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.

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[&]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.

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

NK cells from patients with chronic kidney disease display inflammasome activation

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

Discussion

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

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

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

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

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

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

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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.

FIGURE LEGENDS

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

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

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

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

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

MATERIALS AND METHODS

Human Samples

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

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

Transcriptomic analysis of a publicly available RNAseq data

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

Human NK cell isolation by FACS

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

In vitro inflammasome activation assays

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

Flow cytometry

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

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

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Western blot assays

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

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

Analysis of cytokine and immune factor secretion

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

LDH cytotoxicity assay

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

Quantification and statistical analysis

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

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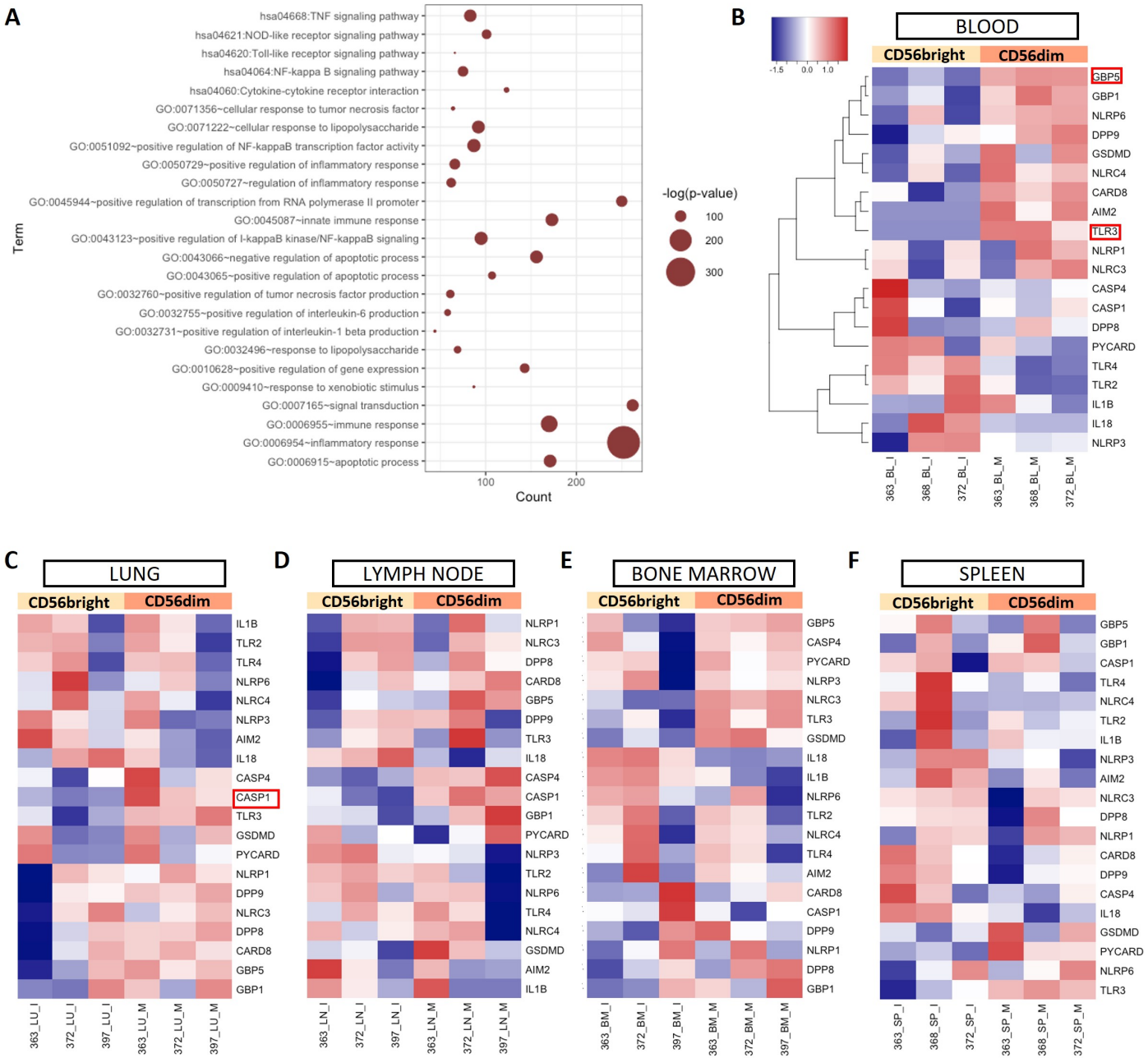
Figure 1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hsa04620:Toll-like receptor signaling pathway
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GO:0071356~cellular response to tumor necrosis factor
GO:0071222~cellular response to lipopolysaccharide
GO:0051092~positive regulation of NF-kappaB 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
CD56bright
CD56dim
-1.5
0.0
1.0
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
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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
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CASP1
GBP1
PYCARD
NLRP3
TLR2
NLRP6
TLR4
NLRC4
GSDMD
AIM2
IL1B
363_LN_I
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397_LN_I
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E
BONE MARROW
CD56bright
CD56dim
GBP5
CASP4
PYCARD
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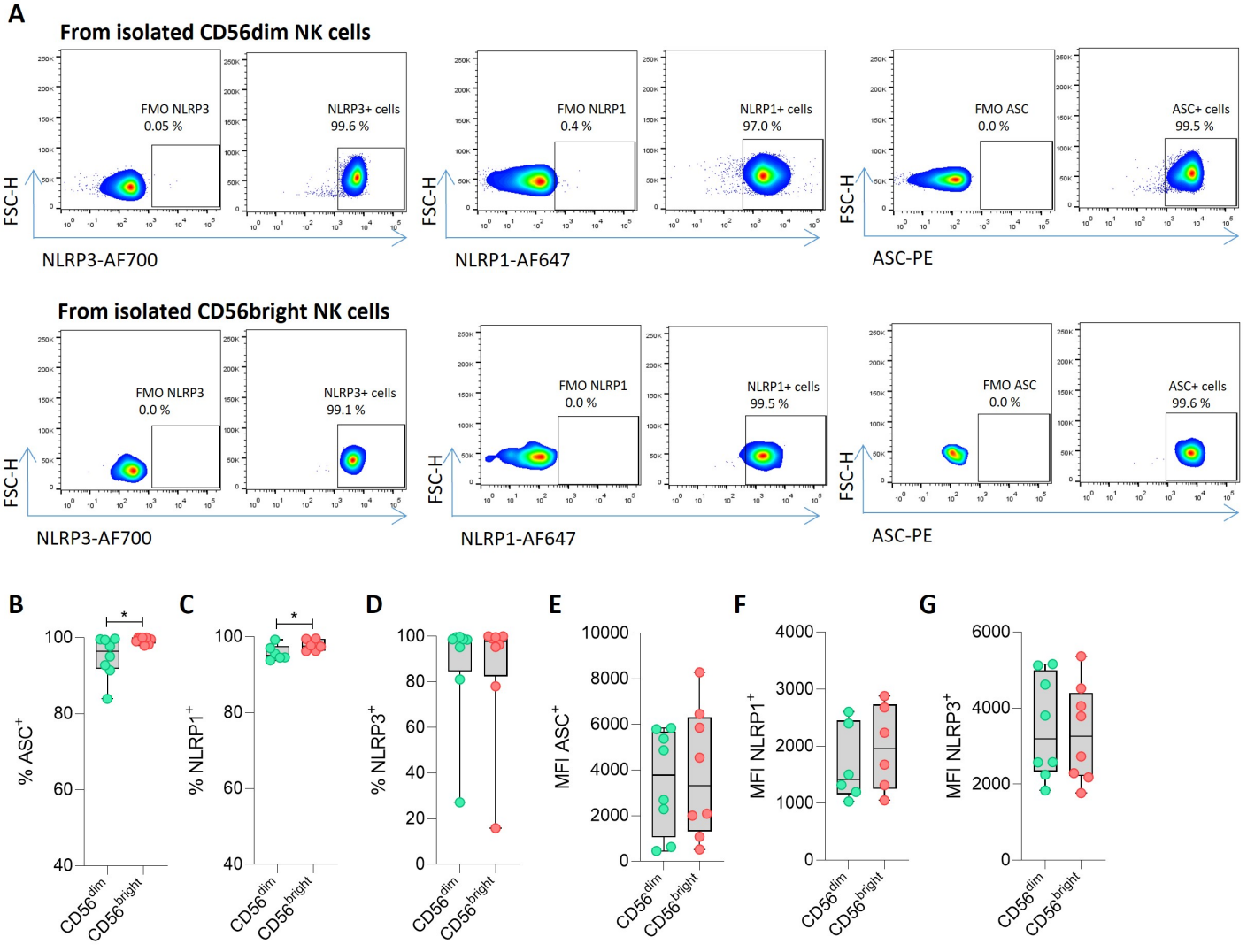
Figure 2


