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## Changes in the Natural Killer cell repertoire and function induced by the cancer immune adjuvant candidate IMMUNEPOTENT-CRP

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15 **Keywords:** Natural Killer Cells, Dialyzable Leukocyte Extract, Cell Activation, NK Cell

16 Receptors Repertoire, Effector Activity, Immunotherapy

17 **Running title:** IMMUNEPOTENT-CRP improves NK cell cytotoxicity

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29 **Abstract**

30 Natural killer (NK) cells are CD3<sup>-</sup>, CD16<sup>+</sup>, CD56<sup>+</sup> that play a crucial role in immune response by  
31 recognizing and eliminating a variety of virus-infected, malignant, and antibody-coated target cells.  
32 We examined activation; repertoire changes and effector functions of human NK cells normal donors  
33 treated with IMMUNEPOTENT-CRP (I-CRP), a bovine dialyzable leukocyte extract (DLE)  
34 containing a mixture of low molecular weight molecules. I-CRP induces human NK cells activation  
35 and increase CD56<sup>Dim</sup> CD16<sup>-</sup> subset, without inducing proliferation. Human NK cells showed an  
36 increase on NKp30, NKp44, NKp46, NKG2D, NKG2C and KIR receptors, whereas no significant  
37 differences on CD160, CD85j and CD226 were observed. I-CRP-treated human NK cells exhibited  
38 an increased degranulation activity against K562 target cells, as shown by CD107a assay, and  
39 correlate with cytotoxicity against K562 cells observed in calcein release assay. These results  
40 indicate that I-CRP can modify human NK cells receptor repertoire leading to an increased cytotoxic  
41 activity, supporting evidence for its use to stimulate NK cells.

42

43 **Highlights**

- 44 • IMMUNEPOTENT-CRP promotes CD56<sup>Dim</sup> CD16<sup>-</sup> NK subset
- 45 • IMMUNEPOTENT-CRP induces changes on receptor repertoire on NK cells
- 46 • IMMUNEPOTENT-CRP improves NK cell cytotoxicity
- 47 • NK increase their cytotoxicity against target cells during IMMUNEPOTENT-CRP treatment

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49

50 **Abbreviations**

51 Antimicrobial peptides (AMP)

52 Carboxyfluorescein succinimidyl ester (CFSE)

53 Concanavalin A (ConA)

54 Dialyzable leukocyte extract (DLE)

55 Fluorescence-Activated Cell Sorting (FACS)

56 Innate lymphoid cells (ILC)

57 IMMUNEPOTENT-CRP (I-CRP)

58 Interferon (IFN)-gamma

59 Major histocompatibility complex (MHC)

60 Natural cytotoxicity receptors (NCR)

61 Killer immunoglobulin-like receptor (KIR)

62 Natural Killer (NK)

63 Tumor necrosis factor (TNF)-alpha,

64 Killer cell lectin-like receptors (KLR)

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68 **1 Introduction**

69 Natural killer (NK) cells are innate lymphoid cells (ILC) commonly defined by the presence of  
70 markers such as CD56<sup>+</sup> (neural cell adhesion molecule) and/or CD16<sup>+</sup> (low-affinity antibody-binding  
71 receptor Fc $\gamma$ - receptor IIIa) and the absence of CD3 molecule on the surface [1,2]. CD56 and CD16  
72 markers are frequently used to define the NK cell functions as immunomodulatory or cytotoxic [3,4].  
73 Biological functions of NK cells play an important role in host defense, because they are involved in  
74 recognizing and killing tumor or pathogen infected cells. They also play a key immunoregulatory  
75 role, as NK cells can secrete various set of cytokines, such as interferon (IFN)-gamma and tumor  
76 necrosis factor (TNF)-alpha, among others [5–7].

77 NK cells constantly interact with other cells in the organism. Normal cells, with the exception of  
78 dendritic cells, cannot be recognized by NK cells because they adequate amounts of major  
79 histocompatibility complex (MHC) class I molecules on their surface. Transformed cells, on the  
80 other hand, have a lowered expression of MHC class I molecules, or have lost them, and thus show  
81 increased expression of stress molecules and can therefore be recognized and killed [8]. The altered  
82 expression of class I antigens or increased expression of stress molecules, a common event in tumor  
83 transformation or following a pathogen infection, leads to NK cell-mediated target cell lysis [9,10].  
84 This “natural” cytotoxicity is controlled by a wide range of receptors expressed on the cell surface,  
85 which can be classified on different families, such as: natural cytotoxicity receptors (NCR; NKp46,  
86 NKp44, NKp30), killer cell lectin-like receptors (NKG2D, NKG2A, CD161), leukocyte inhibitory  
87 receptors (LILRB1 or CD85j), and killer immunoglobulin-like receptor (KIR; CD158a/h, CD158b/j,  
88 CD158d) [7,11,12]. Depending on the subset, NK cells express different combinations of inhibitory  
89 or activating receptors, that result in a diversity within the NK cell populations with the potential to  
90 respond to a variety of stimuli under different pathological conditions [12,13]. A fine regulation  
91 between opposite signals delivered by inhibitory or activating receptors dictates “final” functions of  
92 NK cells effectors [13,14].

93 Tumor progression and chronic infections generally lead to the depletion or exhaustion of NK cells,  
94 limiting their potential for activity [9,15–17]. Multiple mechanisms contribute to these effects, such  
95 as dysregulations in receptor signaling, suppressive effects of regulatory cells, as well as soluble  
96 factors in the microenvironment [16]. This condition could be surpassed by cytokine-mediated  
97 activation and the use of other NK-cell target immunotherapies, increasing immunity against the  
98 tumor or improving the clinical response to conventional treatments [18–20].

99 Immuno-directed therapies have taken an important place in the treatment of several diseases.  
100 Among them, dialyzable leukocyte extracts (DLE), which are a mixture of low molecular weight  
101 substances, obtained from blood or lymphoid tissues, could have immunomodulatory effects [21].  
102 DLE obtained from leukocytes from human, pig, or crocodile blood, or bovine leukocytes from  
103 spleen or colostrum have been shown to modulate multiple molecular targets that promote  
104 immunomodulatory activities in conditions such as immunodeficiencies, autoimmune, bacterial  
105 diseases, asthma, allergies, and some types of cancer [21–26]. The mixture of low molecular weight  
106 molecules obtained from DLE, regardless of the species from which they were obtained, have  
107 demonstrated similar effects on leukocytes from mice and humans, which include the activation of  
108 similar signaling pathways related to their immunomodulatory effects [24,27–30]. In this context,  
109 phase I clinical studies show that IMMUNEPOTENT-CRP (I-CRP) treatment (a bovine dialyzable  
110 leukocyte extract) improves the quality of life of lung and breast cancer patients, increasing the  
111 number of total leukocytes as well as the CD4<sup>+</sup>, CD8<sup>+</sup>, CD16<sup>+</sup> and CD56<sup>+</sup> subpopulations [29,31].  
112 Also, I-CRP is selectively cytotoxic against cancer cell lines *in-vitro*, with no important cytotoxic  
113 affectations in immune system cells [31–35]. However, the direct effect on the functionality of  
114 lymphocytes has not been clearly elucidated, and its effect in NK cells has not been assessed. Thus,  
115 the aim of this study was to analyze the *in-vitro* effects of I-CRP in human NK cells, by testing their  
116 activation, repertoire modifications, and effector activity.

117 **1.1 Material and Methods**

118 **1.1.1 IMMUNEPOTENT-CRP**

119 IMMUNEPOTENT-CRP (I-CRP), was produced by the Laboratorio de Inmunología y Virología of  
120 Facultad Ciencias Biológicas of Universidad Autónoma de Nuevo León (UANL, México), as  
121 previously described [36]. One unit (U) of I-CRP is defined as 24 mg of peptides obtained from  
122  $15 \times 10^8$  leukocytes [36]. I-CRP was aliquoted in culture medium (RPMI 1640 medium; GIBCO  
123 Thermofisher, Waltham, Massachusetts, USA; supplemented with 1  $\mu\text{g}/\text{mL}$  amphotericin B, 1  $\mu\text{g}/\text{mL}$   
124 penicillin and  $2.5 \times 10^{-3}$   $\mu\text{g}/\text{mL}$  streptomycin; GIBCO® by Life technologies; and 10% of FBS;  
125 GIBCO® by Life technologies). The stock solution was stored at 4°C.

126 **1.1.2 Cell isolation**

127 Blood was collected from healthy donors after obtaining written informed consent. This study was  
128 approved by the Institutional Ethics Committee at the Universidad Autónoma de Nuevo León,  
129 College of Biological Sciences. Then, peripheral blood mononuclear cells (PBMC) were obtained  
130 after centrifugation on Ficoll-Paque™ PLUS (GE Healthcare, Chicago, Illinois, USA). PBMC were  
131 maintained at  $1 \times 10^5$  cells/mL at 37°C in 5% CO<sub>2</sub> atmosphere in culture medium. Human  
132 myelogenous leukemia cell line, K562, (ATCC® CCL-243™) and T-acute lymphoblastic leukemia  
133 cell lines CEM (ATCC® CCL-119™) and MOLT-4 (ATCC® CRL-1582™), were obtained from  
134 the American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured as suggested  
135 by the supplier.

136 **1.1.3 NK cells purification**

137 NK cells were purified from PBMC of at least six healthy individuals per study, by negative selection  
138 using magnetic-activated cell sorting (MACS) microbead technology (Miltenyi Biotec, Bergisch  
139 Gladbach, Germany; >90% purity and >90% viability). PBMC were incubated with anti-CD20  
140 microbeads, anti-CD3 microbeads and anti-CD14 microbeads (ref:130-091-104, 130-050-101 and

141 130-050-201; respectively) as stated by manufacturer's instructions. NK cells were put in culture in  
142 similar conditions than PBMC.

#### 143 ***1.1.4 Analysis of lymphocyte populations***

144 The principal lymphocyte subsets (CD3<sup>+</sup>, CD20<sup>+</sup> and CD56<sup>+</sup> CD16<sup>+</sup>) of PBMC were evaluated as  
145 follows: 1×10<sup>5</sup> PBMC per well were seeded in conic 96-well plates (Corning Inc. Costar®, NY,  
146 USA) and treated with different concentrations of I-CRP (0.3, 0.7 and 1.5 U/mL) or with the mitogen  
147 concanavalin A as a control (ConA; 5µg/mL; Sigma-Aldrich, Germany) for 24h and 48h. At the end  
148 of incubation, cells were collected, washed and stained with anti-CD56 (CD56-PE; cat:555516, BD  
149 Biosciences), anti-CD16 (CD16-PerCP-Cy; cat:560717, BD Biosciences), anti-CD3 (CD3-FITC;  
150 cat:555916, BD Biosciences) and anti-CD20 (CD20-APC; cat:559776, BD Biosciences) in 100 µL of  
151 FACS buffer at room temperature in darkness. After 30 min of incubation, cells were washed,  
152 assessed by flow cytometry (BDAccury6; Becton Dickinson, San Jose, CA, USA), and analyzed  
153 using FlowJo Software (Tree Star Inc., Ashland, OR, USA).

#### 154 ***1.1.5 NK-cells receptors repertoire***

155 All phenotypic studies were implemented on PBMC, (1×10<sup>5</sup> per well in 96-well round bottom plate)  
156 and treated with I-CRP (1.5 U/mL), IL-2 (200 U/mL) as positive control, or left unstimulated as  
157 negative control, for 24h and 48h. After, supernatant was discarded, and cells were stained for 30 min  
158 with the following Abs CD45 (clone J33), CD3 (UCHT1), CD16 (3G8), CD160 (191B8), NKp44  
159 (BAB281), NKp30 (Z25), CD85j (HP-F1), CD158e1/2 (Z27.3.7), CD158i (FES172), NKG2D  
160 (ON72), NKp46 (BAB281) from Beckman Coulter, CD56 (B159), CD69 (L78), CD226 (DX11),  
161 CD161 (DX12) from BD Biosciences, NKG2C (134591), CD158d (181703) from R&D Systems,  
162 and CD158b/j (DX27) CD158a/h (11PB6) from Miltenyi Biotec. Analyses were performed by nine-  
163 color staining flow cytometry using a CytoFLEX S cytometer and analyzed using Kaluza Analysis  
164 2.1 software (Beckman Coulter). Lymphocytes were gated in FSC/SSC dot plot and at least 30,000



165 events were collected, then CD45<sup>+</sup> were selected and CD3<sup>+</sup> lymphocytes were eliminated from the  
166 analysis by plotting lymphocytes in a FSC/CD3 expression dot plot. Negative CD3 cells were  
167 analyzed for the expression of CD56 and/or CD16 which defined NK cells and receptor expression  
168 analysis was performed in CD3<sup>-</sup>CD56<sup>+/-</sup>CD16<sup>+/-</sup> population.

#### 169 ***1.1.6 Cell viability and early activation assay***

170 To determine effects on viability and the activation induced by I-CRP on PBMC or NK cells, we  
171 used Calcein-AM (ThermoFisher, Waltham, Massachusetts, USA) staining combined to CD69. First,  
172 PBMC or NK cells were seeded at 1×10<sup>5</sup> cells per well in 96-well conic bottom plates (Corning Inc.  
173 Costar®, NY, USA) and treated for 24h and 48h with different concentrations of I-CRP (0.3, 0.7 and  
174 1.5 U/mL), concanavalin A (ConA; 5 µg/mL; Sigma-Aldrich, Germany), IL-2 (200 U/mL; R&D  
175 Systems®, US) or IL-15 (ng/mL; Gibco®, US). After incubation, cells were collected, washed twice  
176 and then, incubated Calcein-AM (0.25 µM) for 30 min at room temperature in darkness. Finally, after  
177 washings, cells were incubated with an anti-CD69-allophycocyanin antibody (CD69-APC,  
178 cat:555533, BD Biosciences) at room temperature in darkness. Cells were then washed and  
179 resuspended in FACS buffer to be assessed by flow cytometry (Fluorescence-Activated Cell Sorting  
180 [FACS]; BD Accury6; Becton Dickinson, San Jose, CA, USA) and analyzed using FlowJo Software  
181 (Tree Star Inc., Ashland, OR, USA).

