1   **Title**

2   **Neutrophil-extracellular trap-associated RNA (naRNA) and LL37**

3   **enable self-amplifying inflammation in psoriasis**

4

5   **Running title**

6   RNA and LL37 activate neutrophils in psoriasis

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Summary

Human and bacterial RNA in complex with LL37 activates neutrophils via TLR8 to release cytokines, chemokines and neutrophil extracellular traps (NETs). NETs and neutrophil-rich areas in psoriatic skin contain immunostimulatory RNA, termed here NET-associated RNA (naRNA), and LL37, which may fuel a self-sustaining inflammatory cycle in psoriasis.

Abstract

Psoriasis is an inflammatory skin disease with strong immune cell infiltration and high levels of the antimicrobial peptide, LL37. LL37 was shown to complex DNA and RNA to activate plasmacytoid dendritic cells (pDC) in vitro and this has been thought to drive interferon-mediated disease exacerbation. However, the physiological source of such nucleic acids and thus an early initiating event for nucleic-acid mediated inflammation remains unknown. We hypothesized that neutrophils (PMNs), which dominate the psoriatic skin infiltrate, may be the source of DNA and LL37 as well-established components of so-called neutrophil extracellular traps (NETs). We show here that indeed primary murine and human PMNs mount a fulminant NET and cytokine response, albeit not via DNA- but rather RNA-LL37 complexes. This was dependent on TLR8/TLR13 and sensitive to TLR inhibitory oligodeoxynucleotides. Unexpectedly, copious amounts of RNA were identified as a so far unappreciated NET component in vitro and in psoriatic but not healthy skin. Importantly, RNA-LL37-triggered NETs, when transferred to naïve PMNs, prompted additional de novo NET release. Our study highlights TLR-mediated RNA-LL37 sensing by PMNs as parts of a self-propagating vicious cycle that may later engulf pDC and establish chronic inflammation in psoriasis and NET-associated RNA (naRNA) as a physiologically relevant novel component.

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 (Eberle et al., 2016; Griffiths and Barker, 2007). The most common form of psoriasis, plaque psoriasis, is characterized by epidermal hyperplasia due to keratinocyte (KC) hyper-proliferation, increased endothelial proliferation and an infiltrate of leukocytes, such as dendritic cells, T cells and, prominently, polymorphonuclear leukocytes/neutrophils (PMNs) (Griffiths and Barker, 2007). The

70   high and early accumulation of PMNs in psoriatic plaques and micro-abscesses is well documented,

71   as well as an increase of PMNs in the circulation of psoriasis patients, but a specific causative role for

72   PMNs in disease initiation or exacerbation has so far not been defined (Sen et al., 2013) (Schon et al.,

73   2017). Although they are far less abundant in both blood and psoriatic skin, conversely, plasmacytoid

74   dendritic cells (pDCs) have received increased attention in psoriasis. This interest has been fueled by

75   the observations that pDCs can be activated by complexes consisting of either DNA or RNA with a

76   antimicrobial self-peptide that is highly abundant in psoriatic skin, namely LL37: LL37 was shown to

77   form complexes with DNA or RNA that resisted nuclease degradation, were readily taken up by pDCs,

78   and triggered high interferon (IFN) α release from pDCs (Ganguly et al., 2009; Lande et al., 2007).

79   Expression of TLR7 (a pattern recognition receptor for single-stranded RNA) and TLR9 (a receptor for

80   DNA) by pDCs was shown to be vital for the process in vitro.

81   However, as this scenario of pDC activation strictly requires the *prior* presence of DNA-LL37 and/or

82   RNA-LL37 complexes, the above-described pDC-related mechanism does not qualify as an early

83   initiating event in psoriasis and a process providing the three pDC-stimulating ingredients - LL37, DNA

84   and/or RNA – must be upstream. Unfortunately, the nature of this process has not been discovered

85   to date.

86   Conversely to pDCs, PMNs can release DNA via so-called neutrophil extracellular trap (NET)

87   formation (NETosis), an activation-induced process leading to the extrusion of nuclear DNA

88   (Brinkmann et al., 2004). In addition, cellular proteins are important components of NETs, and this

89   includes LL37 (Radic and Marion, 2013) for which PMNs are also the main producers in the skin

90   (Sorensen et al., 1997). LL37 is an amphipathic, positively-charged 37 amino acid peptide generated

91   from a precursor protein, the cathelicidin hCAP18 (Griffiths and Barker, 2007; Morizane and Gallo,

92   2012; Sorensen et al., 2001), that is stored in the secondary granules of PMNs, from where it can be

93   released upon activation (Sorensen et al., 1997).

94   PMNs thus combine the abilities to release (i) DNA and (ii) LL37 as components of immuno-

95   stimulatory ligand complexes via NETosis, although this has not been firmly linked to psoriasis. They

96   may themselves also sense such ligands via TLRs as expression of TLR8, another RNA sensor

97   (Eigenbrod et al., 2015), and TLR9 (Berger et al., 2012) (Lindau et al., 2013), but not TLR3 or TLR7

98   (Berger et al., 2012), has been demonstrated but not functionally evaluated. We therefore

99   hypothesized that PMNs may be the source of at least DNA and LL37 as immuno-stimulatory

100   components and that LL37-mediated DNA sensing via TLRs in PMNs might initiate and fuel

101   inflammatory cytokine production and thus inflammation and immune cell infiltration in psoriatic

102   skin.

103   We here present experimental evidence that human primary PMNs do not sense DNA-LL37 but

104   readily respond to RNA-LL37 complexes, leading to the release of a broad array of chemokines and

105   cytokines and, importantly, NETs, via TLR8 (human) and TLR13 (mouse). Unexpectedly, these NETs

106   contained RNA as a so-far unappreciated component, and they could propagate de novo NETosis in

107   naïve PMNs. PMNs, LL37 and, surprisingly, RNA was also highly abundant in psoriatic but not healthy

108   skin, indicating that PMNs and NET-derived RNA-LL37 complexes may function as integral

109   components of a self-propagating inflammatory cycle.

Results

LL37 promotes RNA uptake and induces PMN activation via endosomal TLRs

Previous results indicated that human primary PMNs can respond to RNA and DNA when stimulated for >12 h, albeit at much lower levels than when stimulated with the nucleoside analog TLR7/8 agonist, R848 (Janke et al., 2009; Lindau et al., 2013). We sought to re-evaluate these findings 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 with LL37 (Fig. 1B). In contrast, in pDCs, LL37 binding of natural DNA triggered potent TLR9 responses (Lande et al., 2007). In the absence of LL37, single-stranded synthetic RNA40 (henceforward referred to as 'RNA') barely caused IL-8 release when applied at equimolar concentrations with R848 (Fig. 1C). This suggests that on their own neither RNA nor DNA are 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 (Kuznik et al., 2011). However, robust IL-8 release and moderate CD62L shedding were observed when RNA was complexed with LL37 (Fig. 1C and *cf.* Fig. 2E). 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 (Schmidt et al., 2015). Flow cytometry using AlexaFluor (AF) 647-labeled RNA showed that LL37 also promoted the uptake of complexed RNA: >20% of PMNs incubated with labeled RNA in the presence of LL37 were AF647-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). Bright-field fluorescence microscopy with Atto488-labeled LL37 showed co-localization of AF647-RNA and Atto488-LL37 in intracellular compartments (Fig. 1H), where endosomal TLRs are expressed (Berger et al., 2012). To pharmacologically explore whether an endosomal TLR might be involved, 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). As chloroquine does not affect cytoplasmic RNA sensors (e.g. RIG-I) (Matsukura et al., 2007), this implicates an endosomal TLR-dependent in RNA-sensing. We next sought to check whether the observed effect on cytokine release extended to normal RNA from

144   mammalian cells which may be more physiologically relevant in the context of psoriasis. As shown in

145   Fig. 1J, human mRNA significantly induced IL-8 release, but only in combination with LL37. Since

146   microbiome analyses have recently revealed alterations in staphylo- and streptococcal skin

147   colonization in psoriasis patients (Alekseyenko et al., 2013) and bacterial RNA is an emerging

148   immuno-stimulatory pattern (Eigenbrod and Dalpke, 2015), we also tested whether LL37 promoted *S.*

149   *aureus*, i.e. bacterial RNA (bRNA) activation of PMNs. Fig. 1K shows that bRNA significantly

150   stimulated IL-8 release similarly to RNA40 when complexed with LL37. Taken together this suggests

151   that uptake of RNA is promoted by LL37 and directs RNA to intracellular compartments from which

152   TLR sensing and activation leading to cytokine release can occur – both in response to mammalian

153   and bacterial/foreign RNA.

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

155   **especially in psoriasis PMNs.**

156   To test whether RNA-LL37 also promoted the release of cytokines other than IL-8, we screened

157   supernatants of stimulated PMNs by Luminex multiplex analysis and detected TNF, IL-1β, IL-6, and –

158   unexpectedly – IL-16 and MIP-1β primarily or exclusively in RNA-LL37-stimulated cell supernatants,

159   respectively (Fig. S2A,B). Despite considerable variation between donors, this was confirmed by

160   cytometric bead arrays analyses (Fig. 2A-C) and ELISA (Fig. 2D, E) in samples from more donors. To

161   our knowledge, receptor-mediated release of the chemoattractants such as IL-16 (also known as

162   Lymphocyte Chemoattractant Factor, LCF) and MIP-1β (also known as Chemokine (C-C motif) Ligand,

163   CCL4) from primary human PMNs has not been reported. In fact, release of IL-16 was also boosted by

164   LL37 alone (Fig. 2D) and MIP-1β was strongly released from PMNs in response to RNA-LL37

165   complexes but not RNA alone (Fig. 2E).

166   Since IL-16 and MIP-1β are known chemoattractants for CD4 T cells and a variety of other immune

167   cells (Cruikshank and Little, 2008; Menten et al., 2002; Roth et al., 2016), we checked whether IL-16

168   or MIP-1β, at the concentrations observed in this study, could influence the migration and infiltration

169   of additional immune cells, which is a hallmark of psoriatic plaques (Schon et al., 2017). Transwell

170   experiments with peripheral blood mononuclear cells (PBMCs) from healthy human donors (upper

171   well) and IL-16 (300 and 1500 pg/ml), MIP-1β (30 and 150 pg/ml) or SDF-1α (100 ng/ml) as a control

172   (lower well) showed that the lowest concentration of both cytokines induced a donor-dependent

173   increase in the number of CD3+CD4+ helper, CD3+CD8+ cytotoxic T cells as well as CD14+HLA-DR+

174   monocytes (Figs. 2F-H and S2C-E). Possibly due to a non-linear dose response relationship observed

175   for some cytokines (Atanasova and Whitty, 2012), higher concentrations had a weaker effect in some

176   donors. Owing to the donor-to-donor variation generally observed throughout in the human system,

177   only a non-significant, moderate effect was observed. We separately tested whether RNA or RNA-

178   LL37 in the lower chamber had any direct influence on the migration of these cell populations and

179   noted an unexpected and significant chemoattractive effect on CD4+ T cells, in response to RNA-LL37

180   (Fig. 2I). Interestingly, the presence of PMNs, regardless of stimulus, was sufficient to attract a variety

181   of other cells (results not shown) due to a so far unknown mechanism.

