Human natural killer cells can activate NLRP1 and NLRP3 inflammasomes and drives pyroptosis

Antonio Astorga-Gamaza[1,2], Inés Muela-Zarzuela[1], Juan Miguel Suárez-Rivero[1], Juan López-Pérez[2,3], Raquel de la Varga-Martínez[2,3], Auxiliadora Mazuecos[4] and Mario D. Cordero[1,5, &]

[1] Department of Molecular Biology and Biochemical Engineering, Universidad Pablo de Olavide, 41013 Seville, Spain.

[2] Instituto de Investigación e Innovación Biomédica de Cádiz, INiBICA, Hospital Universitario Puerta del Mar, Cádiz, Spain.

[3] Department of Immunology, Puerta del Mar Hospital, 11009 Cádiz, Spain.

[4]Department of Nephrology, Hospital Universitario Puerta del Mar, Cadiz, Spain.

[5] CIBER de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III, 28220 Madrid, Spain.

Running tittle: Natural killers can activate inflammasomes

[&]Corresponding author: Mario D. Cordero, PhD

Department of Molecular Biology and Biochemical Engineering, Universidad Pablo de Olavide, 41013 Seville, Spain. Email: mdcormor1@upo.es.

Summary

Innate immunity relies on inflammasomes as key components, defending the host against diverse harmful stimuli by orchestrating the release of pro-inflammatory cytokines and initiating pyroptotic cell death. While extensively studied in myeloid cells, the involvement of natural killer (NK) cells in inflammatory responses through inflammasome signaling remains underexplored. In this study, we elucidate the activation of the inflammasome sensors NLRP1 and NLRP3 in human primary NK cells upon treatment with nigericin and blockade of dipeptidyl peptidases (DPP) using Talabostat (Val-boroPro). Our findings demonstrate the induction of pyroptotic cell death in a subset of NK cells following these stimuli, characterized by the cleavage and activation of gasdermin D, a lytic pore-forming protein. Moreover, we observe the release of lactate dehydrogenase (LDH) and small amounts of interleukin-18 (IL-18). Notably, differential responses are noted between $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets following pro-inflammatory stimulation.

Furthermore, analysis of samples from patients with renal dysfunction reveals sustained inflammasome activation in NK cells, particularly NLRP1 and NLRP3, with a tendency towards a more pro-inflammatory phenotype shortly post-kidney transplantation. These findings underscore the significance of considering NK cells in the context of inflammation studies.

Keywords: Natural Killers, NLRP3-Inflammasome, NLRP1-Inflammasome, Kidney transplantation

Introduction

Inflammation is part of the body's defense response to harmful stimuli. However, exacerbated inflammation is also associated with a range of diseases. In this regard, the activity of natural killer (NK) cells has been shown to highly depend on the context, helping to reduce or even promote inflammatory processes [1].

NK cells are innate lymphoid cells characterized by a huge diversity of phenotypes and functions [2, 3], although primarily categorized into two main subpopulations based on the neural cell adhesion molecule CD56 density. $CD56^{dim}$ NK cells, known for their potent cytolytic activities, and $CD56^{bright}$ NK cells, distinguished by their cytokine secretion capabilities [4]. They play a pivotal role in immune surveillance, effectively targeting malignant and infected cells to control intracellular pathogens and shaping adaptive immune responses [5]. Equipped with a huge array of cell surface receptors, NK cells sense stress signals in target cells, distinguishing between 'self' and 'non-self' or 'aberrant' targets under normal physiological conditions [6]. Upon activation, NK cells release cytotoxic granules containing perforin and granzyme. Also, they secrete interferon (IFN)-γ and tumor necrosis factor (TNF), and immunoregulatory molecules such as interleukin (IL)-10 and the growth factor GM-CSF, as well as chemokines including CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CXCL8 (IL-8) [7]. Through these mechanisms, NK cells can drive inflammation and modulate the activity of other immune cells.

A key part of the inflammatory process and the innate immune response is the assembly of inflammasomes. These cytosolic multi-protein complexes regulate tissue inflammation in response to multiple cellular stress patterns, including pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS), or cellular damage-associated molecular patterns (DAMPs), which include extracellular ATP and changes in intracellular ion concentrations.

59 In this regard, inflammasome biology has been mostly characterized in macrophages, and at least
60 partially studied in other cell types such as endothelial cells, fibroblasts, epithelial cells, and T cells
61 [8-11]. However, it is unknown if NK cells are equipped with functional inflammasome
62 components, and thus, this could represent an underexplored arm of the diverse immune
63 mechanisms mediated by these cells and an important source of inflammation.

64 The first steps in the triggering of inflammasomes comprise the detection of the damage
65 patterns via sensors of several families such as the NOD-like (NLR), Toll-like (TLR), AIM2-like (ALR),
66 or the caspase recruitment domain (CARD)-containing family of proteins, that include the
67 members NLRP1, NLRP3, NLRC4, AIM2, and CARD8, among others [12]. Following activation, some
68 sensors interact with adaptor proteins such as ASC (apoptosis-associated speck-like protein
69 containing a CARD), composing a pre-complex that then recruits inflammatory caspase-1. Next to
70 terminal assembly, the activation of caspase-1 in the inflammasome can lead to the proteolytic
71 cleavage and maturation of the IL-1 family cytokines IL-1β and IL-18 [13]. This pathway is known as
72 the 'canonical' activation. Another usual key event after the inflammasome activation is the
73 cleavage of gasdermins, a family of pore-forming proteins, into its N-terminal active domain which
74 induces a proinflammatory form of regulated cell death termed pyroptosis [14].

75 Here, we conducted a transcriptomic analysis of NK cells derived from various tissues,
76 revealing the expression of genes encoding several inflammasome components. Additionally, we
77 provide proteomic evidence demonstrating the expression of canonical NLRP3 and NLRP1
78 inflammasome components in both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets. More importantly, we
79 demonstrate the functionality of these inflammasomes in NK cells, as evidenced by IL-18 secretion
80 and induction of pyroptotic cell death. Interestingly, we observe distinct responses to pro-
81 inflammatory stimulis between the two NK cell subpopulations. Furthermore, we identify signs of
82 inflammasome activation in NK cells from patients with renal dysfunction, which persist after
83 kidney transplantation and despite immunosuppression. Our findings suggest that NK cells should
84 be considered in inflammation research and as potential therapeutic targets, as they might play an
85 important role in both pathogen defense and inflammatory disorders through inflammasome
86 signaling and pyroptosis.

87 **RESULTS**

88 **Expression of inflammation-related genes in human NK cells from different tissues**

89 To gain knowledge on the potential of human NK cells for shaping inflammatory responses and
90 constituting functional inflammasomes, we conducted a transcriptomic analysis of NK cells from
91 diverse tissues from a publicly available RNA-seq dataset [3], and assessed the expression of a list
92 of genes related to inflammation. As described, donors were free of cancer, chronic diseases, and
93 seronegative for hepatitis B, C, and HIV [3]. From a total of 2,686 genes, we found a significant
94 expression of 1,619 in NK cells from blood, bone marrow, lymph nodes, lungs, and spleen. These
95 genes appeared to be involved in different processes such as the regulation of the NF-κB activity,
96 apoptosis, TNF signaling, cellular response to lipopolysaccharide, NOD-like and Toll-like receptor
97 signaling, IL-6 and IL-1β production (**Figure 1A**). Next, we performed a detailed analysis of the
98 expression of short-listed genes reported to play a role in activating inflammasome multiprotein
99 complexes at different stages (**Figure 1B, C, D, E, and F**). Interestingly, we noticed the expression
100 in NK cells of guanylate-binding proteins (GBP), which target intracellular pathogens and can
101 mediate host defense via inflammasome induction [15, 16]. Moreover, we found expressed other
102 receptors of DAMPs and PAMPs such as TLR-2-3 and 4, NLRP1, NLRP3, NLRP6, NLRC3, and NLRC4,
103 the interferon-inducible protein AIM-2 and CARD. NK cells also expressed PYCARD, the gene
104 coding for ASC protein, required to constitute canonical inflammasomes, and the serin proteases
105 DPP8 and DPP9, whose pharmacological inhibition by Val-boroPro (Vbp) (Talabostat) is reported to
106 activate both NLRP1 and CARD8 inflammasomes [17, 18]. Furthermore, NK cells from all analyzed
107 tissues expressed downstream effectors in the inflammasome pathway such as caspase-1, IL-1β,
108 IL-18, and gasdermin D genes (**Figure 1B, C, D, E, and F**). Moreover, we analyzed differential
109 expression between the NK subpopulations $CD56^{dim}$ and $CD56^{bright}$ and found only a few significant
110 differences that varied depending on the tissue location. This was the case of GBP5 and TLR3 in
111 the blood (**Figure 4B**), and Caspase-1 in the lung (**Figure 4C**), more expressed in $CD56^{dim}$ NK cells.
112 Of note, NK cells exhibit an intrinsic, highly heterogeneous nature intra- and inter-individual [2].
113 Thus, larger datasets are likely needed to reveal more differences between different
114 subpopulations.