#### 182 ***1.1.7 Proliferation assay***

183 To test proliferation of PBMC, we used Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen,  
184 Carlsbad, USA) and 1×10<sup>9</sup> PBMC were stained (1 µM) following the manufacturer's instructions.  
185 PBMC were then washed twice with PBS. CFSE stained PBMC were incubated with I-CRP (1.5  
186 U/mL) or ConA (5 µg/mL) for 96h. At the end of the culture, cells were collected, washed twice with  
187 PBS, and stained with Calcein-AM (as previously described), assessed by flow cytometry

188 (Fluorescence-Activated Cell Sorting [FACS]; BDAccury6; Becton Dickinson, San Jose, CA, USA),  
189 and analyzed using FlowJo Software (Tree Star Inc., Ashland, OR, USA).

#### 190 ***1.1.8 Cytokine release assay***

191 The supernatants from the indicated cultures and co-cultures were collected and concentrations of IL-  
192 2, IL-4, IL-6, IL-10, IL-17a and TNF- $\alpha$  were tested with CBA Human Th1/Th2/Th17 Cytokine Kit  
193 (BD, San Jose, CA, USA) by flow cytometry following the manufacturer's instructions.

#### 194 ***1.1.9 Degranulation assay (CD107a)***

195 After incubation with I-CRP (1.5 U/mL) for 24h, PBMC or NK cells were pelleted, washed twice  
196 with PBS, and co-cultivated with K562 cell line (ratios NK:K562 5:1, 10:1, 15:1, 20:1 and 30:1).  
197 Also, PBMC or NK cells were co-cultured with CEM or MOLT-4 cells, in an independent manner in  
198 the ratio 10:1 (E:T). After 1h, the anti-CD107a (H4A3, CD107a-FITC; cat:555800, BD Biosciences)  
199 was added, and co-cultures were incubated for 3h. Afterwards, cells were recovered, washed (PBS),  
200 assessed by flow cytometry (BDAccury6) and analyzed using FlowJo Software (Tree Star Inc.,  
201 Ashland, OR, USA).

#### 202 ***1.1.10 NK cell cytotoxicity (Calcein-AM release assay)***

203 After PBMC or NK cells were stimulated with I-CRP for 24h, and washed twice, cells were co-  
204 incubated with K562 target cell line labeled with Calcein-AM (2 $\mu$ M) at an E:T ratio of 10:1 for 4h at  
205 room temperature in 5% CO<sub>2</sub>. NK cell-mediated target cell lysis was assessed by flow cytometry and  
206 determined by the loss of Calcein-AM stain-expressing K562 target cells. K562 cells were gated in  
207 FSC/SSC dot plot and 10,000 events were collected K562 cells alone were used as a control.

#### 208 ***1.1.11 Statistical analyses***

209 Statistical analyses were conducted using the nonparametric Mann-Whitney U test (\*p<0.05,  
210 \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001). The level of significance in figures 2 and 3 was adjusted for  
211 multiple testing using the Bonferroni correction. The results are expressed as median of at least three

212 independent experiments (median±SD). The data was analyzed using GraphPad Prism (GraphPad  
213 Software, San Diego, CA, USA).

## 214 **1.2 Results**

### 215 *1.2.1 I-CRP activates and modulates PBMC, but does not promote proliferation*

216 Cell activation was evaluated on peripheral blood mononuclear cells (PBMC) derived from healthy  
217 human donors after treatment with I-CRP at different concentrations (0.3, 0.7 and 1.5 U/mL) or  
218 Concanavalin A (ConA, 5µg/mL) as a positive control. In figure 1A, we show the gating strategy  
219 used to determine cell viability and activation on PBMC. Viability of PBMC stimulated with  
220 different concentrations of I-CRP or ConA is shown in supplementary figure 1. Loss of cell viability  
221 is not observed after 24 or 48h of treatment with I-CRP, even at 0.7 U/mL, a CC<sub>100</sub> (cytotoxic  
222 concentration 100) in leukemic T-cells [33], suggesting that I-CRP is non-toxic to PBMC (middle  
223 panel) (supplementary figure 1). Interestingly, in Figure 1A we can observe that I-CRP incubation  
224 increases the CD69 expression on viable PBMC at 0.7 U/mL and 1.5 U/mL, finding similar values to  
225 those observed after ConA stimulation. The increased expression of CD69 was observed since 24h of  
226 treatment and was more important after 48h of incubation (Fig. 1A).

227 After observing the activation induced by I-CRP, we then assessed if it could modulate different cell  
228 subsets. In figure 1B, we show the representative gating strategy we followed to assess CD3, CD20,  
229 CD16, and CD56 populations. After 24h of culture with different concentrations of I-CRP, we did  
230 not observe significant differences on CD3<sup>+</sup> T cells (Fig. 1C) and CD20<sup>+</sup> B cells (Fig. 1D) of PBMC  
231 from healthy donors, when compared with untreated controls. As shown in figure 1E, we observe a  
232 decrease of CD56<sup>Dim</sup> CD16<sup>+</sup> NK cell subset only after 48h of treatment with 1.5 U/mL of I-CRP. On  
233 the other hand, we can observe a significant augmentation of the CD56<sup>Dim</sup> CD16<sup>-</sup> population after  
234 24h of stimulation with the same concentration as well as with 0.7 U/mL after 48h of treatment (Fig.  
235 1F), when compared to non-stimulated PBMC. After 48h of culture, we observed similar results, and  
236 just a slight decrease in B cells (p<0.05) at the highest concentration of I-CRP tested (Fig. 1D), and a

237 significant increase of CD56<sup>Dim</sup> CD16<sup>-</sup> NK cells at 0.7 U/mL of I-CRP (Fig. 1F). These results show  
238 that I-CRP modulates NK cell subsets by increasing the CD56<sup>Dim</sup> CD16<sup>-</sup> subset. Interestingly, our  
239 cytokine secretion analysis showed that I-CRP promotes TNF-alpha, IL-6, IL-10 and IL-17A  
240 secretion on PBMC, at a higher level than those observed after IL-15 stimulation, and a lower  
241 secretion of IL-2, and no differences in IL-4 production (supplementary figure 2), revealing that I-  
242 CRP-stimulates the release different cytokines.

243 To assess whether I-CRP could induce proliferation, PBMC were stained with CFSE before I-CRP or  
244 ConA incubation, and cells were then cultured for 96h. Results show that PBMC stimulated with I-  
245 CRP do not present any proliferative peaks (Fig. 1G), in contrast with ConA, which showed four new  
246 peaks with lower CFSE fluorescence on histogram. These results revealed that I-CRP induce the  
247 early activation but not proliferation of PBMC.

### 248 *1.2.2 NK cells modulate CD161 accessory molecule and killer cell lectin-like NKG2D and NKG2C* 249 *receptors upon I-CRP stimulation*

250 To get more information about the changes in NK cell CD56<sup>Dim</sup> CD16<sup>-</sup> subsets, we analyzed if I-  
251 CRP activates (CD69 expression) NK cells and if it could modulate accessory receptors and killer  
252 cell lectin-like receptors (KLR), which can mediate NK cell antitumor activity. First, we gated NK  
253 cells (gating strategy on figure 2A), by selecting lymphocytes that were gated in FSC/SSC dot plot  
254 and at least 30,000 events, then CD45<sup>+</sup> cells were selected and CD3<sup>+</sup> lymphocytes were eliminated  
255 from the analysis by plotting lymphocytes in FSC/CD3 expression dot plot. Negative CD3 cells were  
256 analyzed for the expression of CD56 and/or CD16 which were defined as NK cells, and receptor  
257 expression analysis was performed in CD3<sup>-</sup>CD56<sup>+/</sup>-CD16<sup>+/</sup>- population. We observed that I-CRP  
258 increased CD69 expression on NK cells after 24h (p<0.01) and 48h (p<0.01) of treatment, similar to  
259 the results observed on IL-2-treated cells (Fig. 2B), and confirming, that I-CRP induces early  
260 activation on NK cells.

261 Then, we analyzed accessory receptors and KLR that use HLA as a ligand and have inhibiting and  
262 activating types of receptors. In figure 2C we can observe that I-CRP promotes the augmentation of  
263 CD161 expression ( $p < 0.01$ ) in NK cells after treatment, when compared to untreated cells; while no  
264 significant differences were observed on IL-2-stimulated cells when compared to control cells  
265 ( $p > 0.05$ ). However, we did not observe significant differences on CD160, CD85j and CD226  
266 expression of I-CRP or IL-2 treated cells, when compared to control cells (Fig. 2C).

267 On the other hand, when assessing KLR we observed that I-CRP increased NKG2D ( $p < 0.01$ ) and  
268 NKG2C ( $p < 0.01$ ) receptors expression on NK cells, when compared to controls at 24 and 48h (Fig.  
269 2D). In contrast, IL-2 only shows an increase of NKG2D+ cells after 48h of treatment. Taken  
270 together, these results indicate that I-CRP induces early activation of NK cells and an increase of  
271 CD161, NKG2D, and NKG2C in human NK cells from normal donors.

272

273 ***1.2.3 NK cells modulate some killer immunoglobulin-like receptors /CD158a/h, CD158b/j,***  
274 ***CD158d and CD158e1/2) as well as natural cytotoxicity receptors NKp30, NKp44 and***  
275 ***NKp46 after I-CRP treatment***

276 The killer Immunoglobulin-like receptors (KIRs), and natural cytotoxic receptors (NCR) are  
277 fundamental to unravel NK cell function and the molecular mechanisms of tumor cell killing [12,37].  
278 Thus, in view of the modifications observed in CD161 and KLR, we explored if I-CRP-activated NK  
279 cells could also modulate KIR and NCR repertoire to then define if I-CRP could improve antitumoral  
280 activity of NK cells. Gating strategy applied in these analyses was similar to that observed on figure  
281 2A. The KIR analysis shows an increase in their expression compared to control cells, CD158a/h  
282 ( $p < 0.01$ ), CD158b/j ( $p < 0.01$ ), CD158d ( $p < 0.01$ ) and CD158e1/2 ( $p < 0.01$ ) after 24 and 48h of I-CRP  
283 stimulation (Fig. 3A); CD158i did not show a significant difference ( $p > 0.05$ ). In contrast, IL-2 did  
284 not show significant difference in any KIR expression ( $p > 0.05$ ). Moreover, increased NCR  
285 expression is detected after 24h and 48h of I-CRP stimulation when compared to controls ( $p < 0.01$ ). It

286 is important to note that NKp44 ( $p < 0.01$ ) and NKp30 ( $p < 0.01$ ) upregulation is earlier than those  
287 observed after IL-2 stimulation, suggesting a different pathway of activation. These results indicate  
288 that I-CRP modulates KIR and NCR expression on NK cells and suggest that it could improve  
289 antitumoral activity.

#### 290 **1.2.4 I-1.2.4 ICRP induces early activation on isolated NK cells**

291 To determine if the changes observed in NK cells were mediated by a direct effect on NK cells or an  
292 indirect action provided by the effect of I-CRP in other types of cells from PBMC, we isolated NK  
293 cells from PBMC by negative selection (supplementary figure 3). The NK cells (95% pure) obtained  
294 after negative selection (Fig. 4A) were tested for cell viability after treatment with I-CRP or IL-15,  
295 when compared to no stimulation. We found that I-CRP incubation did not affect significantly the  
296 viability of NK cells after 24h of treatment when compared with the untreated control and with IL-15  
297 treated cells (Fig. 4B), confirming the results we observed in total PBMC (Supplementary figure 1).  
298 Concerning CD69 activation marker, we found that I-CRP induces early activation of NK cells  
299 (43%) similar to IL-15 stimulation (45%), when compared to unstimulated control cells (13%) (Fig.  
300 4C). To verify the modulation of NK cell markers, we tested NK subsets according to CD56 and  
301 CD16 expression. In figure 4D, we can observe the dot plots from a representative healthy donor,  
302 indicating the percentages of NK cells subsets in I-CRP and IL-15 stimulated NK cells compared to  
303 non-stimulated cells for 24 and 48h. We found that I-CRP stimulation decreased the CD56<sup>Dim</sup> CD16<sup>+</sup>  
304 subset ( $p < 0.01$ ) and increased CD56<sup>Dim</sup> CD16<sup>-</sup> cells ( $p < 0.01$ ) when compared to control or IL-15  
305 treatment (Fig. 4D), and revealing, that I-CRP directly modulates NK cells.

#### 306 307 **1.2.5 I-CRP enhances natural cytotoxicity activity of NK cells from normal donors**

308 With the purpose of describing the effect of I-CRP on NK cells, next we tested the natural cytotoxic  
309 capacity of unstimulated or I-CRP-stimulated PBMC or isolated NK cells. Thus, to determine if I-  
310 CRP improves the natural cytotoxic activity of NK cells against target cells we tested the

311 degranulation marker expression (CD107a) on NK cells, the killing of target cells, and the activation  
312 (CD69) of NK cells. PBMC or isolated NK cells were stimulated with I-CRP for 24h. After  
313 treatment, cells were co-cultured with K562 target cell line using different ratios of effector to target  
314 cells (5:1, 10:1, 15:1, 20:1 and 30:1) for 4h (schematic set up, figure 5A). First, we measured the  
315 expression of the degranulation marker CD107a (figure 5B) on NK in PBMC and in isolated NK  
316 cells. Lymphocytes were gated in FSC-A/SSC-A dot plot and at least 10,000 events were selected,  
317 then the expression of CD56 and/or CD16 were selected to define NK cells (gating strategy in  
318 supplementary figure 4A). CD107a expression is significantly increased, when compared to  
319 control, in PBMC and in isolated NK cells pre-incubated with I-CRP, after co-culture with K562  
320 cells in the ratios 5:1, 10:1, 15:1, 20:1 and 30:1 (figure 5B). This result is similar to the one observed  
321 after IL-15 stimulation. We observed enhanced CD107a expression at all ratios, but particularly the  
322 10:1 ratio showed low variability among samples and a high CD107a expression. This expression  
323 was significantly increased in PBMC ( $p < 0.0001$ ) (15% to 45%) (left) and NK cells ( $p < 0.0001$ )  
324 (13% to 45%) (right) pre-incubated with I-CRP, before co-culture with K562 cells, while pre-  
325 incubation of PBMC and isolated NK cells with IL-15 induce 18% of CD107a increase, in both cases  
326 (supplementary figure 4B-C). This observation was also observed in MHC class I-expressing target  
327 cells, CEM and MOLT-4 (supplementary figure 5).

