CD38 is a key regulator of enhanced NK cell immune responses during pregnancy through its role in immune synapse formation

Authors

Mathieu Le Gars[1,2,*], Christof Seiler[3], Alexander W. Kay[4], Nicholas L. Bayless[2], Elina Starosvetsky[5], Lindsay Moore[5], Shai S. Shen-Orr[5], Natali Aziz[6], Purvesh Khatri[1], Cornelia L. Dekker[3], Gary E. Swan[6], Mark M. Davis[7,8], Susan Holmes[4], Catherine A. Blish[1,2,9,*]

Departments of [1]Medicine, [4]Pediatrics, [6]Obstetrics & Gynecology, [7]Microbiology & Immunology, [2]Stanford Immunology Program, [6]Stanford Prevention Research Center, Stanford University School of Medicine, [3]Department of Statistics, Stanford University, Palo Alto, California; [5]Faculty of Medicine, Technion - Israel Institute of Technology, Israel; [8]Howard Hughes Medical Institute, [9]Chan Zuckerberg Biohub, San Francisco, California.

*Corresponding authors: Mathieu Le Gars, mlegars@stanford.edu and Catherine Blish, cblish@stanford.edu

Once sentence summary: CD38 is responsible for the enhanced immune responses of NK cells to influenza virus infection during pregnancy through immune synapse formation.

Abstract

Pregnant women are particularly susceptible to complications of influenza A virus infection, which may result from pregnancy-induced changes in the function of immune cells, including natural killer (NK) cells. To decipher mechanisms driving enhanced NK cell activity during pregnancy, we profiled NK cells from pregnant and non-pregnant women, which showed significantly increased CD38 expression during

pregnancy. CD38 expression defines a phenotypically distinct and mature subset of NK cells that display increased ability to secrete IFN-γ and to kill influenza-infected and tumor cells. This enhanced function is based on the ability of CD38 to promote the formation of the NK cell immune synapse. Thus, increased CD38 expression directly promotes enhanced NK cell responses during pregnancy through its role in immune synapse formation. These findings open new avenues in immunotherapeutic development for cancer and viruses by revealing a critical role for CD38 in the formation of the NK cell immune synapse.

32 **Main text**

33 **Introduction**

34 Pregnant women are at high-risk of complications from seasonal and pandemic influenza infections (*1*, *2*).
35 During the 2009 H1N1 influenza virus pandemic, pregnant women in the United States suffered a
36 disproportionately high mortality rate, accounting for 5% of deaths while representing only 1% of the total
37 population (*3*). The majority of pregnant women who died of influenza-related illness during the pandemic
38 were infected in the second and third trimesters of pregnancy (*4*). Influenza infection during second or third
39 trimester of pregnancy is also associated with significant increases in miscarriages, stillbirths, and early
40 neonatal diseases and death (*1*, *5*). However, the mechanisms behind this susceptibility to influenza
41 infection during pregnancy are still poorly understood.

42

43 During pregnancy, the immune system has to finely balance its activity in order to tolerate semi-allogenic
44 fetal antigens, while maintaining the ability to fight microbial challenges (*6-9*). These immune alterations
45 may be at least partially responsible for the increased susceptibility of pregnant women to influenza virus
46 (*10*, *11*). Recent studies have demonstrated enhanced responses to influenza virus by several innate immune
47 cell subsets, including monocytes, dendritic cells and natural killer (NK) cells (*8*, *12-16*). In particular, NK
48 cells play a critical role in virus clearance and regulation of the subsequent adaptive immune response to
49 influenza virus (*17*, *18*). NK cells express an array of inhibitory receptors, including the killer-cell
50 immunoglobulin-like receptors (KIRs) and the heterodimer NKG2A-CD94, and activating receptors,
51 including NKp46, NKp30 and NKG2D, which together define their degree of maturation and
52 responsiveness to stimuli (*19*, *20*). The expression of these receptors enables NK cells to sense altered cells,
53 including virus-infected and cancerous cells *(21-23)*. In response to such threats, NK cells can produce
54 cytokines, such as IFN-γ, which limits viral replication and tumor proliferation, and kill cells via release of
55 cytolytic molecules or through engagement of death receptors. NK cell activation must be tightly regulated
56 to limit tissue damage at the site of infection. The mechanisms by which NK cell activity is altered during

57 pregnancy are unknown but are particularly critical to balance protection from infection with the avoidance
58 of a hyperinflammatory reaction that could cause irreversible injuries to the mother and the fetus.
59
60 The goal of our study was to identify pregnancy-mediated changes in NK cells and their impact on the
61 immune response to influenza infection. Our prior study reported that NK cell responses to influenza virus-
62 infected cells were enhanced during pregnancy (*12*). This finding may at least in part reflect the fact that
63 virus infections were performed in bulk peripheral blood mononuclear cell (PBMC) cultures, where
64 enhanced cytokine responses from monocytes and dendritic cells could have contributed to dramatic NK
65 cell activation (*13*, *21*). In fact, NK cells from pregnant women stimulated directly with phorbol-myristate
66 acetate and ionomycin in our experiments, or with IL-12/IL-15 by Kraus et al. (*15*), have suppressed
67 responses compared to those of non-pregnant women. In this study, we aim to resolve these contradictory
68 findings by determining the mechanisms behind changes in cell-intrinsic NK cell function during
69 pregnancy. To do so, we used mass cytometry paired with logistic regression to predict pregnancy-related
70 changes in NK cells between pregnant and non-pregnant women in two independent cohorts. We performed
71 functional assays to define the role of specific NK cell receptors in the responsiveness to influenza-infected
72 cells, uncovering a surprising new role for the cell surface molecule CD38 in the formation of the immune
73 synapse that is required for NK cell cytotoxic activity. We then explored whether this phenomenon extended
74 beyond the setting of influenza-infected cells and discovered that CD38 also promotes immune synapse
75 formation between NK cells and cancer cells, potentially presenting new therapeutic targets in cancer and
76 infectious diseases.

77 **Results**

78 *NK cell immune response to influenza virus and cancer cells during pregnancy.*

79 To investigate how pregnancy alters NK cell phenotype and function, we recruited two cohorts of pregnant
80 and non-pregnant (control) women in subsequent years (Tables S1 and S2 for cohort 1 and 2 demographics).
81 Pregnant women were enrolled in their second or third trimesters, and blood samples were collected at
82 enrollment and 6 weeks postpartum. To investigate intrinsic NK cell function during pregnancy, NK cells
83 and autologous monocytes were sorted from the PBMCs of controls (n=10), pregnant women (n=10), and
84 postpartum women (n=10) from cohort 1 (Fig. 1A, experimental workflow). Monocytes were infected with
85 2009 pandemic H1N1 influenza virus strain and then co-cultured with the autologous NK cells before
86 functional assessment by flow cytometry. We observed that the frequency of NK cells expressing CD107a
87 as a marker of cytolytic activity (Fig. 1B) and IFN-γ production (Fig. 1C) was significantly greater in
88 pregnant women than in controls or in postpartum women. These data confirm our earlier findings that
89 influenza-specific NK cell responses are enhanced during pregnancy (*12*). However, monocytes
90 demonstrate enhanced anti-influenza responses during pregnancy, which could activate NK cells through
91 inflammatory cytokine production (*13*). We hypothesized that if NK cell function was intrinsically elevated
92 during pregnancy, we should observe enhanced anti-tumor responses as well. We therefore exposed sorted
93 NK cells from controls and pregnant women to the K562 tumor cell line (Fig. 1A), which represents a
94 homogenous, identical target for NK cells from controls and pregnant women. NK cells from pregnant
95 women demonstrated significantly greater CD107a expression and tumor cell killing than NK cells from
96 non-pregnant women (Fig. 1, D and E). Interestingly, though tumor killing activity was enhanced during
97 pregnancy, there were no significant differences in the frequency of IFN-γ-producing NK cells (Fig. 1F),
98 possibly reflective of the lack of inflammatory cytokine production by these tumor targets. These data
99 indicate that NK cells have an intrinsically enhanced ability to kill both infected and tumor targets during
100 pregnancy.

101

102 *Deep profiling of NK cell phenotype from healthy pregnant and non-pregnant women*

103 To understand potential drivers of this enhanced NK cell function during pregnancy, we next profiled the
104 expression patterns of inhibitory and activating surface receptors on NK cells in control non-pregnant
105 women, pregnant women, and postpartum women. PBMCs from women in both cohorts were evaluated by
106 mass cytometry as outlined in Fig. 2A and Tables S3 and S4 (antibody panels for cohort 1 and 2,
107 respectively). NK cells were identified as $CD3^{-}CD19^{-}CD20^{-}CD14^{-}CD56^{+/-}CD16^{+/-}$ cells (Fig. S1A). The
108 frequency of NK cells did not significantly differ between pregnant and control women, nor in pregnant vs.
109 postpartum women in either cohort (Fig. S1, B and C). To identify NK cell markers predictive of pregnancy,
110 we used a generalized linear model (GLM) with bootstrap resampling to account for correlations between
111 cells and inter-individual variability (Figure 2A, and Materials and Methods). This method was selected
112 because markers expressed on cells from the same subject are usually more highly correlated than marker
113 expression patterns between subjects. Thus, accounting for correlated data reduces the number of false
114 positives.

115 In both cohorts, the expression of CD38 was significantly predictive of pregnant vs. non-pregnant
116 women (Fig. 2, B and C). CD38 also strongly predicted pregnancy in a paired comparison within women
117 between their pregnant and post-partum time points (Fig. 2, D and E). To confirm these results, CD38
118 expression on NK cells was assessed by manual gating, revealing that NK cells from pregnant women
119 express significantly more CD38 than NK cells from non-pregnant or postpartum women (Fig. S2, A to F).
120 Several other markers, including PD-1, CD27, CD94, LILRB1, NKp46, NKp44, NKG2A, and CD244,
121 were predictive of pregnancy in one of the cohorts or analyses (Fig. 2, B to E). Manual gating analyses
122 similarly confirmed enhanced expression of several of these markers within individual cohorts and
123 comparisons, yet none of the markers was consistently predictive of pregnancy across cohorts or analyses
124 (Fig. S3 to S7). As CD38 was the only marker consistently predictive of pregnancy across both cohorts and
125 all analyses, we elected to explore how its enhanced expression during pregnancy might alter NK cell
126 function.