115 Our results indicate that NK cells, both the two main subpopulations $CD56^{dim}$ and $CD56^{bright}$,
116 express the genetic elements needed to constitute functional inflammasomes.

117

118 **Protein expression of NLRP1 and NLRP3 and the adaptor ASC in NK cells**

119 Next, we aimed to assess the expression of the protein sensors NLRP1 and NLRP3, which have
120 been implicated in the regulation and pathogenesis of many conditions. We studied the
121 expression of the proteins NLRP1, NLRP3, and the adaptor ASC in NK cells by flow cytometry, using
122 blood-derived primary NK cells previously isolated by FACS (fluorescence-activated cell sorting)
123 (**Supplementary Figure 1**). The median purity of the cells was 90.7% and 94.8% for $CD56^{dim}$ and
124 $CD56^{bright}$ NK cells, respectively. A representative example of the staining can be found in **Figure**
125 **2A**. These proteins were widely expressed in NK cells, with slightly higher frequencies of ASC and
126 NLRP1 in the $CD56^{bright}$ subpopulation (**Figure 2B and C**), but no differences in the percentage of
127 NLRP3 (**Figure 2D**) nor in the intensity of expression (mean fluorescence intensity, MFI) of any of
128 them (**Figure 2E, F, and G**). Therefore, NLRP3 and NLRP1 inflammasomes may play a role in NK
129 cell-mediated immunity if functional.

130 **Activation of the NLRP3 and NLRP1 inflammasome complexes in NK cells triggered by specific**
131 **activators**

132 As a next step, we directly tested the functionality of inflammasomes in NK cells. For that, we
133 treated NK cells previously isolated by FACS with known inductors of the NLRP3 and NLRP1-
134 inflammasomes. We included conditions of cells cultured with lipopolysaccharide (LPS) plus ATP
135 (LPS-ATP), nigericin (Nig), and the DPP8/DPP9 inhibitor Vbp for both $CD56^{dim}$ and $CD56^{bright}$ NK
136 subpopulations separately. Inflammasome activation was first assessed by measuring the content
137 of inflammatory cytokines in culture supernatants. Remarkably, we detected secretion of pro-
138 inflammatory IL-18 upon stimulation with nigericin of $CD56^{dim}$ (**Figure 3A**), but not $CD56^{bright}$ NK
139 cells (**Figure 3B**). The opposite pattern was obtained for Vbp, which triggered IL-18 production in
140 $CD56^{bright}$ (**Figure 3C**) but not in $CD56^{dim}$ cells (**Figure 3D**). No increment was detected after LPS-ATP
141 stimulation (**Supplementary Figures A and B**). IL-1β was not induced in general in these in vitro
142 assays, with the exemption of one donor that also showed higher levels of IL-6 (**Supplementary**
143 **Figures C, D, and E**). Besides, we measured other immune mediators by a multiplex Luminex
144 immunoassay. We failed to detect secretion of IL-10, GM-CSF, and the NK-associated cytotoxic and
145 regulatory molecules IFN-γ, IFN-α, or TNF-α in our assayed conditions, suggesting that in vitro
146 treatment with nigericin and Vbp does not induce NK unspecific cytotoxicity and inflammatory

147 response, and that an inflammasome specific response is triggered. Interestingly, ICAM-1, the
148 adhesion receptor and regulator of inflammation, seemed to increase its concentration after LPS-
149 ATP and nigericin treatment of CD56^{dim} cells (**Supplementary Figures F and G**). This suggests that
150 different pro-inflammatory stimuli affect differently distinct NK subpopulations.

151 Second, we assessed cell death by pyroptosis, another key process that follows inflammasome
152 activation. We detected by western blot the activation of the N-terminal fragment of Gasdermin D
153 after nigericin and Vbp treatment of NK cells (**Figure 3E**). Of note, this was successfully detected
154 only in CD56^{dim} NK cells. However, the release of LDH, which confirms cell death, was more
155 prominent in the CD56^{bright} subpopulation (**Figure 3F and G**), raising the possibility that more
156 aggressive death of CD56^{bright} NK cells may hinder the detection of active gasdermin D. Both pro-
157 inflammatory cytokine secretion and gasdermin-dependent pyroptosis rely on the previous
158 cleavage of inflammatory caspases into its active form. Importantly, we observed increased active
159 fragments of caspase-1 in NK cells stimulated with nigericin and Vbp (**Figure 3H**).

160 To obtain more clues about the activation of inflammasomes in NK cells, we assessed the
161 expression of proteins within control and treated cells by flow cytometry. The purity of the cells
162 employed in these assays was always higher than 90.0%. Moreover, any remaining cell
163 contaminants in the culture were discarded from our analysis using lineage markers for NK cells in
164 the flow panel. Treatment of NK cells, CD56^{dim} or CD56^{bright}, with LPS-ATP, nigericin, or Vbp, did not
165 alter the frequency of cells expressing NLRP3 (**Figures 3I and J**), NLRP1 (**Figures 3K and L**), or ASC
166 (**Figures 3M and N**). However, changes in the intensity of expression (MFI) were detected in the
167 case of nigericin exposure of CD56^{bright} NK cells, with a reduction in NLRP3 levels (**Figure 3O**). This
168 was not observed for the rest of the proteins or stimulus (**Supplementary Figures 3H, I, J, K, and**
169 **L**). This may indicate that nigericin and Vbp promote the reorganization of these molecules to
170 assemble the inflammasomes rather than drastically changing their expression levels. Also,
171 nigericin may promote more aggressive pyroptotic death of CD56^{bright} NK cells, explaining the
172 lower detected levels of NLRP3 and higher LDH release. Moreover, we studied the formation of
173 specks by flow cytometry as previously reported [19]. In the formation of inflammasomes, ASC
174 interacts with upstream sensors such as NLRP1 and NLRP3 that subsequently trigger its assembly
175 into a structure known as a speck composed of multimers of ASC dimers. The speck can be
176 detected by flow cytometry using the time of flight (TOF) signal of ASC, as it concurs with higher
177 pulse height to area (H:A) (**Figure 3P)**. We consistently observed signs of speck in CD56^{dim} NK cells

178 after nigericin stimulation (**Figure 3Q**). These results resembled that of IL-18 secretion, as
179 treatment with Vbp also induced speck in 5 out of 7 independent assays in CD56^{bright} NK cells
180 (**Figure 3R**). Notably, we did not detect speck in LPS-ATP or Vbp treated CD56^{dim} NK cells
181 (**Supplementary Figures 2N and O**), nor consistently in LPS-ATP and nigericin treated CD56^{bright}
182 subpopulation (**Supplementary Figures 2P and Q**). Of note, we believe that pyroptosis of NK cells
183 activating the inflammasomes may be hindering/underestimating the quantification of specks in
184 these assays.