182   When repeating the above described stimulation experiments with psoriasis PMNs compared to

183   PMNs from healthy donors, both IL-8 and MIP-1β release was significantly increased two-fold in

184   response to RNA-LL37 (Fig. 2J, K), but not to LPS (Fig. S2F, G). Whereas IL-8 release in response to

185   'RNA alone' (i.e. without LL37) was indistinguishable from 'unstimulated' samples in healthy donor

186   PMNs (*cf.* also Fig. 1C), psoriasis PMNs treated with 'RNA alone' (Fig. 2J, arrow) produced significantly

187   more IL-8 than both 'unstimulated' psoriasis PMNs and healthy donor PMNs stimulated with 'RNA

188   alone') Since in healthy donors, RNA seemed to strongly depend on LL37 for efficient PMN uptake

189   and stimulation (*cf*. Fig. 1E, F) and PMNs are the major contributors to elevated LL37 in psoriasis, we

190   speculated whether psoriasis PMNs might constitutively secrete LL37 and this endogenous LL37

191   might promote an increased responsiveness to exogenously added RNA alone in psoriasis PMNs.

192   Psoriasis PMNs constitutively showed an increased baseline release of LL37 compared to healthy

193   donors (Fig. 2L). We conclude that the combination of RNA with LL37 triggers the release of an

194   extended array of pro-inflammatory cytokines and chemoattractants not triggered by RNA alone.

195   This was more prominent in psoriasis PMNs, potentially due to a higher abundance of PMN-derived

196   LL37.

RNA-LL37 complexes trigger the release of RNA- and LL37-containing NETs (naRNA)

198   Our data so far indicate that RNA and LL37 might contribute to cytokine-mediated inflammation and

199   immune infiltration in neutrophil-containing skin lesions in psoriasis patients. Such neutrophil

200   responses would probably be temporary, unless RNA-LL37 triggered the release of additional RNA

201   and LL37. PMN NETs are known to contain extracellular DNA and DNA can act as an immune

202   stimulant of pDCs when complexed with LL37 (Lande et al., 2011). But DNA-LL37 cannot activate

203   PMNs (*cf.* Fig. 1B) and whether RNA is released by NETosis has not been shown. NETs would thus

204   only propagate PMN activation in case they also contained RNA. We therefore next tested (i)

205   whether RNA-LL37 induces NETosis and (ii) whether NETs contain RNA. Interestingly, electron

206   microscopy (Fig. 3A) and a elastase-based assay (see Methods) confirmed significant neutrophil

207   elastase release, a hallmark of NETosis, in response to RNA-LL37 and PMA (positive control), but also

208   with LL37 alone (Fig. 3B). However, fluorescence microscopy analysis of fixed PMN samples

209   established that only RNA-LL37 complexes, and not LL37 alone, prompted the formation of LL37-

210   positive NET-like structures (Fig. 3C). To check whether the NETs also contained RNA, an RNA-

211   selective fluorescent dye, SYTO RNAselect, was used to stain NETs. This staining showed released

212   endogenous RNA (green) in both PMA and RNA-LL37-mediated NETs (Fig. 3C). Although the dye's

213   specificity for RNA has been confirmed already (Li et al., 2006) we confirmed that RNA staining, but

214   not DNA staining, was sensitive to RNase A and thus RNA-specific (Fig. S3A, B). The presence of

215   cellular RNA in NET structures was also confirmed by the use of specific Abs against pseudo-uridine

216   (ΨU), a nucleotide absent from both DNA and also the synthetic RNA40 used for LL37 complex

217   formation and stimulation (Fig. 3D). Confirming the specificity of the stain, the signal was completely

218   RNase sensitive. Furthermore, using AF647- or AF488-labeled synthetic RNA for in vitro stimulation

219   both the extracellular SYTO RNAselect and anti-ΨU signals could be unequivocally attributed to de-

220   novo released 'cellular RNA' and distinguished from exogenously added 'stimulant (synthetic) RNA'

221   by (Fig. S3C, D). The same applied to LL37, using Atto488-LL37 (Fig. S3E). To further exclude artefacts

222   that might arise from staining fixed samples, RNA-LL37 mediated NETosis was also analyzed and

223   confirmed using live-cell time-lapse analysis of PMNs (Fig. 3E and Movies S1-S3, quantified in Fig. 3F).

224   Interestingly, in PMNs captured on the verge of NETosis, RNA staining in a granula-like fashion could

225   be clearly observed (Fig. S3F). Since increased levels of NETs containing DNA have already been

226   reported in the blood and skin of psoriasis patients (Hu et al., 2016), we next investigated whether

227   this was also the case for RNA. We therefore stained skin sections with SYTO RNAselect, anti-LL37

228   and anti-NE Abs. Indeed, psoriatic skin was highly positive for LL37; interestingly, the RNA signal was

229   also generally stronger when compared to healthy skin, using identical staining and microscopy

230   settings (Fig. 3G). RNA and LL37 were frequently co-localized in samples with high PMN infiltration

231   (anti-NE staining), (Fig. 3H and Movie S4). SYTO RNAselect specificity was also confirmed in skin

232   sections using anti-ΨU staining with both stains clearly overlapping (Fig. S3G). This indicates that

233   RNA-LL37 complexes may act as physiologically relevant activators in psoriatic skin. Further, the

234   ability of RNA-LL37 to drive primary PMNs to extrude NETs containing RNA in addition to DNA and

235   LL37 may represent the so far unknown activating event upstream of pDC activation.

236   Based on these results it also appeared plausible that RNA-LL37 complexes might trigger a NET-

237   mediated self-propagating inflammatory loop of repetitive PMN activation, in which NET material

238   would act in a manner similar to RNA-LL37 and activate unstimulated/naïve PMNs to undergo

239   NETosis, leading to repetitive cycles of immune activation. Indeed, when we transferred NETs

240   harvested from PMNs stimulated with PMA or RNA-LL37 complexes to unstimulated/naïve PMNs,

241   these responded with de-novo NET release in turn (Fig. 3J, quantified in I). Conversely, material

242   harvested from LL37 or untreated PMNs, which did not contain NETs, did not. Collectively, NETs

243   generated by initial RNA-LL37 activation had the capacity to activate naive PMNs to extrude further

244   DNA, RNA and LL37, thus providing the requirements for a self-propagating feed forward loop of

245   immune activation that might apply to skin PMNs. At the same time, we provide evidence for the so

246 far unappreciated existence and physiological relevance of RNA in NETs, which we would like to term

247 NET-associated RNA or naRNA.

RNA-LL37 complexes and NETs activate PMNs via TLR8/TLR13

249 In order to gain an insight which receptor mediates the observed effects of RNA-LL37 in primary

250 PMNs, we first compared BM-PMNs from WT mice and mice deficient for *Unc93b1*, a critical

251 chaperone of endosomal TLRs (Eigenbrod et al., 2012). Evidently, not only the cytokine response of

252 BM-PMNs to bRNA-LL37 (and the control stimulus CpG) was strictly dependent on *Unc93b1* and thus

253 endosomal TLRs (Fig. 4A); NETosis triggered by bRNA-LL37 complexes, but not the TLR-independent

254 control stimulus PMA, was also *Unc93b1* dependent (Fig. 4B).Given that RNA is a known dual TLR7

255 and TLR8 agonist (Colak et al., 2014) and primary human PMNs do not show *TLR7* expression (Berger

256 et al., 2012), we suspected TLR8 to mediate RNA-LL37-mediated PMN activation. Unfortunately,

257 human primary PMNs are not amenable to RNAi or genetic editing. Therefore, we first tested the

258 responsiveness of BM-PMNs from mice deficient for TLR13, the functional counterpart of human

259 TLR8 and primary bRNA sensor in mice (Eigenbrod and Dalpke, 2015). In murine PMNs both cytokine

260 and NET responses to bRNA-LL37 were strictly dependent on TLR13 (Fig. 4A, C), whereas the negative

261 controls LPS, *E. c*oli or PMA were not, respectively. Complementarily, the failure of *TLR8* CRISPR-

262 deleted BlaER1 monocytes (Vierbuchen et al., 2017) to respond to RNA-LL37 complexes (Fig. 4D)

263 indicated a dependence of RNA-LL37 on TLR8 in the human system. Thus the stimulatory potential of

264 RNA-LL37 complexes can be considered to be dependent on the TLR8/TLR13 RNA sensing system

265 (Eigenbrod et al., 2015).

PMN cytokine release and NET formation by RNA-LL37 can be blocked by iODNs

267 Having identified TLR8 as the responsible receptor in humans, we next wondered whether PMN

268 activation by RNA-LL37 could be blocked at the level of TLR activation by so-called inhibitory

269 oligonucleotides (iODNs). IRS869, IRS661 and IRS954 represent such TLR7 and 9-inhibitory iODNs that

270 were recently proposed for the treatment of another autoimmune disease, namely systemic lupus

271 erythematosus (SLE), whereas IRS546 was used as a non-inhibitory 'control' ODN (Barrat and

272 Coffman, 2008; Barrat et al., 2005; Duramad et al., 2005; Guiducci et al., 2010). Given the similarities

273 between endosomal TLRs (Colak et al., 2014), we speculated that some of these iODNs could

274 potentially also block TLR8, but the effects of iODNs on this receptor have not been studied

275 systematically. HEK293T cells do not express TLR7-9 but can be rendered responsive to their agonists

276 by exogenously expressing TLR7, TLR8 or TLR9 upon transfection (Colak et al., 2014). In HEK293T cells

277 transfected and stimulated in this way, IRS869 and IRS954 blocked NF-κB activation in response to

278 R848 for TLR7 and 8 (Figs. S4A and B) and CpG for TLR9 (Fig. S4C). IRS661 effectively blocked TLR7-

279 but not TLR9-mediated activation. IRS661 strongly increased R848-mediated NF-κB activation via

280   TLR8 (Fig. S4B), similar to observations made for T-rich ODNs combined with R848 (Jurk et al., 2006)

281   and highlighting the differences between R848 and RNA reported previously (Colak et al., 2014).