328 To determine if I-CRP-treated NK cells enhanced their cytotoxicity against a target cell we tested the  
329 cell viability in K562 cells, using calcein-AM staining assessment. We stained K562 cells with  
330 calcein-AM and then we co-cultured them with PBMC (figure 5C left) or with purified NK cells  
331 (figure 5C right), in the 10:1 ratio (NK cells to K562). We gated at least 10,000 events of target cells  
332 (K562) and then we quantified calcein negative cells (dying cells) (Supplementary figure 6). We  
333 observed that when PBMC or isolated NK cells were stimulated with I-CRP they induced loss of cell  
334 viability of K562 cells. This result was similar to the one observed in IL-15 stimulated cells. These  
335 data demonstrate that I-CRP improves the natural cytotoxic activity of NK cells against target cells.

336 In figure 5D (left) we can observe that CD69 expression increases, after K562 stimulation, in PBMC  
337 (16.8% to 38.1%) and in isolated NK cells (31% to 53%) pre-incubated with I-CRP; this result was  
338 similar to that observed in IL-15 stimulated PBMC (16.8% to 31.7%) and NK cells (31% to 42%).  
339 Significant differences were observed on PBMC ( $p < 0.001$ ) and isolated NK cells ( $p < 0.05$ ) stimulated  
340 with I-CRP, when compared to their respective control. This observation was showed in other target  
341 cells, CEM and MOLT-4 cells lines in the ratio 10:1 (supplementary figure 7). Taken together, these  
342 data demonstrate that I-CRP increases the effector activity of isolated NK cells against target cells,  
343 through their activation, release of cytotoxic granules (CD107a expression), and killing of target  
344 cells, with no apparent implication of other cell populations present in PBMC.

345

### 346 **1.3 DISCUSSION**

347 We examined changes in the NK cell repertoire and functions occurring in response to I-CRP  
348 treatment. We show that I-CRP does not affect PBMC viability and induces activation of NK cells,  
349 modifications on the repertoire of NK markers, and an augmented recognition against the K562 cell  
350 line. These processes are closely linked to the changes on the expression of the activation and  
351 inhibitory NK cell receptors, which control activation and provide NK cell diversity and functionality  
352 [38], indicating that I-CRP improves NK cells functional activity.

353 Several markers of early NK cell activation have been described, including the C-type lectin receptor,  
354 CD69 [39]. CD69 is not expressed on resting NK cells but is highly upregulated on NK cells after  
355 activation [40]. In this study CD69 was consistently higher in I-CRP-treated NK cells, even when  
356 NK cells were previously isolated. The increase of the NK cells CD56<sup>Dim</sup> CD16<sup>-</sup> subset was observed  
357 after I-CRP treatment, this can be related to an observation reported by Lajoie et al. in 2014, who  
358 indicated that CD16 shedding is restricted to activated cells and this process is partially ADAM-17  
359 dependent [41]. Despite ADAM-17 inhibitors were not implemented in our study, CD16 shredding is  
360 strongly correlated with degranulation [41], which was observed with a high constitutive potential on



361 I-CRP-treated NK cells, using the CD107a functional assay. Fogel et al. [40] observed that CD16  
362 shedding increased NK cell motility and facilitate detachment of NK cells from target cells [40,42].  
363 We observed an increase in target cell recognition and cytotoxic activity in I-CRP-stimulated NK  
364 cells, suggesting that CD16 shedding could be implicated in these processes.

365 The phenotypic analysis of the NK cell repertoire identified patterns of NK cell receptors that may  
366 define NK cell activation by DLEs, such as enhanced CD161, NKG2D, NCR and KIR expression.  
367 CD161 engagement appears to have a complex role in the modulation of immune NK functions with  
368 both activating and inhibitory effects [43]. CD161 is involved in triggering the NK cell cytotoxicity  
369 against human melanoma [44] as well as in the ability to respond to IL-12 and IL-18, upregulating  
370 NKp30, CD160 and CD25 [45]. Similarly, I-CRP induces an increase in the expression of NKp30  
371 and CD69, but not in CD160, suggesting a different kind of activation. Increased NKG2D expression  
372 is related to NK cell activation, as it can have functions as an activating and a co-stimulatory  
373 receptor, playing a fundamental role in the surveillance of microbial infections and cancer [46].  
374 NKG2D was increased after stimulation with I-CRP, supporting the activation of NK cells.  
375 Moreover, NKp44, an NCR which is only up regulated in activated NK cells, was also increased.  
376 Taken together, these results indicate that I-CRP can activate NK cells, isolated or in PBMC. This  
377 activation does not involve proliferation, but involves NKG2C and general KIR expression,  
378 characteristics mostly observed in adaptive-like NK cells, which are well studied in HCMV-  
379 seropositive patients and are defined as CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells [47,48]. In this study CD57 was  
380 not analyzed, but the I-CRP-activation pattern we report here has important similarities with the  
381 adaptive-like NK cells, like CD16 decrement, which is observed after acquisition of NKG2C in  
382 CD56<sup>dim</sup> cells [49], and a better degranulation activity, also reported in NKG2C<sup>+</sup> NK cells, compared  
383 to NKG2C<sup>-</sup> NK cells [49]. Another similarity is the expression of inhibitory and activating KIR in  
384 NK cells, which has been related to the adaptation to HCMV-infection in addition to NKG2C [50]. It  
385 has been determined that adaptive NK cells possess unique KIR repertoire, and display strong

386 cytotoxicity against tumor cells [50–52], and, on the other hand, the KIR expression have been  
387 shown to be clinically relevant in allogeneic transplantations to treat acute leukemia [53]. It remains  
388 unclear if these cells can surpass NK cell-resistant tumor cells, but it suggests a better cytotoxic  
389 activity as seen in matured adaptive-like NK cells.

390 In this context, other reports on DLEs revealed *in vitro* differentiation of functional  
391 CD56<sup>+</sup>CD16<sup>+</sup>CD11c<sup>+</sup> NK-like cells from CD34<sup>+</sup> cells [26], this supports the hypothesis that I-CRP  
392 activation of NK cells might involve a maturation mechanism. The receptors CD161 and NCR are  
393 not usually observed in HCMV-adaptive-like NK cells, but are expressed in I-CRP-treated NK cells;  
394 recently, these receptors were related to a BMPRIA<sup>+</sup> (type IA BMP receptor) immature thymocytes,  
395 which able to generate higher numbers of NK cells [54] and promote maturation of a resulting  
396 CD94<sup>+</sup>CD161<sup>+</sup>CD56<sup>+</sup>NKp46<sup>+</sup> NK cells [55]. The increased expression of NKG2C and CD161, and  
397 CD16 loss could be the result of the heterogeneous peptide mixture composing I-CRP, which could  
398 include peptides derived from MHC class I molecules [56], as well as peptides encoding by UL40 in  
399 HCMV [57], which can activate adaptive-like NKG2C NK cells. Furthermore antimicrobial peptides  
400 (AMP), like LL-37, can mediate ADAM activation [58] and BMP pathway engagement [59].  
401 However, further studies about the exact peptides contained in DLEs and inhibition studies must be  
402 done to better describe these mechanisms of action.

403

#### 404 **1.4 Conclusion**

405 Overall, our results show that I-CRP induces NK cell activation (CD69 expression), increasing the  
406 CD56<sup>Dim</sup> CD16<sup>-</sup> subset and modulating the expression of the main NK receptors repertoire (Fig.6A),  
407 resulting in a NKG2C<sup>+</sup>CD161<sup>+</sup>NK cells with improvements in their cytotoxic response against  
408 target cells (Fig.6B). This work helps to understand the mechanism of I-CRP as immunostimulant  
409 and its possible implications on NK cell differentiation/activation. In addition, these results pointed-

410 out the possible use of I-CRP in diseases having a mayor dysfunction of NK cells like viral and  
411 bacterial infections and cancer.

412

## 413 **2 Conflict of Interest**

414 The authors declare no conflict of interest. C.R.-P. is employee of LONGEVEDEN SA de CV; the  
415 company had no role in the design of the study, in the collection, analyses, or interpretation of data;  
416 in the writing of the manuscript; or in the decision to publish the results.

417

## 418 **3 Author Contributions**

419 HYLA, ABML, RTG, DSA, ACMT, and CRP analyzed and interpreted data. HYLA, and ABML  
420 performed all the experimental work. DSA, ACMT, and CRP conceived and supervised the project.  
421 HYLA, ABML and ACMT wrote the manuscript. RTG, DSA, and CRP revised the manuscript. All  
422 authors approved the final manuscript.

423

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- 650

## 651 6.1 Figures Legends

652 **Figure 1. I-CRP increases CD69 expression modulating CD56Dim CD16<sup>+/−</sup> NK subset on**  
653 **PBMC. A)** Gating strategies for flow cytometry analysis of Calcein-AM and CD69 on PBMC.  
654 Quantification of CD69 expression by flow cytometry in PBMC from 6 healthy subjects treated with  
655 different concentrations of I-CRP (0.3 U/mL, 0.7 U/mL and 1.5 U/mL) for 24 and 48h or  
656 Concanavalin-A (ConA, 5 mg/mL). **B)** Gating strategy for the analysis of CD3<sup>+</sup> (T cells), CD20<sup>+</sup> (B  
657 cells) and CD56/CD16 (NK cells) lymphocytes on PBMC by flow cytometry. **C)** Quantification of  
658 CD3<sup>+</sup> T Cells on PBMC treated by I-CRP or Concanavalin A, with different concentrations as



659 indicated for 24 and 48h (middle left). **D)** Quantification of CD20+ B Cells on PBMC treated with  
660 different concentrations of I-CRP or Concanavalin A, as indicated during 24 and 48h (middle right).  
661 **E)** Quantification of CD56Dim CD16+ subset of NK cells on PBMC treated with different  
662 concentrations of I-CRP or Concanavalin A, as indicated for 24 and 48h (down left). **F)**  
663 Quantification of CD56Dim CD16–NK cell subset on PBMC treated by I-CRP or Concanavalin A,  
664 with different concentrations as indicated for 24 and 48h (down right). **G)** Representative histograms  
665 (left) and quantification (right) of proliferation pics by flow cytometry through CFSE staining in  
666 PBMC 4 healthy individuals treated with I-CRP (1.5 U/mL) or Concanavalin A (Con A, 5 mg/mL)  
667 for 96h. All data shown is median and range. Statistics were performed by non-parametric Mann–  
668 Whitney U-test (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001).

669 **Figure 2. I-CRP promotes early activation and enhances NKG2C expression on NK cells. A)**  
670 Representative gating strategy used in analysis. **B)** Quantification of CD69 expression on NK cells  
671 after PBMC incubation with I-CRP (1.5 U/mL) or IL-2 (200 U/mL) during 24 and 48h. **C)**  
672 Quantification of accessory receptors (CD161, CD161, CD85j and CD226) on NK cells after PBMC  
673 stimulation with I-CRP (1.5 U/mL) or IL-2 (200 U/mL) during 24 and 48h. **D)** Quantification of  
674 Killer cell lectin-like receptors (NKG2D and NKG2C) on NK cells after PBMC incubation with I-  
675 CRP (1.5 U/mL) or IL-2 (200 U/mL) during 24 and 48h. All data shown is median and range from  
676 healthy donors n=6. Statistics were performed by non-parametric Mann–Whitney U-test with  
677 Bonferroni correction (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001).

678 **Figure 3. I-CRP modulates Killer Immunoglobulin-like receptors and Natural cytotoxicity**  
679 **receptors expression on NK cells. A)** Quantification of Killer Immunoglobulin-like receptors (KIR,  
680 CD158a/h, CD158b/j, CD158d, CD158e1/2 and CD158i) on NK cells after PBMC incubation with I-  
681 CRP (1.5 U/mL) or IL-2 (200 U/mL) during 24 or 48h. **B)** Quantification of Cytotoxicity receptors  
682 (NKp30, NKp44 and NKp46) on NK cells after PBMC stimulation with I-CRP (1.5 U/mL) or IL-2

683 (200 U/mL) at 24 and 48h. All data shown is median and range from healthy donors n=6. Statistics  
684 were performed by non-parametric Mann–Whitney U-test with Bonferroni correction (\*p<0.05,  
685 \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001).

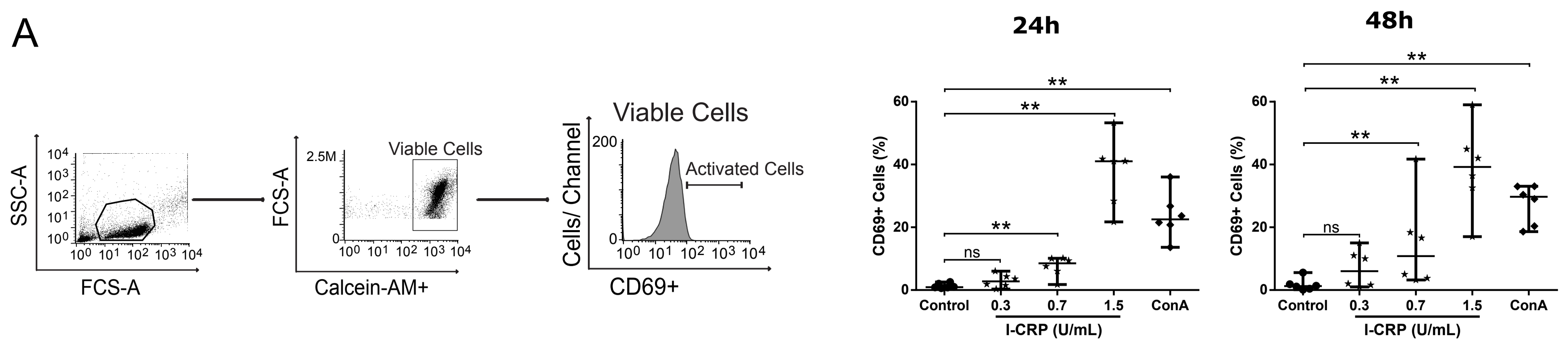
686 **Figure 4. I-CRP modulates CD56Dim CD16+/- NK.** **A)** Representative gating strategy and dot  
687 plot (CD20 vs CD3) obtained from one healthy donor after negative NK cell isolation from PBMC.  
688 The median of purity efficacy in NK cell population was 95%. **B)** Quantification of cell viability by  
689 flow cytometry using calcein-AM staining in isolated NK cells treated with I-CRP (1.5 U/mL) or IL-  
690 15 (15 ng/mL) during 24h (data from 6 healthy donors). **C)** Quantification of CD69 expression by  
691 flow cytometry on isolated NK cells from 7 healthy individuals. Isolated NK cells were incubated  
692 with I-CRP (1.5 U/ml) or IL-15 (15 ng/mL) during 24 h. **D)** Representative dot plots of isolated NK  
693 cells subsets using CD56 and CD16 markers (I:CD56BrightCD16-, II:CD56BrightCD16+,  
694 III:CD56DimCD16-, IV:CD56DimCD16+ and V:CD56-CD16+) untreated or treated with I-CRP  
695 (1.5 U/mL) or IL-15 (15 ng/mL) for 24h. Quantification of CD56DimCD16+ and CD56DimCD16-  
696 subsets after I-CRP or IL-15 stimulation for 24h (data from 6 healthy donors). All data shown is  
697 median and range. Statistics were performed by non-parametric Mann–Whitney U-test (\*p<0.05,  
698 \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001).