A
From isolated CD56dim NK cells
FMO NLRP3
0.05 %
NLRP3+ cells
99.6 %
FSC-H
NLRP3-AF700
FMO NLRP1
0.4 %
NLRP1+ cells
97.0 %
NLRP1-AF647
FMO ASC
0.0 %
ASC+ cells
99.5 %
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+
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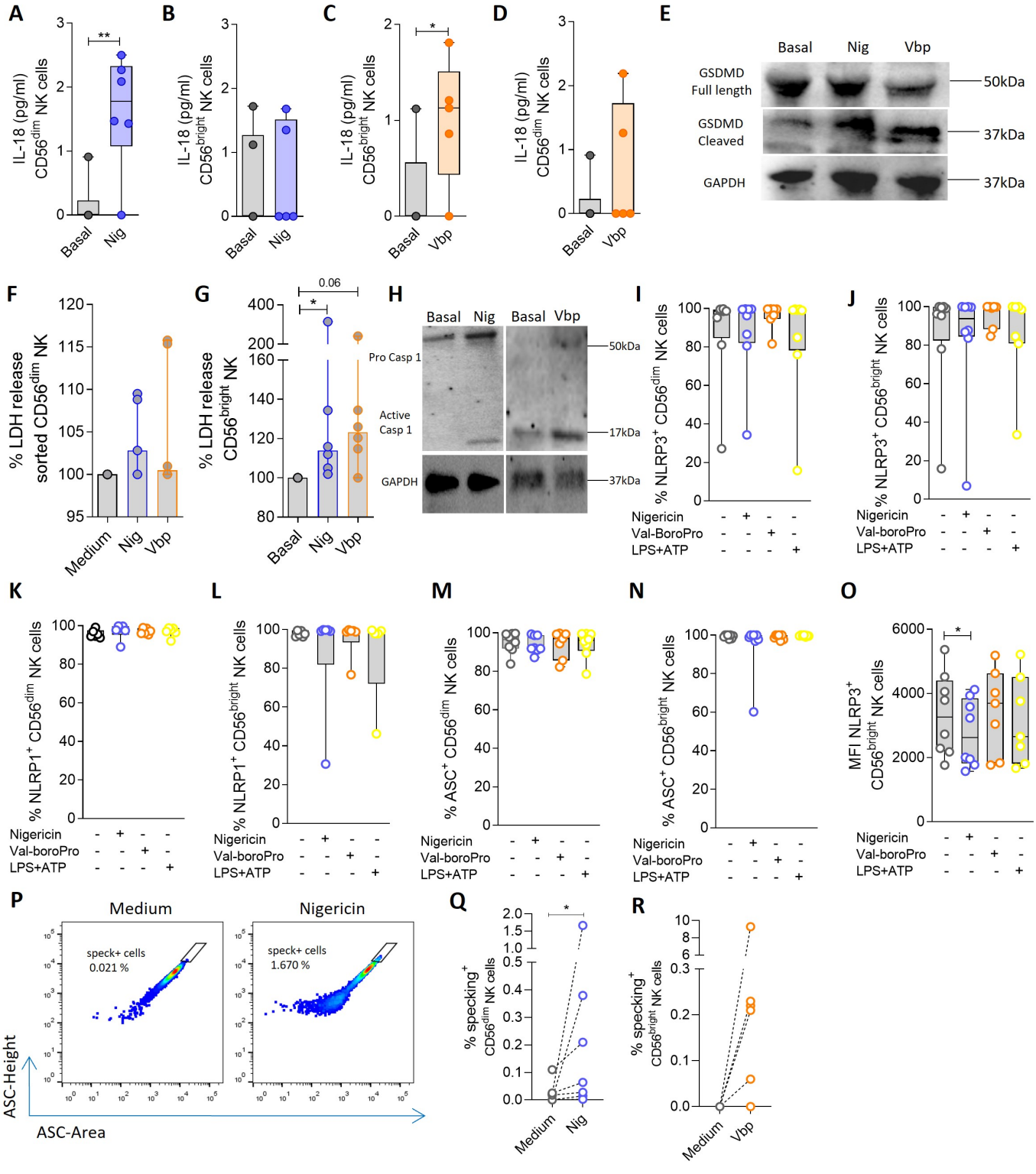
MFI NLRP1+
G
MFI NLRP3+
CD56dim
CD56bright

