Title

Complexes of RNA and the antimicrobial peptide, LL37, trigger TLR-mediated cytokine release from psoriasis neutrophils

Running title

RNA and LL37 activate neutrophils in psoriasis

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Human neutrophils respond to host and foreign RNA when complexed to the psoriasis-associated antimicrobial peptide, LL37

The resulting release of cytokines requires TLRs, is elevated in psoriasis patients and can be blocked using inhibitor oligodeoxynucleotides

Abstract

Psoriasis is an inflammatory autoimmune disease characterized by skin lesions showing strong immune cells infiltration and high levels of the antimicrobial peptide, LL37. LL37 binds RNA and DNA and these complexes trigger Toll-like receptors (TLRs) and subsequent interferon release from plasmacytoid dendritic cells. Polymorphonuclear leukocytes (PMNs) are abundant in psoriatic skin and are primary sources for LL37, extracellular nucleic acids and inflammatory cytokines. We hypothesized that LL37-nucleic acid complexes sustain a self-amplifying inflammatory loop via TLR signaling in PMNs but this hypothesis has not been investigated. We here show that LL37 potentiated TLR8-mediated IL-8 release by PMNs in response to synthetic, human and bacterial RNA, by promoting its uptake. Additionally, RNA complexed to LL37 prompted PMNs to produce TNF, IL-6 and IL-1β, as well as the chemoattractants IL-16 and MIP-1β. Interestingly, in PMNs from psoriasis patients, TLR-triggered cytokine and chemokine release and constitutive LL37 secretion were generally elevated compared to PMNs from healthy donors and RNA triggered IL-8 even in the absence of exogenous LL37. Finally, we observed that inhibitory oligodeoxynucleotides (iODNs), previously used to block TLR7 and TLR9 responses, prevented the TLR response of PMNs to RNA-LL37 complexes. Collectively, our data suggest that in psoriatic lesions RNA-LL37-driven PMN activation may contribute to a vicious cycle of inflammation and immune cells attraction, and that TLR blockade could be used to limit PMN-mediated inflammation and immune cell infiltration in this context.

Introduction

Psoriasis is an autoimmune disease of the skin with high incidence in Western countries (1.5-3%), causing high socioeconomic and disease burden with limited but increasing treatment options [1,2]. The most common form of psoriasis, plaque psoriasis, is characterized by epidermal hyperplasia due to keratinocyte (KC) hyperproliferation, increased endothelial proliferation and an infiltrate of leukocytes, such as dendritic cells, T cells and, prominently, polymorphonuclear neutrophils (PMNs) [1]. The high and early accumulation of PMNs in psoriatic plaques and micro-abscesses is well documented, as well as an increase of PMNs in the circulation of psoriasis patients [3 4]. However, it is unclear whether PMN infiltration represents a bystander phenomenon or has a causative role in the development of the disease.

PMNs are known as source of LL37, an amphipathic, positively-charged 37 amino acid peptide generated from a precursor protein, the cathelicidin hCAP18, that is highly abundant in psoriatic lesions [1,5]. LL37 is stored in the secondary granules of PMNs from which it can be released upon activation [6]. Apart from acting as a self-antigen for CD4 helper T cells in psoriasis patients [7], LL37 was previously shown to form a complex with DNA and RNA that resisted nuclease degradation, was readily taken up by plasmacytoid dendritic cells (pDCs), and triggered high Interferon (IFN) α and low Tumor necrosis factor (TNF) or Interleukin (IL) 6 secretion from these cells [8,9]. Although type I IFNs are considered to promote psoriasis [10,11], chemokines and pro-inflammatory cytokines such as TNF, IL-1β or IL-23 are additionally required for immune cell infiltration and T cell polarization, respectively [12]. Since pDCs are comparatively low producers of these psoriasis-initiating inflammatory cytokines and do not accumulate to high numbers in psoriatic skin, this raises the question whether nucleic acid-LL37 sensing by other immune cells, particularly PMNs, may contribute more prominently to initial inflammatory cytokine/chemokine milieu observed in psoriasis.

In contrast to pDCs, PMNs are known producers of considerable quantities of various cytokines and chemokines [13-15]. Additionally, PMNs are able to undergo neutrophil extracellular trap (NET) formation (NETosis), an activation-induced process leading to the extrusion of nuclear DNA [16], as well as cellular proteins, including LL37 [17]. Like pDCs, PMNs express multiple pattern recognition receptors (PRRs) of the Toll-like receptor (TLR) family, namely TLR2, 4, 8 [18] and 9 [19], but not TLR3 or TLR7 [18]. TLRs directly bind activating ligands, typically so-called microbe-associated molecular patterns (MAMPs) absent from the host [20]; however, if they can gain access to endosomal compartments - for example by complex formation with LL37 - host nucleic acids are able to trigger the endosomal TLRs 3, 7, 8 (sensing double-stranded or single-stranded RNA, respectively) or TLR9 (sensing DNA). TLR7 expression in humans is restricted to pDC and (only inducibly) B cells. TLR8 thus appears the primary receptor for foreign (and potentially host-derived) single-stranded RNA in humans [21].

Since PMNs theoretically combine the abilities (i) to release LL37 and nucleic acids as components of immunostimulatory ligands, (ii) to sense such ligands via TLRs, and (iii) to secrete chemo-/cytokines, we speculated whether LL37-mediated DNA or RNA sensing via TLRs in PMNs might initiate and fuel inflammatory cytokine and chemokine production and thus inflammation and immune cell infiltration in psoriatic skin. We here present experimental evidence that human primary PMNs readily respond to RNA-LL37 complexes, but not RNA on its own, leading to the release of a broad array of chemokines and cytokines – a process that induces chemoattraction of other immune cells and that is elevated in psoriasis patients and can be therapeutically blocked by iODNs.

Materials and Methods

Reagents

All chemicals and PRR ligands were from Sigma or Invivogen, respectively, unless otherwise stated in Supplementary materials or Tables S2 and S3. Antibodies and recombinant cytokines are listed in Table S4.

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. Psoriasis patients had a median age of 39.1 years (range 23 to 59) with PASI scores >10 and did not receive any systemic treatments at the time of blood sampling. All samples obtained from psoriasis patients (obtained at the University Hospital Tübingen, Department of Dermatology) were processed simultaneously with samples from at least one healthy donor (obtained at University of Tübingen, Department of Immunology) matched for age and sex.