185 Overall, our results indicate that both NLRP3 and NLRP1-inflammasomes are functional in NK
186 cells, particularly under nigericin and Vbp treatment. CD56^{dim} and CD56^{bright} NK cells behave
187 differently under pro-inflammatory stimulation.

188 **NK cells from patients with chronic kidney disease display inflammasome activation**

189 Recent data support the hypothesis that imbalanced inflammation might be an important
190 driver of kidney disease that may persist even after organ transplantation. We wondered if NK
191 cells may contribute to the inflammatory milieu in patients with kidney dysfunction. We collected
192 PBMCs from patients with chronic kidney disease listed in **Supplementary Table 1**. Western
193 blotting studies showed high levels of NLRP1, active caspase-1, and gasdermin-D in these patients
194 before and during the first week after kidney transplantation (**Figure 4A and Supplementary**
195 **Figure 3**), confirming the activation of inflammasomes and pro-inflammatory cell death by
196 pyroptosis of circulating immune cells. We isolated by FACS the CD56^{dim} and CD56^{bright}
197 subpopulations from a fraction of these PBMC, but it was technically challenging to perform the
198 same western blot assays likely due to low cell numbers. Importantly, flow cytometry analyses (as
199 represented in **Supplementary Figures 4A and B**) confirmed that nearly 100.0 % of NK cells in
200 these patients expressed NLRP1 (**Figure 4B**), about 50.0 % expressed ASC (**Figure 4C**), and a
201 smaller fraction NLRP3, which increased three days after transplantation (**Figure 4D**). We also
202 detected a fraction of NK cells with signs of speck, being enriched three days post-transplantation
203 (**Figure 4E and Supplementary Figure 4C**). Speck levels in NK cells were much lower than those
204 detected in monocytes, as expected (**Supplementary Figure 4D**). Additional analyses distinguishing
205 between specking versus non-specking cells showed higher levels of NLRP1 and NLRP3 in speck^{+}
206 cells (**Figures 4F, G, H and I**), suggesting the activation of both inflammasomes in NK cells from
207 patients with renal dysfunction.

208

209

210

211 **Discussion**

212 In this study, we try to shed light on the biology of inflammasomes in human NK cells, as
213 dedicated studies are lacking. These multiprotein complexes are involved in innate defense and
214 inflammatory responses to pathogens. Similarly, NK cells constitute a first line of defense against
215 aberrant targets. However, whether inflammasomes encompass one of their multiple immune
216 mechanisms of action and how it may impact health are unknown. Inflammasomes are composed
217 of sensors, adaptors, and effectors components, and their activation triggers the release of
218 inflammatory cytokines and pyroptosis [20]. Here, we show the expression of transcripts encoding
219 several inflammasome-forming proteins in NK cells distributed across different tissues. Sensors of
220 several inflammasomes, including the members of the NALP family NLRP3 and NLRP1 were found
221 expressed. Consistent with a prior report [21], we found a high constitutive expression of NLRP3.
222 We also noticed high levels of NLRP1 and the adaptor protein ASC in NK cells. More importantly,
223 we prove here that the NLRP3 and NLRP1 inflammasomes can be assembled and are functional in
224 these cells.

225 Several activators of the NLRP3 and NLRP1 inflammasomes have been identified, being
226 reported potassium ion (K^+) efflux as one of the most potent in the case of NLRP3 [22], and
227 recently also proven to activate NLRP1 [23], and inhibition of the serin dipeptidyl peptidases DPP8
228 and DPP9 for NLRP1 activation [17, 18]. Here, we used Nigericin, a toxin derived
229 from *Streptomyces hygroscopicus* that facilitates H^+/K^+ anti-port across cell membranes and
230 thereby causes potassium efflux [24], and Vbp, an inhibitor of the inhibitory interaction between
231 DPP8/DPP9 and NLRP1 [25], to assess the functionality of both inflammasomes in NK cells. Our
232 results revealed changes that are compatible with inflammasome activation, including secretion of
233 IL-18 and more notable pyroptosis. Also seemed to indicate that the components of the NLRP3
234 and NLRP1 inflammasomes more likely undergo a reorganization rather than upregulation upon
235 stimulation with nigericin or Vbp. Moreover, we found differences in the response of $CD56^{dim}$ and
236 $CD56^{bright}$ NK cells to both compounds. This is not completely unexpected given the high plasticity
237 and diversity in phenotypes and functions of NK cells [2, 3]. Nigericin induced the secretion of IL-

238 18 by CD56^{dim} NK cells while Vbp did the same with the CD56^{bright} subpopulation. Regarding the
239 pore-forming protein gasdermin D, capable of inducing pyroptosis, its active form was detected in
240 CD56^{dim} NK cells treated with nigericin and Vbp. LDH levels were also increased in the culture of
241 these cells, particularly in those treated with nigericin. Of note, although we were not able to
242 detect active gasdermin D in CD56^{bright} cells, a notable release of LDH by these cells was observed.
243 The release of LDH is considered a surrogate of cell pyroptosis as large proteins are usually filtered
244 out in the absence of cell death [26]. Thus, it may be plausible that CD56^{bright} NK cells undergo a
245 more aggressive pyroptosis upon *in vitro* activation of the NLRP3/NLRP1 inflammasomes,
246 hindering the detection of active gasdermin-D. Also, the low numbers of these cells in the blood,
247 which usually represent less than 10% of circulating NK cells [27], may suppose a technical
248 challenge for the detection of proteins. Different frequencies and proportions of NK cell
249 subpopulations can be found in tissues like the bone marrow, spleen, lung, liver, lymph nodes,
250 tonsils and intestines [3]. Thus, further studies with different tissue samples, NK subpopulations
251 and inflammasome-triggering agents or conditions could add valuable insight in the biology of
252 these lymphocytes and the inflammatory field.

253 To our knowledge, this is the first report clearly showing that NLRP3/NLRP1 inflammasome
254 activation and pyroptosis take place in human NK cells. Nevertheless, prior results in mice support
255 our results that canonical activation of inflammasomes occur in these immune cells, showing that
256 burn injury and radiation exposure induce the activation of caspase-1 in many cell subsets
257 including NK cells [28, 29]. Importantly, Fong et al also showed that the human pathogen group B
258 *Streptococcus* directly interacts with NK cells and suppress a pyroptotic-like response via the
259 interaction with siglec-7 [30]. In this regard, our results did not show a typical inflammasome
260 response after the LPS-ATP stimulation of NK cells. However, this could be explained by the low
261 surface expression of TLR4 in these cells [31]. Thus, we do not rule out the possibility that direct
262 bacterial infection might activate some of the inflammasomes in NK cells. In fact, our
263 transcriptomic analysis showed that many other inflammasome sensors are transcriptionally
264 expressed in these cells, including NLRP6, NLRC4 or AIM-2, although if they are functional remain
265 unknown and could be more suitable after direct infections. Thus, the results presented here
266 provides new insight in the biology of NK cells that might support a role of these lymphocytes in
267 different contexts via inflammasome activation. Elucidating when these responses take place and
268 whether are beneficial or harmful could help in the identification of new targets in inflammatory
269 disorders.