127

128 *CD38 expression on NK cells correlates with the magnitude of the influenza-specific immune response*

129 We assessed CD38 expression by conventional flow cytometry on NK cells during their response to
130 influenza-infected cells as outlined in Fig. 1A. Consistent with our mass cytometry results, the frequency
131 of CD38-expressing NK cells as well as the intensity of CD38 expression on NK cells was significantly
132 greater in pregnant women compared to controls or postpartum women (Fig. 3, A and B). Further, CD38
133 expression was significantly correlated with the magnitude of the NK cell response as measured by CD107a
134 expression (Fig. 3C) and IFN-γ production (Fig. 3D). This raises the possibility that the increased responses
135 in pregnant women are driven primarily by their enhanced CD38 expression. We therefore evaluated the
136 function of $CD38^-$ vs. $CD38^+$ NK cells. Regardless of pregnancy status, CD38-expressing NK cells
137 displayed a higher CD107a and IFN-γ expression compared to $CD38^-$ NK cells (Fig. 3, E and F). These data
138 are consistent with the idea that expansion of a highly functional subset of NK cell expressing CD38 is
139 responsible for the enhanced influenza-specific NK cell responses during pregnancy.

140 To further confirm that CD38 expression is associated with enhanced NK cell responses to influenza
141 regardless of pregnancy status, we used NK cells from healthy blood bank donors to compare the ability of
142 $CD38^-$ versus total NK cells to respond to pH1N1-infected monocytes. We elected to compare $CD38^-$ NK
143 cells to total NK cells (of which 45-95% express CD38, Fig. S2) rather than to sort on CD38 because its
144 ligation could alter NK cell function. Total NK cells displayed significantly greater CD107a and IFN-γ
145 responses to autologous influenza-infected monocytes than did $CD38^-$ NK cells (Fig. 3, G and H).
146 Moreover, the frequency of dead monocytes was significantly increased after co-culture with total NK cells
147 compared to $CD38^-$ NK cells, suggesting that $CD38^+$ NK cells are enhanced in killing activity (Fig. 3I).
148 These data confirm that $CD38^+$ NK cells have increased responses to influenza-infected cells.

149

150 *Profiling $CD38^-$ and $CD38^+$ NK cells.*

151 To better understand the mechanisms driving enhanced function of CD38-expressing NK cells, we
152 examined the phenotype of $CD38^+$ NK cells. We used GLM with bootstrap resampling to evaluate markers

153 predictive of CD38$^+$ vs. CD38$^-$ NK cells (Fig. 4A, for cohort 2 and fig. S8A, for cohort 1), which differed
154 in the specific NK cell markers examined (Fig. 2A). As CD38 expression was associated with enhanced
155 responsiveness in all subjects regardless of pregnancy status, we examined NK cells from both pregnant
156 and control women together. We observed that CD38 expression marked a unique phenotype of NK cells
157 in cohort 2 (Fig. 4A). Specifically, several activating (CD244 (2B4), CD11b, CD57, NKp30 and NKp46)
158 and inhibitory (KIR2DL1, KIR2DL2/L3/S2, KIR3DL1, KIR3DL2 and LILRB1) receptors were
159 significantly predictive of CD38$^+$ NK cells. CD38$^-$ NK cells were more likely to express CD94, CD27, and
160 KIR2DL1. A similar analysis of cohort 1 confirmed the increased expression of CD57, NKp30 and NKp46
161 in CD38$^+$ NK cells compared to CD38$^-$ NK cells (Fig. S8A). Expression of perforin, which was not included
162 in the panel to evaluate cohort 2, was significantly increased on CD38$^+$ NK cells (Fig. S8A). Manual gating
163 to examine the frequency of NK cells expressing specific receptors, as well as their mean signal intensity,
164 confirmed these results (Fig. 4, B and C). Similar results were obtained when the control and pregnant
165 women were evaluated independently (Fig. S9, A and B). Overall, the increased expression of several KIRs,
166 NKp30, NKp46, CD57, CD11b, and perforin suggest that CD38$^+$ NK cells are a mature subset with high
167 cytotoxic potential.
168
169 *CD38 plays a direct role in NK cell immune response to influenza infection.*
170 The mature phenotype of CD38-expressing NK cells could explain their enhanced responsiveness if CD38
171 marks cells with greater functional potential. Yet it is also possible that CD38 plays a direct role in the NK
172 cell immune response to influenza-infected cells. CD38 has two known functions, neither of which have
173 been studied in antiviral or antitumoral NK cell function. First, it is an extracellular ectoenzyme that can
174 drive intracellular calcium flux through i) catalyzing the synthesis of cyclic Adenosyl-di-phosphate ribose
175 from Nicotinamide Adenine Dinucleotide$^+$ or ii) catalyzing the hydrolysis of cyclic Adenosyl-di-phosphate
176 ribose into Adenosyl-di-phosphate ribose (*22*, *23*). Second, it is an adhesion molecule that binds to CD31
177 and likely other ligands (*24*, *25*). We therefore used an inhibitor of CD38 enzymatic activity, kuromanin,

178 to assess the role of CD38 enzymatic activity in the NK cell response to influenza-infected monocytes. We
179 observed no significant differences in CD107a or IFN-γ responses in the presence or absence of this
180 inhibitor at concentrations known to inhibit CD38 function in T cells (*26*) (Fig. S10, A and B). Thus, the
181 enzymatic activity of CD38 does not appear to play a role in altering influenza-specific NK cell responses.
182 As monocytes express CD31 (Fig. S10C), we used CD38 and CD31 blocking antibodies to assess
183 the role of CD38-CD31 interactions in the immune response to influenza. The addition of CD38 blocking
184 antibodies significantly inhibited NK cell CD107a and IFN-γ responses (Fig. 5, A and B). Further, blocking
185 CD38 diminished the frequency of dead monocytes, suggesting that NK cell killing is diminished (Fig. 5C).
186 Blocking the CD38 ligand, CD31, also significantly abrogated NK cell CD107a and IFN-γ responses
187 compared to the incubation with an isotype control antibody. These data indicate that CD38, in addition to
188 marking a mature subset of NK cells, plays a direct role in NK cell responses to influenza, likely by the
189 binding to its ligand, CD31.
190
191 *CD38 is crucial to the establishment of immune synapse with influenza-infected cells.*
192 We next investigated whether CD38-CD31 interactions might play a role in the formation of the immune
193 synapse between the NK cell and the infected cell. We therefore examined whether CD38-CD31
194 interactions contributed to the ability of NK cells to make conjugates with influenza-infected monocytes.
195 Conjugation between fluorophore-labelled NK cells and monocytes were assessed by flow cytometry (Fig.
196 5D and fig. S11). In presence of blocking antibodies to both CD38 and LFA-1 (which was used as a positive
197 control), we observed a significant reduction in the formation of NK cell-monocyte conjugates compared
198 to untreated cells or isotype control antibody-treated cells. This suggests that CD38 plays a role in immune
199 synapse formation. To confirm this, using confocal microscopy, we observed that CD38 is co-localized
200 with the adhesion molecule LFA-1 in the contact zone between NK cells and H1N1-infected monocytes
201 (Fig. 5, E and F). Minimal accumulation of CD38 was observed when NK cells were in contact with mock-
202 infected monocytes (Fig. 5, E and F). In presence of a blocking CD38 or CD31 antibody, the formation of

203 the immune synapse was strongly and significantly inhibited compared to non-treated cells or cells
204 incubated with an isotype control antibody (Fig. 5, G and H). Similarly, immune synapse formation was
205 rarely observed between H1N1-infected cells and CD38$^-$ NK cells. As a positive control, blocking LFA-1
206 also led to a significant decrease in the immune synapse formation between NK cells and pH1N1-infected
207 monocytes compared to non-treated cells or cells treated with an isotype control antibody (Fig. 5, G and
208 H). Together, these data indicate that CD38 contributes to the establishment of the immune synapse between
209 NK cells and influenza-infected cells, which is pivotal for NK cell immune response.
210
211 *CD38 contributes to immune synapse formation between NK cells and cancer cells.*
212 To explore whether the role of CD38 in NK cell responses extends beyond the response to influenza-
213 infected cells, we examined NK cell responses to K562 tumor cells. We exposed total or CD38$^-$ NK cells
214 from healthy donors to K562 cancer cells in the presence of CD38 or CD31 blocking antibodies. Total NK
215 cells had significantly greater CD107a responses than did CD38$^-$ NK cells and resulted in higher levels of
216 tumor cell killing (Fig. 6, A and B). Blocking CD38 significantly diminished NK cell CD107a expression
217 and tumor cell killing (Fig. 6, A and B). The frequency of IFN-γ^+ cells did not significantly differ between
218 CD38$^-$ and total NK cells, and blocking CD38 and CD31 did not significantly alter the frequency of IFN-γ^+
219 NK cells responding to tumor targets (Fig. 6C). Blocking CD31 had no significant effect on CD107a
220 expression, K562 cell death, or IFN-γ production, suggesting that an alternate ligand may be used. Thus,
221 these data indicate that CD38 also plays a critical role in NK cell cytolytic responses to cancer cells. To
222 demonstrate the direct role of CD38 in NK cell anti-tumor responses, we assessed NK cell-K562 cell
223 conjugate formation, and found that blocking CD38 or LFA-1 (positive control) significantly diminished
224 conjugate formation compared to isotype control or without antibody (Fig. 6D). Finally, we visualized
225 CD38 in the formation of immune synapses between NK cells and K562 tumor cells. As depicted in Fig. 6,
226 E and F, CD38 polarizes at the contact zone between NK cells and K562 cells. Together, our data indicate

227 that CD38 plays a crucial role in the establishment of the immune synapse and in NK cell cytotoxic function

228 towards cancer cells.

229 **Discussion**

230 During pregnancy, the maternal immune system is engaged in a fine balance: tolerance is required to
231 preserve the fetus while defenses must be maintained to protect mother and baby from microbial challenges.
232 NK cells play a critical role in this balance as their job is to patrol the body for 'altered self'(*27*). NK cell
233 activity had been thought to be suppressed during pregnancy to protect the fetus (*28*), yet recent studies
234 have suggested a more nuanced view. NK cells from pregnant women display diminished responses to
235 stimulation with cytokines and phorbol-myristate acetate and ionomycin (*12*, *15*, *16*), yet NK cell responses
236 to influenza-infected cells are enhanced (*12*). Here we profiled NK cells from pregnant and non-pregnant
237 women to better understand how NK cell phenotype and function are altered during pregnancy. We
238 discovered that pregnancy is characterized by increased expression of the multi-faceted surface protein
239 CD38 on NK cells. CD38 is expressed on a large proportion of NK cells even in non-pregnant individuals
240 and is significantly increased in cell surface density during pregnancy. $CD38^{+}$ NK cells display a mature
241 phenotype and kill both infected and tumor cells more effectively than $CD38^{-}$ NK cells. The basis of this
242 enhanced responsiveness is the ability of CD38 to promote immune synapse formation between NK cells
243 and infected and tumor cells. These observations not only demonstrate a new role for CD38 in promoting
244 NK cell functional responses through immune synapse formation, but also provide a mechanistic
245 understanding of why NK cells display enhanced responses to influenza virus during pregnancy.