Neutrophil and PBMC isolation and stimulation, ELISA, cytometric bead array and Luminex analysis

PMNs and PBMC were isolated from whole blood (anticoagulant: EDTA) using Ficoll density gradient centrifugation. After resting for 30 min at 37°C, 5% CO_2 they were pre-treated with inhibitors (where indicated) for another 30 min and subsequently stimulated with the indicated agonists for 4 h (for ELISA, cytometric bead array or Luminex analysis according to standard protocols, see Supplementary methods) or for 30 min to 2 h (for FACS analysis or microscopy). The purity and activation status of neutrophils were always determined as described in Supplementary methods.

Fluorescence and Electron Microscopy and ImageStream analysis

The cells were seeded and stimulated for 1 hour using RNA-AF647 and/or LL37-Atto488. After FcR block, staining, fixation and permeabilization as for flow cytometry, the cells were analyzed by ImageStream cytometry or mounted and analyzed on a Nikon Ti2 eclipse bright-field fluorescence microscope (100x magnification). For electron microscopy (EM, performed by J. Berger, Max-Planck-Institute, Tübingen, Germany), specimen preparation and EM analysis was done according to standard procedures. See Supplementary methods for further details.

Transwell experiments

PBMCs (inserts) and Neutrophils (lower chamber) were used from the same donor. 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 (controls, 100 ng/ml) and no PMNs were added to the lower chamber. After 4 h, the total number of migrated cells in the lower compartment was determined using counting beads (Biolegend), see supplementary methods.

Transient transfection of HEK293T cells and Dual luciferase assay

Human embryonic kidney (HEK) 293T cells were transfected using the $CaPO_4$ method with TLR/MyD88 plasmids, firefly luciferase NF-κB reporter, *Renilla* luciferase control reporter, and Enhanced green fluorescence protein (EGFP) plasmids (Table S5), stimulated as described [22] and NF-κB activity (Promega) measured as detailed in supplementary methods.

Statistics

Experimental data was analyzed using Excel 2010 (Microsoft) and/or GraphPad Prism 6 or 7 (GraphPad Software), microscopy data with ImageJ/Fiji, flow cytometry data with FlowJo 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 test. p-values ($\alpha=0.05$) 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 bybrackets.

Results

LL37 promotes RNA uptake and induces TLR8-mediated PMN activation

Previous results indicated that human primary PMNs can respond to RNA and DNA when stimulated for >12 h, albeit at much lower levels than the nucleoside analog TLR7/8 agonist, R848 [19,23]. We sought to re-evaluate these results using highly purified primary PMNs assayed within a short time period (4 h) that excludes secondary release effects, e.g. by apoptosis (Fig. S1A). The TLR7/8 agonist R848, like the TLR4 agonist, LPS, elicited robust IL-8 release but only LPS triggered CD62L shedding; phospho-thioate (PTO) synthetic CpG ODN, a typical TLR9 agonist, also strongly activated IL-8 release and CD62L shedding (Fig. 1A, Table S1). However, unmodified, natural phospho-diester DNA ODN or human genomic DNA elicited neither IL-8 release nor CD62L shedding, irrespective of whether they were complexed to LL37 (Fig. 1B). In contrast, in pDC LL37 binding of natural DNA triggered potent TLR9 responses [8]. In the absence of LL37, single-stranded synthetic RNA40 (henceforward referred to as 'RNA') was also barely active regarding IL-8 release at equimolar concentrations with R848 (Fig. 1C). This suggests that on their own RNA and DNA are not able to trigger primary PMN responses. This may be due to the endosomal localization of TLR8 and 9 which R848 and CpG can apparently access, whereas RNA and normal DNA cannot [24]. However, robust IL-8 release and moderate CD62L shedding were observed when RNA was complexed with LL37 (Fig. 1C). To check whether PMNs could engage RNA-LL37 complexes, live PMNs were seeded and RNA-LL37 complexes added for 20 min before fixing the cells for electron microscopy analysis. As shown in Fig. 1D, PMNs were found in proximity to fiber-like structures corresponding to RNA-LL37 complexes [25]. Flow cytometry using Alexa647-labeled RNA showed that LL37 also promoted the uptake of complexed RNA: more than 20% of PMNs incubated with labeled RNA in the presence of LL37 were Alexa647-positive within 60 min, compared to 5% in the absence of LL37 (Fig. 1E). ImageStream bright-field cytometry confirmed that this was not due to external/surface binding of labeled RNA but rather internalization (Fig. 1F, G) and bright-field fluorescence microscopy with Atto488-labeled LL37 showed co-localization of Alexa647-RNA and Atto488-LL37 in intracellular compartments (Fig. 1H) where TLR8 is expressed [18]. To pharmacologically confirm the role of the endosomal TLR8, we investigated the effect of chloroquine, a well-known inhibitor of endosomal TLRs that was non-toxic for PMNs (Fig. S1B). Indeed IL-8 release from primary PMNs in response to RNA-LL37 and CpG ODN (positive control) was significantly reduced by chloroquine addition (Fig. 1I and S1C). Since TLR7 is not expressed in PMNs [18] and chloroquine does not affect cytoplasmic RNA sensors (e.g. RIG-I) [26], we conclude TLR8 to be the receptor to mediate the observed effects in PMNs. We next sought to check whether the observed effect on cytokine release extended to normal RNA from mammalian cells which may be more physiologically relevant in the psoriatic context. As shown in Fig. 1J, human mRNA induced significant

IL-8 release, but only in combination with LL37. Since microbiome analyses have recently revealed alterations in staphylo- and streptococcal skin colonization in psoriasis patients [27] and bacterial RNA is an emerging immuno-stimulatory pattern [28], we also tested whether LL37 promoted *S. aureus*, i.e. bacterial, RNA (bRNA) activation of PMNs. Fig. 1K shows that bRNA significantly stimulated IL-8 release similarly to RNA40 when complexed to LL37. Taken together this suggests that uptake of RNA is promoted by LL37 and directs RNA to intracellular compartments from which TLR8 sensing and consequently activation leading to cytokine release can occur – both in response to mammalian and bacterial/foreign RNA.