270 Inflammasome activity has been associated to chronic kidney disease and the rejection of
271 the graft in kidney-transplanted patients, particularly linked to AIM-2, NLRP3, and more recently
272 NLRP1 [32-36]. Kidney transplantation is considered the best therapy for patients with end-stage
273 kidney disease, being associated with better quality of life and patient survival compared with
274 dialysis. However, a fraction of kidney transplant recipients still faces short-term or long-term graft
275 rejection, in which inflammation seems to play a relevant role [37]. Here, we observed signs of
276 NLRP3 and NLRP1 inflammasome activation as well as activation of gasdermin-D in circulating
277 immune cells, including NK cells, in patients with end-stage kidney disease before and after
278 receiving a donor kidney. Previously, it has been shown that post-transplantation NK cells subsets
279 can change even at the peripheral level, with variations in number and phenotype [38]. Here, we
280 show the acquisition of a more proinflammatory phenotype in NK cells shortly post-
281 transplantation, characterized by higher levels of NLRP1, NLRP3 and ASC. This suggests that
282 different inflammatory milieus in vivo could induce the activity of inflammasomes not only in
283 classically studied subsets such as monocytes and macrophages, but also innate lymphoid cells
284 such as NK cells. Until now, two mechanisms are considered the main drivers for the graft
285 rejection in transplanted patients, the T-cell mediated rejection, and/or the antibody-mediated
286 rejection. In both, NK cells have been shown to cooperate, through maturation of dendritic cells
287 and activation of T cells and mediating a pathogenic ADCC response triggered by anti-HLA
288 antibodies in $CD56^{dim}$ CD16-expressing NK cells [39]. Our results add evidence that NK cells might
289 also contribute to pathogenic inflammation in renal injured patients through pro-inflammatory
290 activity via inflammasome signaling. Although larger studies and longer follow-up periods are
291 needed, our results are tempting to speculate that inflammasome is a pathway by which NK cells
292 might influence renal disease and the graft outcome. Moreover, they might play a role in several
293 other inflammatory diseases such as ischemic stroke, sepsis or cancer, where persistent
294 inflammasome activity and activation of gasdermin D has been observed to cause undesired
295 effects including organelle dysfunction, cell lysis and persistent release of cytokines [14]. Also, it
296 will be relevant to determine other contexts where inducing inflammasome activity of NK cells
297 could be beneficial.

298 In conclusion, our findings underscore the importance of including NK cell subpopulations in future
299 studies investigating inflammasome functionality.

300

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Author contributions

Conceptualization: A.A.G, M.D.C; Methodology: A.A.G, I.M.Z; Investigation: A.A.G, JM.S.R, M.D.C; Resources: A.A.G, J.L.P, R.V.M, M.D.C; Visualization: A.A.G, I.M.Z, M.D.C; Supervision: A.A.G, M.D.C; Funding acquisition: M.D.C; Writing – Original Draft: A.A.G; Writing –Review & Editing: A.A.G, M.D.C.

DECLARATION OF INTEREST

The authors declare no competing interest.

319 **FIGURE LEGENDS**

320 **Figure 1. RNA-Seq analysis of inflammatory transcriptional signatures in circulating and tissue**
321 **resident human NK cells.** Cells were isolated from donors without cancer or any other chronic
322 disease, and seronegative for hepatitis B, C, and HIV viral infections. **A)** Significance of the most
323 represented immune pathways according to the expression of a list of 2,686 genes related to
324 inflammation. Functional gene annotation was performed using DAVID. **B)** Heatmap of the
325 expression of short-listed genes key in the activation and triggering of effector functions of
326 inflammasomes in circulating NK cells. An analysis of the differential expression of these genes
327 between $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cell subsets is also shown, and genes showing a
328 different pattern of expression between both NK subpopulations are marked with a red box. The
329 same is shown for **C)** NK cells in the lung, **D)** NK cells in the lymph nodes, **E)** NK cells in the bone
330 marrow, and **F)** NK cells in the spleen. Differentially expressed genes were identified using linear
331 models (Limma-Voom) and P values adjusted for multiple comparisons by applying the Benjamini-
332 Hochberg correction.

333 **Figure 2. Expression of the inflammasome sensors NLRP1 and NLRP3 and the adaptor protein**
334 **ASC in human NK cells.** Two subpopulations of NK cells: $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-/dim}$,
335 were isolated by cell sorting and interrogated for the expression of the mentioned proteins by
336 flow cytometry (N=8). **A)** Flow-cytometry plots showing a representative example for the
337 expression of NLRP1, NLRP3 and ASC in $CD56^{dim}$ and $CD56^{bright}$ NK cells, and the corresponding
338 Fluorescence Minus One (FMO) control for the proper gating. **B)** Frequency (%) of expression of
339 ASC, **C)** Frequency of NLRP1, **D)** Frequency of NLRP3, and Mean Fluorescence Intensity (MFI) signal
340 for each marker in **E)**, **F)** and **G)**. Median with range is represented. Statistical comparisons were
341 performed using the Wilcoxon matched-pairs signed-rank test.

342 **Figure 3**. **Activation of the NLRP1 and NLRP3 inflammasomes and induction of pyroptosis in**
343 **human NK cells ex vivo.** Isolated NK cells from blood samples were cultured in the presence of the
344 inflammasome activators LPS (1 µg/ml for 4 hours) and ATP (5 mM for 30 mins), nigericin (10 µM
345 for 30 mins) or Vbp (1 µM for 4 hours). After that, the concentration of cytokines of the IL-1 family
346 IL-18 and IL1β as well as lactate dehydrogenase (LDH) release were measured in the culture
347 supernatants. Changes in the cell expression of inflammasome sensors and downstream effectors
348 in the inflammatory cascade such as caspase-1 and gasdermin D were also measured by western
349 blot and flow cytometry. **A)** Concentration of IL-18 in cell cultures of untreated $CD56^{dim}$ NK cells or
350 treated with nigericin (n=6). **B)** The same for $CD56^{bright}$ NK cells. **C)** IL-18 concentration in cell
351 cultures of $CD56^{bright}$ NK cells stimulated with Vbp (n=5). **D)** IL-18 levels after stimulation with Vbp
352 of $CD56^{dim}$ NK cells. **E)** Western blot analysis of the full length and cleaved form of gasdermin D in
353 $CD56^{dim}$ NK cells in basal conditions and after nigericin and Vbp exposure. Representative result
354 from n=5 independent experiments. **F)** LDH release in cell cultures of stimulated $CD56^{dim}$ NK cells
355 (n=6), and **G)** stimulated $CD56^{bright}$ NK cells (n=6). **H)** Western blot analysis of the precursor and
356 active forms of caspase-1 in $CD56^{dim}$ NK cells in basal conditions and after nigericin and Vbp
357 exposure. Representative result from n=3 independent experiments. **I)** to **O)** represent the

358 frequency and MFI signal for the expression of NLRP1, NLRP3 and ASC in CD56^{dim} and CD56^{bright} NK
359 cells in non-stimulated and under pro-inflammatory stimuli with LPS-ATP, nigericin and Vbp
360 conditions (n=8). Graphs represent median with range. Statistical comparisons were performed
361 using the Wilcoxon matched-pairs signed-rank test. **P)** Representative flow cytometry plots of the
362 time-of-flight signal for ASC in NK cells, showing an increase in ASC area: height pulse, indicative of
363 specking, after nigericin in vitro stimulation. **Q)** Graph showing the frequency of CD56^{dim} NK cells
364 with signs of ASC specking after nigericin stimulation. **R)** The same for CD56^{bright} NK cells stimulated
365 with Vbp. Statistical comparisons were performed using the Wilcoxon matched-pairs signed-rank
366 test.