246

247 Pregnant women are significantly more likely to suffer adverse consequences from influenza infection than
248 are the general population. During the 1918 influenza pandemic, the case fatality rate for influenza infection
249 was estimated to be 27-75% among pregnant women but only 2-3% among the general population (*29*).
250 Even with improved supportive care, the case-fatality rate among pregnant women was twice that of the
251 general population during the 2009 pandemic (*3*). Thus, an understanding of the mechanisms driving this
252 enhanced susceptibility to influenza infection during pregnancy represents an important challenge for the
253 scientific community. During influenza virus infection, the recruitment of peripheral NK cells into the lungs

254 represents one of the first lines of defense following influenza infection (*30*). Though isolated NK cells
255 stimulated with cytokines or chemicals have suppressed responses during pregnancy, our data here confirm
256 earlier findings that NK cell responses to autologous influenza-infected cells are enhanced during pregnancy
257 (*12*). This enhanced responsiveness could be deleterious to lung integrity and drive pathogenesis. Consistent
258 with this idea, Kim and colleagues demonstrated that pregnant mice infected by influenza virus have an
259 increased lung inflammation and damage compared to non-pregnant mice (*31*). Further, Littauer and
260 colleagues suggested that innate immune responses play a role in the initiation of pregnancy complications
261 such as pre-term birth and stillbirth following influenza virus infection (*10*). Finally, the idea that enhanced
262 NK cell responses could be detrimental in pregnant women is consistent with observations that
263 hyperinflammatory responses are a driving force behind severe influenza disease in humans (*32-34*). Thus,
264 to better understand the potential contributions of NK cells to influenza pathogenesis during pregnancy, we
265 sought to define exactly how pregnancy altered human NK cells.
266
267 We were surprised to discover that the most dramatic and consistent difference between NK cells of
268 pregnant and non-pregnant women was an increase in CD38 expression during pregnancy. While CD38 is
269 most commonly viewed as an activation marker on T cells, it is more highly expressed on NK cells and has
270 several important functions. First, CD38 confers lymphocytes with the ability to adhere to endothelial cells
271 through its binding to CD31, a necessary step in extravasation. CD38 also functions as an ectoenzyme,
272 converting extracellular NAD^+ to cADPR through its cyclase activity or cADPR to Adenosyl-di-phosphate
273 ribose through its hydrolase activity (*35*). These molecules in turn can diffuse into the cell and promote its
274 activation by driving intracellular calcium increase, phosphorylation of signaling molecules, production of
275 cytokines, and vesicular transport (*35*). The diverse roles and functions raised the question of whether
276 increased CD38 expression during pregnancy is merely a marker of a more 'activated' state or whether it
277 was playing a direct role in modulating NK cell function. Thus, we first explored whether CD38 expression
278 marked a unique subset of NK cells. We found that expression of CD38 marks a population of NK cells

279 with a differential expression of KIRs, CD11b, CD57, perforin and CD94 compared to CD38$^-$ NK cells,
280 indicating a higher degree of cell maturation (*36*). In particular, the high expression of one or several KIRs
281 on CD38$^+$ NK cells is an essential feature characterizing NK cell maturation and terminal differentiation
282 (*37*, *38*). This suggests that CD38$^+$ NK cells are functionally competent, with high cytotoxic abilities, but
283 also likely self-tolerant since they display at least one inhibitory receptor for Major Histocompatibility
284 Complex. The high degree of co-expression between KIR and CD38 could reflect a need to assure tolerance
285 towards fetal semi-allogenic antigens, while maintaining a high degree of cytotoxicity towards virus-
286 infected and transformed cells, as demonstrated here.
287
288 Our demonstration here that CD38 contributes to the formation of immune synapse between NK cells and
289 their targets represents a novel role for this molecule. While much is known about its enzymatic activities
290 and adhesion functions through interaction with CD31, CD38 has not been extensively studied in terms of
291 the biology of immune cells, especially cytotoxic ones. We show here that CD38-CD31 interactions are
292 necessary to establish the NK immune synapse and for its cytotoxic activity against influenza-infected target
293 cells. Importantly, this function for CD38 was not restricted to NK cells role in responding to influenza-
294 infected cells, as we also show that blocking CD38 diminishes the ability to kill tumor targets. Thus, CD38
295 has a previously unrecognized, but critical, role in facilitating cytolytic activity against both infected cells
296 and tumor targets. Interestingly, blocking CD38 diminished both IFN-γ production and cytolytic activity in
297 response to influenza-infected cells, but diminished only cytolytic activity, and not IFN-γ production, in
298 response to K562 cells. This is likely due to a requirement for both cytokines (primarily type I interferons)
299 and cellular contact to obtain maximal IFN-γ production by NK cells (Kronstad et al., in revision and
300 bioRxiv). While infected monocytes produce abundant IFN-α, K562 cells do not.
301
302 This study leaves open the question of the mechanism by which CD38 is increased during pregnancy. CD38
303 protein expression increases with age (*39*). Our pregnant and non-pregnant women were well-matched for

304 age, so this cannot account for the differences between cohorts. CD38 gene and protein expression is
305 regulated by transcription factors induced by diverse potential signals such as cytokines and hormones (*40*,
306 *41*). Thus, the altered hormonal and inflammatory environments during pregnancy could contribute to
307 enhanced CD38 expression. In particular, NK cells express the estrogen receptor beta (*42*). Estrogen binding
308 to its receptor in peripheral NK cells could increase CD38 expression during pregnancy, especially during
309 second and third trimester where a substantial increase in estrogen concentration in the blood is observed
310 (*43*). While this has not been explored in humans or in pregnancy, CD38 protein expression on
311 cardiomyocytes was increased following injection of systemic estrogen, but not progesterone, in a rat model
312 (*41*).
313
314 Harnessing NK cell cytotoxic power has been of great interest within the scientific community within the
315 last several years. Several studies suggest that CAR-modified NK cell immunotherapy may be as effective
316 as CAR-T cells in recognizing and killing target cells after genetic modification (*44*, *45*). Thus, the
317 identification of a new role for CD38 in immune synapse formation has significant implications for these
318 therapies. For immunotherapy approaches in which cytotoxic activity is desired, it will be important to
319 assure that these cells express CD38. Further, it will be important to understand the factors that control
320 CD38 expression in vivo to assure its retention on effector cells. For instance, we find that both IL-2 and
321 IL-15, two important cytokines for NK cell homeostasis, promote increased CD38 expression *in vitro* (data
322 not shown). It is equally important that we assess the role of CD38 on $CD8^+$ cytotoxic T cells. Finally, our
323 data raise interesting implications for the CD38-targeting agents that are currently in clinical use, primarily
324 for multiple myeloma and chronic lymphocytic leukemia (*46*, *47*). Clinical treatment with daratumumab, a
325 specific human CD38 binding antibody, improves patient outcomes (*46*), but also may lead to NK cell
326 fratricide as most NK cells express CD38 (*48*, *49*). It is currently unclear how daratumumab affects CD38-
327 mediated immune synapse formation, and how this influences NK cell killing. This will be an important
328 area of future investigation.

329

330

331 There are several limitations of our study, including the fact that our mass cytometry panels differed
332 between the two cohorts and remain limited to ~40 markers. Thus, we may have excluded other molecules
333 involved in NK cell immune responses during pregnancy, including critical NK cell surface molecules such
334 as DNAM-1, TIGIT and Siglec-7. We also did not follow-up on other differences that were seen in only
335 one cohort. Further, here we studied peripheral blood NK cells and were not able to sample lung resident
336 NK cells or uterine NK cells. Finally, we had limited data reflecting the prior history of the infected and
337 control women in terms of their prior vaccination status, prior influenza infection status, cigarette and drug
338 use, and others. We cannot exclude that unmeasured factors could influence the quality of the NK cell
339 response to influenza.

340

341 Here, our goal was to better understand NK cell biology and activity in the context of pregnancy and
342 influenza virus infection. Our work reveals that CD38 expression is increased on NK cells during
343 pregnancy, and that CD38 marks a mature NK cell subset. Further, we demonstrate a novel role for CD38
344 in the enhancement of NK cell responses through its role in immune synapse formation. This new pathway
345 controlling NK cell function could be used as a target in future therapeutics to modulate NK cell killing
346 activity.

347

348 **Materials and Methods**

349 *Study design*

350 The goal of this study was to determine changes in the phenotype and function of NK cells during
351 pregnancy. Two cohorts of pregnant women enrolled during their second and third trimester and control
352 non-pregnant women were recruited in separate years. In cohort 1, twenty-one healthy pregnant women
353 were recruited between October 2013 and March 2014 from the Obstetrics Clinic at Lucile Packard
354 Children's Hospital at Stanford University. Twenty-one non-pregnant (control) women were recruited at
355 Stanford's Clinical and Translational Research Unit (NCT number: NCT03020537, NCT03022422 and
356 NCT02141581). In cohort 2, thirty-two non-pregnant (control) women were recruited at Stanford's Clinical
357 and Translational Research Unit (NCT numbers: NCT01827462 and NCT03022422) and twenty-one
358 healthy pregnant women were recruited between October 2012 and March 2013 from the Obstetrics Clinic
359 at Lucile Packard Children's Hospital at Stanford University. No difference in terms of age has been
360 observed in both cohort between control and pregnant women (*t test*: p=0.1967 for cohort 1; *t test*: p= 0.1697
361 for cohort 2). Venous blood was collected from all participants at baseline; pregnant women also provided
362 a sample at six weeks postpartum. Participant criteria are listed in Supplemental Table S1 and S2. Exclusion
363 criteria for all participants included concomitant illnesses, immunosuppressive medications, or receipt of
364 blood products within the previous year. Pregnant women were also excluded for known fetal abnormalities
365 and morbid obesity (pre-pregnancy body mass index > 40). This study was performed in accordance with
366 the Declaration of Helsinki and approved by the Stanford University Institutional Review Board; written
367 informed consent was obtained from all participants.

368 In addition, blood from healthy donors at the Stanford blood bank center was obtained to perform several
369 functional assays in the study.