282   However, for RNA complexed with DOTAP (a synthetic complexing agent promoting endosomal

283   uptake (Yasuda et al., 2005), similarly to LL37), all three iODNs blocked TLR8-mediated NF-κB

284   activation (Fig. 4E). As the 'control' ODN IRS546 blocked NF-κB activation in response to TLR7-9

285   stimulation, in our hands it did not represent a proper control unlike previously published (Barrat et

286   al., 2005). Nevertheless, the effects of all iODNs seemed specific for endosomal TLR signaling as the

287   effect of all iODNs on NF-κB activity induced by TNF (a non-TLR receptor pathway activating NF-κB,

288   Fig. S4D) or MyD88 overexpression (TLR receptor-independent NF-κB activation, Fig. S4E) were

289   minor. Having excluded a toxic effect on primary PMNs (Fig. S4F), we investigated whether the

290   observed effects could be transferred to endogenous TLR8 activation in primary PMNs, where LL37

291   as a physiologically relevant uptake reagent was again used. Here, we found that iODNs IRS661 and

292   IRS954 were able to inhibit IL-8 release by primary PMNs from healthy donors in response to RNA-

293   LL37 at nanomolar iODN concentrations so that IL-8 levels were not statistically above background

294   (Fig. 4F). On the other hand, LPS-mediated IL-8 release was unaffected (Fig. S4G and (Saitoh et al.,

295   2012)). The blocking effect extended to MIP-1β (Fig. 4G), similar to the effect observed for

296   chloroquine (Fig. S4H). Importantly, NETosis by primary human PMNs could be effectively blocked by

297   nanomolar concentrations of IRS661 (Fig. 4H, quantified in Fig. 4I). These results are in agreement

298   with the observed TLR8-dependence and iODN-mediated inhibition of NETosis in HIV infections

299   (Saitoh et al., 2012) and indicate an applicability to RNA-LL37 complexes. In conclusion, known pre-

300   clinical iODNs are able to block RNA-LL37-mediated cytokine and NET release.

Discussion

Although systemic therapies with biologicals have brought benefit for many psoriasis patients in industrialized countries (Eberle et al., 2016), psoriasis remains a significant health problem worldwide. One reason is that early triggers and mechanisms leading to chronic disease, exacerbation and flare-ups are not well understood. Effects of DNA-/RNA-LL37 complexes on pDCs were reported as a novel mechanism in psoriasis inflammation (Ganguly et al., 2009; Lande et al., 2007). However, since pDCs are only a minor cell population even in psoriatic skin and they cannot release nucleic acids or LL37, their contribution to cell-mediated inflammation and the provision of self-ligands had to be restricted to amplifying an already ongoing pathology, characterized by pDC-attracting chemokines and an existing presence of self-ligands in the skin. This begged the question of how nucleic acid-LL37 complexes may be released in the skin to trigger subsequent immune activation. Since PMNs can release nucleic acids, constitute the main sources of LL37 in humans and congregate in psoriatic skin, we chose to study PMN RNA-LL37-mediated responses after TLR8 was identified as an RNA receptor on PMNs (Berger et al., 2012). We now provide novel evidence that PMNs are a likely early source of DNA, RNA and LL37 that may be at the heart of inflammation in situ.

We here found that the combination of RNA and LL37 produces an inflammatory ligand that is not only sensed by PMNs but it also prompted the release of further components for additional RNA-LL37 and DNA-LL37 complexes from PMNs. Whereas for pDCs both "inflammatory ingredients", RNA or DNA and LL37, required the presence of another cellular source first, a small number of activated and NETing PMNs – by releasing both, LL37 and RNA/DNA – may spark potent, self-propagating inflammation amongst PMNs and *in situ.* Following an initial activation of PMNs by endogenous RNA from damaged skin cells (so-called Köbner phenomenon), immune activation may get out of control due to chemokine release and NET formation (and thus nucleic acid and LL37 extrusion (Neumann et al., 2014a) which has been observed in the skin of psoriasis patients (Hu et al., 2016). Although this will require further investigation, our data for the first time raise the possibility that not only self-RNA, as previously proposed (Ganguly et al., 2009), but also foreign, e.g. bacterial RNA (Eigenbrod and Dalpke, 2015) (*cf.* Fig. 1K) or possibly fungal RNA may unfold immunostimulatory properties in the presence of LL37 triggered during minor skin injury. Although the concentration of the cytokines released after 4 h stimulation 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 over time. The PMN-mediated ongoing release of NET DNA, RNA and LL37 would eventually enable pDCs and other immune cells to join the vicious cycle of activation fueled by endogenous nucleic acids (Chamilos et al., 2012; Ganguly et al., 2009; Lande et al., 2007).

335   Although KC- and pDC-released type I IFNs contribute to psoriasis (Afshar et al., 2013; Yao et al.,

336   2008), TNF and IL-1β are additionally required for immune cell infiltration and T cell polarization,

337   respectively (Ghoreschi et al., 2007) and RNA-LL37-mediated activation of PMNs may be the so far

338   missing source of these cytokines. Subsequent cytokine-mediated inflammation is known to favor

339   hyper-proliferation (Hanel et al., 2013), LL37 production (Schauber et al., 2007), TLR9 responsiveness

340   (Morizane et al., 2012) and IFN production (Zhang et al., 2016) in KC, as well as T cell and monocyte

341   attraction and polarization (Ghoreschi et al., 2007). Our study thus raises the possibility that self-

342   amplifying inflammation mediated by RNA and LL37 via TLRs recognition and NETosis of PMNs may

343   represent an early and vital step in psoriasis development (Fig. S5).

344   An unexpected and novel finding that appears vital for this process and may have wide ramifications

345   in many other NET-related processes, is the observation that RNA is an abundant component of

346   mammalian NETs. Several hundred reports on NETs have so far not provided experimental evidence

347   that NET-associated RNA (naRNA) of physiological importance, let alone is contained in NETs. Our

348   data now demonstrate that naRNA is an abundant component of both RNA-LL37 and PMA-triggered

349   NETs. The observation that it also triggers further immune activation of PMNs additionally

350   demonstrates physiological relevance.   NaRNA could thus represent as a novel immunostimulatory

351   component within NETs, which has not been appreciated so far. Interestingly, insect NETs contain

352   primarily RNA rather than DNA (Altincicek et al., 2008), and eosinophils were reported to pre-store

353   RNA within their granules (Behzad et al., 2010), possibly for later extrusion. Of course, free RNA is not

354   stable over long periods, but LL37 may confer protection not only for NETs in general (Neumann et

355   al., 2014b) but also naRNA. NaRNA may thus be a common and functionally important NET

356   component which remains to be explored further. Given that extracellular RNA can also amplify

357   responses to other PRR ligands (Noll et al., 2017), the role of naRNA will thus be interesting to study

358   in other NET-related diseases, e.g. SLE (Gestermann et al., 2018), atherosclerosis (Warnatsch et al.,

359   2015) and even cancer (Gregoire et al., 2015), where the focus so far has been exclusively on NET

360   DNA. Furthermore, given that NETosis first and foremost has been described as an important host-

361   defense mechanism (Brinkmann et al., 2004), it remains to be investigated whether naRNA also

362   executes or participates in antimicrobial activities.

363   In conclusion, our study offers insights into how PMN RNA-LL37 sensing and NET propagation may

364   contribute to early disease development and, based on the observed effects of iODNs on PMN

365   activation, warrant the further exploration of TLR pathways and PMNs as targets for restricting

366   innate immune activation in psoriatic skin. Additionally, our study also calls for an exploration of

367   naRNA as a novel and intriguing new component of NETs in psoriasis and other processes.

Materials and Methods

Reagents

All chemicals were from Sigma unless otherwise stated. PRR agonists and LL37 were from Invivogen except RNA40 (iba Lifescience, normal and AF647- or AF488-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 was isolated as described (Eigenbrod et al., 2015). 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 (10 µg/ml) was mixed together with 10 µg LL37 (see Table S2, Atto-488 where indicated) and left for one hour at RT. For experiments with BM-PMNs, 1.25 µg bacterial RNA was complexed with 2.5 µg LL37. 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, ImageStream analysis or fluorescence microscopy 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 committees at the University Hospitals of Tübingen and Heidelberg, in accordance with the principles laid down in the Declaration of Helsinki as well as applicable laws and regulations. All blood or skin samples obtained from psoriasis patients (median age 41.8 years, PASI >10, no systemic treatments at the time of blood/skin sampling) were obtained at the University Hospitals Tübingen or Heidelberg, Departments of Dermatology, respectively, and were processed simultaneously with samples from at least one healthy donor matched for age and sex (recruited at the University of Tübingen, Department of Immunology). Skin sections were from 12 patients with Plaque Psoriasis and 1 patient with Psoriasis guttata.

Mice and isolation of bone-marrow derived PMNs (BM-PMNs)

Unc93b1$^{3d/3d}$-(Tabeta et al., 2006), *Tlr13*-deficient (Li and Chen, 2012) mice (both C57BL/6 background) and WT C57BL/6 mice between 8 and 20 weeks of age were used in accordance with local institutional guidelines on animal experiments and under specific locally approved protocols for sacrificing and *in vivo* work. All mouse colonies were maintained in line with local regulatory guidelines and hygiene monitoring. Bone-marrow (BM)-PMNs were isolated from the bone marrow using magnetic separation (mouse Neutrophil isolation kit, Miltenyi Biotec, 130-097-658) and following the manufacturer's instructions. PMN stimulation was carried out for 5 hours at 37 °C and 5% CO_2. Thereafter supernatants were harvested and used for ELISA. For microscopy the cells were stimulated for 16 h and subsequently stained.

Neutrophil isolation and stimulation

Whole blood (EDTA-anticoagulated) was diluted in PBS (Thermo Fisher, 14190-169), loaded on Ficoll (1.077 g/ml, Biocoll, ab211650) and centrifuged for 25 min at 506 x g at 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, sterile filtered, TH Geyer, 11682258)) and 1.6 x 10^6 cells/ml were seeded (24 well plate, 96 well plate). After resting for 30 min at 37°C, 5% CO_2, the cells were pre-treated with inhibitors (where indicated) for 30 min and subsequently stimulated with the indicated agonists for 4 hours (for ELISA) or for 30 min to 3 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 then carefully transferred into a new reaction tube and diluted in PBS (1:1). The cell suspension was spun down at 645 x g 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.