367 **Figure 4**. **Inflammasome activation in NK cells from patients with chronic kidney disease.** Blood
368 samples from patients with renal disease were collected at different time points, before and after
369 being subjected to kidney transplantation (n=7). Activation of the NLRP1 and NLRP3
370 inflammasomes in these patients was studied by western blotting and flow cytometry. **A)** Western
371 blot representative results in total peripheral blood mononuclear cells (PBMCs) from one renal-
372 injured patient (#FJP in **Supplementary Table 1**), showing the expression of NLRP1, NLRP3, pro-
373 active caspase-1, the active fragment of caspase-1, full-length gasdermin D, active gasdermin D,
374 and the control β-actin, pre-transplantation and 3 and 7 days after being transplanted. **B)**
375 Frequency of expression of NLRP1 in total CD56^{+} NK cells. **C)** Frequency of ASC. **D)** Frequency of
376 NLRP3. **E)** Percentage of ASC specking in NK cells. Graphs represent median with range. Statistical
377 comparisons were performed using the Wilcoxon matched-pairs signed-rank test. Graphs in **F)** and
378 **G)** show the percentage and MFI of NLRP1 within speck^{-} and speck^{+} NK cells at the different time
379 points. The same for NLRP3 in **H)** and **I).** Graphs represent median with range. P values shown in
380 the graphs represent ANOVA Friedman test, and asterisks denote the multiple comparison Dunn's
381 test. *$p<0.05$; **$p<0.01$. Moreover, comparisons between the speck^{-} and speck^{+} NK cells in the
382 same time points were performed using the Wilcoxon matched-pairs signed-rank test.

383 **MATERIALS AND METHODS**

384 Human Samples

385 In this study, we used primary cells obtained from blood samples from healthy donors or people
386 with kidney disease and undergoing transplantation. Peripheral blood mononuclear cells (PBMCs)
387 were obtained from the Hospital Puerta del Mar in Cádiz, Spain. Study protocols were approved by
388 the corresponding Ethical Committees (Institutional Review Board numbers). All subjects recruited
389 for this study were adults who provided written informed consent. Information on renal disease
390 parameters from affected patients is summarized in Table S1. Gender and age are also indicated in
391 Table S1 unless not available. Since we utilized samples from donors of different genders in our
392 experiments, we can conclude that the results reported here apply to both men and women.
393 PBMCs were obtained by Ficoll-Paque density gradient centrifugation. PBMCs were cultured in

394 RPMI medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco), 1% streptomycin-
395 penicillin (Thermo Fisher, Waltham, MA, USA, 11548876) (R10 medium), and maintained at 37ºC
396 in a 5% CO_2 incubator, when needed. NK cells were isolated from PBMCs by FACS (fluorescence-
397 activated cell sorting) (MELODY sorter) or using a commercial kit (MagniSort™ Human NK cell
398 Enrichment; eBioscience). The purity of the cells was always higher than 90%.

399 Transcriptomic analysis of a publicly available RNAseq data

400 We analyzed publicly available RNA-seq datasets. Data are available from Gene Expression
401 Omnibus (GEO) corresponding to the study with accession number GSE133383. This study
402 provides valuable transcriptomic data of two subpopulations of NK cells, $CD56^{dim}$ and $CD56^{bright}$, in
403 tissues difficult to obtain [3]. R version 4.1.2 was used to perform all bioinformatic analyses. Count
404 values were imported and processed using edgeR
405 [https://academic.oup.com/bioinformatics/article/26/1/139/182458?login=false]. Expression
406 values were normalized using the trimmed mean of M values (TMM) method and lowly-expressed
407 genes (<1 counts per million) were filtered out. Differentially expressed genes were identified
408 using linear models (Limma-Voom) [https://academic.oup.com/nar/article/43/7/e47/2414268],
409 and P values adjusted for multiple comparisons by applying the Benjamini-Hochberg correction.
410 Heatmaps were generated using the heatmap3 package [https://github.com/slzhao/heatmap3].
411 Glimma [https://github.com/Shians/Glimma] was used for interactive visualizations. Functional
412 gene annotation was performed using DAVID [https://david.ncifcrf.gov].

413 Human NK cell isolation by FACS

414 For Fluorescence Activated Cell Sorting (FACS), 80 million PBMCs were stained with LIVE/DEAD
415 AQUA viability (Invitrogen) for 20 minutes at RT. After washing with staining buffer (PBS 3% FBS),
416 cells were surface stained with anti-CD56-FITC (B159, Becton Dickinson), anti-CD3-PE-Cy7 (SK7,
417 Becton Dickinson), anti-CD16-BV786 (3G8, Becton Dickinson) and anti-CD4-APC (RPA-T4, Becton
418 Dickinson) antibodies for 20 minutes at RT. Cells were then washed with staining buffer and
419 immediately sorted using the MELODY Cell Sorter. We sorted the live populations $CD4^{-}CD3^{-}CD56^{dim}$
420 ($CD56^{dim}$ NK cells) and $CD4^{-}CD3^{-}CD56^{bright}$ ($CD56^{bright}$ NK cells). The purity of the cells was >90% in all
421 cases.

422 In vitro inflammasome activation assays

423 For in vitro pro-inflammatory stimulation, NK cells were treated with 1µg/ml LPS (Sigma-Aldrich,
424 St. Louis, MI, USA, L4391-1MG) for 4 hours and 5 mM ATP (Santa Cruz Biotechnology, Santa Cruz,
425 CA, USA, sc-214507A) for 30 mins. NK cells were also stimulated with 1 µM Val-boroPro (Sigma-
426 Aldrich, St. Louis, MI, USA, 5314650001) for 4 hours or 10 µM nigericin for 30 mins. As the number
427 of isolated NK cells varied between donors, we maintained the condition of the cell culture,
428 stimulating the cells at a concentration of approximately 0,1M/ml, in 24-well plates for the
429 $CD56^{dim}$ subpopulation and 96-well plates for the $CD56^{bright}$ subpopulation. After the incubation,
430 we collected culture supernatants for LDH and cytokine measures, and cells for proteomics
431 analyses by flow cytometry or western blot.

432 Flow cytometry

433 For studying the protein expression, before and after pro-inflammatory stimulation, NK cells were
434 stained with LIVE/DEAD AQUA viability (Invitrogen) for 20 minutes at room temperature (RT).
435 After washing once with staining buffer (1X PBS 3% FBS), cells were surface stained with anti-
436 CD56-FITC (B159, Becton Dickinson), and anti-CD3-PE-Cy7 (SK7, Becton Dickinson) antibodies for
437 20 mins at RT. Next, we performed a washing step and cells were fixed and permeabilized with
438 Fixation/Permeabilization Solution (Becton Dickinson) for 20 minutes at 4ºC and then washed with
439 BD Perm/Wash buffer. After, cells were stained with rabbit anti-NLRP1 (A16212 ABclonal) for 20
440 mins at RT, washed, and detected by staining with an anti-rabbit secondary AF750 (ab175735,
441 Abcam) antibody for an additional 20 mins at RT. Another washing step was performed and
442 staining with anti-NLRP3-AF700 (768319, R&D Systems) and anti-ASC-PE (HASC-71, Biolegend) was
443 carried out for 20 minutes at RT. Finally, cells were washed with BD Perm/Wash.

444 We also studied the expression of the inflammasome components in NK cells and monocytes from
445 kidney-injured patients, before and after being subjected to kidney transplantation. PBMCs from
446 these patients were stained with LIVE/DEAD AQUA viability (Invitrogen), and then, with anti-CD56-
447 FITC (B159, Becton Dickinson), anti-CD3-PE-Cy7 (SK7, Becton Dickinson), anti-CD4-BV605 (RPA-T4,
448 Becton Dickinson), anti-HLA-DR-PE-Da594 (L243, Biolegend), and anti-CD14-APC-H7 (M5E2, Becton
449 Dickinson) antibodies. Cells were subsequently fixed and permeabilized with
450 Fixation/Permeabilization Solution (Becton Dickinson) and intracellularly stained with anti-NLRP1-
451 AF647 (vwr), anti-NLRP3-AF700 (768319, R&D Systems) and anti-ASC-PE (HASC-71, Biolegend).
452 Samples were acquired on a CELESTA flow cytometer, and data was analyzed using FlowJo V10
453 software. Gating was performed according to the different FMO controls.

454 Samples were acquired on a CELESTA flow cytometer, and data was analyzed using FlowJo V10
455 software. Gating was performed according to the different FMO controls.

