pEF-SEM	hTLR9
MyD88	pTO-N-SH Streptag N-terminal Gateway	hMyD88 FL aa13-296 L265P, Stop-codon
pcDNA3.1	pcDNA3.1 (+)	Empty
pEF-SEM	pEF-SEM	Empty

Table S6: 10x Ammoniumchloride erythrocyte lysis buffer

Compound	**company**	**Product no.**
1.54 M NH_4Cl	Roth	5470.1
100 mM $KHCO_3$	Fluka	60220
1 mM EDTA; pH 8	ThermoFisher	15575020
dissolved in Ampuwa water	Fresenius Kabi	1833
pH adjusted to 7.3, sterile filtered (0.22 µm)		