RNA-LL37 complexes prompt release of multiple pro-inflammatory cytokines and chemokines

To test whether RNA-LL37 also promoted release of cytokines other than IL-8 we screened two supernatants of stimulated PMNs by Luminex multiplex analysis and detected TNF, IL-1β, IL-6, and – unexpectedly – IL-16 and MIP-1β primarily or exclusively in RNA-LL37 stimulated supernatants, respectively (Fig. S2). Despite considerable variation between donors, this was confirmed by cytometric bead arrays analyses (Fig. 2A-C) and ELISA (Fig. 2D, E) in samples from more donors. To our knowledge, active receptor-mediated release of the chemoattractants, IL-16 (also known as Lymphocyte Chemoattractant Factor, LCF) and MIP-1β (also known as Chemokine (C-C motif) Ligand, CCL4) from primary human PMNs has not been reported before. In fact, release of IL-16 was also boosted by LL37 alone (Fig. 2D) and MIP-1β was highly released from PMNs in response to RNA-LL37 complexes but not RNA alone (Fig. 2E). We conclude that the combination of RNA with LL37 can stimulate not only IL-8 release from primary PMNs, but also induced additional pro-inflammatory cytokines and chemoattractants not triggered by RNA alone. To test whether this was relevant for the psoriasis context, we next analyzed cyto-/chemokine release in response to RNA-LL37 complexes on PMNs from patients with moderate to severe psoriasis (here referred to as 'psoriasis PMNs') without systemic treatment and a psoriasis area and severity (PASI) score ≥10. Additionally, the effect of PMN-produced cytokines on the attraction of additional immune cells known to infiltrate psoriatic skin and contribute to pathogenesis was tested.

Psoriasis PMNs respond more potently to RNA-LL37

When repeating the above described stimulation experiments with psoriasis PMNs compared to healthy donor PMNs, both IL-8 and MIP-1β release were up to two-fold higher in response to RNA-LL37 (Fig. 3A, B). Whereas IL-8 release in response to 'RNA alone' (i.e. without LL37) was indistinguishable from 'unstimulated' in healthy donor PMNs (*cf.* also Fig. 2), psoriasis PMNs treated with 'RNA alone' (Fig. 3A, arrow) produced more IL-8 release than both 'unstimulated' psoriasis PMNs and 'RNA alone'-stimulated healthy donor PMNs. Since in healthy donors, RNA seemed to strongly require LL37 for efficient PMN uptake and stimulation (*cf*. Fig. 1) and PMNs are the major

contributors to elevated LL37 levels in psoriasis, we speculated whether psoriasis PMNs might constitutively secrete LL37 and this endogenous LL37 might promote this increased responsiveness to exogenously added RNA alone in psoriasis PMNs. We indeed observed that psoriasis PMNs constitutively had a higher baseline of LL37 release than healthy donors (Fig. 3C) but were not pre-activated prior to stimulation (no CD62L shedding observable). Although the response of psoriasis PMNs to LPS was also elevated, this difference was not significant and for MIP-1β psoriasis and healthy donor PMNs produced the same levels of cytokine (Fig. S3A, B). Thus, in psoriasis PMNs TLR-mediated inflammatory mediator release was noticeably increased, especially for RNA and potentially due to a higher abundance of PMN-derived LL37.

RNA-LL37 complexes induce migration of mononuclear immune cells

IL-16 is a known modulator and chemoattractant for CD4 T cells [29,30] and MIP-1β acts as a chemoattractant for a variety of immune cells and stimulates further pro-inflammatory cytokine production [31]. We therefore next checked whether IL-16 or MIP-1β, at the concentrations observed in this study could influence the migration and infiltration of additional immune cells, a hallmark of psoriatic plaques [4]. Transwell experiments with peripheral blood mononuclear cells (PBMCs) from healthy human donors (upper well) and IL-16 (300 and 1500 pg/ml), MIP-1β (30 and 150 pg/ml) or SDF-1α as a control (lower well) were conducted (Fig. 4A, B and S4). We specifically focused on T cells as known mediators of the psoriatic Th17 phenotype [12] and CD14 MHC II (HLA-DR)-expressing monocytes as inflammatory cells and potential APCs for LL37 [7]. As evident in Fig. 4A-C, the lowest concentration of both cytokines led to a donor-dependent increase in the number of $CD3^+CD4^+$ helper, $CD3^+CD8^+$ cytotoxic T cells and $CD14^+HLA\text{-}DR^+$ monocytes, whereas higher concentrations had a weaker effect, i.e. showed a bell-shaped dose response curve not uncommon for cytokines [32]. Due to donor-to-donor variation observed throughout in the human system, only a non-significant, moderate effect of MIP-1β was observed (Fig. 4C). We also separately tested whether RNA or RNA-LL37 in the lower chamber had any direct influence on the migration of these cell populations and noted an unexpected and significant chemoattractive effect on $CD4^+$ T cells, and to a lesser extent $CD8^+$ T cells and $CD14^+HLA\text{-}DR^+$ monocytes, in response to RNA-LL37. Interestingly, the presence of PMNs, regardless of stimulus, was enough to attract a variety of other cells (results not shown) due to a so far unknown mechanism. Taken together IL-16 and MIP-1β from PMNs or RNA-LL37 itself may contribute to cellular migration of T cells and monocytes with APC capacity.

Inhibitory Oligonucleotides are able to block immune responses to RNA-LL37 complexes