456 Western blot assays

457 Western blotting was performed using standard methods. Total PMBCs or NK cell extracted
458 proteins were used for standard protein electrophoresis and Western blot assays. Gel
459 electrophoresis was performed using 4–20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels, Biorad
460 at 200V in Tris-Glycine-SDS buffer for 40 mins. Protein transfer was made using a TurboTransfer
461 (Biorad, Hercules, CA, USA) at 25V for 7 mins. After transferring the proteins to 0.45 µM
462 nitrocellulose membranes (Biorad, Hercules, CA, USA), these were incubated for 1 hour in BSA 5%
463 in PBS-Tween20 0.05% and then overnight at 4ºC with primary antibodies at 1:1,000 dilution. Then
464 washed twice with PBS-Tween20 and incubated with the corresponding secondary antibody
465 coupled to horseradish peroxidase diluted 1:10,000 for 1h at RT. Protein loading was checked
466 using stain-free gel activation and tubulin protein amount. Stripping was not used.

467 The following primary antibodies were used: NLRP1 (ABclonal, A16212), NLRP3 (ABclonal, A5652),
468 Caspase-1 (Cell signaling, 3866S), Gasdermin D (Santacruz, sc-393581), GADPH (Cell signaling,
469 5174S). Anti-rabbit or anti-mouse IgG secondary antibody from Calbiochem was used.

470 Analysis of cytokine and immune factor secretion

471 Concentrations of the cytokines and molecules GM-CSF, IFN-γ, IL-1β, IL-6, IL-10, TNF-α, IL-18, IFN-
472 α, and ICAM-1 were quantified in 50 µL of supernatant from stimulated NK cells using a bead-
473 based multiplex immunoassay (ProcartaPlex; Invitrogen) according to the manufacturer's
474 recommendations. Measurements were performed using a Luminex Intelliflex instrument
475 (ThermoFisher Scientific) and analyzed using a standard curve for each cytokine.

476 LDH cytotoxicity assay

477 Cell death was measured by lactate dehydrogenase (LDH) release in the supernatant following the
478 manufacturer's instructions (abcam). In these experiments, cells were plated and stimulated in
479 RPMI 1640 without FBS to not interfere with the assay.

480 Quantification and statistical analysis

481 Statistical analyses were performed with Prism software, version 6.0 (GraphPad). A P value <0.05
482 was considered significant. The statistical details for the different experiments can be found in
483 each figure legend.

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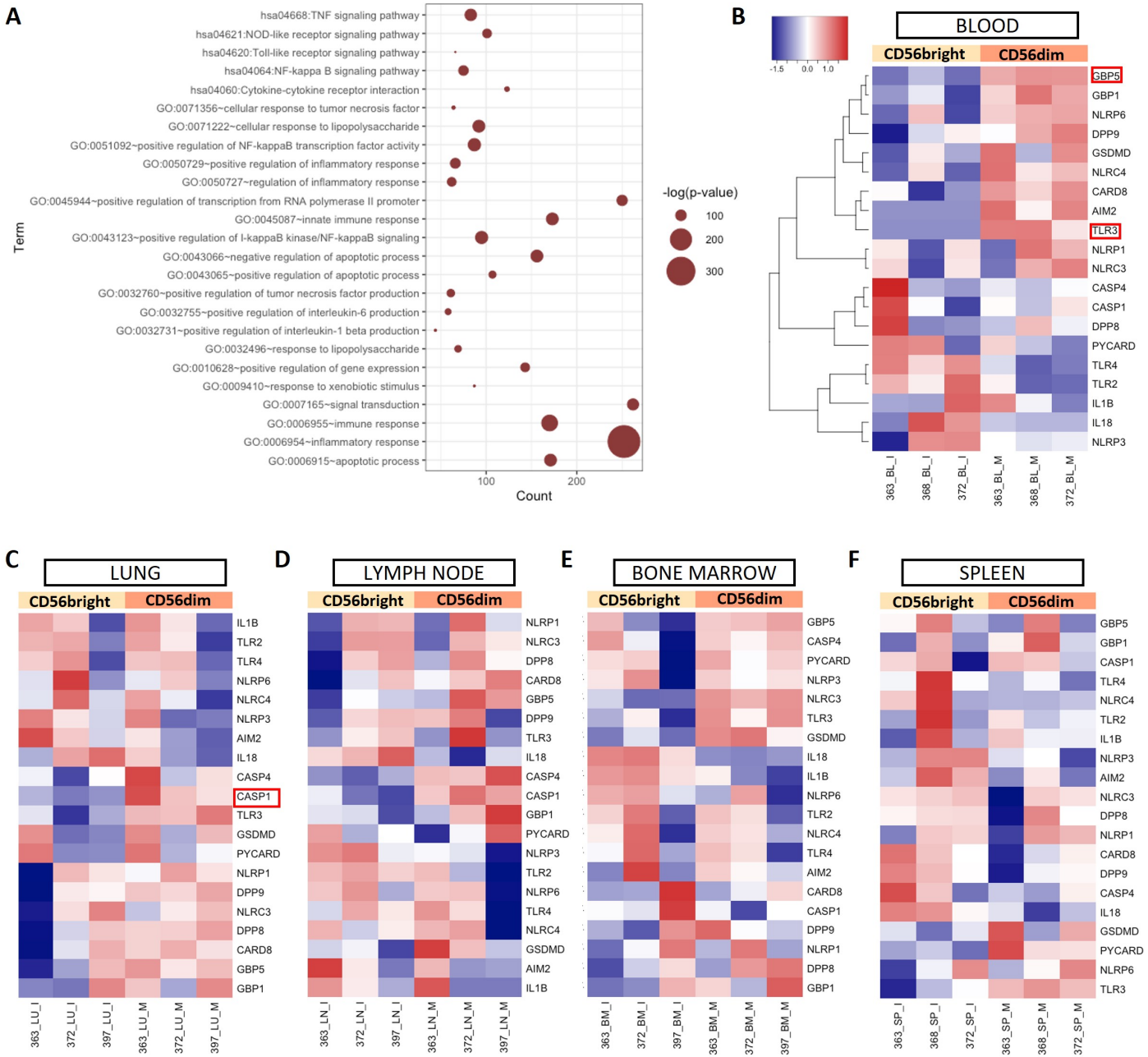
567

Figure 1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hsa04668:TNF signaling pathway
hsa04621:NOD-like receptor signaling pathway
hsa04620:Toll-like receptor signaling pathway
hsa04064:NF-kappa B signaling pathway
hsa04060:Cytokine-cytokine receptor interaction
GO:0071356~cellular response to tumor necrosis factor
GO:0071222~cellular response to lipopolysaccharide
GO:0051092~positive regulation of NF-kappaB transcription factor activity
GO:0050729~positive regulation of inflammatory response
GO:0050727~regulation of inflammatory response
GO:0045944~positive regulation of transcription from RNA polymerase II promoter
GO:0045087~innate immune response
GO:0043123~positive regulation of I-kappaB kinase/NF-kappaB signaling
GO:0043066~negative regulation of apoptotic process
GO:0043065~positive regulation of apoptotic process
GO:0032760~positive regulation of tumor necrosis factor production
GO:0032755~positive regulation of interleukin-6 production
GO:0032731~positive regulation of interleukin-1 beta production
GO:0032496~response to lipopolysaccharide
GO:0010628~positive regulation of gene expression
GO:0009410~response to xenobiotic stimulus
GO:0007165~signal transduction
GO:0006955~immune response
GO:0006954~inflammatory response
GO:0006915~apoptotic process
Term
100
200
Count
-log(p-value)
100
200
300
B
BLOOD
-1.5
0.0
1.0
CD56bright
CD56dim
GBP5
GBP1
NLRP6
DPP9
GSDMD
NLRC4
CARD8
AIM2
TLR3
NLRP1
NLRC3
CASP4
CASP1
DPP8
PYCARD
TLR4
TLR2
IL1B
IL18
NLRP3
363_BL_I
368_BL_I
372_BL_I
363_BL_M
368_BL_M
372_BL_M
C
LUNG
CD56bright
CD56dim
IL1B
TLR2
TLR4
NLRP6
NLRC4
NLRP3
AIM2
IL18
CASP4
CASP1
TLR3
GSDMD
PYCARD
NLRP1
DPP9
NLRC3
DPP8
CARD8
GBP5
GBP1
363_LU_I
372_LU_I
397_LU_I
363_LU_M
372_LU_M
397_LU_M
D
LYMPH NODE
CD56bright
CD56dim
NLRP1
NLRC3
DPP8
CARD8
GBP5
DPP9
TLR3
IL18
CASP4
CASP1
GBP1
PYCARD
NLRP3
TLR2
NLRP6
TLR4
NLRC4
GSDMD
AIM2
IL1B
363_LN_I
372_LN_I
397_LN_I
363_LN_M
372_LN_M
397_LN_M
E
BONE MARROW
CD56bright
CD56dim
GBP5
CASP4
PYCARD
NLRP3
NLRC3
TLR3
GSDMD
IL18
IL1B
NLRP6
TLR2
NLRC4
TLR4
AIM2
CARD8
CASP1
DPP9
NLRP1
DPP8
GBP1
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372_BM_M
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F
SPLEEN
CD56bright
CD56dim
GBP5
GBP1
CASP1
TLR4
NLRC4
TLR2
IL1B
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AIM2
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Figure 2