BlaER1 cells culture, transdifferentiation and stimulation

BlaER1 cells (WT and TLR8 -/- , kindly provided by Tatjana Eigenbrod from Heidelberg) were cultured in culture medium (VLE-RPMI (Merck Biochrom, FG1415) + 10 % FBS, 1 % Pen/Strep (Gibco, 15140122), 1 % sodium pyruvate (100 mM, Gibco, 11360070), 1 % HEPES soluation (Sigma, H0887)). After reaching a cell concentration not higher than 2 x 106/ml the cell were seeded in a 6 well plate (1 x 106 cells per well) and transdifferentiated in culture medium adding 150 nM β-estradiol, 10

432   ng/ml M-CSF and 10 ng/ml hIL-3 for 6 days including 2 medium changes (on day 2 and 5). On day 7

433   the adherent cells were counted again, seeded (96 well plate, 5 x 104 cells per well in culture

434   medium) and left to rest for 1h. Subsequently, they were stimulated for 18 h and the supernatants

435   were harvested and collected for ELISA measurements. The transdifferentiation efficiency was

436   verified by FACS analysis, using CD19, CD14 and CD11b as cell surface markers.

Flow cytometry

438   After PMN isolation and stimulation, the purity and activation status of neutrophils was determined

439   by flow cytometry. 200 µl of the cell suspension was transferred into a 96 well plate (U-shape) and

440   spun down for 5 min at 448 x g, 4 °C. FcR block was performed using pooled human serum diluted

441   1:10 in FACS buffer (PBS, 1 mM EDTA, 2% FBS heat inactivated) for 15 min at 4 °C. After washing, the

442   samples were stained for approximately 20-30 min at 4°C in the dark. Thereafter, fixation buffer (4%

443   PFA in PBS) was added to the cell pellets for 10 min at RT in the dark. After an additional washing

444   step, the cell pellets were resuspended in 150 µl FACS buffer. Measurements were performed on a

445   FACS Canto II from BD Bioscience, Diva software. Analysis was performed using FlowJo V10 analysis

446   software.

ELISA

448   Cytokines were determined in half-area plates (Greiner, Bio-one) using duplicates or triplicates and

449   measuring with a standard plate reader. The assays were performed according to the manufacturer's

450   instructions (Biolegend, R&D systems), using appropriate dilutions of the supernatants. For LL37

451   determination a kit from HycultBiotech (HK321-02) was used following the manufacturer's

452   instructions.

ImageStream analysis

454   ImageStream analysis was used to analyze internalization of RNA-LL37 complexes using spot-counts

455   and tracking single cells. The cells were first seeded in a 96 well plate, 8 x 10^6 cells/ml, 125 µl per

456   well. Subsequently, they were stimulated for 1 hour with RNA-AF647 (IBA technologies) and/or LL37-

457   Atto488 (kindly provided by Hubert Kalbacher, University of Tübingen). FcR block and surface

458   staining (here CD15 PE) was performed as described above. After fixation, the cells were

459   permeabilized with 0.05 % Saponin (Applichem, A4518.0100) for 15 min at RT in the dark. After

460   washing, nuclei were stained with Hoechst 33342 (Sigma, B2261, 1 µg/ml) for 5 min at RT in the dark,

461   washed and resuspended in 50 µl FACS buffer and transferred into a 1.5 ml Eppendorf tube. At least

462   10.000 cells were acquired for each sample with 40x magnification using an ImageStream X MKII with

463   the INSPIRE instrument controller software (Merck-Millipore/Amnis). Data were analyzed with IDEAS

464   Image analysis software. All samples were gated on single cells in focus.

Fluorescence Microscopy of fixed neutrophils

465

466 The cells were seeded in a 96 well plate at 1.6 x 10^6 cells/ml, 125 µl per well. Subsequently they were

467 stimulated with the complexes for 30 min and 1 h using RNA-AF647/AF488 and/or LL37-Atto488. FcR

468 block, staining, fixation and permeabilization were performed as for Flow cytometry. The cell pellets

469 were resuspended in 50-100 µl FACS buffer. 40 µl of the cell suspension was pipetted on a Poly-L-

470 Lysine coated coverslip (Corning, 734-1005) and the cells were left to attach for one hour in the dark.

471 ProLong Diamond Antifade (Thermo Fisher, P36965) was used to mount the coverslips on uncoated

472 microscopy slides. For NET analysis PMNs were seeded in 24 well plates, containing Poly-L-Lysine

473 coated coverslips and stimulated with RNA-LL37 complexes or PMA (600 nM) for 3 hours. NETs were

474 fixed and stained using the protocol from Brinkmann et al.(Brinkmann et al., 2010). Where indicated,

475 100 µg/ml RNase A (DNase, protease-free, ThermoFisher, EN0531) was added after fixation and

476 incubated overnight at 37°C. RNA was stained using SYTO RNAselect Green fluorescent dye (Thermo

477 Fisher, 50 µM) or anti-ΨU antibody (see Table S4) and nuclear DNA was stained with Hoechst33342

478 (Thermo Fisher, 1 µg/ml). LL37 and PMNs were visualized using an unconjugated rabbit anti-LL37

479 antibody or unconjugated mouse anti-NE with subsequent staining with an AF647-conjugated anti-

480 rabbit or an AF594-conjugated anti-mouse antibody, respectively (see Supplementary Table S4), after

481 blocking with pooled human serum (1:10 in PBS). Secondary antibodies alone did not yield any

482 significant staining. The slides were left to dry overnight at RT in the dark and were then stored at 4

483 °C before microscopy. The measurements were conducted with a Nikon Ti2 eclipse (100x

484 magnification) and the analysis was performed using Fiji analysis software.

Fluorescence Microscopy of tissue samples

485

486 Skin samples from psoriasis patients with a PASI ≥ 10 and without systemic treatment, and healthy

487 skin samples paraffin-embedded according to standard procedures were deparaffinized and

488 rehydrated using Roti Histol (Roth, 6640.1) and decreasing concentrations of ethanol (100%, 95%,

489 80% and 70%). After rinsing in ddH$_2$O, antigen retrieval was performed by boiling for 10-20 min in

490 citrate buffer (0.1 M, pH=6). The skin tissue was then washed 3 times for 5 min with PBS. Blocking

491 was performed using pooled human serum (1:10 in PBS) for 30 min at RT. The primary antibody was

492 added either overnight at 4°C or for 1 hour at RT. After 3 washes, the secondary antibody was added

493 for 30 min at RT in the dark. After another 3 washes, SYTO RNAselect Green fluorescent dye (Thermo

494 Fisher, 50 µM) was added for 20 min at RT in the dark. Thereafter, the samples were washed again

495 and Hoechst 33342 (ThermoFisher, 1 µg/ml) was added for 5 min. Then 3 last washes were

496 performed before using ProLong Diamond Antifade (Thermo Fisher, P36965) for mounting. The

497 samples were left to dry overnight at RT in the dark before being used for microscopy or stored at

498 4°C. The specimens were analyzed on a Nikon Ti2 eclipse bright-field fluorescence microscope (10x-

499 60x magnification) and the analysis was performed using Fiji analysis software. Autofluorescence in
500 multiple channels typical for the stratum corneum was labeled "AF".

Live cell imaging of primary neutrophils

502 Human neutrophils were isolated by magnetic separation using MACSxpress whole blood neutrophil
503 isolation kit (Miltenyi Biotec, 130-104-434) and were seeded into a micro-insert 4 well dish (Ibidi
504 80406). Hoechst 33342 (1 µg/ml) and SYTO RNAselect Green fluorescent dye (50 µM) were added to
505 the cells and incubated for 20 min at 37°C, 5 % CO_2. Live cell imaging was performed by using Nikon
506 Ti2 eclipse bright-field fluorescence microscope (40x magnification) including a CO_2-O_2 controller
507 from Okolab. Measurements were started immediately after adding of stimuli. Time-lapse analysis
508 was performed by taking pictures every 3 min for at least 2 hours. Image analysis was performed
509 using NIS Elements from Nikon and Fiji analysis software.

Luminex cytokine multiplex analysis

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

Cytometric bead array

520 A cytometric bead array was performed using the "Human inflammatory cytokine kit" from BD
521 Bioscience (551811) and following the manufacturer's instruction. 25 µl of samples and standards
522 were added to 25 µl of the capturing bead mixture. Additionally, 25 µl of PE detection reagent was
523 added to all tubes and incubated for 3 h at RT in the dark. Thereafter, 1 ml of wash buffer was added
524 to each tube and centrifuged at 200 x g for 5 minutes. The supernatant was carefully removed and
525 the pellet was resuspended in 300 µl wash buffer. Measurements were performed with the FACS
526 Canto II from BD Bioscience operated using Diva software. Analysis was performed with Soft Flow
527 FCAP Array v3 analysis software from BD Bioscience.

Transwell experiments

529 PBMCs and neutrophils were always used from the same donor in each experiment. Transwell inserts
530 were loaded with 100 µl of PBMC suspension (0.8 x 10^6 cells/insert). In the lower chamber, either
531 PMNs were seeded as described above (same plate size, same volume, same cell concentration)

532   using polycarbonate, 24 well plates, 3 µm pores, Corning, 734-1570) and stimulated for 4 h with

533   stimuli as indicated. Alternatively, media containing the stimuli only (i.e. no PMNs) or media

534   containing only MIP-1β (30 and 150 pg /ml), IL-16 (300 and 1500 pg/ml) or SDF-1α (control, 100

535   ng/ml) and no PMNs were added. After 4 h, the lower compartment was harvested and FACS staining

536   was performed as described above. The total number of migrated cells was acquired using counting

537   beads (Biolegend, 424902) on a FACS Canto II (BD Bioscience) with Diva software. Analysis was

538   performed using FlowJo V10 analysis software.

Neutrophil elastase NETosis assay

540   Neutrophil extracellular trap formation was determined using the colorimetric NETosis Assay Kit from

541   Cayman Chemicals based on the enzymatic activity of NET-associated neutrophil elastase. PMNs from

542   various healthy donors were isolated as described above and stimulated with RNA-LL37 complex, or

543   PMA and a calcium ionophore (A-23187) as positive controls for 1 or 3 hours. The assay was

544   performed following the manufacturer's instructions. The absorbance was then measured at 405 nm

545   using a standard plate reader.