The pathological potential of endosomal TLR triggering by host nucleic acids, albeit not linked to LL37, has been demonstrated for another autoimmune disease, namely systemic lupus erythematosus (SLE) [33]. Consequently, potential new therapeutic approaches in SLE include the

blocking of endosomal nucleic-acid sensing TLR7 and 9 signaling by so-called inhibitory oligonucleotides (iODNs) [33-35], for example, IRS869 [34], IRS661 and IRS954 [36]. In these studies IRS546 was used as a non-inhibitory 'control' ODN. Given the similarities between endosomal TLRs [22], we speculated that some of these iODNs could potentially also block TLR8, but the effects of iODN on this receptor have not been studied systematically. In HEK293T cells exogenously expressing TLR7, TLR8 or TLR9 (control) upon transfection [22], IRS869 and IRS954 blocked NF-κB activation in response to R848 for TLR7 and 8 (Fig. 5A,B) and CpG for TLR9 (Fig. S5A). IRS661 strongly blocked TLR7- but not TLR9-mediated activation. Surprisingly, IRS661 strongly increased R848-mediated NF-κB activation via TLR8 (Fig. 5B), similar to observations made for T-rich ODNs combined with R848 [37] and highlighting the differences between R848 and RNA reported before [22]. However, for RNA complexed to DOTAP (a synthetic complexing agent promoting endosomal uptake [38], similarly to LL37), all three iODNs blocked TLR8-mediated NF-κB activation (Fig. 5C). Since it blocked NF-κB activation in response to TLR7-9 stimulation, the 'control' IRS546 in our hands did not represent a proper control ODN unlike previously published. Nevertheless, the effects of all iODNs on NF-κB activity mediated on control activation by TNF (a non-TLR receptor pathway activating NF-κB, Fig. S5B) or MyD88 overexpression (TLR receptor-independent NF-κB activation, Fig. S5C) were minor, suggesting a direct inhibitory effect on TLR8-RNA signaling. First excluding a toxic effect (Fig. S5D), we investigated whether these effects could be transferred to endogenous TLR8 activation in primary PMNs. Here, we could observe that at nanomolar concentrations, iODNs IRS661 and IRS954 were able to inhibit IL-8 release by primary PMNs from healthy donors in response to RNA-LL37 (Fig. 5D), whereas LPS-mediated IL-8 release was unaffected (Fig. S5E). The blocking effect extended to MIP-1β (Fig. 5E), similar to the effect of chloroquine (Fig. S5F). Thus, known pre-clinical iODNs are able to block RNA-LL37-mediated TLR8 activation and cytokine release in primary PMNs.

Discussion

Although systemic therapies with biologicals have brought benefit to many psoriasis patients in industrialized countries [2], psoriasis continues to be a significant health problem with high socioeconomic burden worldwide. One reason is that triggers and mechanisms leading to chronification, exacerbation and flare-ups of the disease are not well understood. This uncertainty also still applies to the role played by PMNs in these processes [4]. Given the contribution of pDC activation by nucleic acid-LL37 complexes to the production of IFNα (but not pro-inflammatory cytokine) production in the psoriatic skin context [8,9], we chose to study PMN RNA-LL37 responses after identifying TLR8 as an RNA receptor on PMNs [18]. This seemed intriguing since PMNs can release nucleic acids themselves, are the main sources of LL37 in humans and congregate in psoriatic skin.

The Gilliet lab elegantly showed the presence of DNA-/RNA-LL37 complexes in psoriatic skin and their effect on pDCs [8,9]. We found that the combination of RNA and LL37 represents an inflammatory ligand that is also sensed by TLR8 in PMNs. For pDCs both "inflammatory ingredients", RNA and LL37, will have to be provided first by another cellular source; on the other hand, a small number of activated, apoptotic and/or NETing PMNs – by releasing both, LL37 and RNA – may spark potent inflammation *inter pares* and *in situ.* This initial inflammation may quickly get out of control due to LL37 promoting further chemokine release and NET formation (and thus nucleic acid and LL37 extrusion [39]) which was observable in the skin of psoriasis patients [40]. Thus a vicious cycle of RNA-LL37 activation may rapidly engulf bystander PMNs congregating in Munro's microabscesses or pustules of Kagoj - especially since neutrophils in patients are more easily activated and respond more strongly (*cf.* Fig. 3). Although the concentration of PMN cytokines measured after 4 h stimulation here was moderate, release over longer time periods and the sheer number of PMNs found in psoriatic skin may nevertheless contribute substantially to local inflammation and attraction of additional leukocytes. Our study thus provides a plausible explanation how PMNs may contribute to early disease development and progression via an LL37-host RNA/NET feed-forward loop resulting in local inflammation. The latter is known to favor KC hyperproliferation [41], LL37 production [42], TLR9 responsiveness [43] and IFN production [44], as well as T cell and monocyte attraction and polarization [12]. Additional chemokines may also attract pDCs that could then fuel the vicious cycle of activation by endogenous nucleic acids [8,9] even further.

Although endogenous RNA is a likely internal trigger, bacterial RNA [28] may also function as a complexing partner for LL37 and as an initial trigger or additional fuel (*cf.* Fig. 1K). Given the differences in the TLR8-immunostimulatory potential of different bacterial RNAs (e.g. tRNAs) due to strain-specific nucleotide modifications [45], it could be speculated that certain skin microbiota may especially favor LL37-mediated immune activation and thus psoriatic inflammation via PMNs or

pDCs. Interestingly, differences in the abundance of the *Staphylococcus* and *Streptococcus* genera - whose RNA is immunostimulatory [21,46] - were already reported in psoriasis [27]. It may therefore be interesting to investigate correlations between PMN infiltration and/or activation, LL37 levels and streptococcal and staphylococcal abundance in future clinical studies.

Our data also show interesting blocking effects of iODNs for TLR8 PMN responses. The broad and receptor-unrestricted effects of published iODNs on both TLR7, TLR8 and TLR9 (and preferably also TLR3 which can be triggered by RNA-LL37 in KC [47]) could potentially block LL37-mediated TLR activation of both PMNs, pDCs and KCs [43], cutting out a major part of innate immune contributions to skin inflammation. This is intriguing since a potential topical application of iODNs would likely exclude systemic toxicities. This is in line with a recent phase 2a study (NCT01899729), showing a positive influence of another TLR7-9 blocking iODN, IMO-8400, on PASI scores. Thus TLR blockade may indeed be beneficial and therapeutically viable in psoriasis [48]. Our study provides a rationale for exploring to which extent the blockade of PMN responses contribute to the observed improvement and how PMN responses may be targeted even more effectively in the future.

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Authorship contributions

FH, ZB, SD, DE, TK and NSM performed experiments; FH, ZB, SD, DE, NSM and ANRW analyzed data; MH, MWL, KG and TE were involved in sample and reagent acquisition; DH, KG and TE contributed to the conceptual development of the study; FH and ANRW wrote the manuscript and all authors commented on an revised the manuscript; ANRW coordinated and supervised the entire study.

Conflict of Interest Disclosures

None.