Figure 3


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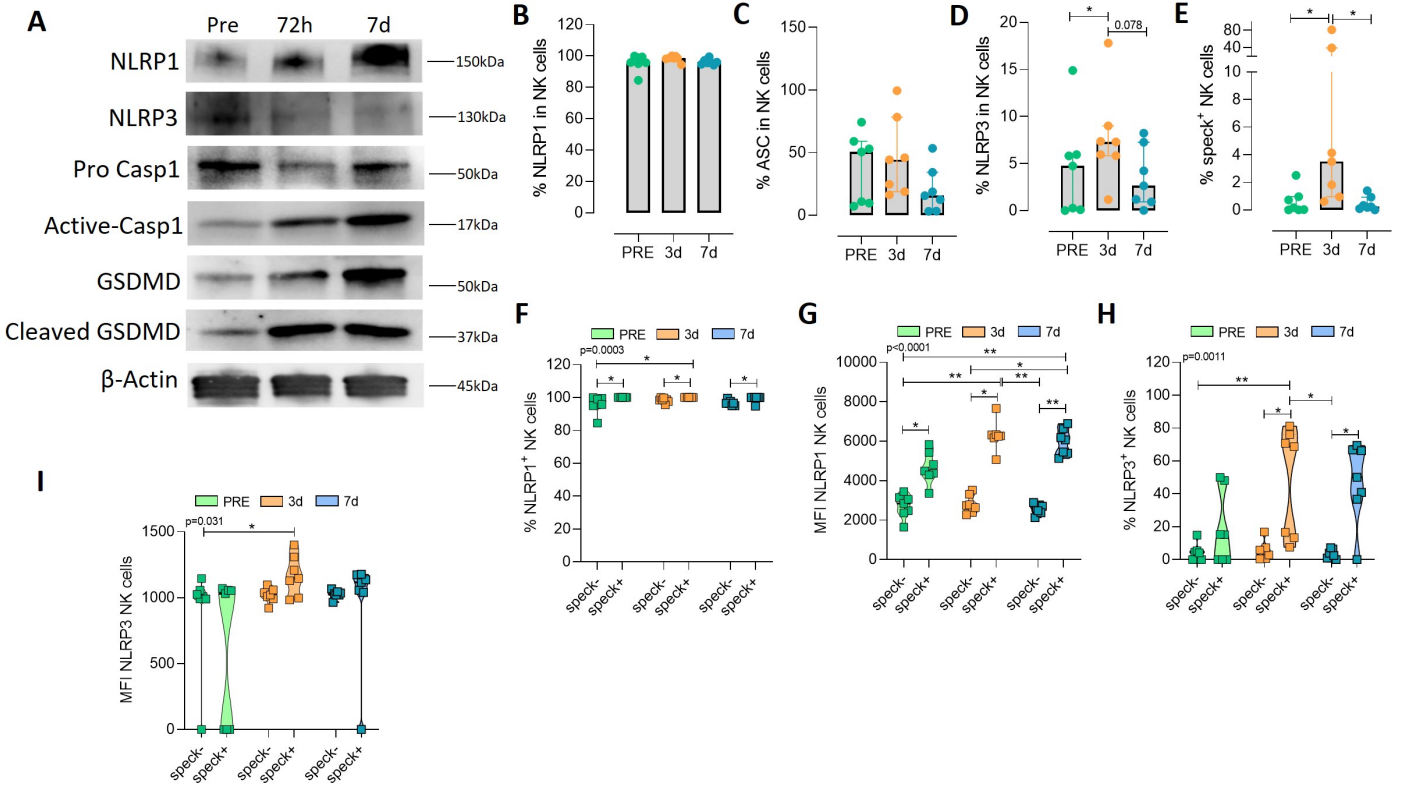
speck+ cells 1.670 %
ASC-Height
ASC-Area
% specking+ CD56dim NK cells
% specking+ CD56bright NK cells

Figure 4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Pre
72h
7d
NLRP1
NLRP3
Pro Casp1
Active-Casp1
GSDMD
Cleaved GSDMD
β-Actin
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130kDa
50kDa
17kDa
50kDa
37kDa
45kDa
B
% NLRP1 in NK cells
PRE 3d 7d
C
% ASC in NK cells
PRE 3d 7d
D
% NLRP3 in NK cells
0.078
PRE 3d 7d
E
% speck+ NK cells
PRE 3d 7d
F
PRE 3d 7d
p=0.0003
% NLRP1+ NK cells
speck- speck+
G
PRE 3d 7d
p<0.0001
MFI NLRP1 NK cells
speck- speck+
H
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p=0.0011
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speck- speck+
I
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