Transient transfection of HEK293T cells

547   HEK293T cells were transiently transfected using the $CaPO_4$ method as described (Colak et al., 2014).

548   Cells were seeded in 24-well plates at a density of 14×10^5 cells/ml 2-3 h prior to transfection. For the

549   transfection of one well, 310 ng of plasmid DNA (100 ng TLR plasmid, 100 ng firefly luciferase NF-κB

550   reporter, 10 ng *Renilla* luciferase control reporter, and 100 ng EGFP plasmid) was mixed with 1.2 µl of

551   a 2 M $CaCl_2$ solution and filled up with sterile endotoxin-free H_2O to obtain a total reaction volume of

552   10 µl. After the addition of 10 µl of 2X HBS solution (50 mM HEPES (pH 7.05), 10 mM KCl, 12 mM

553   Glucose, 1.5 mM Na_2HPO_4), the mixture was then added to the cell suspension. As negative controls,

554   TLR coding plasmids were replaced by empty vectors carrying the appropriate backbone of the TLR

555   plasmids. After the addition of the transfection complexes, the cells were incubated either for 24 h

556   followed by stimulation, or kept for 48 h without stimulation (MyD88 expression). For stimulation,

557   the media was aspirated and replaced by fresh growth medium in which TNFα or the different TLR

558   ligands (R848, CpG, RNA40) with or without IRS were diluted to appropriate concentrations. TLR8

559   activation with RNA40 was facilitated by complexation of the RNA with DOTAP (L787.1, Roth). RNA40

560   and DOTAP were first diluted in 1X HBS separately. Next, RNA40/HBS was diluted 1:3 in DOTAP/HBS.

561   The solution was carefully mixed by pipetting up and down. After 15 min of incubation at RT, the

562   mixture was 1:6.7 diluted in growth medium (with or without IRS) and finally dispensed (500 µl/well)

563   into the wells containing transfected HEK293T cells. Each tested condition was measured in

564   triplicates. The cells were stimulated and inhibited for 24 h at 37°C.

565 **Dual Luciferase Reporter Assay**

566 After checking transfection efficiency via EGFP fluorescence microscopy, HEK293T supernatants were

567 aspirated and 60 µl of 1X passive lysis buffer (E194A, Promega) added per well. The plate was then

568 incubated for 15 min at RT on the plate shaker and subsequently stored at -80 °C for at least 15

569 minutes to facilitate complete cell lysis. After thawing, 60 µl were transferred into a V-bottom 96-

570 well plate and centrifuged for 10 min at 2500 rpm and 4 °C to pellet cell debris. 10 µl supernatant

571 were then transferred into a white microplate and each condition was measured in triplicates using

572 the FLUOstar OPTIMA device (BMG Labtech). Firefly and Renilla luciferase activity were determined

573 using the Promega Dual luciferase kit. Both enzyme activities were measured for 12.5 s with 24

574 intervals of 0.5 s, respectively. The data was analyzed by calculating the ratio of the two measured

575 signals, thereby normalizing each firefly luciferase signal to its corresponding Renilla luciferase signal.

576 The ratios were represented as the relative light units (RLU) of NF-κB activation.

577 **Statistics**

578 Experimental data was analyzed using Excel 2010 (Microsoft) and/or GraphPad Prism 6 or 7,

579 microscopy data with ImageJ/Fiji, flow cytometry data with FlowJo 10. In case of extreme values,

580 outliers were statistically identified using the ROUT method at high (0.5%) stringency. Normal

581 distribution in each group was always tested using the Shapiro-Wilk test first for the subsequent

582 choice of a parametric (ANOVA, Student's t-test) or non-parametric (e.g. Friedman, Mann-Whitney U

583 or Wilcoxon) test. p-values ($\alpha=0.05$) were then calculated and multiple testing was corrected for in

584 Prism, as indicated in the figure legends. Values < 0.05 were generally considered statistically as

585 significant and denoted by * throughout. Comparisons made to unstimulated control, unless

586 indicated otherwise, denoted by brackets.

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Supplementary Material

Is uploaded separately and includes 5 Supplementary figures and 6 supplementary tables.

Authorship contributions

F.H., Z.B., S.D., D.E., T.K. and N.S.M. performed experiments; F.H., Z.B., S.D., D.E., N.S.M. and A.N.R.W. analyzed data; M.H., H. K., M.W.L., L.F., K.S., K.G. and T.E. were involved in sample and reagent acquisition; F.H., D.H., K.G. and T.E. contributed to the conceptual development of the study; F.H. and A.N.R.W. wrote the manuscript and all authors commented on an revised the manuscript; A.N.R.W. coordinated and supervised the entire study. None of the authors declare competing interests.

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References

Afshar, M., A.D. Martinez, R.L. Gallo, and T.R. Hata. 2013. Induction and exacerbation of psoriasis with Interferon-alpha therapy for hepatitis C: a review and analysis of 36 cases. J Eur Acad Dermatol Venereol 27:771-778.

Alekseyenko, A.V., G.I. Perez-Perez, A. De Souza, B. Strober, Z. Gao, M. Bihan, K. Li, B.A. Methe, and M.J. Blaser. 2013. Community differentiation of the cutaneous microbiota in psoriasis. Microbiome 1:31.

Altincicek, B., S. Stotzel, M. Wygrecka, K.T. Preissner, and A. Vilcinskas. 2008. Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. J Immunol 181:2705-2712.

Atanasova, M., and A. Whitty. 2012. Understanding cytokine and growth factor receptor activation mechanisms. Crit Rev Biochem Mol Biol 47:502-530.

Barrat, F.J., and R.L. Coffman. 2008. Development of TLR inhibitors for the treatment of autoimmune diseases. Immunol Rev 223:271-283.

622 Barrat, F.J., T. Meeker, J. Gregorio, J.H. Chan, S. Uematsu, S. Akira, B. Chang, O. Duramad, and R.L.
623 Coffman. 2005. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like
624 receptors and may promote systemic lupus erythematosus. The Journal of experimental medicine
625 202:1131-1139.

626 Behzad, A.R., D.C. Walker, T. Abraham, J. McDonough, S. Mahmudi-Azer, F. Chu, F. Shaheen, J.C.
627 Hogg, and P.D. Pare. 2010. Localization of DNA and RNA in eosinophil secretory granules. Int Arch
628 Allergy Immunol 152:12-27.

629 Berger, M., C.Y. Hsieh, M. Bakele, V. Marcos, N. Rieber, M. Kormann, L. Mays, L. Hofer, O. Neth, L.
630 Vitkov, W.D. Krautgartner, D. von Schweinitz, R. Kappler, A. Hector, A. Weber, and D. Hartl. 2012.
631 Neutrophils express distinct RNA receptors in a non-canonical way. The Journal of biological
632 chemistry 287:19409-19417.

633 Brinkmann, V., B. Laube, U. Abu Abed, C. Goosmann, and A. Zychlinsky. 2010. Neutrophil
634 extracellular traps: how to generate and visualize them. J Vis Exp

635 Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, and A.
636 Zychlinsky. 2004. Neutrophil extracellular traps kill bacteria. Science 303:1532-1535.

637 Chamilos, G., J. Gregorio, S. Meller, R. Lande, D.P. Kontoyiannis, R.L. Modlin, and M. Gilliet. 2012.
638 Cytosolic sensing of extracellular self-DNA transported into monocytes by the antimicrobial peptide
639 LL37. Blood 120:3699-3707.

640 Colak, E., A. Leslie, K. Zausmer, E. Khatamzas, A.V. Kubarenko, T. Pichulik, S.N. Klimosch, A. Mayer, O.
641 Siggs, A. Hector, R. Fischer, B. Klesser, A. Rautanen, M. Frank, A.V. Hill, B. Manoury, B. Beutler, D.
642 Hartl, A. Simmons, and A.N. Weber. 2014. RNA and imidazoquinolines are sensed by distinct TLR7/8
643 ectodomain sites resulting in functionally disparate signaling events. J Immunol 192:5963-5973.

644 Cruikshank, W., and F. Little. 2008. Interleukin-16: The Ins and Outs of Regulating T-Cell Activation.
645 Critical Reviews in Immunology 28:467-483.

646 Duramad, O., K.L. Fearon, B. Chang, J.H. Chan, J. Gregorio, R.L. Coffman, and F.J. Barrat. 2005.
647 Inhibitors of TLR-9 act on multiple cell subsets in mouse and man in vitro and prevent death in vivo
648 from systemic inflammation. J Immunol 174:5193-5200.

649 Eberle, F.C., J. Bruck, J. Holstein, K. Hirahara, and K. Ghoreschi. 2016. Recent advances in
650 understanding psoriasis. F1000Res 5:

651 Eigenbrod, T., and A.H. Dalpke. 2015. Bacterial RNA: An Underestimated Stimulus for Innate Immune
652 Responses. J Immunol 195:411-418.

653   Eigenbrod, T., L. Franchi, R. Munoz-Planillo, C.J. Kirschning, M.A. Freudenberg, G. Nunez, and A.
654   Dalpke. 2012. Bacterial RNA mediates activation of caspase-1 and IL-1beta release independently of
655   TLRs 3, 7, 9 and TRIF but is dependent on UNC93B. J Immunol 189:328-336.

656   Eigenbrod, T., K. Pelka, E. Latz, B. Kreikemeyer, and A.H. Dalpke. 2015. TLR8 Senses Bacterial RNA in
657   Human Monocytes and Plays a Nonredundant Role for Recognition of Streptococcus pyogenes. J
658   Immunol 195:1092-1099.

659   Ganguly, D., G. Chamilos, R. Lande, J. Gregorio, S. Meller, V. Facchinetti, B. Homey, F.J. Barrat, T. Zal,
660   and M. Gilliet. 2009. Self-RNA-antimicrobial peptide complexes activate human dendritic cells
661   through TLR7 and TLR8. The Journal of experimental medicine 206:1983-1994.

662   Gestermann, N., J. Di Domizio, R. Lande, O. Demaria, L. Frasca, L. Feldmeyer, J. Di Lucca, and M.
663   Gilliet. 2018. Netting Neutrophils Activate Autoreactive B Cells in Lupus. J Immunol 200:3364-3371.

664   Ghoreschi, K., C. Weigert, and M. Rocken. 2007. Immunopathogenesis and role of T cells in psoriasis.
665   Clin Dermatol 25:574-580.

666   Gregoire, M., F. Guilloton, C. Pangault, F. Mourcin, P. Sok, M. Latour, P. Ame-Thomas, E. Flecher, T.
667   Fest, and K. Tarte. 2015. Neutrophils trigger a NF-kappaB dependent polarization of tumor-
668   supportive stromal cells in germinal center B-cell lymphomas. Oncotarget 6:16471-16487.

669   Griffiths, C.E., and J.N. Barker. 2007. Pathogenesis and clinical features of psoriasis. Lancet 370:263-
670   271.

671   Guiducci, C., M. Gong, Z. Xu, M. Gill, D. Chaussabel, T. Meeker, J.H. Chan, T. Wright, M. Punaro, S.
672   Bolland, V. Soumelis, J. Banchereau, R.L. Coffman, V. Pascual, and F.J. Barrat. 2010. TLR recognition of
673   self nucleic acids hampers glucocorticoid activity in lupus. Nature 465:937-941.