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Abbreviations

Enhanced green fluorescence protein – EGFP; Human embryonic kidney – HEK; Microbe-associated molecular pattern – MAMPs; Inhibitory oligodeoxynucleotides – iODNs; Interferon – IFN; Interleukin – IL; Keratinocytes – KC; Plasmacytoid dendritic cells – pDCs; Pattern recognition receptor – PRR; Polymorphonuclear leukocytes – PMNs; Neutrophil extracellular trap - NET; Systemic lupus erythematosus – SLE; TLR – Toll-like receptor; Tumor necrosis factor (TNF)

Figure legends

Figure 1: Human neutrophils take up and respond to RNA when complexed to LL37

(A-C) IL-8 release (ELISA, 4 h stimulation) and CD62L shedding (flow cytometry, 1 or 2h) by PMNs from healthy donors stimulated with LPS, R848 or CpG ODN (A, n=6-7), ssDNA and genomic DNA (B, n=5), or RNA40 (C, n=8), with or without LL37. (D) Electron microscopy of PMNs incubated with RNA-LL37 for 20 min (n=1). (E-G) FACS (E, n=6), ImageStream cytometry (F, G, scale bar = 10 µm) or conventional bright-field microscopy (H, n=2, scale bar = 10 µm) of PMNs incubated for 60 min with RNA-AlexaFluor647 complexed with LL37 (n=6). In F the % of cells with 'internalized' features are shown (n=2, see Methods), in G selected cells from one representative donor are shown. In H unmodified LL37 was replaced with Atto488-labeled LL37, one representative donor shown. (I) as in C but including chloroquine (CQ) pre-incubation for 30 min (n=6-7). (J) and (K) as in C but using total human RNA from HEK293T cells (J, n=4-6) or bacterial RNA isolated from *S. pyogenes* (K, n=4) with and without LL37. A-C, E, F, I-K represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). In D, G and H one representative of 'n' biological replicates (donors) is shown (mean+SD). * $p<0.05$ according to one-way ANOVA with Dunnett's correction (A, B, C, I, J) or Friedman test with Dunn's correction (E, K).

Figure 2: RNA-LL37 complexes promote release of multiple pro-inflammatory cytokines and chemokines

Cytometric bead array (A-C) or ELISA (D, E) for TNF (A), IL-6 (B), IL-1β (C), IL-16 (D) or MIP-1β (E) secreted from PMNs stimulated for 4 h as indicated (n=4-7). A-E represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor). * $p<0.05$ according to one-way ANOVA with Dunnett's correction (E), Kruskall-Wallis (D) or Friedman test with Dunn's correction (A-C).

Figure 3: Psoriasis PMNs are more efficiently stimulated by RNA-LL37 complexes

(A-B) ELISA of IL-8 and MIP-1β secreted from psoriasis PMNs (n=3 patients, 9 healthy controls, squares, chequered bars) or PMNs from sex-and age-matched healthy donors (n=3 patients, 10 healthy controls, circles, filled bars). (C) as in A or B but ELISA for LL37 (n=4 patients, 7 healthy donors). A-C represent combined data (mean+SD) from 'n' biological replicates (each dot represents one donor/patient). * $p<0.05$ according to one-way ANOVA with Dunnett's correction.

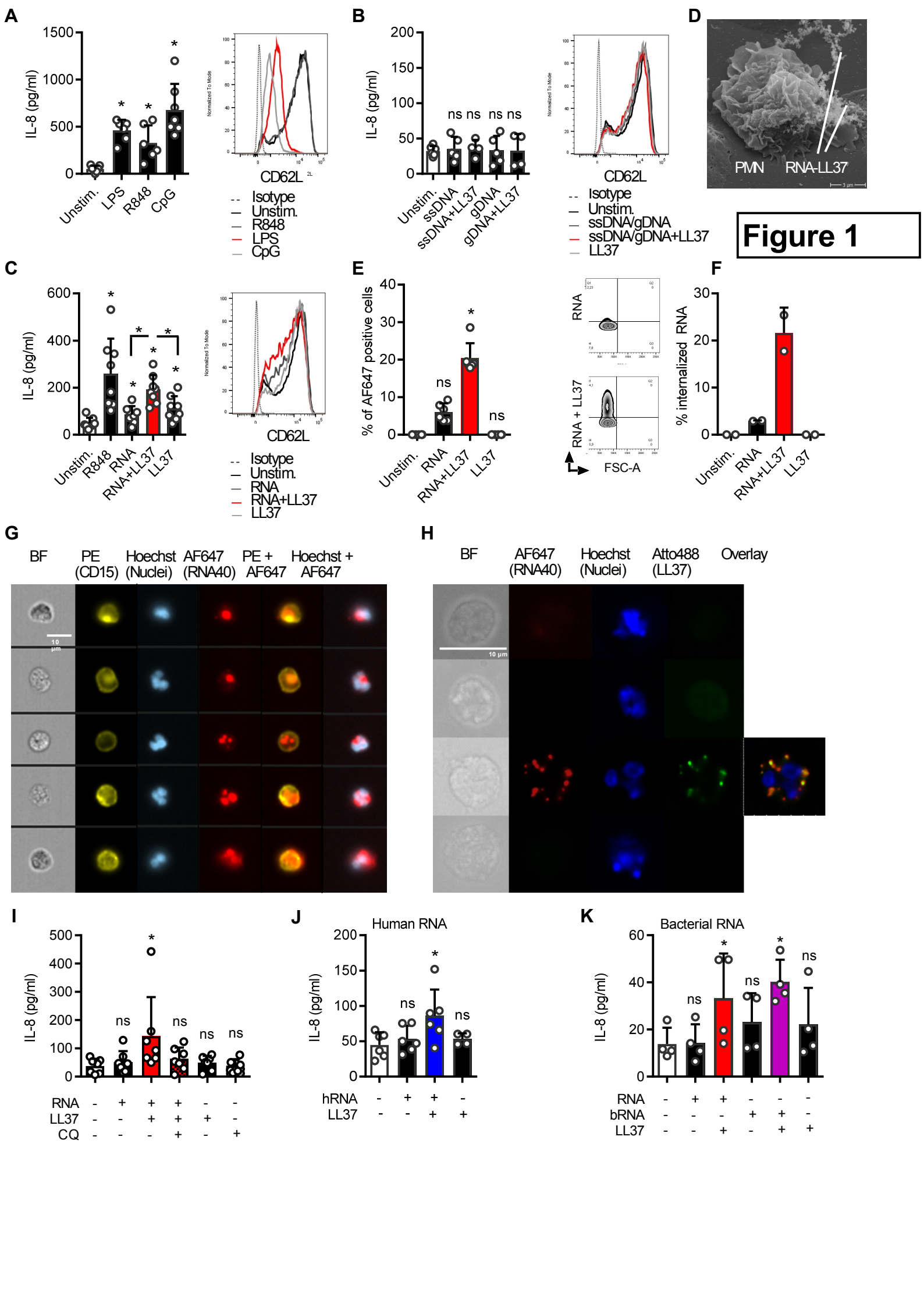
Figure 4: Cytokines released by human PMNs and LL37-complexed RNA induce migration of T cells and monocytes