699 **Figure 5. I-CRP increases NK cells effector activity.** **A)** Schematic representation of experiment  
700 design. **B)** Quantification of CD107a expression by flow cytometry on PBMC (left) and isolated NK  
701 cells (right) from 6 healthy individuals, pre-stimulated with I-CRP (1.5 U/ml) or IL-15 (15 ng/mL)  
702 for 24h and then co-cultured with K562 cells in different ratios 5:1, 10:1, 15:1, 20:1 and 30:1  
703 (NK:K562) during 4h. **C)** Quantification of the percentage of calcein-AM negative K562 cells co-  
704 cultured during 4h with PBMC (left) or isolated NK cells (right) from 8 healthy individuals pre-  
705 stimulated with I-CRP (1.5 U/ml) or IL-15 (15 ng/mL) for 24h, and then co-cultured with K562 cells

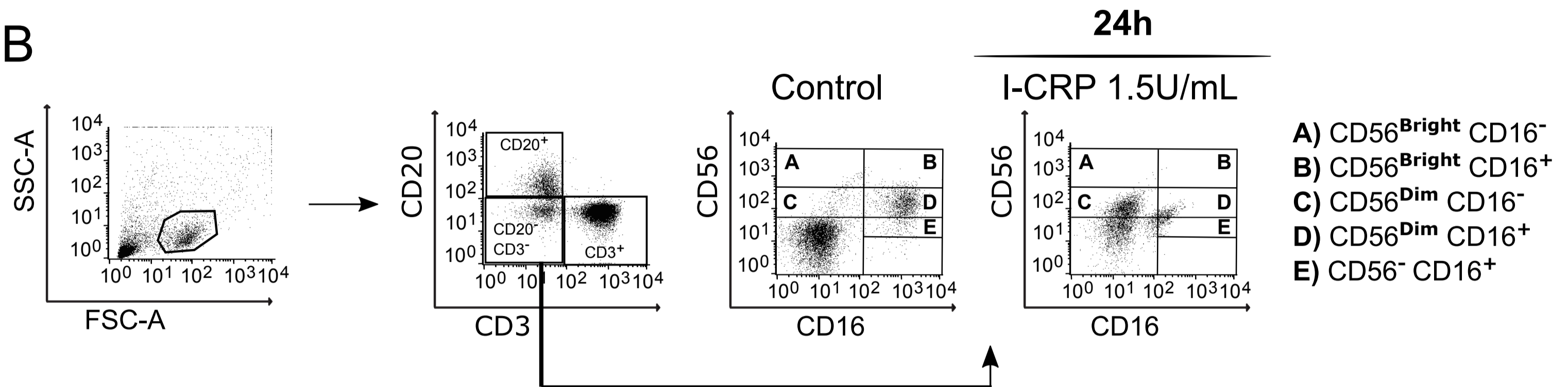
706 in a 10:1 (NK:K562) ratio. **D)** Representative histograms of CD69 expression by flow cytometry on  
707 PBMC and isolated NK cells from 11 healthy individuals, pre-stimulated with I-CRP (1.5 U/ml) or  
708 IL-15 (15 ng/mL) for 24h and then co-cultured with K562 cells in a 10:1 (NK:K562) ratio for 4h, and  
709 quantification (right). All data shown is median and range. Statistics were performed by non-  
710 parametric Mann–Whitney U-test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

711 **Figure 6. Schematic representation of I-CRP effect on NK cells.** **A)** I-CRP increase early  
712 activation marker CD69, promotes overexpression of CD161 an accessory/activator receptor, killer  
713 cell lectin-like receptors (KLR) NKG2D and NKG2C, Killer Immunoglobulin-like receptors (KIR)  
714 and enhances natural cytotoxicity receptors (NCR, NKp30, NKp44 and NKp46) on NK cells. **B)** I-  
715 CRP improves the effector activity of NK cells by enhancing degranulation marker (CD107a),  
716 promoting cytokines release and improving cytotoxicity on K562 target cells.