674   Hanel, K.H., C. Cornelissen, B. Luscher, and J.M. Baron. 2013. Cytokines and the skin barrier. Int J Mol
675   Sci 14:6720-6745.

676   Hu, S.C., H.S. Yu, F.L. Yen, C.L. Lin, G.S. Chen, and C.C. Lan. 2016. Neutrophil extracellular trap
677   formation is increased in psoriasis and induces human beta-defensin-2 production in epidermal
678   keratinocytes. Sci Rep 6:31119.

679   Janke, M., J. Poth, V. Wimmenauer, T. Giese, C. Coch, W. Barchet, M. Schlee, and G. Hartmann. 2009.
680   Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8. The
681   Journal of allergy and clinical immunology 123:1026-1033.

682   Jurk, M., A. Kritzler, B. Schulte, S. Tluk, C. Schetter, A.M. Krieg, and J. Vollmer. 2006. Modulating
683   responsiveness of human TLR7 and 8 to small molecule ligands with T-rich phosphorothiate
684   oligodeoxynucleotides. European Journal of Immunology 36:1815-1826.

685   Kuznik, A., M. Bencina, U. Svajger, M. Jeras, B. Rozman, and R. Jerala. 2011. Mechanism of
686   endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. Journal of Immunology
687   186:4794-4804.

688   Lande, R., D. Ganguly, V. Facchinetti, L. Frasca, C. Conrad, J. Gregorio, S. Meller, G. Chamilos, R.
689   Sebasigari, V. Riccieri, R. Bassett, H. Amuro, S. Fukuhara, T. Ito, Y.J. Liu, and M. Gilliet. 2011.
690   Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in
691   systemic lupus erythematosus. Sci Transl Med 3:73ra19.

692   Lande, R., J. Gregorio, V. Facchinetti, B. Chatterjee, Y.H. Wang, B. Homey, W. Cao, B. Su, F.O. Nestle,
693   T. Zal, I. Mellman, J.M. Schroder, Y.J. Liu, and M. Gilliet. 2007. Plasmacytoid dendritic cells sense self-
694   DNA coupled with antimicrobial peptide. Nature 449:564-569.

695   Li, Q., Y. Kim, J. Namm, A. Kulkarni, G.R. Rosania, Y.H. Ahn, and Y.T. Chang. 2006. RNA-selective, live
696   cell imaging probes for studying nuclear structure and function. Chem Biol 13:615-623.

697   Li, X.D., and Z.J. Chen. 2012. Sequence specific detection of bacterial 23S ribosomal RNA by TLR13.
698   Elife 1:e00102.

699   Lindau, D., J. Mussard, B.J. Wagner, M. Ribon, V.M. Ronnefarth, M. Quettier, I. Jelcic, M.C. Boissier,
700   H.G. Rammensee, and P. Decker. 2013. Primary blood neutrophils express a functional cell surface
701   Toll-like receptor 9. European Journal of Immunology 43:2101-2113.

702   Matsukura, S., F. Kokubu, M. Kurokawa, M. Kawaguchi, K. Ieki, H. Kuga, M. Odaka, S. Suzuki, S.
703   Watanabe, T. Homma, H. Takeuchi, K. Nohtomi, and M. Adachi. 2007. Role of RIG-I, MDA-5, and PKR
704   on the expression of inflammatory chemokines induced by synthetic dsRNA in airway epithelial cells.
705   Int Arch Allergy Immunol 143 Suppl 1:80-83.

706   Menten, P., A. Wuyts, and J. Van Damme. 2002. Macrophage inflammatory protein-1. Cytokine
707   Growth Factor Rev 13:455-481.

708   Morizane, S., and R.L. Gallo. 2012. Antimicrobial peptides in the pathogenesis of psoriasis. J Dermatol
709   39:225-230.

710   Morizane, S., K. Yamasaki, B. Muhleisen, P.F. Kotol, M. Murakami, Y. Aoyama, K. Iwatsuki, T. Hata,
711   and R.L. Gallo. 2012. Cathelicidin antimicrobial peptide LL-37 in psoriasis enables keratinocyte
712   reactivity against TLR9 ligands. The Journal of investigative dermatology 132:135-143.

713   Neumann, A., E.T. Berends, A. Nerlich, E.M. Molhoek, R.L. Gallo, T. Meerloo, V. Nizet, H.Y. Naim, and
714   M. von Kockritz-Blickwede. 2014a. The antimicrobial peptide LL-37 facilitates the formation of
715   neutrophil extracellular traps. Biochem J 464:3-11.

716   Neumann, A., L. Vollger, E.T. Berends, E.M. Molhoek, D.A. Stapels, M. Midon, A. Friaes, A. Pingoud,

717   S.H. Rooijakkers, R.L. Gallo, M. Morgelin, V. Nizet, H.Y. Naim, and M. von Kockritz-Blickwede. 2014b.

718   Novel role of the antimicrobial peptide LL-37 in the protection of neutrophil extracellular traps

719   against degradation by bacterial nucleases. J Innate Immun 6:860-868.

720   Noll, F., J. Behnke, S. Leiting, K. Troidl, G.T. Alves, H. Muller-Redetzky, K.T. Preissner, and S. Fischer.

721   2017. Self-extracellular RNA acts in synergy with exogenous danger signals to promote inflammation.

722   PLoS One 12:e0190002.

723   Radic, M., and T.N. Marion. 2013. Neutrophil extracellular chromatin traps connect innate immune

724   response to autoimmunity. Seminars in immunopathology 35:465-480.

725   Roth, S., W. Solbach, and T. Laskay. 2016. IL-16 and MIF: messengers beyond neutrophil cell death.

726   Cell Death Dis 7:e2049.

727   Saitoh, T., J. Komano, Y. Saitoh, T. Misawa, M. Takahama, T. Kozaki, T. Uehata, H. Iwasaki, H. Omori,

728   S. Yamaoka, N. Yamamoto, and S. Akira. 2012. Neutrophil extracellular traps mediate a host defense

729   response to human immunodeficiency virus-1. Cell Host Microbe 12:109-116.

730   Schauber, J., R.A. Dorschner, A.B. Coda, A.S. Buchau, P.T. Liu, D. Kiken, Y.R. Helfrich, S. Kang, H.Z.

731   Elalieh, A. Steinmeyer, U. Zugel, D.D. Bikle, R.L. Modlin, and R.L. Gallo. 2007. Injury enhances TLR2

732   function and antimicrobial peptide expression through a vitamin D-dependent mechanism. The

733   Journal of clinical investigation 117:803-811.

734   Schmidt, N.W., F. Jin, R. Lande, T. Curk, W. Xian, C. Lee, L. Frasca, D. Frenkel, J. Dobnikar, M. Gilliet,

735   and G.C. Wong. 2015. Liquid-crystalline ordering of antimicrobial peptide-DNA complexes controls

736   TLR9 activation. Nat Mater 14:696-700.

737   Schon, M.P., S.M. Broekaert, and L. Erpenbeck. 2017. Sexy again: the renaissance of neutrophils in

738   psoriasis. Exp Dermatol 26:305-311.

739   Sen, B.B., E.N. Rifaioglu, O. Ekiz, M.U. Inan, T. Sen, and N. Sen. 2013. Neutrophil to lymphocyte ratio

740   as a measure of systemic inflammation in psoriasis. Cutan Ocul Toxicol

741   Sorensen, O., K. Arnljots, J.B. Cowland, D.F. Bainton, and N. Borregaard. 1997. The human

742   antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to

743   specific granules in neutrophils. Blood 90:2796-2803.

744   Sorensen, O.E., P. Follin, A.H. Johnsen, J. Calafat, G.S. Tjabringa, P.S. Hiemstra, and N. Borregaard.

745   2001. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular

746   cleavage with proteinase 3. Blood 97:3951-3959.

747 Tabeta, K., K. Hoebe, E.M. Janssen, X. Du, P. Georgel, K. Crozat, S. Mudd, N. Mann, S. Sovath, J.
748 Goode, L. Shamel, A.A. Herskovits, D.A. Portnoy, M. Cooke, L.M. Tarantino, T. Wiltshire, B.E.
749 Steinberg, S. Grinstein, and B. Beutler. 2006. The Unc93b1 mutation 3d disrupts exogenous antigen
750 presentation and signaling via Toll-like receptors 3, 7 and 9. Nature Immunology 7:156-164.

751 Vierbuchen, T., C. Bang, H. Rosigkeit, R.A. Schmitz, and H. Heine. 2017. The Human-Associated
752 Archaeon Methanosphaera stadtmanae Is Recognized through Its RNA and Induces TLR8-Dependent
753 NLRP3 Inflammasome Activation. Front Immunol 8:1535.

754 Warnatsch, A., M. Ioannou, Q. Wang, and V. Papayannopoulos. 2015. Inflammation. Neutrophil
755 extracellular traps license macrophages for cytokine production in atherosclerosis. Science 349:316-
756 320.

757 Yao, Y., L. Richman, C. Morehouse, M. de los Reyes, B.W. Higgs, A. Boutrin, B. White, A. Coyle, J.
758 Krueger, P.A. Kiener, and B. Jallal. 2008. Type I interferon: potential therapeutic target for psoriasis?
759 PLoS One 3:e2737.

760 Yasuda, K., P. Yu, C.J. Kirschning, B. Schlatter, F. Schmitz, A. Heit, S. Bauer, H. Hochrein, and H.
761 Wagner. 2005. Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-
762 dependent and -independent pathways. J Immunol 174:6129-6136.

763 Zhang, L.J., G.L. Sen, N.L. Ward, A. Johnston, K. Chun, Y. Chen, C. Adase, J.A. Sanford, N. Gao, M.
764 Chensee, E. Sato, Y. Fritz, J. Baliwag, M.R. Williams, T. Hata, and R.L. Gallo. 2016. Antimicrobial
765 Peptide LL37 and MAVS Signaling Drive Interferon-beta Production by Epidermal Keratinocytes
766 during Skin Injury. Immunity 45:119-130.