Flow cytometric cell count of migrated CD4 T cells (A, D), CD8 T cells (B, E) and $CD14^{+}HLA\text{-}DR^{+}$ monocytes (C, F) quantified in transwell assays with total PBMCs in the upper and either MIP-1β or IL-16 (A-C, n=6-7, $p>0.05$ for treatments vs media according to Friedman or one-way ANOVA test,

respectively) or R848, RNA with and without LL37 (D-F, n=3-7) in the lower chamber. A-F 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, D, E), or one-way ANOVA with Dunnett's correction for multiple testing (C, F).

Figure 5: Inhibitory oligodeoxynucleotides are able to inhibit RNA-LL37 cytokine release from human PMNs

NF-κB dual luciferase reporter assay in HEK293T cells, transfected NF-κB firefly luciferase reporter, *Renilla* control reporter and plasmids for either TLR7 (A) or TLR8 (B, C), subsequently stimulated with R848 (A, B) or RNA (C) without (arrow) or with IRS661, IRS954, IRS869 and IRS546 (0.15-3.5 µM, n=2 each). IL-8 (D, n=6) or MIP-1β (E, n=4) release from stimulated PMNs with or without IRS661 (1 nM) or IRS954 (50 nM) pre-incubation (30 min) quantified by ELISA. D and E 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 of technical triplicates). * $p<0.05$ according to two-way ANOVA with Dunnett correction for multiple testing (A-C, comparison against 'no inhibitor' condition, arrow), one-way ANOVA with Sidak correction (D) or Friedmann test with Dunn correction (E).


A
IL-8 (pg/ml)
1500
1000
500
0
Unstim.
LPS
R848
CpG
CD62L
Isotype
Unstim.
R848
LPS
CpG
B
IL-8 (pg/ml)
200
150
100
50
0
ns ns ns ns
Unstim.
ssDNA
ssDNA+LL37
gDNA
gDNA+LL37
CD62L
Isotype
Unstim.
ssDNA/gDNA
ssDNA/gDNA+LL37
LL37
D
PMN
RNA-LL37
Figure 1
C
IL-8 (pg/ml)
600
400
200
0
Unstim.
R848
RNA
RNA+LL37
LL37
CD62L
Isotype
Unstim.
RNA
RNA+LL37
LL37
E
% of AF647 positive cells
40
30
20
10
0
ns
ns
Unstim.
RNA
RNA+LL37
LL37
RNA
RNA + LL37
FSC-A
F
% internalized RNA
30
20
10
0
Unstim.
RNA
RNA+LL37
LL37
G
BF
PE (CD15)
Hoechst (Nuclei)
AF647 (RNA40)
PE + AF647
Hoechst + AF647
10 µm
H
BF
AF647 (RNA40)
Hoechst (Nuclei)
Atto488 (LL37)
Overlay
10 µm
I
IL-8 (pg/ml)
500
400
300
200
100
0
ns
ns
ns
ns
RNA - + + + - -
LL37 - - + + + -
CQ - - - + - +
J
Human RNA
IL-8 (pg/ml)
200
150
100
50
0
ns
ns
hRNA - + + -
LL37 - - + +
K
Bacterial RNA
IL-8 (pg/ml)
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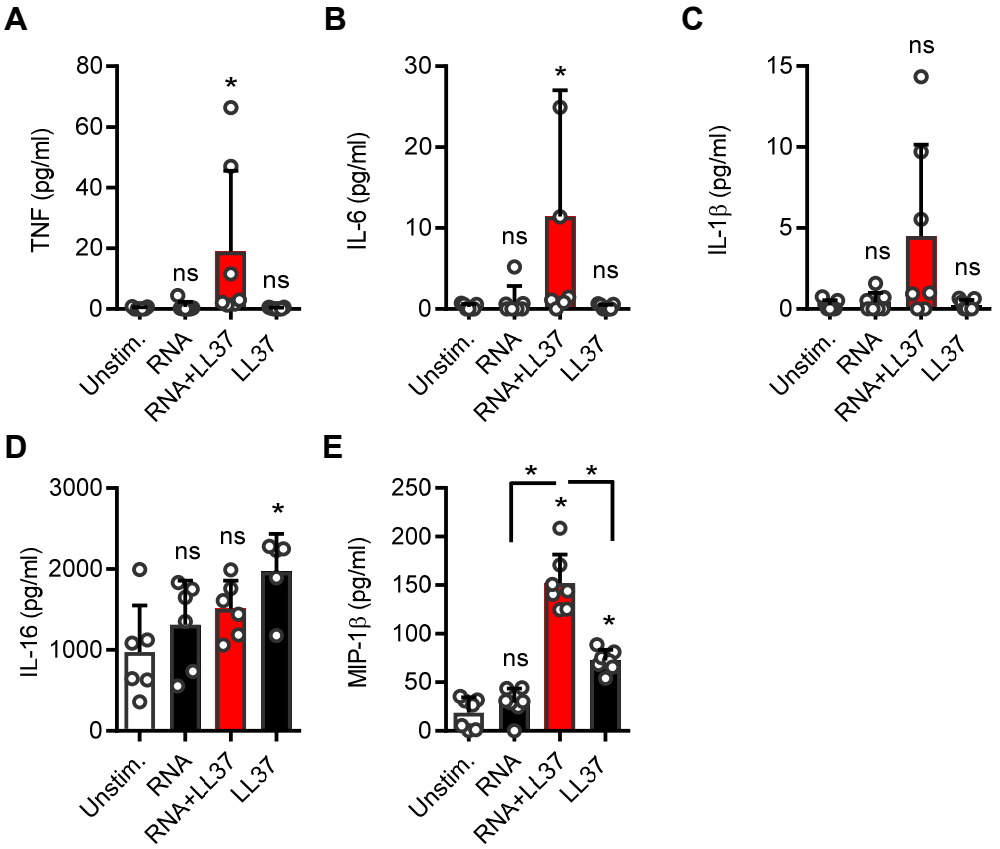
40
20
0
ns
ns
ns
RNA - + + - - -
bRNA - - - + + -
LL37 - - + - + +