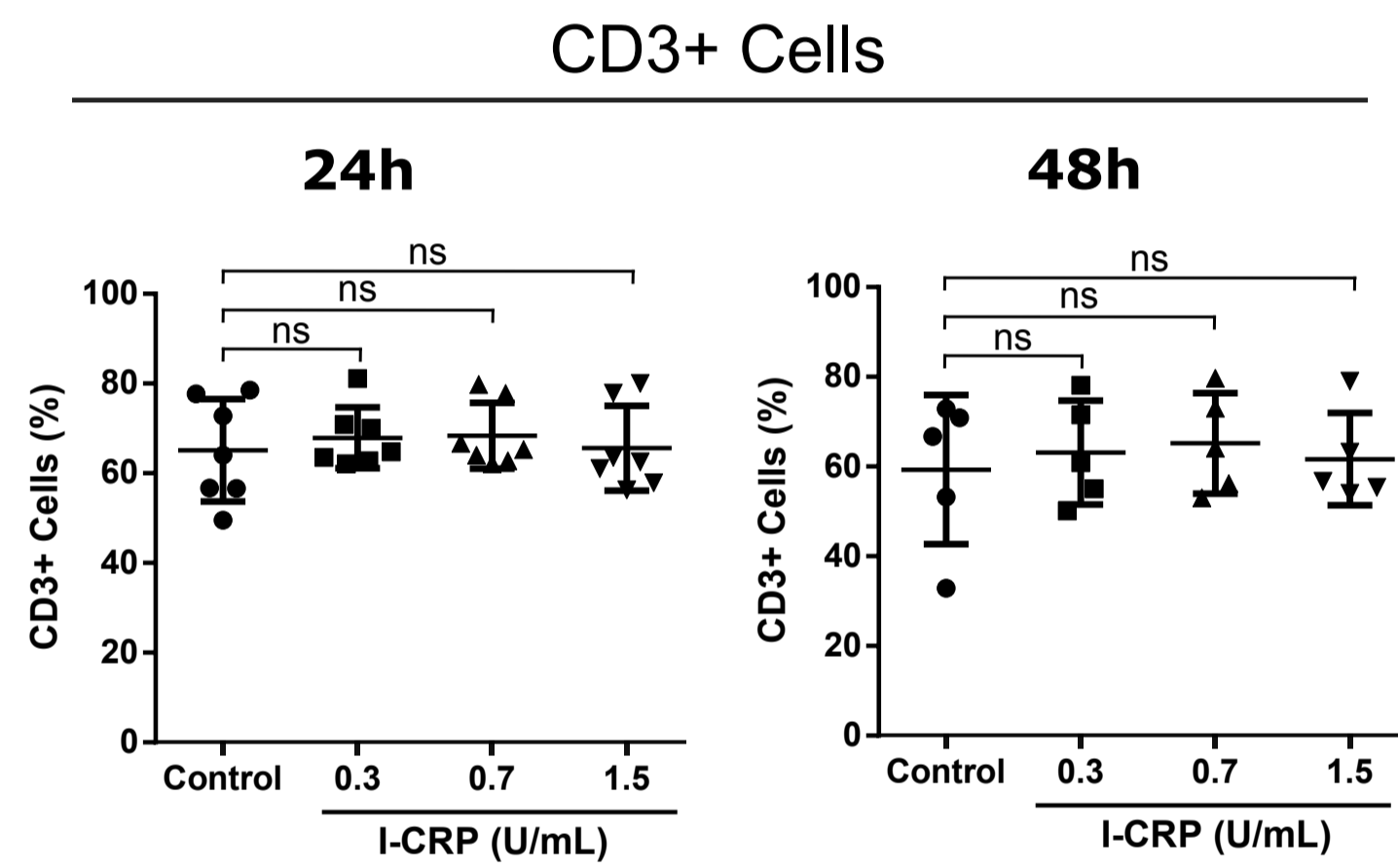
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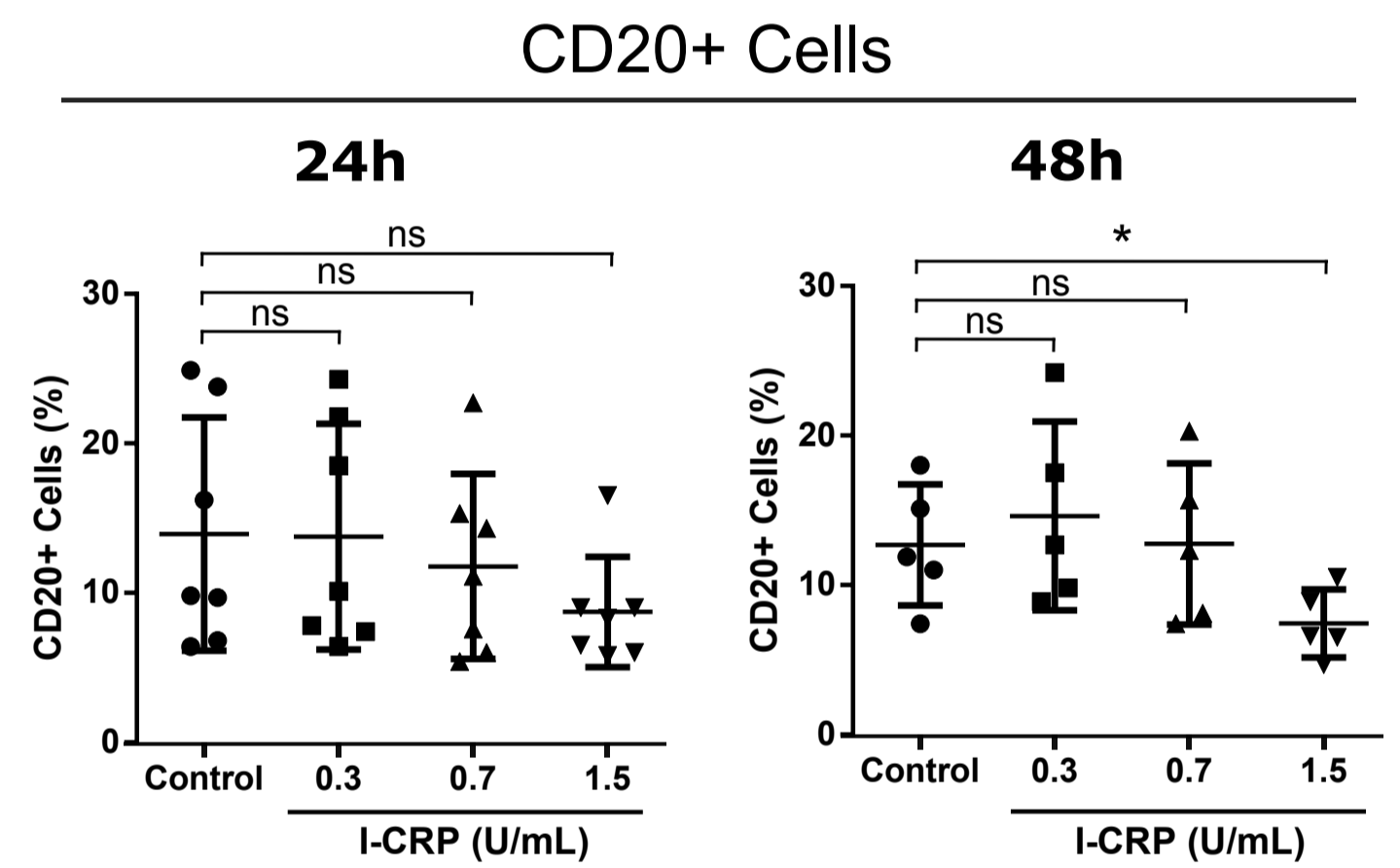
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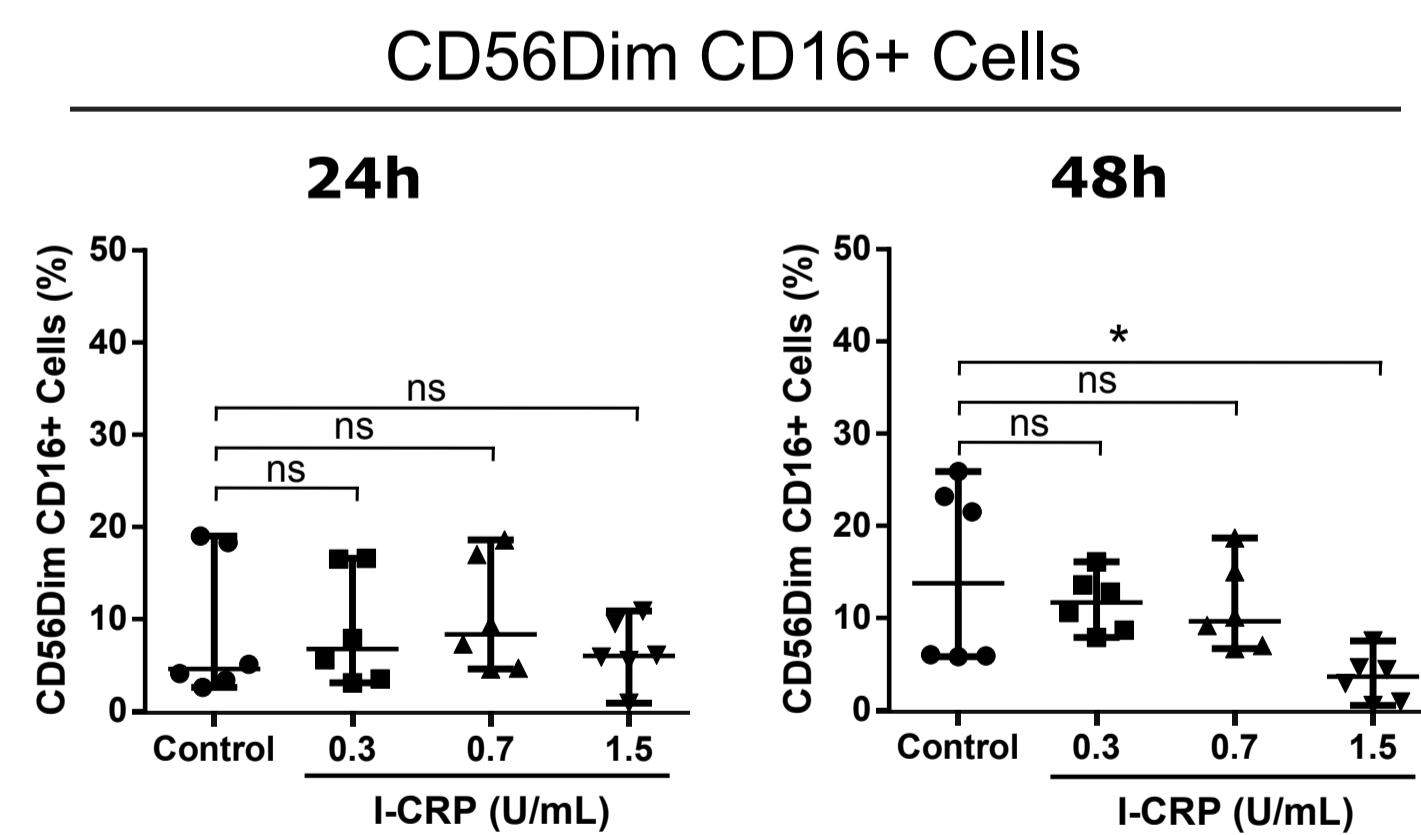
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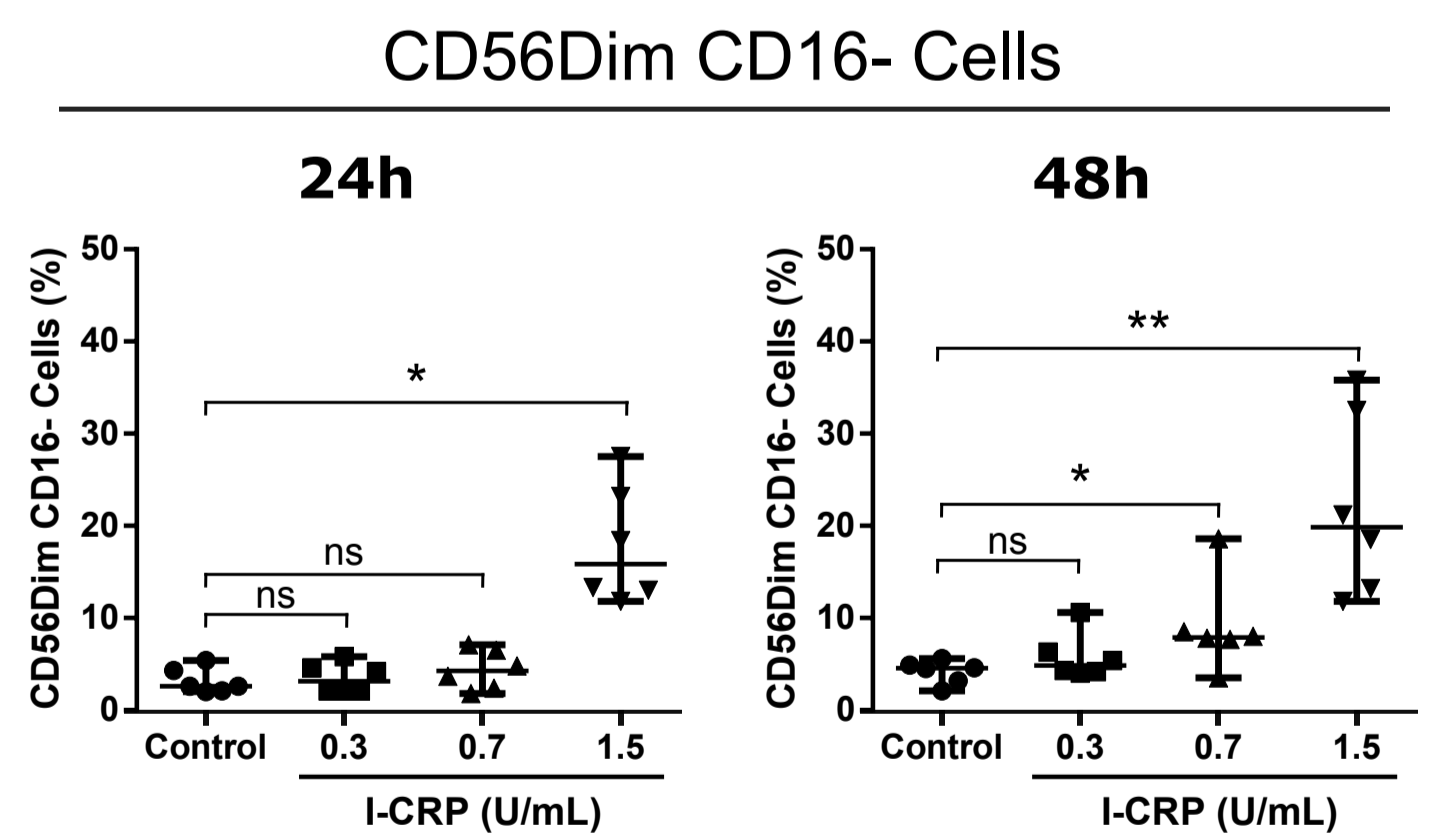
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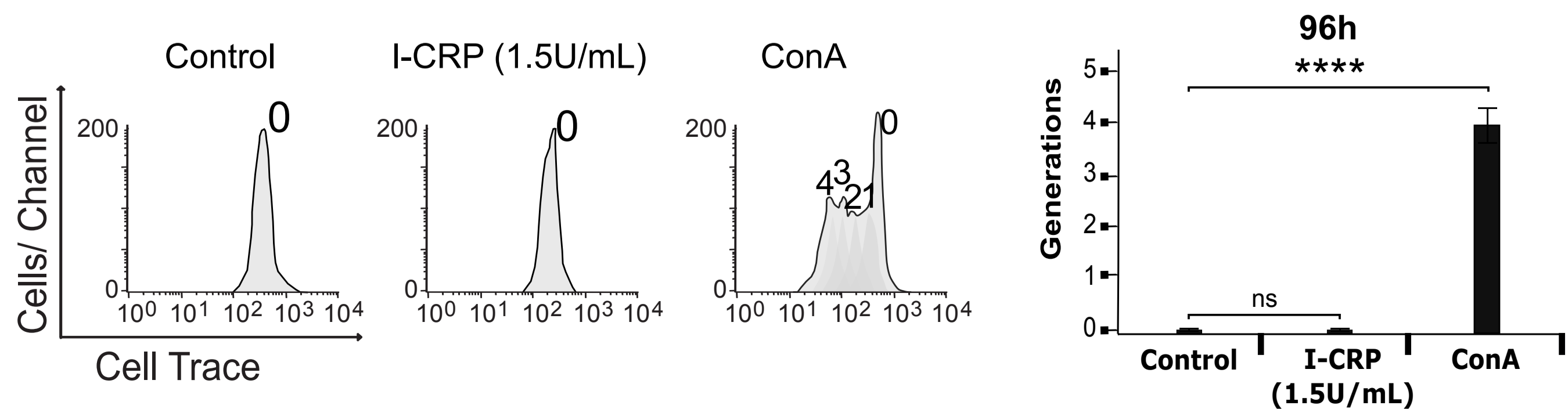
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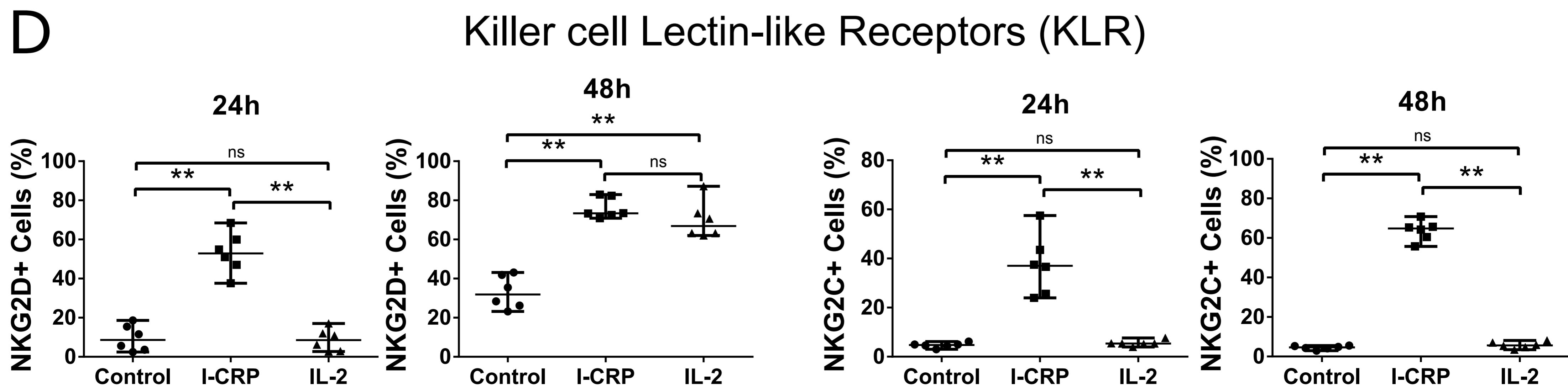
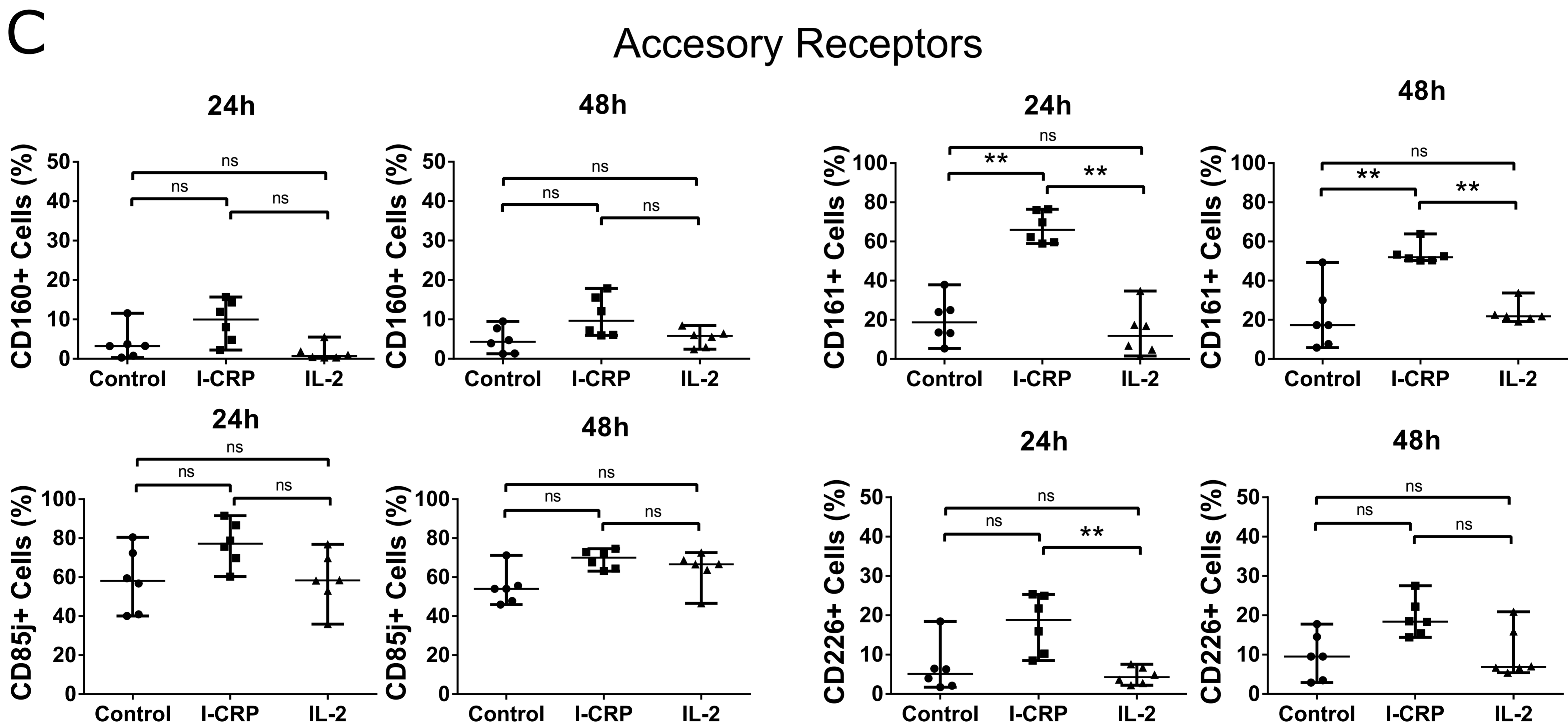
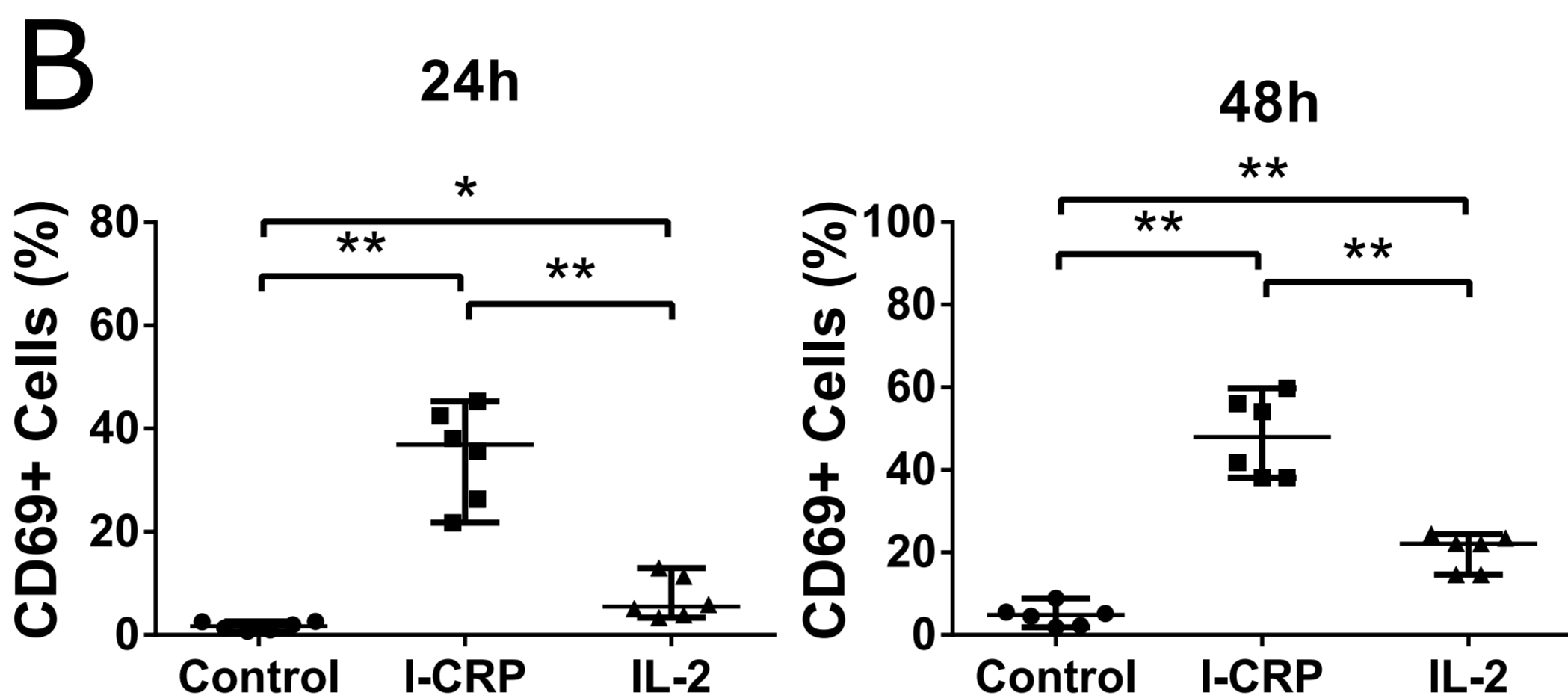
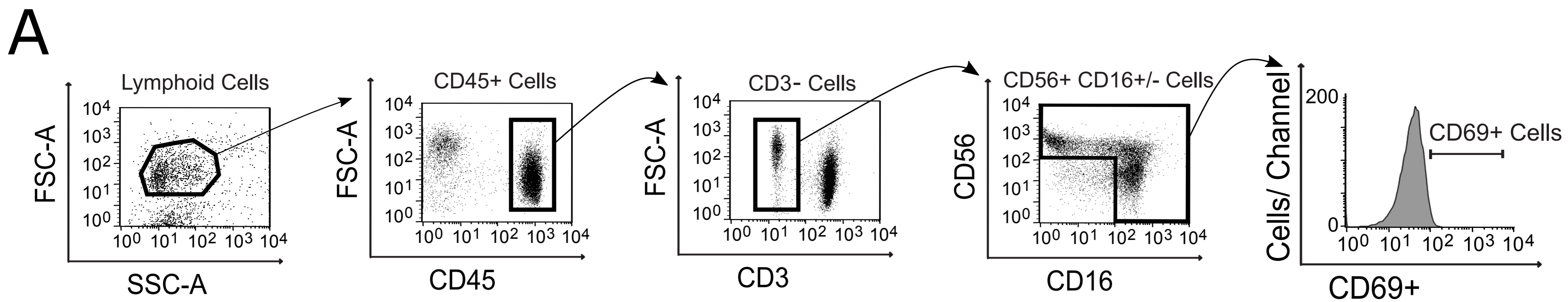