767

768   Abbreviations

769 APC – antigen-presenting cell; bRNA – bacterial ribonucleic acid; Enhanced green fluorescence
770 protein – EGFP; Human embryonic kidney – HEK; HLA – Human leucocyte antigen; iODN – inhibitory
771 oligonucleotides; Microbe-associated molecular pattern – MAMPs; MHC – Major histocompatibility
772 complex; Inhibitory oligodeoxynucleotides – iODNs; Interferon – IFN; Interleukin – IL; Keratinocytes –
773 KC; PASI – psoriasis area and severity; Plasmacytoid dendritic cells – pDCs; PMA – phorbol myristate
774 acetate; Pattern recognition receptor – PRR; Polymorphonuclear leukocytes – PMNs; NE - Neutrophil
775 elastase; Neutrophil extracellular trap - NET; Scanning electron microscopy – SEM; Systemic lupus
776 erythematosus – SLE; TLR – Toll-like receptor; Tumor necrosis factor (TNF).

777   **Figure legends**

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

779   (A-C) IL-8 release (ELISA, 4 h stimulation) and CD62L shedding (flow cytometry, 1 or 2h) by PMNs

780   from healthy donors stimulated with LPS, R848 or CpG ODN (A, n=6-7), ssDNA and genomic DNA (B,

781   n=5), or RNA40 (C, n=8-15), with or without LL37. (D) Electron microscopy of PMNs incubated with

782   RNA-LL37 for 20 min (n=1). (E-G) FACS (E, n=6), ImageStream cytometry (F, G, scale bar = 10 μm) or

783   conventional bright-field microscopy (H, n=2, scale bar = 10 μm) of PMNs incubated for 60 min with

784   RNA-AF647 complexed with LL37 (n=6). In F the % of cells with 'internalized' features are shown

785   (n=2, see Methods), in G selected cells from one representative donor are shown. In H unmodified

786   LL37 was replaced with Atto488-labeled LL37, one representative donor shown. (I) as in C but

787   including chloroquine (CQ) pre-incubation for 30 min (n=6-7). (J) and (K) as in C but using total human

788   RNA from HEK293T cells (J, n=4-6) or bacterial RNA isolated from *S. aureus* (K, n=4) with and without

789   LL37. A-C, E, F, I-K represent combined data (mean+SD) from 'n' biological replicates (each dot

790   represents one donor). In D, G and H one representative of 'n' biological replicates (donors) is shown

791   (mean+SD). * p<0.05 according to one-way ANOVA with Dunnett's correction (A, B, C, I, J) or

792   Friedman test with Dunn's correction (E, K).

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

794   **chemokines, especially in psoriasis PMN**

795   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)

796   secreted from PMNs stimulated for 4 h as indicated (n=6). Flow cytometric cell counts of migrated

797   CD4 T cells (F, I), CD8 T cells (G) and CD14^{+}HLA-DR^{+} monocytes (H) quantified in transwell assays with

798   total PBMCs in the upper and either MIP-1β (30 and 150 pg/ml) or IL-16 (300 and 1500 pg/ml) (F-H,

799   n=6-7, p>0.05 for treatments vs. media) or R848, RNA with and without LL37 (I, n=3-7) in the lower

800   chamber. (J-K) ELISA of IL-8 and MIP-1β secreted from psoriasis PMNs (n=3 patients, 10 healthy

801   controls, squares, chequered bars) or PMNs from sex-and age-matched healthy donors.(L) as in J or K

802   but ELISA for LL37 (n=4 patients, 7 healthy donors). A-K represent combined data (mean+SD) from 'n'

803   biological replicates (each dot represents one donor). * p<0.05 according to Friedmann test with

804   Dunn's correction (A, B, D-H), or one-way ANOVA with Dunnett's correction for multiple testing (C, I,

805   J-L).

806   **Figure 3: RNA-LL37 trigger the release of NETs containing further RNA, DNA and LL37**

807   (A) EM pictures from PMNs stimulated with RNA-LL37. (B) Neutrophil elastase (NE) release from

808   PMNs stimulated for 3 h (n=8, each dot represents one donor). Fluorescence microscopy of fixed and

809   Hoechst, anti-LL37, SYTO RNAselect- (C) and/or anti-ΨU-stained (D, with or without RNase A

810   treatment) or live (E) PMNs stimulated as indicated (n=6, scale bar = 10 µm or n=4, scale bar = 20

811   µm). (F) Quantification of live cell microscopy), see also Movies S1-S3. (G, H) Skin sections from

812   healthy (G) or psoriasis- (G, H) affected skin (n=12 patients and 3 healthy controls, scale bar = 20 µm).

813   (I, J) as in C (left panels) and stimulated with transferred NETs (right panels). AF = autofluorescence.

814   B, F and I represent combined data (mean+SD) from 'n' biological replicates. In A, C-H and J

815   representative samples of 'n' replicates or donors are shown. Arrowheads indicate released

816   RNA/NETs (C-E) or RNA-LL37 co-localization (H). * $p<0.05$ according to one-way ANOVA (B), two-way

817   ANOVA (F) with Dunnett's correction or Kruskal-Wallis test with Dunn's correction (I).

Figure 4: RNA-LL37 effects in primary PMNs are mediated by TLR8/TLR13 and can be inhibited by

Inhibitory oligodeoxynucleotides

820   (A) Tnf release (ELISA, 5 h stimulation as indicated) by BM-PMNs from different mouse strains (n=4-8

821   each). (B, C) Fluorescence microscopy of fixed and Hoechst and SYTO RNAselect-stained stimulated

822   BM-PMNs from different mouse strains (n=4 Tlr13$^{-/-}$, n=5 Unc93b1$^{3d/3d}$, n=8 WT; scale bar = 10 µm or

823   n=4, scale bar = 20 µm). (D) IL-8 and TNF release (ELISA, 18 h stimulation as indicated) by WT and

824   *TLR8* CRISPR-edited BlaER1 cells (n=7). (E) NF-κB dual luciferase reporter assay in HEK293T cells,

825   transfected with NF-κB firefly luciferase reporter, *Renilla* control reporter and TLR8 plasmid and

826   subsequently stimulated with RNA without (arrow) or with IRS661, IRS954, IRS869 and IRS546 (0.15-

827   3.5 µM, n=2 each). IL-8 (F, n=6) or MIP-1β (G, n=4) release from stimulated PMNs with or without

828   IRS661 (1 nM) or IRS954 (50 nM) pre-incubation (30 min) quantified by ELISA. (H) Fluorescence

829   microscopy of fixed and Hoechst and anti-ΨU-stained RNA-LL37-stimulated PMNs (n=3, scale bar =

830   10 µm). (I) Quantification of H. A, D, E, F, G and I represent combined data (mean+SD) from 'n'

831   biological replicates (each dot represents one mouse or donor). In B, C, E and H one representative of

832   'n' replicates is shown (mean+SD of technical triplicates). * $p<0.05$ according to two-way ANOVA with