Figure 2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TNF (pg/ml)
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60
40
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ns
*
ns
Unstim.
RNA
RNA+LL37
LL37
B
IL-6 (pg/ml)
30
20
10
0
ns
*
ns
Unstim.
RNA
RNA+LL37
LL37
C
IL-1β (pg/ml)
15
10
5
0
ns
ns
ns
Unstim.
RNA
RNA+LL37
LL37
D
IL-16 (pg/ml)
3000
2000
1000
0
ns
ns
*
Unstim.
RNA
RNA+LL37
LL37
E
MIP-1β (pg/ml)
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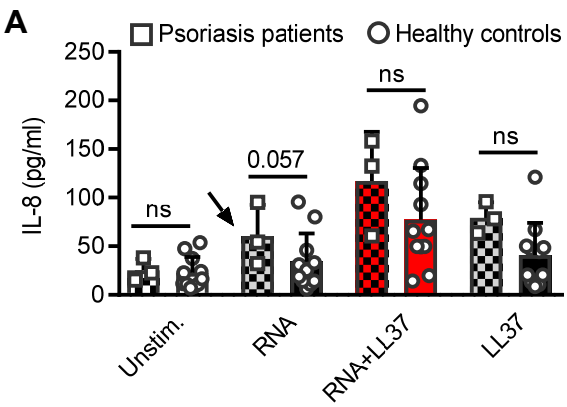
200
150
100
50
0
*
*
*
ns
*
Unstim.
RNA
RNA+LL37
LL37

Figure 3


A
Psoriasis patients
Healthy controls
IL-8 (pg/ml)
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200
150
100
50
0
ns
0.057
ns
ns
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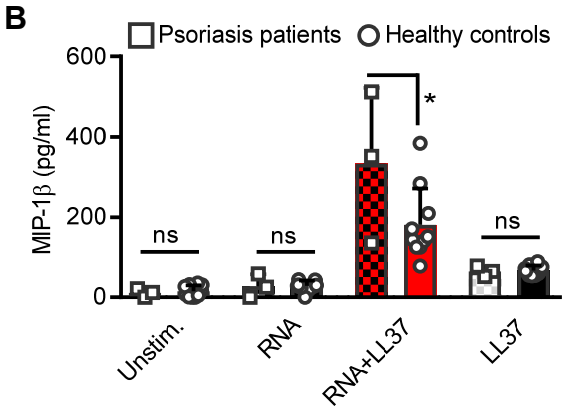
RNA
RNA+LL37
LL37


B
Psoriasis patients
Healthy controls
MIP-1β (pg/ml)
600
400
200
0
ns
ns
*
ns
Unstim.
RNA
RNA+LL37
LL37