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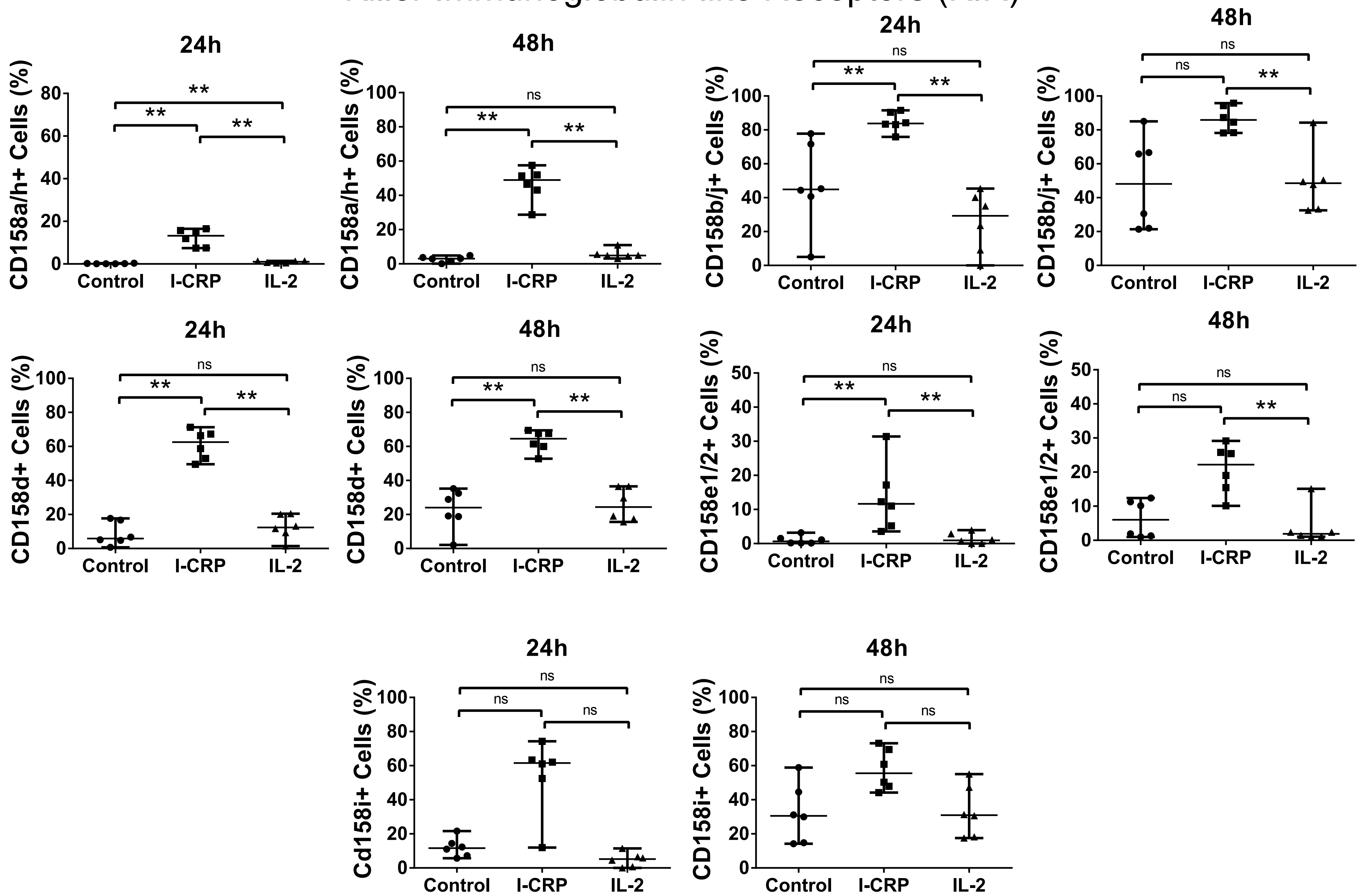


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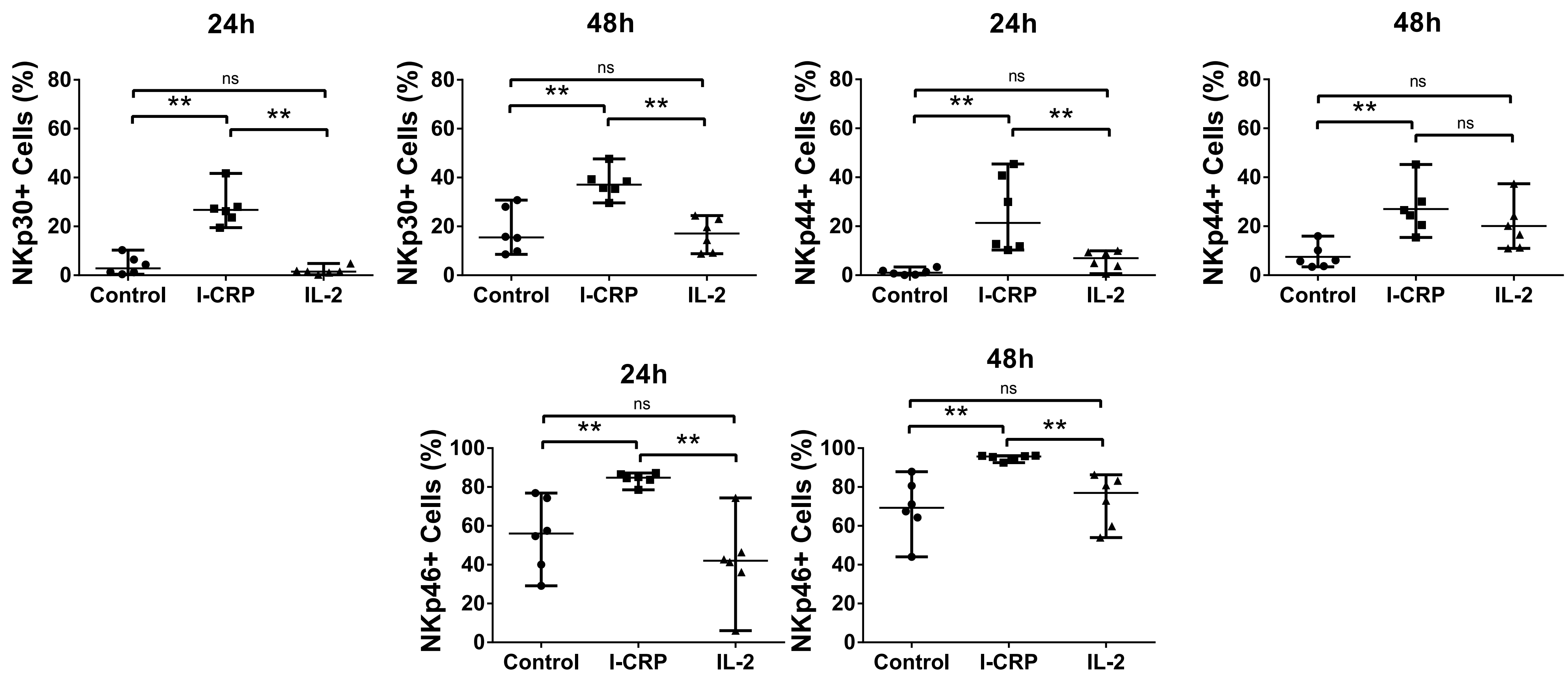




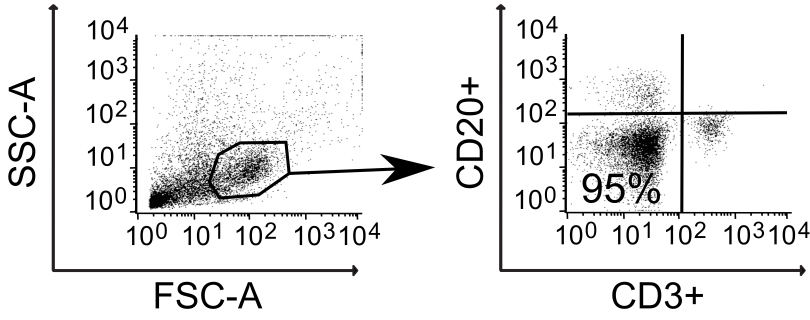
# A Killer Immunoglobulin-like Receptors (KIR)



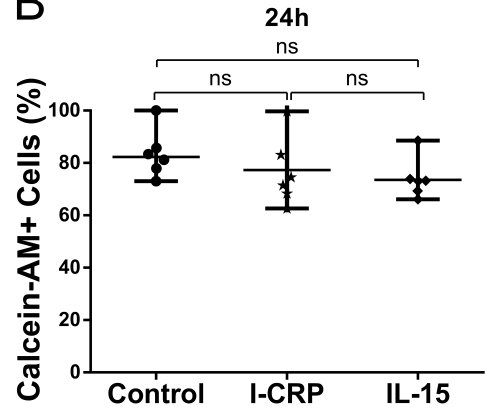
# B Natural Cytotoxicity Receptors (NCR)



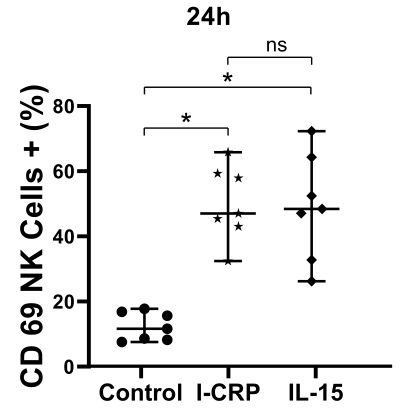
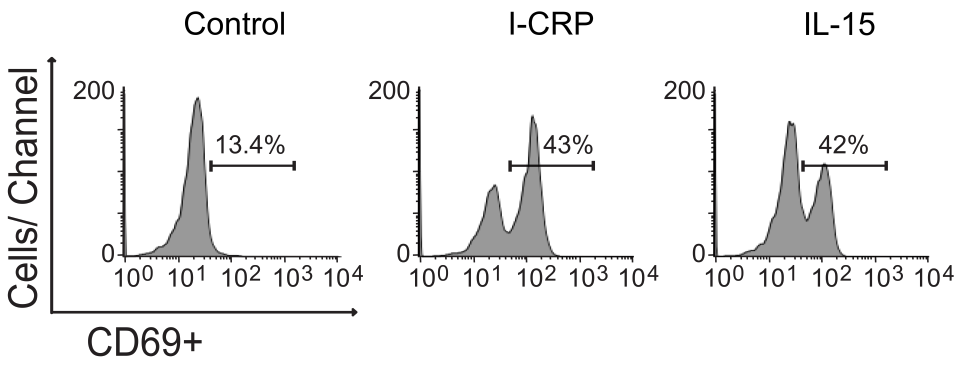
### A NK Isolation Control