370

371 *PBMC Isolation and cryopreservation*

372 PBMCs from healthy donors and from individuals belonging to cohort 1 and 2 were isolated from whole
373 blood by Ficoll-Paque (GE Healthcare) and cryopreserved in 90% fetal bovine serum (Thermo
374 Scientific)/10% dimethyl sulfoxide (Sigma-Aldrich).
375
376
377 *PBMC Staining and CyTOF Acquisition*
378 Cryopreserved PBMCs from non-pregnant and pregnant women in cohort 1 and 2 were thawed and cells
379 were transferred to 96-well deep-well plates (Sigma), resuspended in 25 μM cisplatin (Enzo Life Sciences)
380 for 1 min and quenched with 100% serum. Cells were stained for 30 min on ice, fixed (BD FACS Lyse),
381 permeabilized (BD FACS Perm II), and stained with intracellular antibodies for 45 min on ice. Staining
382 panels are described in Tables S3 and S4. All antibodies were conjugated using MaxPar X8 labeling kits
383 (DVS Sciences). Cells were suspended overnight in iridium interchelator (DVS Sciences) in 2%
384 paraformaldehyde in phosphate-buffered saline (PBS) and washed 1× in PBS and 2× in H_2O immediately
385 before acquisition on a CyTOF-1 (Fluidigm).
386
387 *Preprocessing of Mass Cytometry Data*

388 Prior to analysis, we transform protein counts using the inverse hyperbolic sine function. This
389 transformation assumes a two-component model for the measurement error (*50*, *51*): small counts are less
390 noisy than large counts. This model can be justified due to CyTOF2's dual count scale (CyTOF2 Mass
391 Cytometer User Manual): less abundant proteins can be measured more precisely than high abundant
392 proteins due to pulse overlap in the detector.

393 *Generalized Linear Mixed Model*

394 The response y_i specifies the experimental group for the ith cell, e.g. encoding whether the cell i was
395 stimulated ($y_i = 0$) or unstimulated ($y_i = 1$), and the explanatory variables are inverse hyperbolic sine

transformed protein counts $\boldsymbol{x}_i$. Each vector $\boldsymbol{x}_i$ is of length p equal to the number of measured proteins. Each experiment will produce n pairs of $(y_1, \boldsymbol{x}_1), \ldots, (y_n, \boldsymbol{x}_n)$ coming from different donors. Our statistical model is

$$y_i \sim \text{Bernoulli}(\pi_i)$$

$$\log\left(\frac{\pi_i}{1-\pi_i}\right) = \beta_{\text{donor}[i],0} + \sum_{j=1}^{p} \beta_{\text{donor}[i],j}\, x_{i,j} = \boldsymbol{\beta}^{\boldsymbol{T}}_{\text{donor}[i]} \boldsymbol{x}_i$$

$$\boldsymbol{\beta}_{\text{donor}[i]} \sim \text{Normal}(\boldsymbol{\theta}, \boldsymbol{\Sigma})\,.$$

In our notation for regression coefficients $\beta_{\text{donor}[i],j}$, the first subscript donor$[i]$ maps the ith cell to its donor, and the second subscript indexes the jth protein marker. We assume that the donor specific coefficients are distributed according to a multivariate normal distribution. This model is often called a Generalized Linear Mixed Model (GLMM). Using the GLMM terminology, the unknown parameters that we need to estimate are the fixed effects $\boldsymbol{\theta}$, the random effects $\boldsymbol{\beta}_{\text{donor}[i]}$, and the covariance matrix $\boldsymbol{\Sigma}$ of the random effect. GLMMs can account for donor specific heterogeneity by separating donor specific variation from overall variation. As it is common in single cell datasets, we observe 10,000 to 100,000 cells per donor and measure 30 to 40 proteins. To handle such large amounts of data, we use the R package *mbest* that implements a fast moment-based estimation to fit GLMM models (*52*). One key assumption in GLMMs is the modeling of random effects by a multivariate normal distribution.

Generalized Linear Model with Bootstrap Resampling

Our GLMM approach can be directly applied to paired experiments. In immunology, we often have a paired experimental design when comparing stimulated with unstimulated cells taken from the same blood sample. We also have paired samples, if we compare the functional response of different cell types within the sample blood sample. In contrast, in our pregnancy study, we have blood samples from pregnant and not pregnant donors. Furthermore, we do not have any additional covariates that we could use to match samples. In such

420 cases, the GLMM approach might provide conservative results, therefore, we propose to use a
421 nonparametric bootstrap resampling (*53*) approach using standard Generalized Linear Models (GLMs) (*54*),
422

424 $$y_i \sim \text{Bernoulli}(\pi_i)$$

425 $$\log\left(\frac{\pi_i}{1-\pi_i}\right) = \beta_0 + \sum_{j=1}^{p} \beta_j \, x_{i,j} = \boldsymbol{\beta}^{\boldsymbol{T}} \boldsymbol{x}_i \, .$$

423

426 In this approach, we handle the donor heterogeneity without explicitly defining a parametric mixed effects
427 distribution. One bootstrap draw is taken by sampling donors with replacement. For each bootstrap sample,
428 we fit a GLM using the base R implementation. We repeat this procedure B times resulting in B coefficient
429 vectors $\boldsymbol{\beta}_1^*, \ldots, \boldsymbol{\beta}_B^*$. We can then compute component-wise quantiles of these vectors to obtain confidence
430 intervals.
431

432 *R Package: CytoGLMM*

433

434 All previously mentioned regression methods and additional exploratory data analysis tools are available in
435 our new R package *CytoGLMM*.
436 The package can be installed from GitHub (https://github.com/ChristofSeiler/CytoGLMM).
437

438 *Virus Preparation*

439 A/California/7/2009 influenza (pH1N1) wild-type influenza A viruses obtained from Kanta Subbarao at the
440 National Institutes of Health were propagated in embryonated chicken eggs.
441

442 *Cell purification for functional assays*

443 Cryopreserved PBMCs from healthy donors, or non-pregnant and pregnant women from cohort 1, were
444 thawed and washed with complete RP10 media (RPMI 1640 (Invitrogen) supplemented with 10% fetal
445 bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (Life
446 Technologies)) and 50 U/mL benzonase (EMD Millipore).
447 Cell isolation. Autologous NK cells and/or monocytes were purified by magnetic-activated cell sorting via
448 negative selection (Miltenyi). In several experiments, $CD38^-$ NK cells were further isolated from total NK
449 cells by magnetic-activated cell sorting via negative selection (Miltenyi).
450 Cell sorting. Autologous NK cells and/or monocytes were sorted using Sony sorter SH800 (Sony). The
451 following antibodies were used to perform NK cell and monocyte sorting: CD3-Allophycocyanine (clone
452 OKT3; BioLegend), CD14-Brilliant Violet 421 (clone HCD14; BioLegend), CD19-Alexa Fluor 488 (clone
453 HIB19; Biolegend) and CD56-Phycoerythrin Cyanine 7 (clone NCAM; BioLegend).
454
455 *Monocyte infection by influenza virus*
456 Following isolation or purification, monocytes were washed and re-suspended in serum-free RPMI media
457 at 1×10^5 per 100 μL and infected at a multiplicity of infection (MOI) of 3 for 1 h at 37°C with 5% carbon
458 dioxide. One-hour post-infection, viral inoculum was removed and cells were re-suspended in 100 μL of
459 complete RP10. Autologous NK cells were incubated separately for 5 min in Fc Block, washed, and
460 incubated for 15 min with CD38 (clone AT1/3, Bio-Rad), CD31 (clone WM59, Biolegend) or LFA-1 (clone
461 TS1/22, Invitrogen) blocking antibodies or isotype control antibodies. NK cells were then exposed to
462 pH1N1-infected monocytes at a effector:target (E:T) ratio 1:1. After a further 2-hour incubation, 2 μM
463 monensin, 3 μg/mL brefeldin A (eBiosciences), and anti-CD107a-allophycocyanin-H7 (BD Pharmingen)
464 were added to the co-culture for 4 hours, followed by cell staining for flow cytometry analysis.
465
466 *NK cell exposure to autologous K562 tumor cell line.*

467 Following isolation or purification, NK cells were incubated for 5 min in Fc Block, washed, and incubated
468 for 15 min with CD38, CD31 or LFA-1 blocking antibodies or isotype control antibodies. NK cells were
469 then exposed to pH1N1-infected monocytes at a Effector:Target (E:T) ratio 1:1. Immediately following co-
470 incubation, 2 μM monensin, 3 μg/mL brefeldin A, and anti-CD107a-allophycocyanin-H7 were added to the
471 co-culture for 4 hours, followed by cell staining for flow cytometry analysis.
472
473 *Cell staining and Flow-Cytometry Analysis*
474 Cells were stained with LIVE/DEAD fixable Aqua Stain (Life Technologies), followed by surface staining
475 and then fixed and permeabilized with FACS Lyse and FACS Perm II (BD Pharmingen) according to the
476 manufacturer's instructions. Cells were stained with anti-CD3-PE or -APC, anti-CD16-PerCPCy5.5 (clone
477 3G8; BioLegend), anti-CD38-PE or FITC (clone HIT2 Biolegend), anti-IFNγ-FITC or V450 (clone B27;
478 BD Biosciences), anti-CD56-PEcy7, or anti-CD14-APC or -APC-H7 and fixed using 1%
479 paraformaldehyde. Uncompensated data were collected using a three-laser MACSQuant® Analyser
480 (Miltenyi). Analysis and compensation were performed using FlowJo flow-cytometric analysis software,
481 version 9.9.4 (Tree Star).
482
483 *Confocal microscopy*
484 Isolated NK cells were stained using CellTrace Violet dye (Thermofischer) for 20 min at RT then washed
485 twice in PBS. Isolated NK and mock- or pH1N1-infected monocytes were incubated on a Poly-L-Lysine
486 pre-coated 8 well μ-slide (Ibidi) for 2 hours (enough time to let the cells settle down). Cells were then
487 washed in PBS-FBS 2%, fixed in PFA 4% for 15 min and washed twice in PBS-FBS 2%. Cells were then
488 stained with mouse anti-CD38 and/or rabbit anti-LFA-1 antibody for 30 min at RT, then washed twice in
489 PBS-FBS 2%. Secondary staining was performed using a goat anti-mouse AlexaFluor594 or a goat anti-
490 rabbit AlexaFuor488 antibody for 30 min at RT. After washing the cells twice in PBS-FBS 2%, cell mount

491 media (Ibidi) was added on cells for microscopy. Images were acquired using LSM880 Meta (Zeiss) laser
492 scanning confocal microscope equipped with a 63× (NA 1.4) DIC oil objective.
493
494 *Flow cytometry-based conjugation assay*
495 Isolated NK cells were labeled with CellTrace CFSE for 20 min at 37°C, washed and incubated with Fc
496 Block for 5 min. NK cells were then incubated with no antibody, an isotype control or CD38 blocking
497 antibody for 15 min at 37°C. pH1N1-infected monocytes or K562 cells were labelled with CellTrace violet
498 for 20 min at RT. 10^5 NK cells and 2×10^5 target cells (E:T ratio 1:2) were mixed in 200 μl complete RP10,
499 incubated for the indicated times (0, 20 or 40 min) at 37°C, vortexed, and fixed with 1% PFA in PBS. Cell
500 mixtures were run on a three-laser MACSQuant® Analyser (Miltenyi). Analysis and compensation were
501 performed using FlowJo flow-cytometric analysis software, version 9.9.4 (Tree Star).
502
503 *Statistical Analysis*
504 Modeling was discussed above. Other statistical analyses were performed using GraphPad Prism, version
505 6.0d (GraphPad Software). Pregnant and control participant characteristics were compared using Mann–
506 Whitney *U* Tests for continuous variables and Fisher's exact test for discrete variables. Pregnant and
507 postpartum samples were compared using Wilcoxon match-paired test. Functional results were compared
508 between groups using Mann–Whitney *U* tests.
509

510 **Supplementary Materials**

511 Fig. S1Figure S1. NK cell gating strategy.

512 Fig S2. CD38 expression in control, pregnant and postpartum women from cohort 1 and 2.

513 Fig S3. Gating strategy for each marker on NK cells from cohort 1.

514 Fig S4. Gating strategy for each marker on NK cells from cohort 2.

515 Fig S5. Percentage of expression for each marker on NK cells from cohort 1 and 2 using conventional
516 gating.

517 Fig S6. Mean Signal intensity of NK cell marker in control, pregnant and postpartum from cohort 1 using
518 conventional gating.