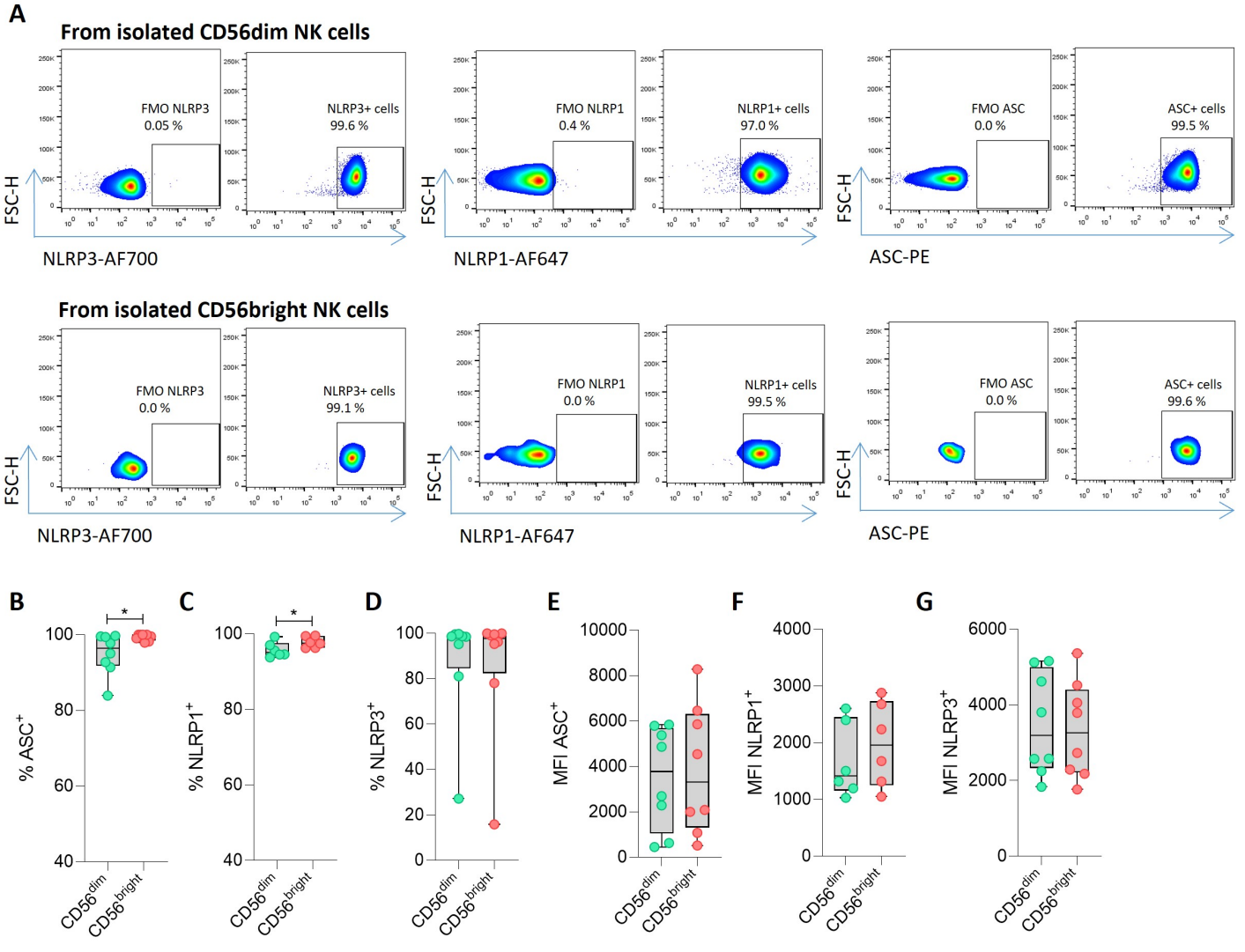

A
From isolated CD56dim NK cells
FMO NLRP3
0.05 %
NLRP3+ cells
99.6 %
FMO NLRP1
0.4 %
NLRP1+ cells
97.0 %
FMO ASC
0.0 %
ASC+ cells
99.5 %
FSC-H
NLRP3-AF700
NLRP1-AF647
ASC-PE
From isolated CD56bright NK cells
FMO NLRP3
0.0 %
NLRP3+ cells
99.1 %
FMO NLRP1
0.0 %
NLRP1+ cells
99.5 %
FMO ASC
0.0 %
ASC+ cells
99.6 %
B
% ASC+
C
% NLRP1+
D
% NLRP3+
E
MFI ASC+
F
MFI NLRP1+
G
MFI NLRP3+
CD56dim
CD56bright