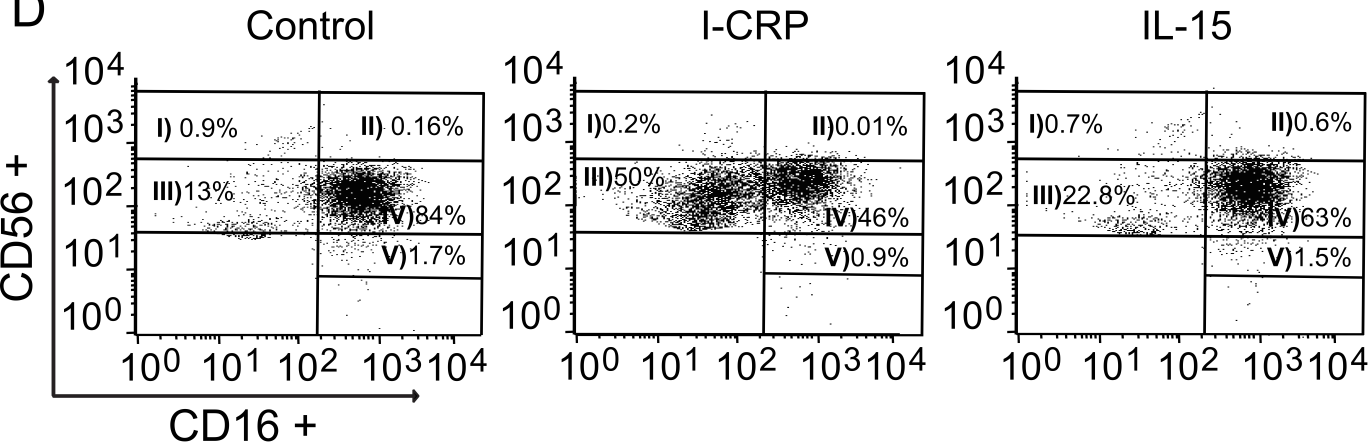
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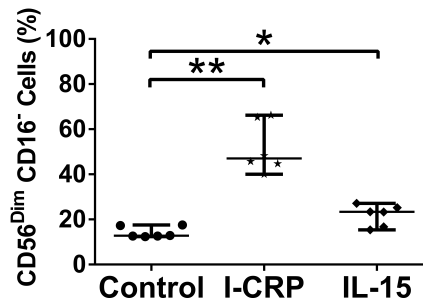
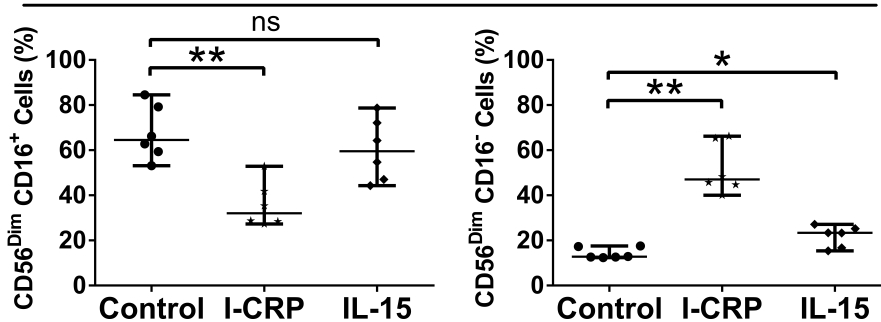
### C

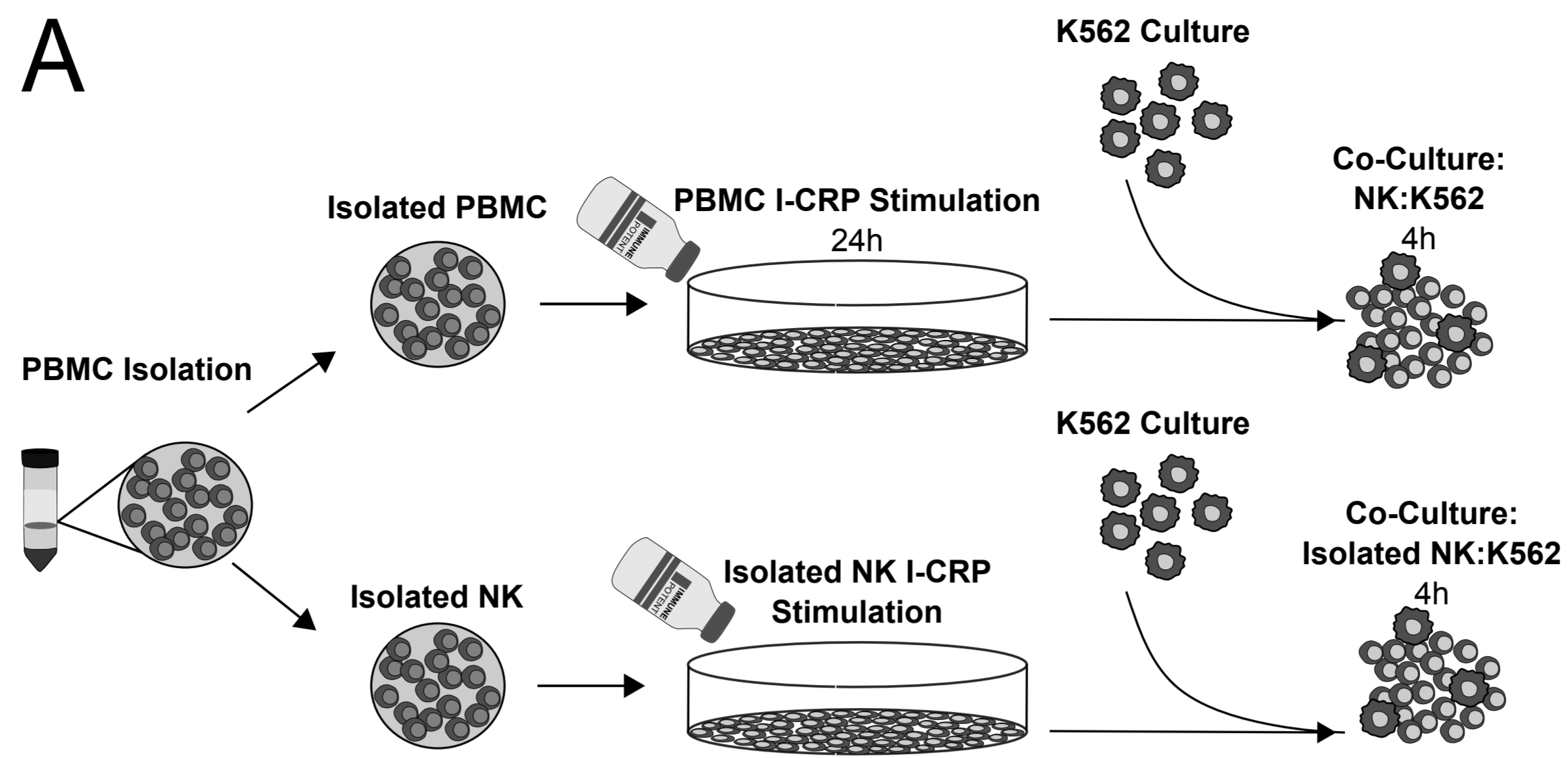
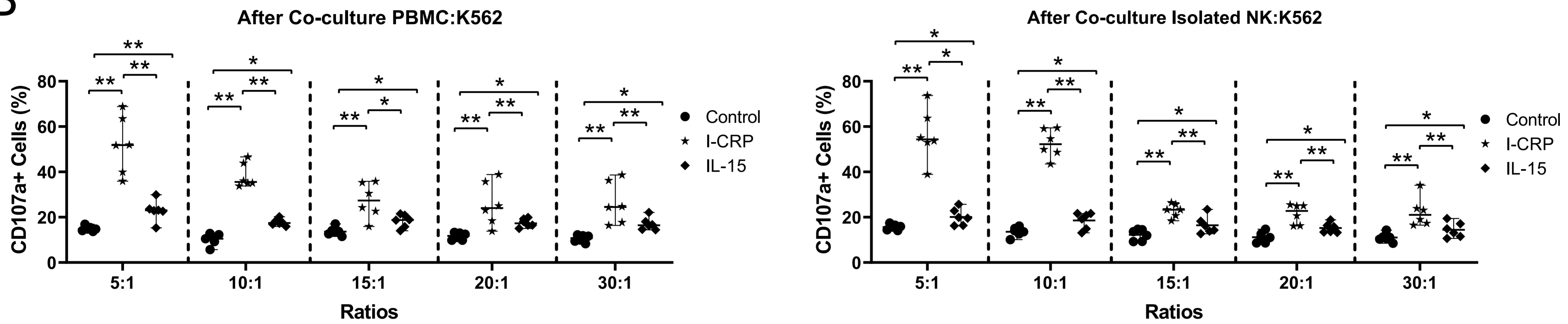
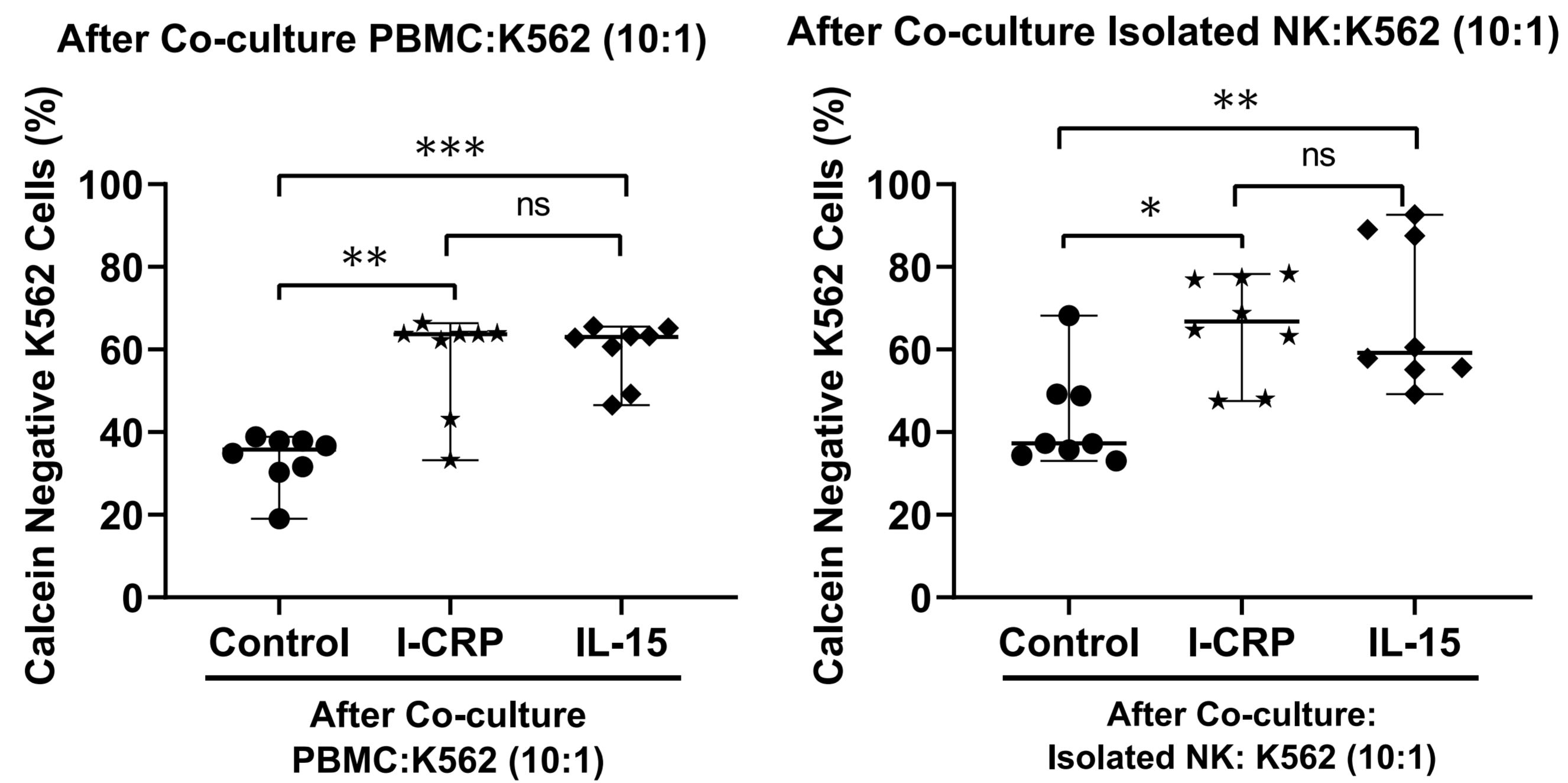
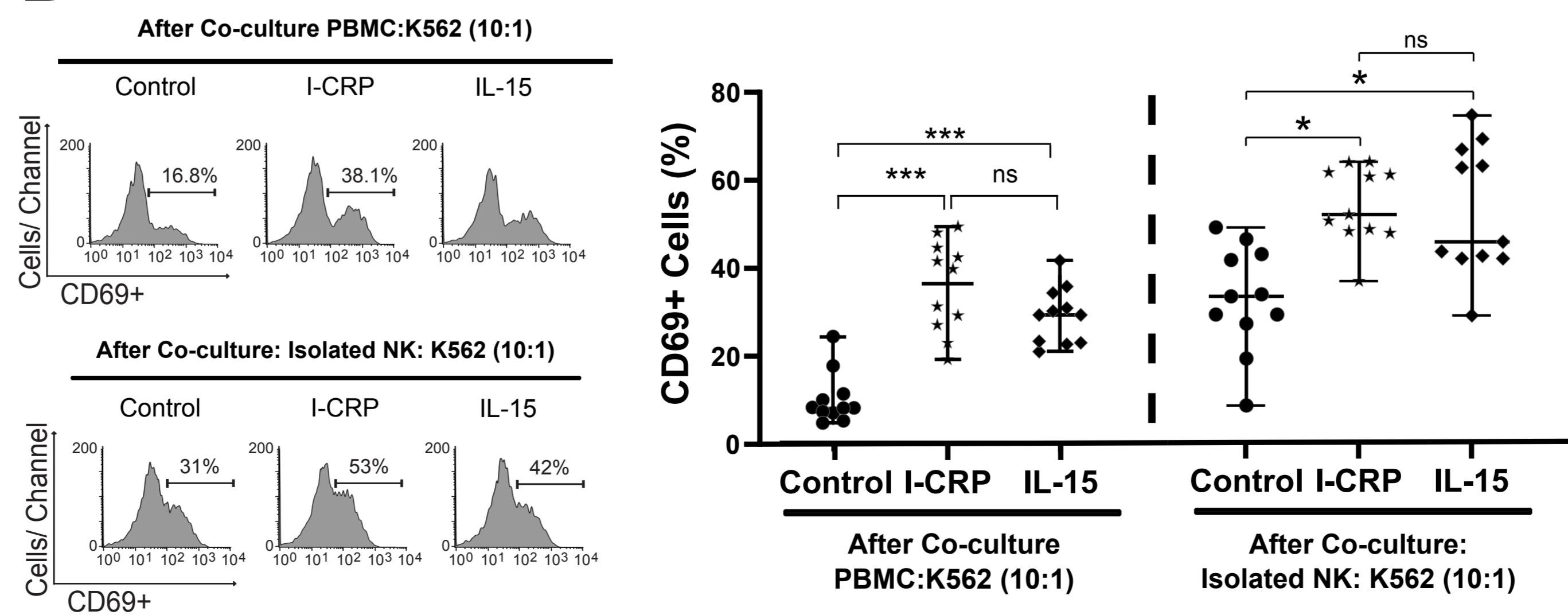