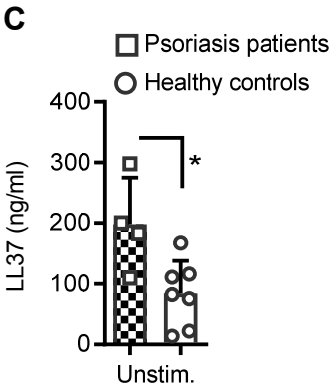
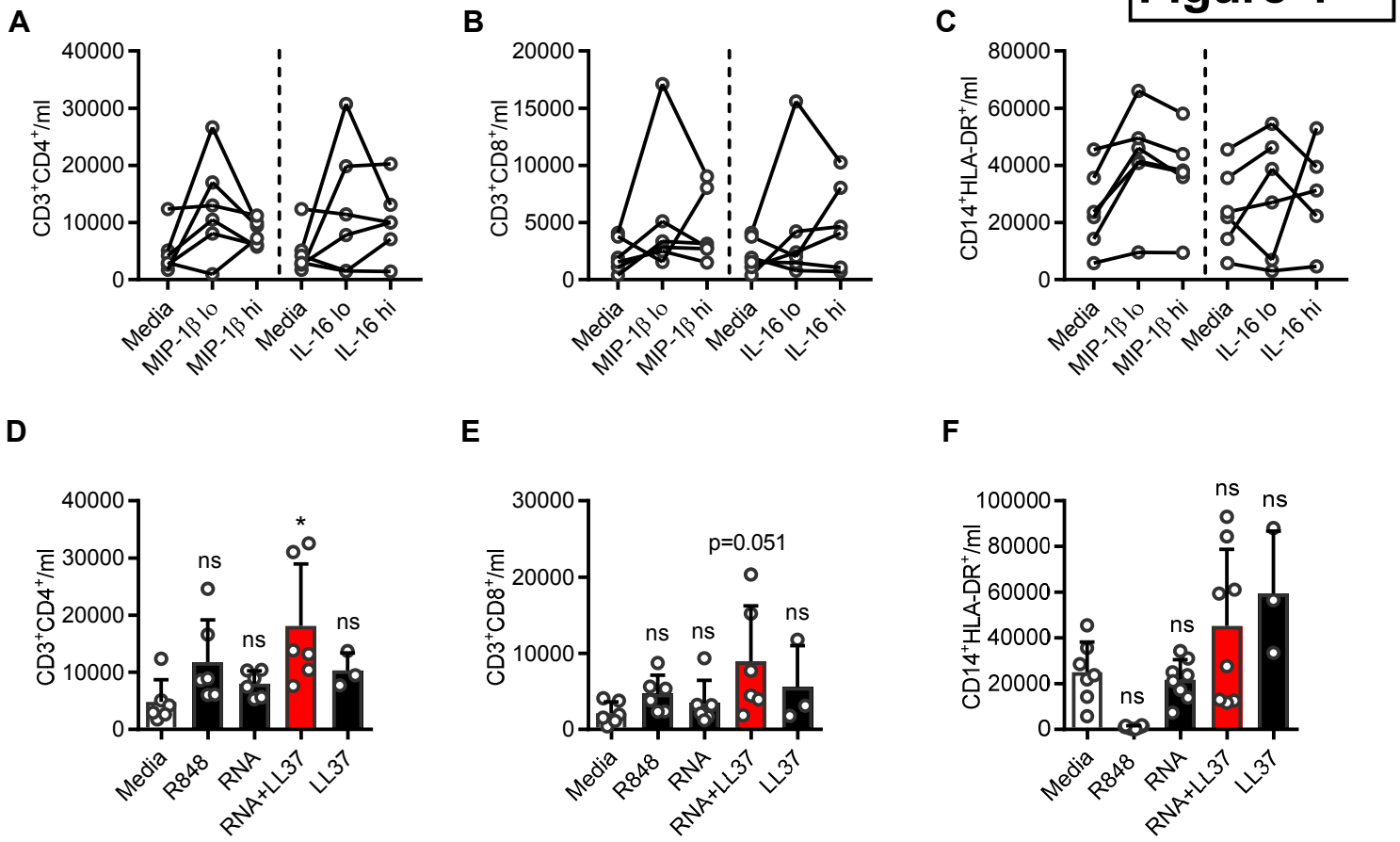
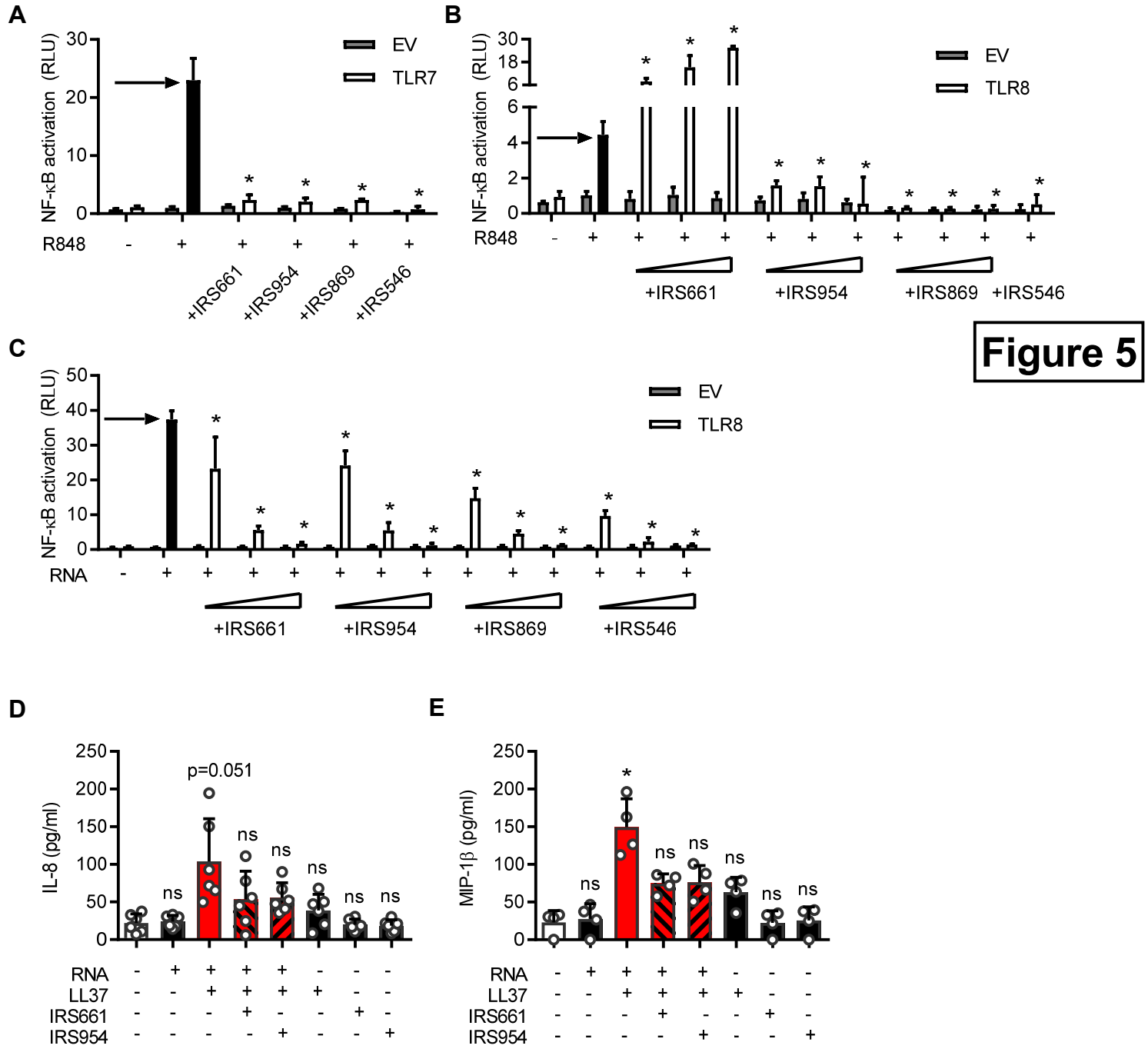

C
Psoriasis patients
Healthy controls
LL37 (ng/ml)
400
300
200
100
0
*
Unstim.

Figure 4


A
B
C
D
E
F
CD3+CD4+/ml
CD3+CD8+/ml
CD14+HLA-DR+/ml
Media
MIP-1β lo
MIP-1β hi
IL-16 lo
IL-16 hi
R848
RNA
RNA+LL37
LL37
ns
*
p=0.051


A
NF-κB activation (RLU)
EV
TLR7
R848
+IRS661
+IRS954
+IRS869
+IRS546
B
NF-κB activation (RLU)
EV
TLR8
R848
+IRS661
+IRS954
+IRS869
+IRS546
Figure 5
C
NF-κB activation (RLU)
EV
TLR8
RNA
+IRS661
+IRS954
+IRS869
+IRS546
D
IL-8 (pg/ml)
p=0.051
ns
RNA
LL37
IRS661
IRS954
E
MIP-1β (pg/ml)
ns
RNA
LL37
IRS661
IRS954