519 Fig S7. Mean Signal intensity of NK cell marker in control, pregnant and postpartum from cohort 2 using
520 conventional gating.

521 Fig S8. Comparison of $CD38^-$ and $CD38^+$ NK cell expression of each marker in control and pregnant women
522 from cohort 1 using logistic regression model and conventional gating.

523 Fig S9. Comparison of activating and inhibitory receptor expression on $CD38^-$ and $CD38^+$ NK cells from
524 cohort 2 in control or pregnant women by conventional gating.

525 Fig S10. Effect of the CD38 enzyme inhibitor, kuromanin, on the NK cell response to H1N1-infected
526 monocytes.

527 Fig S11. CD38 contributes to NK cell - influenza-infected monocytes conjugation.

528 Table S1. Demographics for cohort 1.

529 Table S2. Demographics for cohort 2.

530 Table S3. Antibody panel for mass cytometry in cohort 1.

531 Table S4. Antibody panel for mass cytometry in cohort 2.

532

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550
551 **Author contributions:** M.L.G., A.W.K., N.L.B. and C.A.B. designed experiments. M.L.G., A.W.K. and
552 N.L.B. conducted experiments. M.L.G. and C.S. analyzed the data. E.L., L.M., S.S.S.O., P.K., collaborated
553 and provided advice in the analysis of the data. M.L.G and C.A.B. wrote the manuscript. M.M.D., C.L.D.,
554 G.E.S. and N.A. coordinated and provided human samples. M.M.D., C.S., and S.H. revised the manuscript.
555
556 **Competing interests:** All authors have no competing interests to declare.
557

558 **Data and materials availability:** Materials are available from the corresponding authors upon request.

559 All .FCS files will be uploaded to Immport upon publication.

560

561

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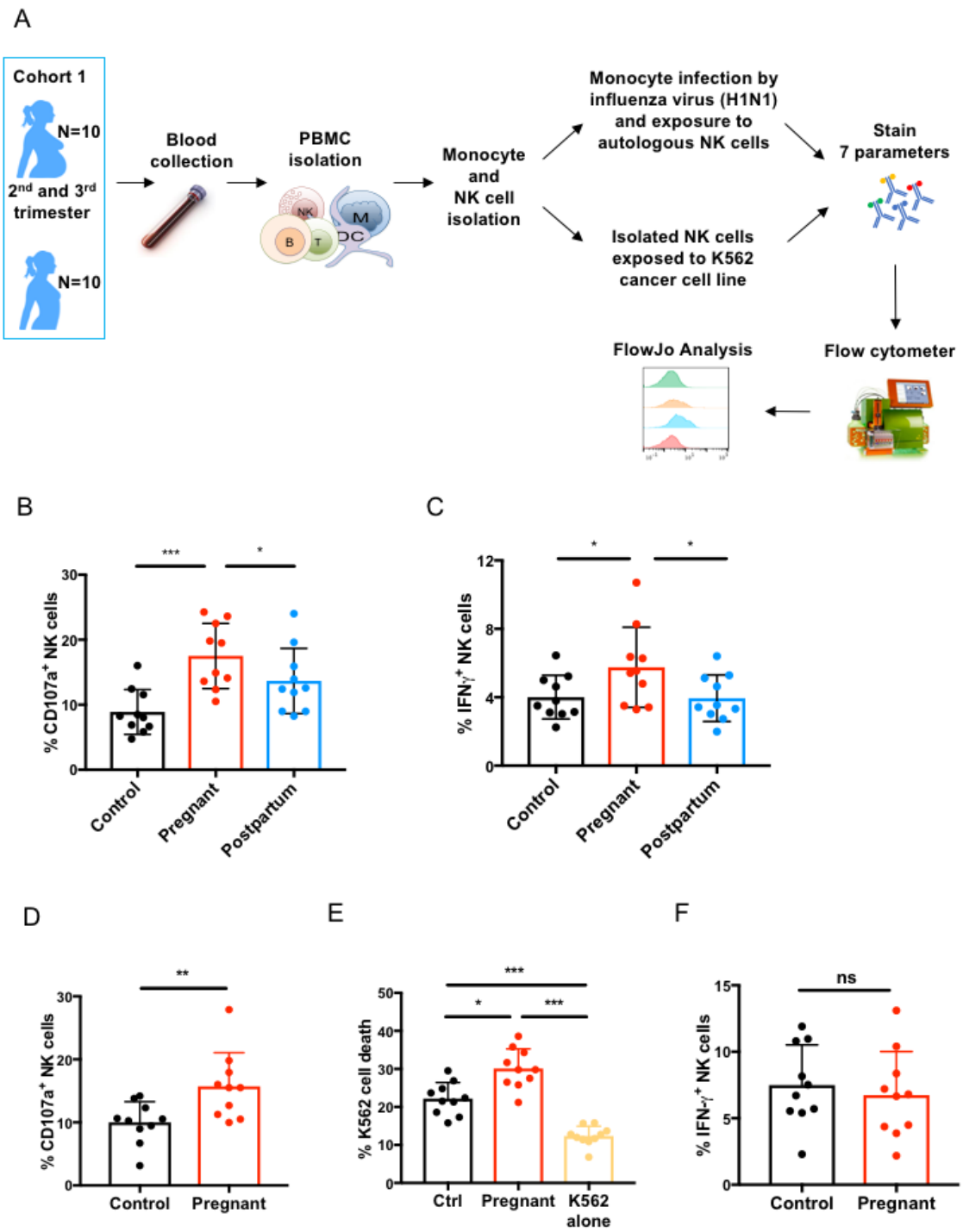
675 **Figures**

676 **Figure 1. NK cell immune response to influenza infected and tumor cells during pregnancy.**



677

678 **(A)** Experimental workflow: PBMCs from controls (N=10), pregnant women (N=10) and postpartum

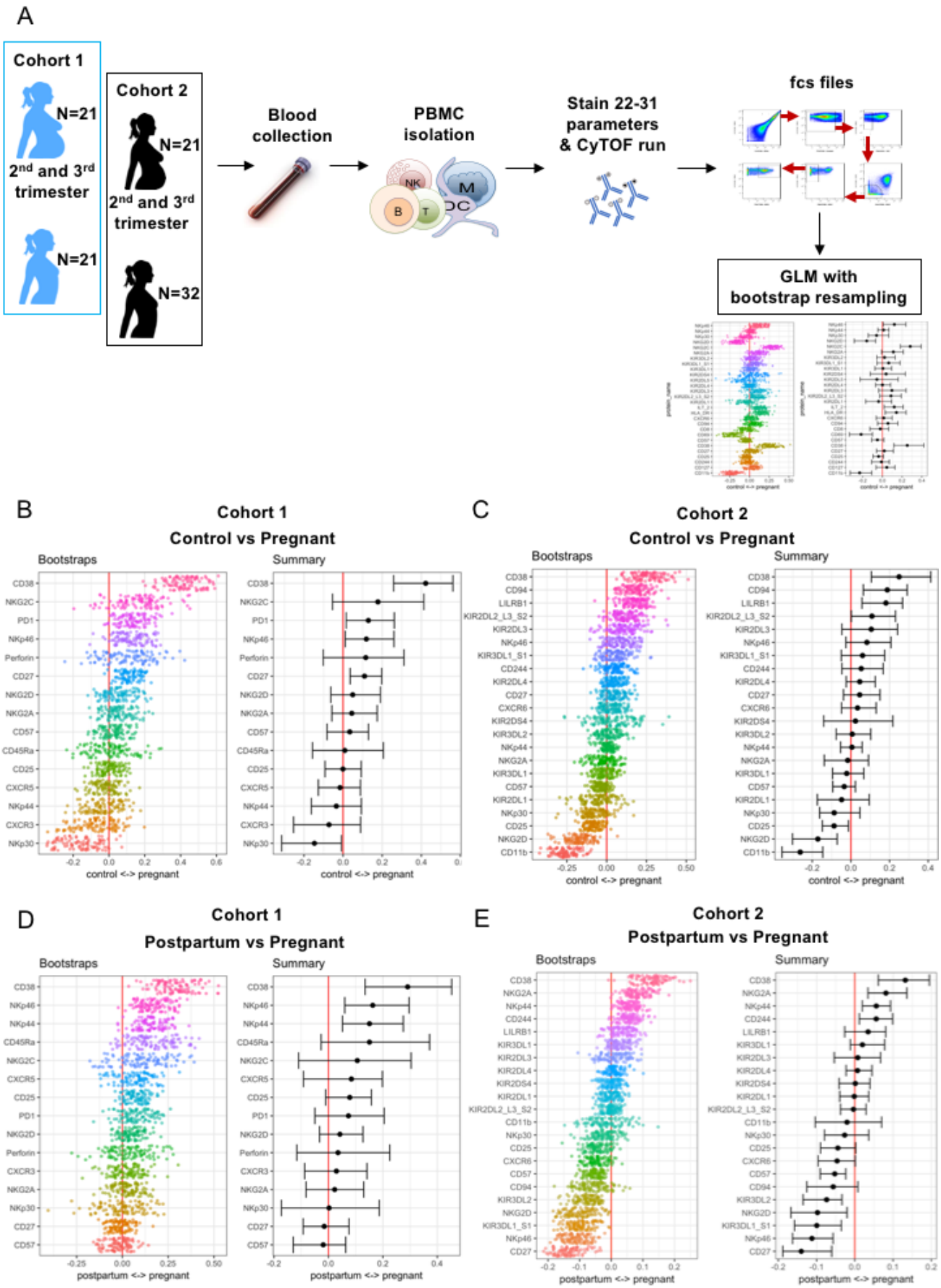
679 women (N=10) in cohort 1 were isolated from blood samples. Monocytes and NK cells were sorted and