Figure 3

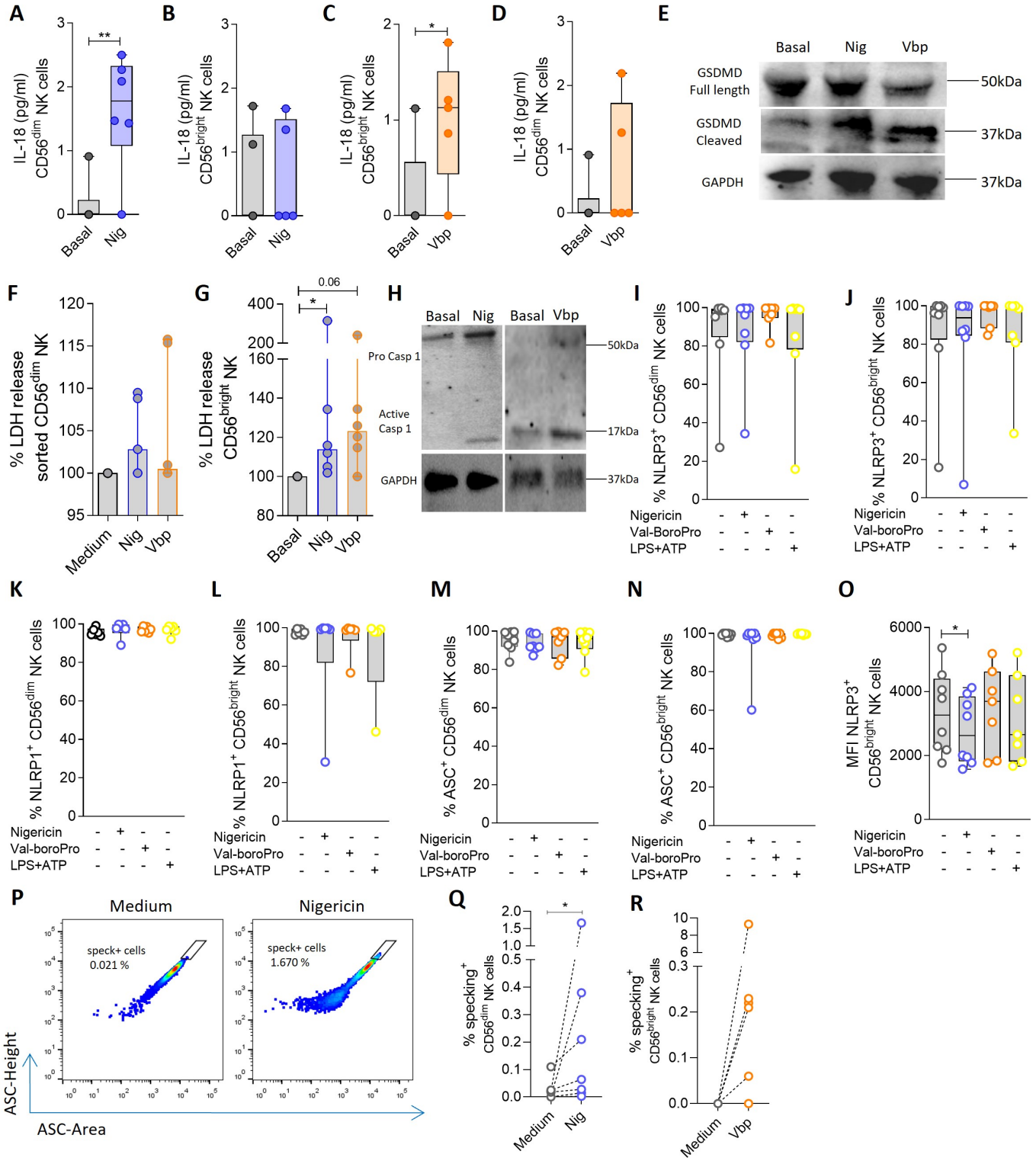
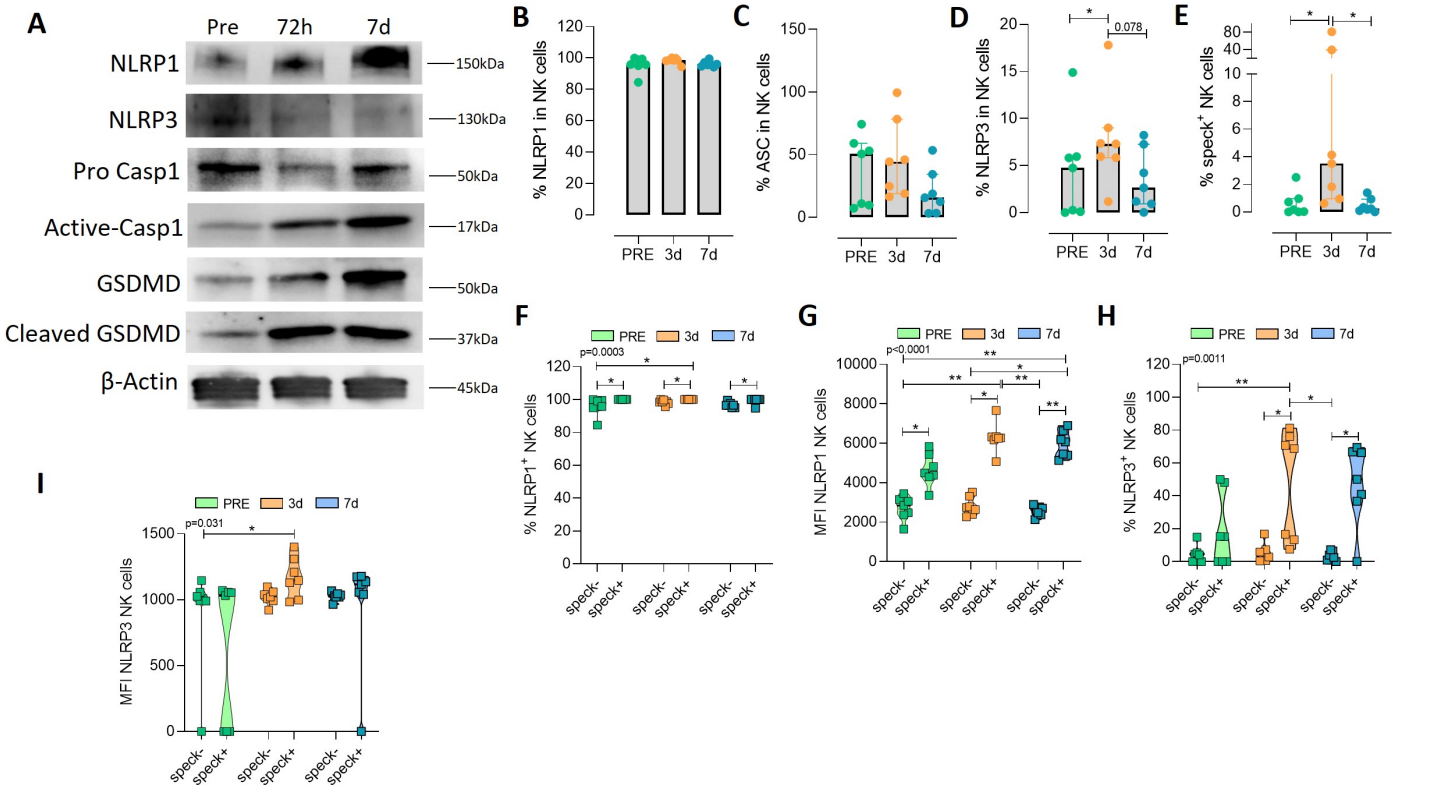

A
IL-18 (pg/ml) CD56dim NK cells
Basal
Nig
B
IL-18 (pg/ml) CD56bright NK cells
C
IL-18 (pg/ml) CD56bright NK cells
Vbp
D
IL-18 (pg/ml) CD56dim NK cells
E
Basal
Nig
Vbp
GSDMD Full length
GSDMD Cleaved
GAPDH
50kDa
37kDa
37kDa
F
% LDH release sorted CD56dim NK
Medium
G
% LDH release CD56bright NK
0.06
H
Pro Casp 1
Active Casp 1
50kDa
17kDa
37kDa
I
% NLRP3+ CD56dim NK cells
Nigericin
Val-BoroPro
LPS+ATP
J
% NLRP3+ CD56bright NK cells
K
% NLRP1+ CD56dim NK cells
L
% NLRP1+ CD56bright NK cells
M
% ASC+ CD56dim NK cells
N
% ASC+ CD56bright NK cells
O
MFI NLRP3+ CD56bright NK cells
P
Medium
Nigericin
speck+ cells 0.021 %
speck+ cells 1.670 %
ASC-Height
ASC-Area
Q
% specking+ CD56dim NK cells
R
% specking+ CD56bright NK cells

Figure 4


A
Pre
72h
7d
NLRP1
NLRP3
Pro Casp1
Active-Casp1
GSDMD
Cleaved GSDMD
β-Actin
150kDa
130kDa
50kDa
17kDa
50kDa
37kDa
45kDa
B
% NLRP1 in NK cells
C
% ASC in NK cells
D
% NLRP3 in NK cells
0.078
E
% speck+ NK cells
PRE
3d
7d
F
% NLRP1+ NK cells
p=0.0003
G
MFI NLRP1 NK cells
p<0.0001
H
% NLRP3+ NK cells
p=0.0011
I
MFI NLRP3 NK cells
p=0.031
speck-
speck+