### D



24h

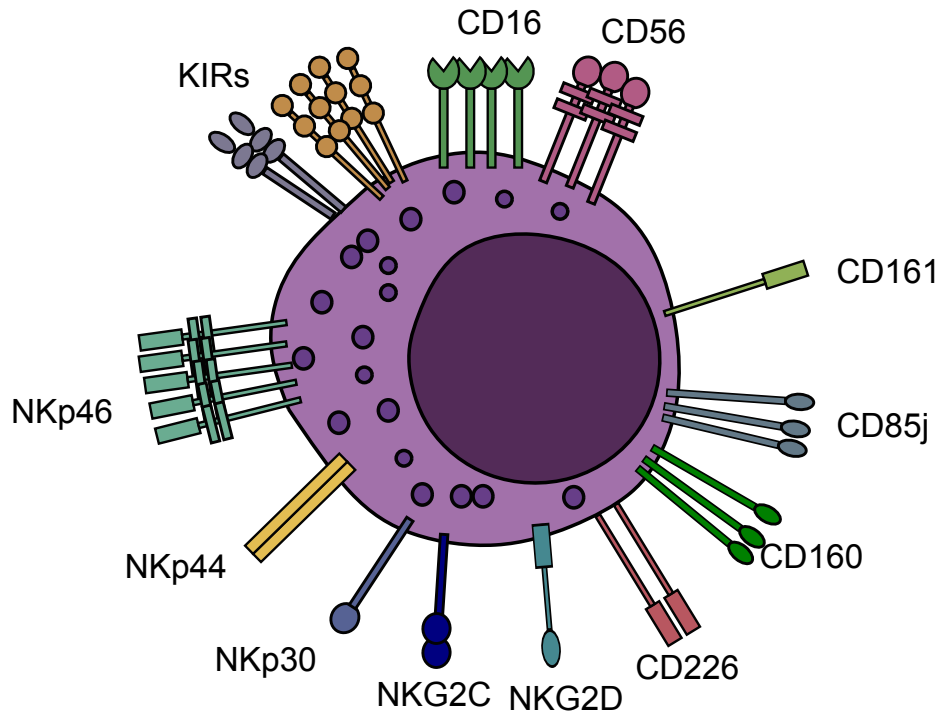


**A****B****C****D**

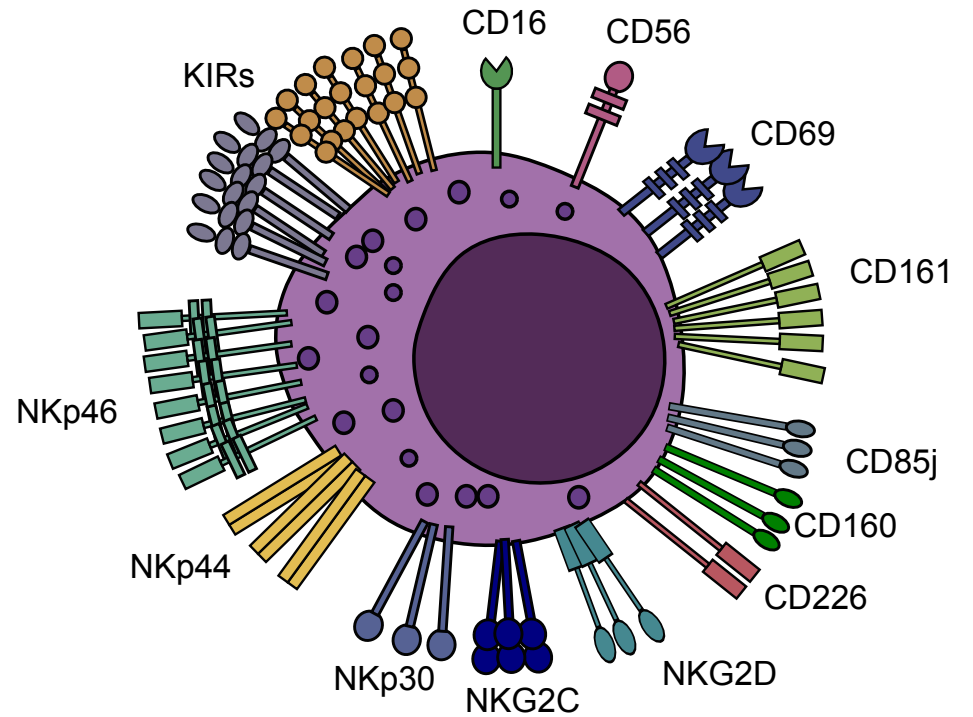


# A NK Cells Receptors Repertoire

## Control

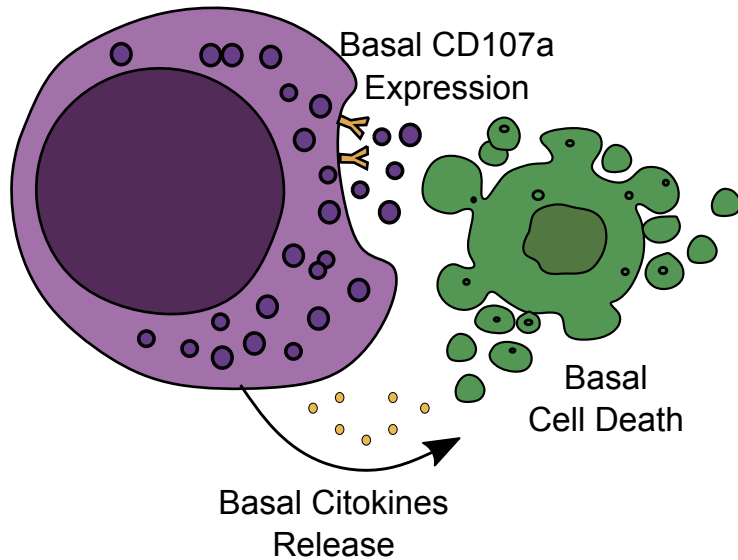


## I-CRP

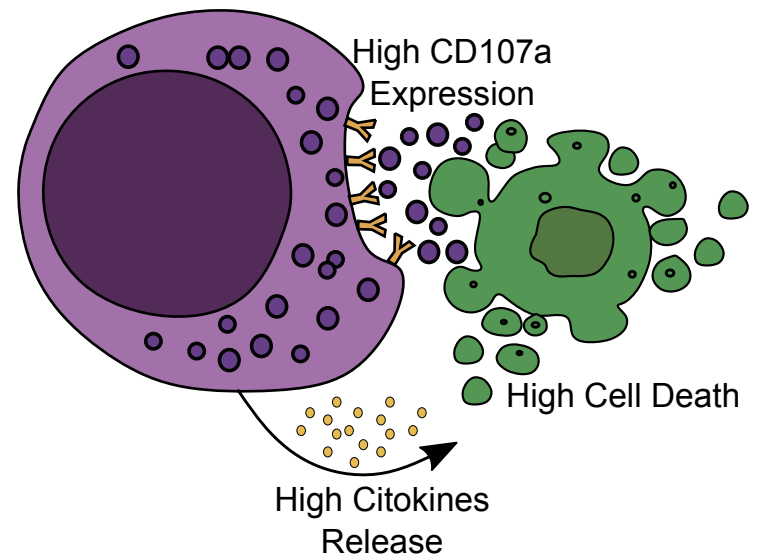


# B NK Cells Effector Activity

## Control

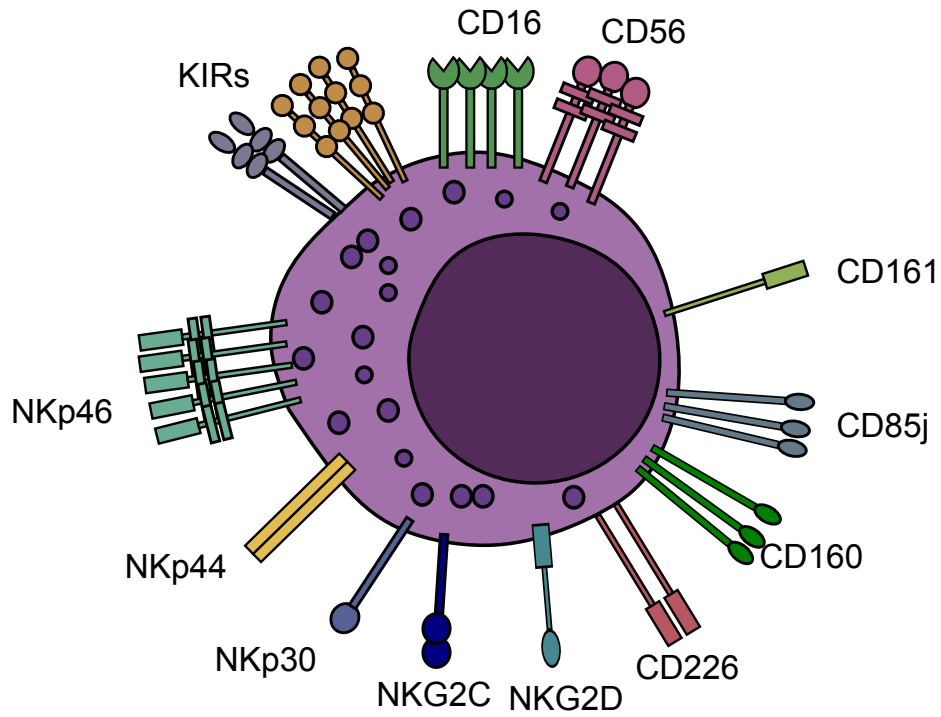


## I-CRP

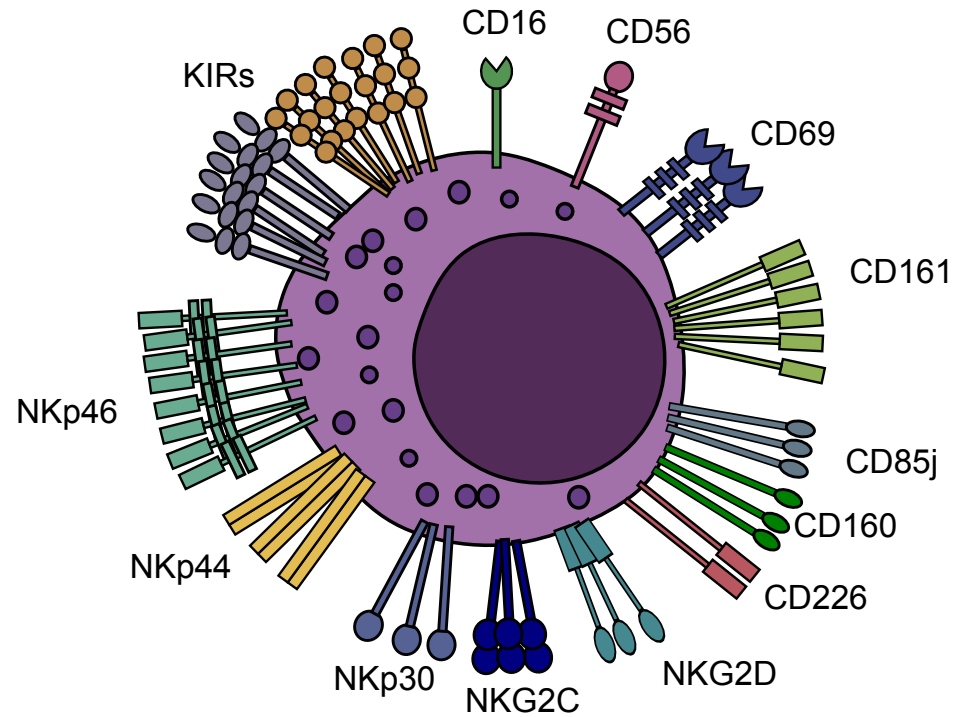


# A NK Cells Receptors Repertoire

## Control

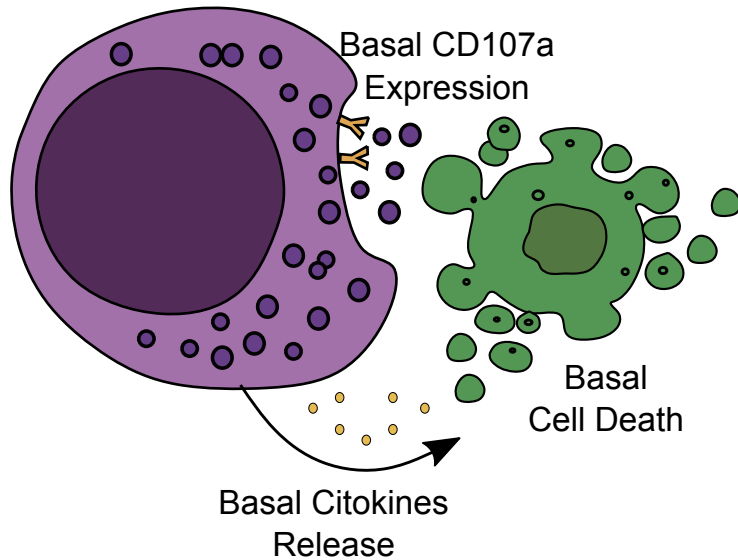


## I-CRP



# B NK Cells Effector Activity

## Control



## I-CRP