680 monocytes were infected with the H1N1 influenza virus strain. NK cells were either exposed to H1N1-

681 infected monocytes or to K562 tumor cells for 7h or 4h, respectively. The NK cell immune response was

682 then determined by flow cytometry. (B) The frequency of **(B)** CD107a- and **(C)** IFN-γ-expressing NK cells
683 in response to influenza-infected monocytes. **(D)** The frequency of CD107a-expressing NK cells in
684 response to K562 cells. **(E)** The frequency of dead K562 cells as assessed by viability stain following co-
685 culture with NK cells. **(F)** The frequency of IFN-γ-production NK cells in response to K562 cells. *P<0.05,
686 **P<0.01 and ***P<0.001 (Mann–Whitney *U* Tests to compare controls vs. pregnant and control vs.
687 postpartum; Wilcoxon matched-paired test to compare pregnant vs. postpartum).
688

689 **Figure 2. Deep profiling NK cells in non-pregnant and pregnant women.**

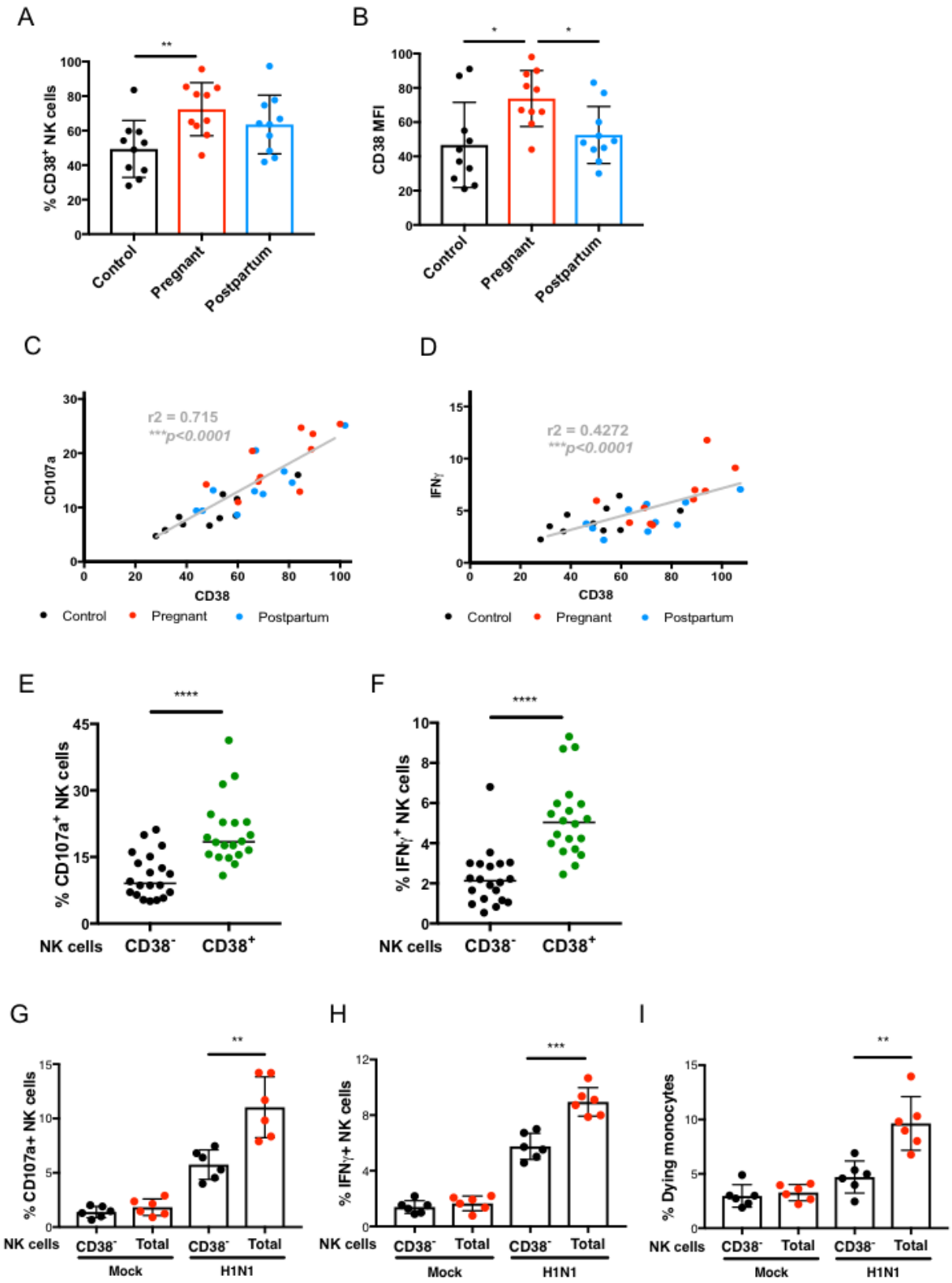


690

691 **(A)** Experimental workflow. PBMCs from controls, pregnant, and postpartum women were isolated and
692 labeled using a 22- or 31-parameter antibody panel for cohort 1 and 2, respectively. **(B)** Markers predictive
693 of control vs. pregnant women in Cohort 1 were assessed by GLM with bootstrap resampling. The markers
694 are listed on the y-axis and the x-axis represents the log-odds that the marker expression levels predict the
695 outcome (pregnancy on the left vs. control on the right). Dots in the left graph represent individual bootstrap
696 samples, with summary data in the right panel showing the 95% confidence interval. Markers in which the
697 bar does not cross zero are significantly predictive of one state vs. the other with a false discovery rate of
698 5%. **(C)** Evaluation of markers predictive of pregnancy vs. postpartum in cohort 1. **(D)** Markers predictive
699 of pregnancy vs. control and **(E)** pregnant vs. postpartum in cohort 2.
700
701

702 **Figure 3. NK cell immune response to influenza virus infection in non-pregnant, pregnant women**

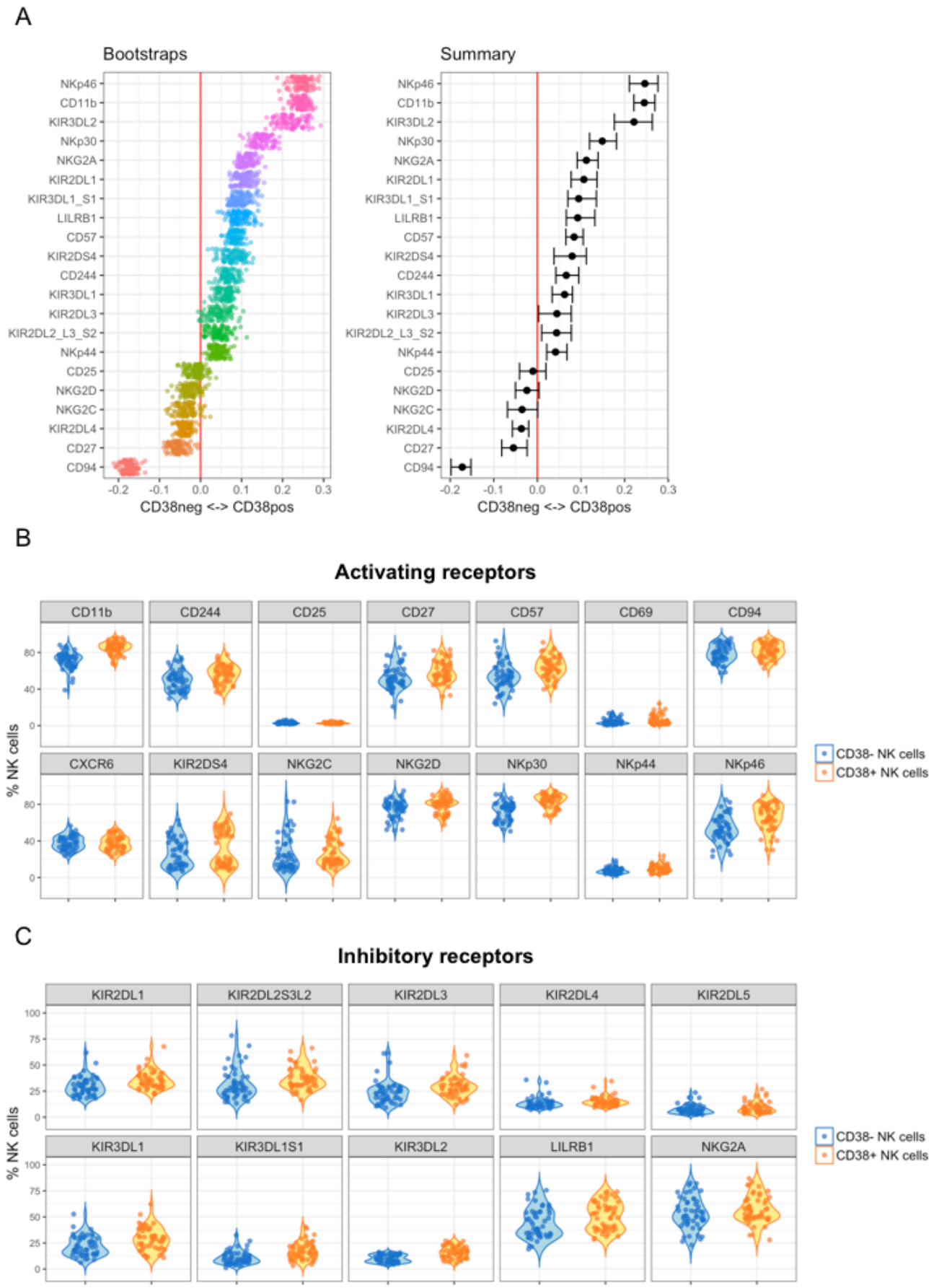
703 **and postpartum.**



704

705 NK cells and autologous monocytes were sorted from the PBMCs of controls (N=10), pregnant women
706 (N=10) and postpartum women (N=10). Monocytes were infected by H1N1 influenza virus and exposed to
707 autologous NK cells for 7h. The frequency of NK cells expressing CD38 **(A)** and the CD38 MFI **(B)** on NK
708 cells is shown. **P<0.01 (Mann–Whitney *U* Tests to compare controls vs. pregnant). Correlation by linear
709 regression between CD38 and CD107a expression **(C)** or IFN-γ expression **(D)** was determined on NK
710 cells. **(E)** CD107a expression and **(F)** IFN-γ production were measured in $CD38^-$ and $CD38^+$ subsets of NK
711 cells exposed to autologous influenza-infected monocytes. Data from both pregnant and control women
712 together were analyzed. **(G, H and I)** $CD38^-$ and total NK cells, and autologous monocytes were isolated
713 from healthy blood donors (N=6). Monocytes were infected by H1N1 virus and exposed to autologous
714 $CD38^-$ or total NK cells for 7h. CD107a expression **(G)** and IFN-γ production **(H)** by $CD38^-$ or total NK
715 cells were determined by flow cytometry as well as monocyte cell death **(I)** as measured by a viability stain.
716 ****P<0.0001 (Wilcoxon matched-paired test to compare $CD38^-$ NK cells vs. $CD38^+$ NK cells).
717