833   Dunnett correction for multiple testing (E, comparison against 'no inhibitor' condition, arrow), one-

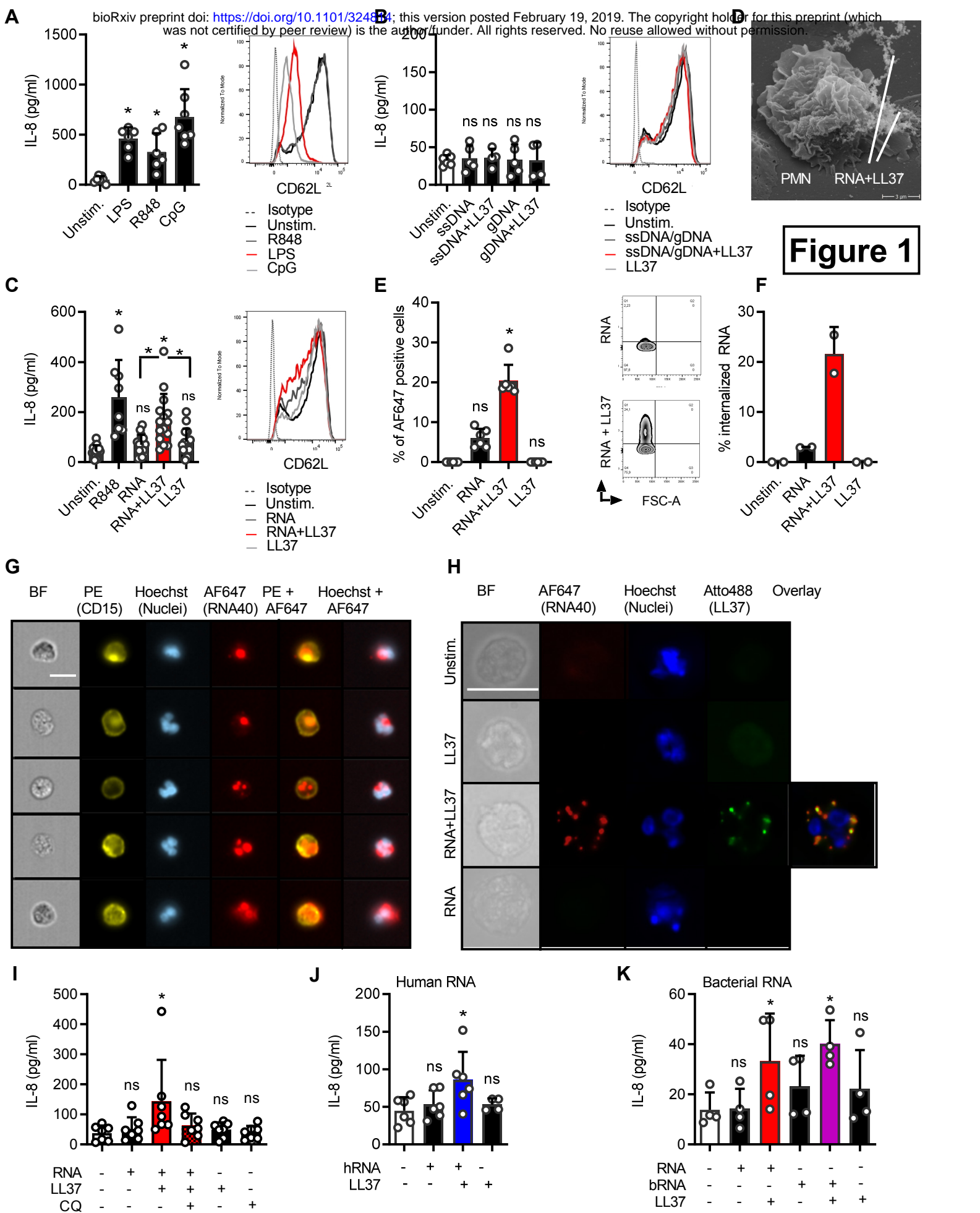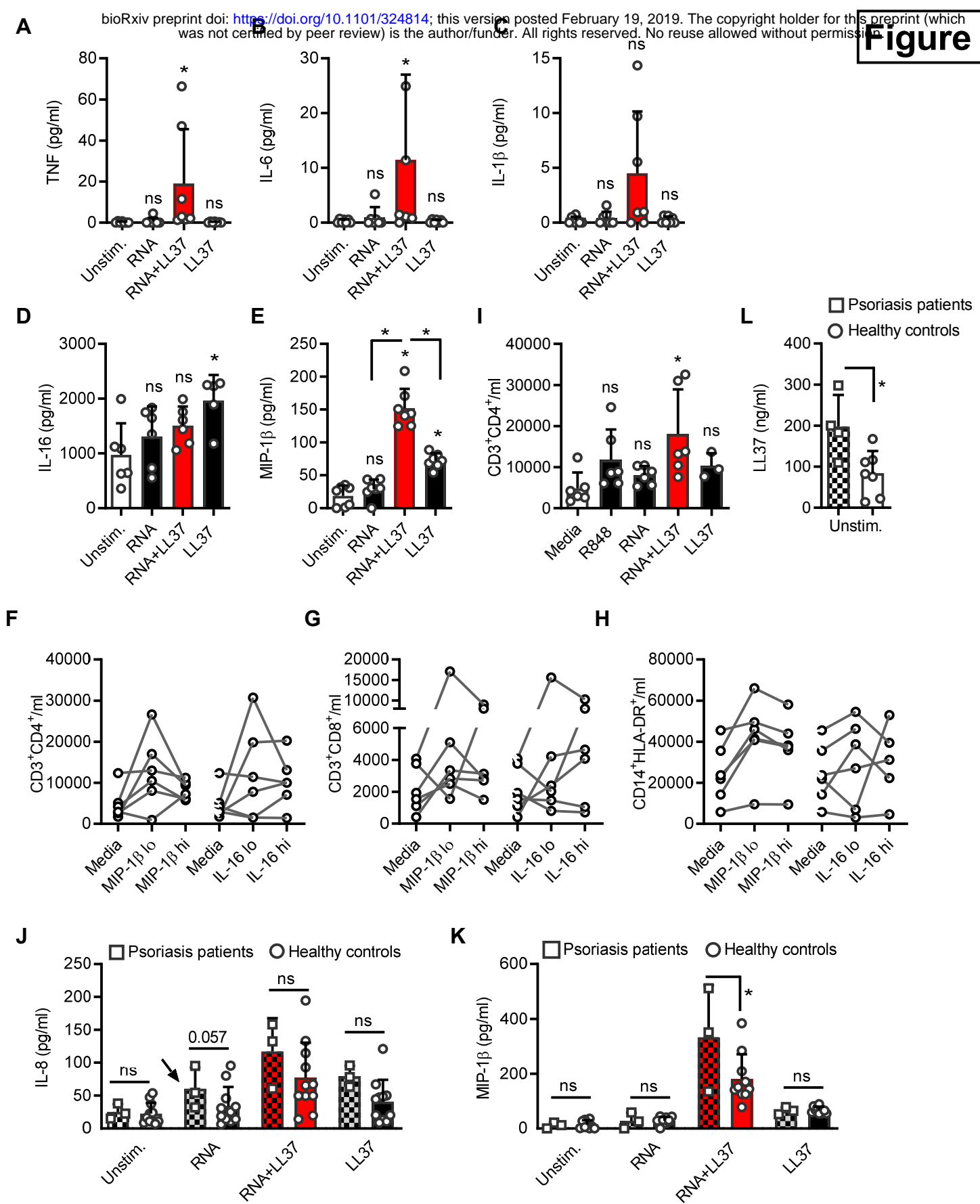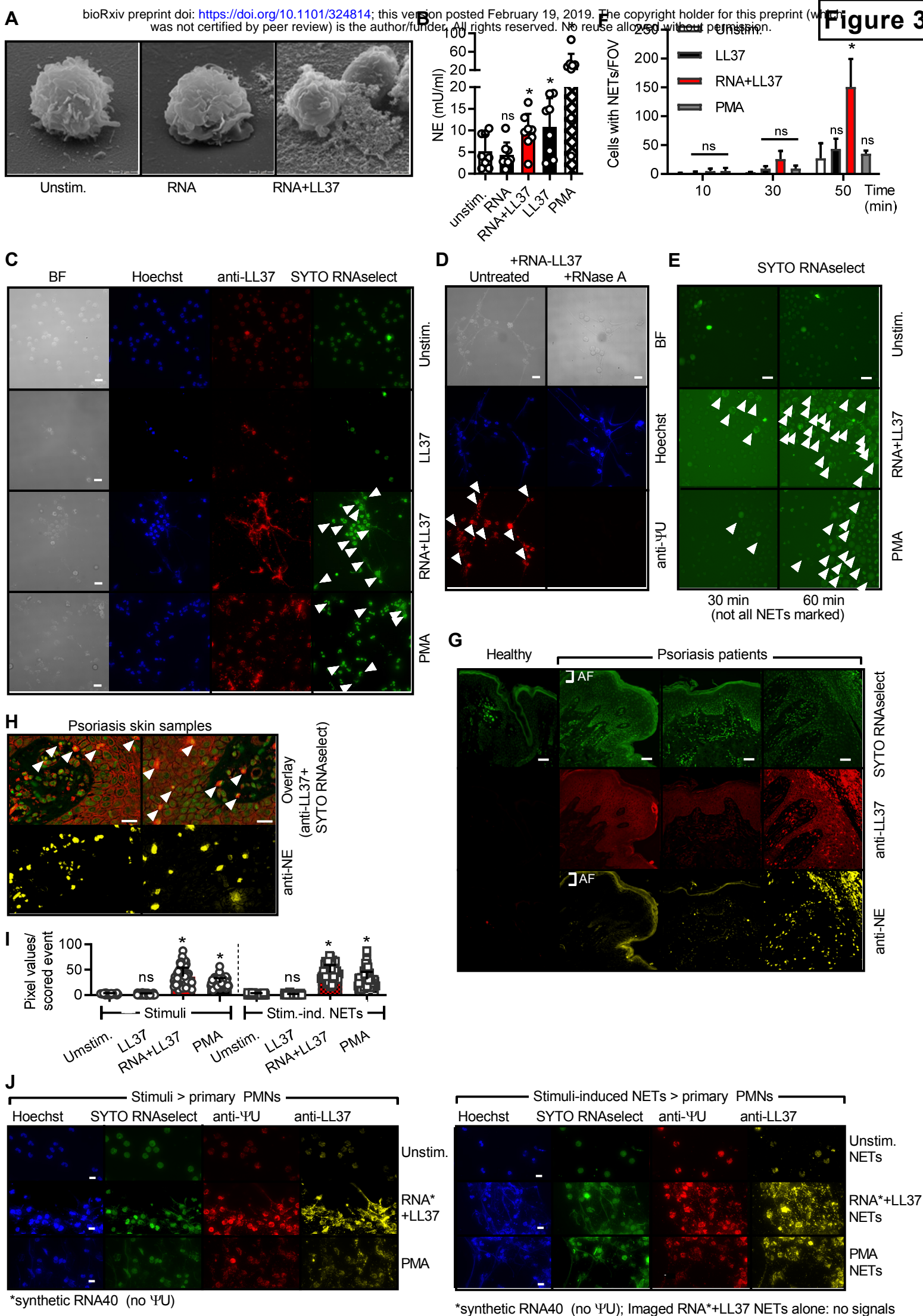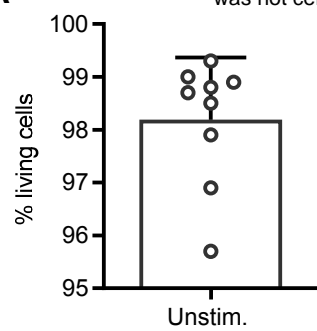834   way ANOVA with Sidak correction (A, F) or Friedmann test with Dunn correction (G).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 4 panels:

A — BM-PMNs, Tnf (pg/ml): Unstim., bRNA, bRNA+LL37, LL37, CpG, LPS, E. coli. WT, Unc93b1[3d/3d], Tlr13.

B — WT vs Unc93b1[3d/3d]; PMA, bRNA+LL37. Overlay: Hoechst (blue) Syto RNAselect (green)

C — WT vs Tlr13^{-/-}; PMA, bRNA+LL37. Overlay: Hoechst (blue) Syto RNAselect (green)

D — IL-8 (ng/ml): Unstim., RNA, RNA+LL37, LL37, E. coli, TL8. WT, TLR8^{-/-}

E — NF-κB activation (RLU): RNA, +IRS661, +IRS954, +IRS869, +IRS546. EV, TLR8

F — IL-8 (pg/ml): RNA, LL37, IRS661, IRS954. p=0.051

G — MIP-1β (pg/ml): RNA, LL37, IRS661, IRS954.

H — Overlay (Hoechst + anti-ΨU): Unstim., RNA+LL37, RNA+LL37 +IRS661 (50 nM)

I — RNA-LL37 stimulated PMNs; Pixel values/extracellular event: No iODN, +IRS661

Figure S1

A

SYTO RNAselect signal

RNA+LL37 — Nucleoli, Nucleoli+RNase, NETs, NETs+RNase
PMA — Nucleoli, Nucleoli+RNase, NETs, NETs+RNase

B

Hoechst DNA signal

RNA+LL37 — Nuclei, Nuclei+RNase, NETs, NETs+RNase
PMA — Nuclei, Nuclei+RNase, NETs, NETs+RNase

C

BF | DNA (Hoechst) | Total RNA (SYTO RNAselect) | Stimulus RNA40 (AF647) | Overlay

+Unlabeled LL37 +RNA40-AF647 (High magnification)

+Unlabeled LL37 +RNA40-AF647

D

DNA (Hoechst) | Stimulus RNA40* (AF488) | Cellular/native RNA (AF594) | Overlay

+Unlabeled LL37 +RNA40-AF488* (High magnification)

+Unlabeled LL37 +RNA40-AF488*

* does not contain ΨU

E

BF | DNA (Hoechst) | Stimulus LL37 (LL37-Atto488) | Total LL37 (1° ab > 2° Ab-AF647) | Overlay

+Unlabeled RNA40 +LL37-Atto488

F

+RNA+LL37

Overlay:
SYTO RNAselect
Hoechst
LL37 (Ab)

G

SYTO RNAselect | anti-ΨU | overlay

Psorisis skin sample

Figure S3

Figure S4

Host-derived?
Bacterial?
LL37
RNA
LL37

Neutrophil

iODN

TLR

Self-propagating
inflammatory loop

Infiltration & Activation
of additional leukocytes

pDC

IFNs

Pro-
inflammatory
cytokines

NETs

LL37 DNA RNA LL37