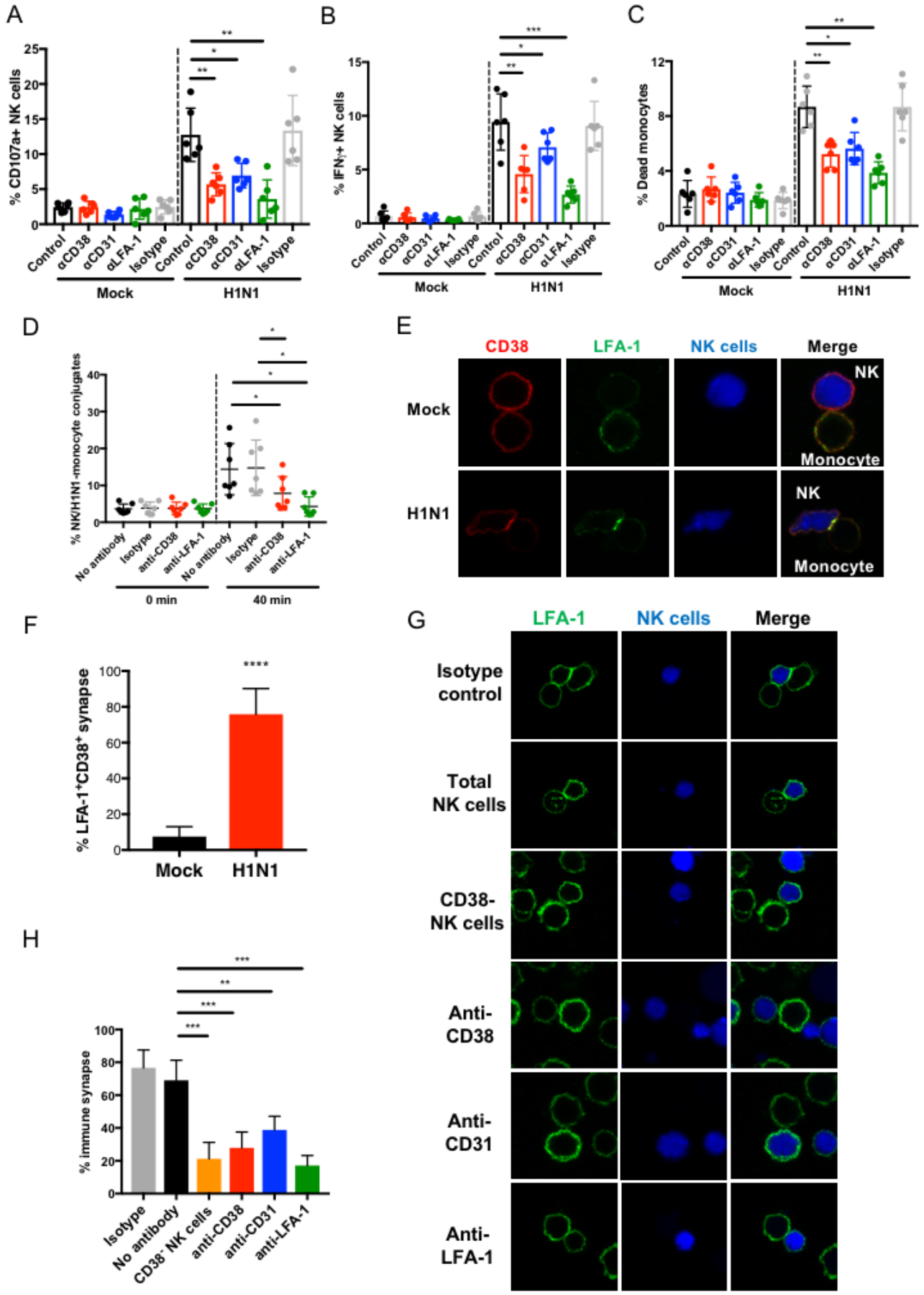
718 **Figure 4. Deep profiling of CD38⁻ and CD38⁺ NK cells in control and pregnant women.**



719

720 PMBCs from controls and pregnant women in cohort 2 were isolated and labeled using a 31-parameter
721 antibody panel (see Table S3). $CD38^-$ and $CD38^+$ NK cells were separated by manual gating and analyzed
722 using GLM with bootstrap resampling **(A)** and conventional gating strategy for each marker (**B**, for
723 activating receptor expression, and **C**, for inhibitory receptor expression).
724

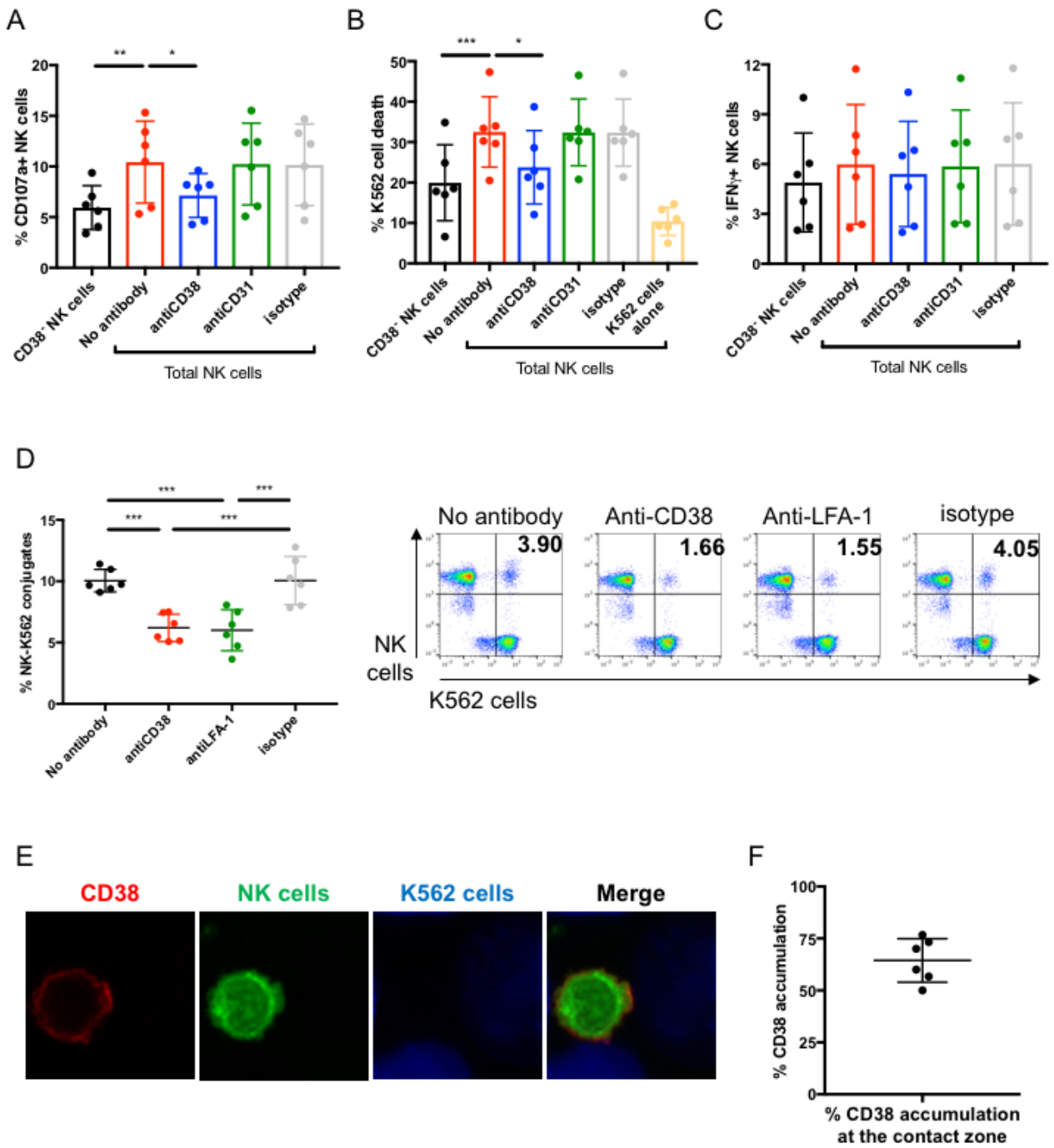
725 **Figure 5. CD38 contributes to the immune synapse between NK cells and H1N1-infected cells.**



726

727 **(A to C)** Isolated NK cells from healthy donors (N=6) were exposed to autologous H1N1-infected
728 monocytes in presence or absence of an isotype control antibody (grey dots) or a CD38 (red dots), CD31
729 (blue dots) or LFA-1 (green dots) blocking antibody or not (black dots). CD107a expression **(A)**, monocyte
730 killing **(B)** and IFN-γ production **(C)** by NK cells were measured by flow cytometry. *P<0.05, **P<0.01,
731 ***P<0.001 (Wilcoxon matched-paired test). **(D)** Conjugate formation was assessed by labeling NK cells
732 with and monocytes with Cell Trace Violet. Monocytes were infected with H1N1 influenza and exposed to
733 autologous NK cells for 0 or 40 min in presence or absence of a CD38 or LFA-1 blocking antibody or an
734 isotype control antibody. NK cell-monocyte conjugation was determined by the accumulation of the double
735 positive $CFSE^{+}$Cell Trace $Violet^{+}$ population by flow cytometry. *P<0.05 and **P<0.01 (Wilcoxon
736 matched-paired test to compare each condition). **(E and F)** Total NK cells and monocytes from healthy
737 blood donors (N=8) were isolated, monocytes were either mock- or H1N1-infected and exposed to
738 autologous NK cells. **(E)** CD38 (in red) and LFA-1 (in green) localization was determined by confocal
739 microscopy. A representative image is depicted for each condition. **(F)** Immune synapse formation was
740 determined by the accumulation of LFA-1 at the contact zone (N=30) between NK cells (in blue) and mock-
741 or H1N1-infected monocytes, and CD38 co-localization at the immune synapse was quantified. The
742 percentage of immune synapses with co-localization is represented based on blinded counting.
743 ****P<0.0001 (Wilcoxon matched-paired test). **(G and H)** H1N1-infected monocytes were exposed to
744 $CD38^{-}$ NK cells or total NK cells in presence or absence of an isotype control, CD38, CD31 or LFA-1
745 blocking antibody. The accumulation of LFA-1 (in green) was measured to evaluate the number of immune
746 synapses **(G)**. A representative image is depicted for each condition. 60 contacts between NK cells and
747 H1N1-infected monocytes were observed and LFA-1 accumulation was used to identify immune synapse
748 formation in a blinded fashion **(H)**. The percentage of immune synapse for each condition is represented.
749 **P<0.01 and ***P<0.001 (Wilcoxon matched-paired test to compare each condition).
750

751 **Figure 6. CD38 contributes to immune synapse formation between NK cells and cancer cells.**



752

753 **(A to C)** from PBMCs from healthy blood donors (N=6) were collected, CD38⁻ or total NK cells were

754 sorted, incubated with an isotype control (grey dots), CD38 (blue dots) or CD31 (green dots) blocking

755 antibody or without antibody (red dots), and exposed to K562 tumor cells for 4h. CD107a expression **(A)**,

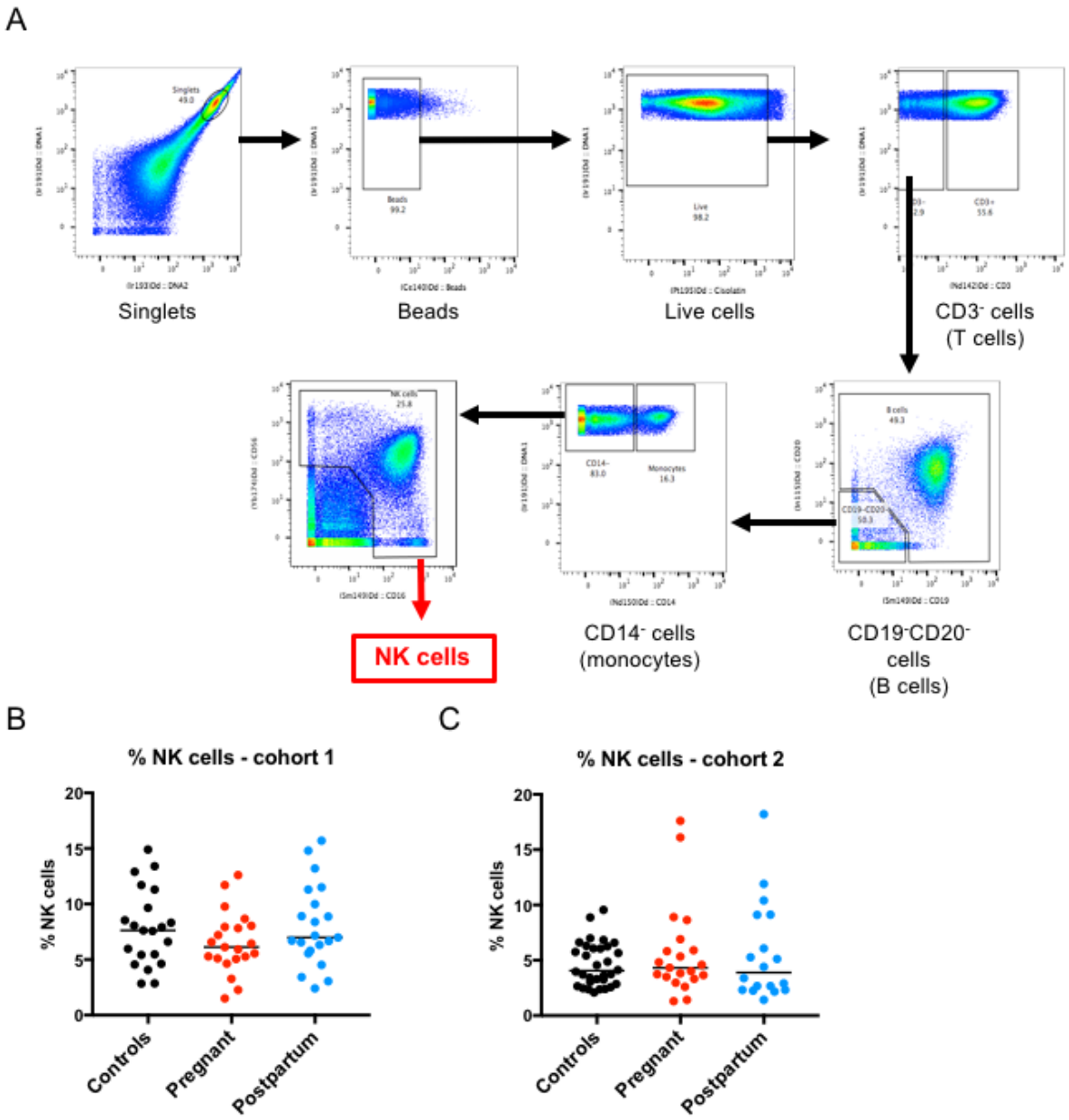
756 K562 cell killing **(B)** and IFN-γ production **(C)** by NK cells was measured by flow cytometry. *P<0.05,

757 **P<0.01, ***P<0.001 (Wilcoxon matched-paired test). **(D)** NK cells from 6 healthy donors were isolated

758 and stained with CFSE. In parallel, K562 cells were stained with Cell Trace Violet. NK cells were then
759 exposed to K562 cells for 0 or 40 min in presence or absence of a CD38 or LFA-1 blocking antibody or an
760 isotype control antibody. NK cell-K562 cell conjugation was determined by the accumulation of the double
761 positive $CFSE^{+}$Cell Trace $Violet^{+}$ population by flow cytometry. Left graph represents a summary of the
762 experiment and the right panel shows representative plots from one individual. ***P<0.001 (Wilcoxon
763 matched-paired test). **(E and F)** Total NK cells from healthy blood donors (N=6) were isolated and exposed
764 to K562 tumor cells for 1h. **(E)** CD38 (in red) and LFA-1 (in green) localization was determined by confocal
765 microscopy. A representative image is depicted for each condition. **(F)** Accumulation of CD38 in 30 contact
766 zones between NK and K562 cells was determined. The percentage of CD38 accumulation at the contact
767 zone was determined.
768

769 **Supplemental Materials.**

770 **Figure S1. NK cell gating strategy.**



771

772 **(A)** Two-dimensional mass cytometry plots are shown for a representative patient sample. Gating was

773 performed using FlowJo software (FlowJo, LLC). Negative lineage gating was performed to exclude CD3+

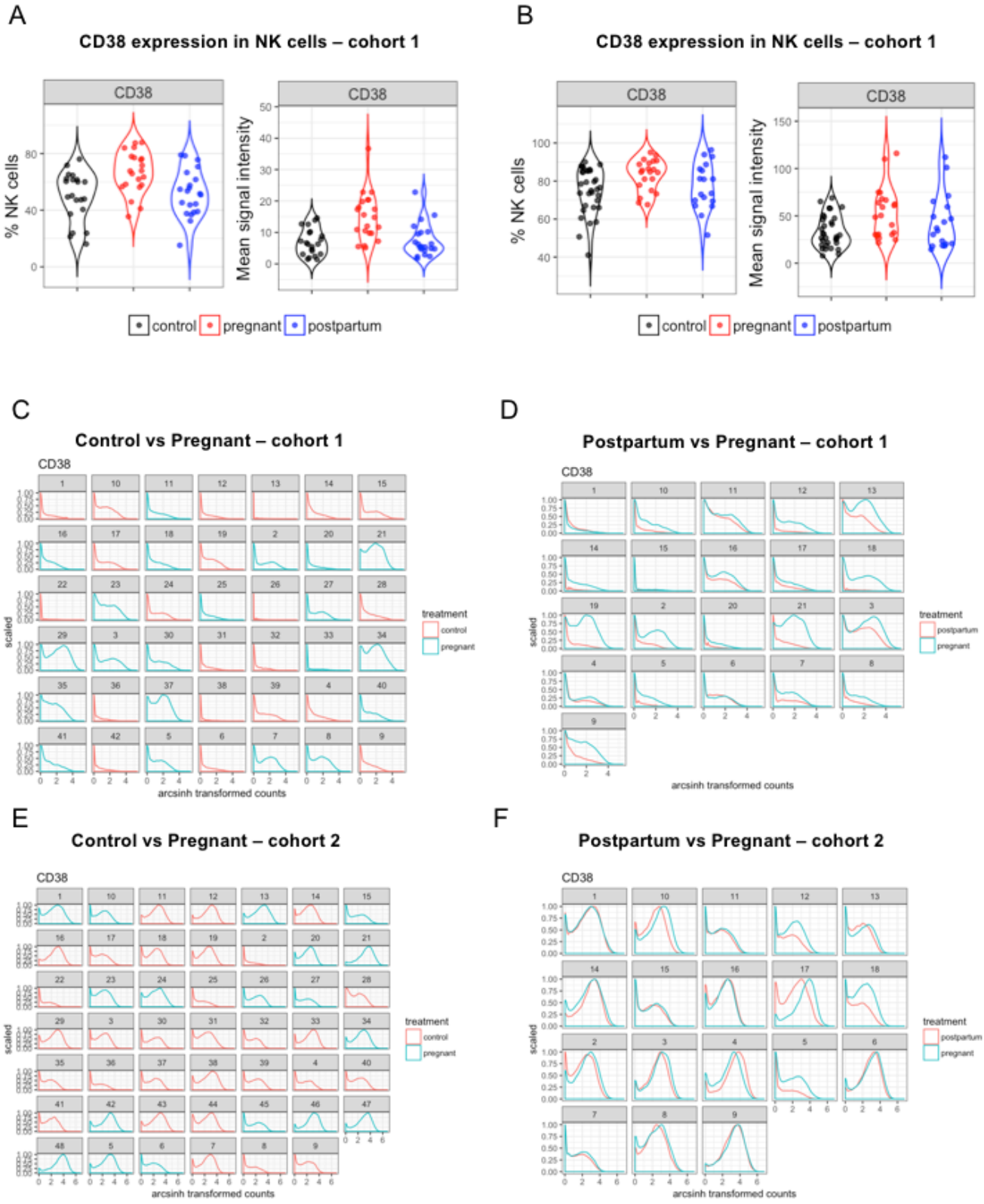
774 T cells, CD19+CD20+ B cells and CD14+ monocytes, followed by a positive gating on $CD56^{+/-}CD16^{+/-}$ NK

775 cells. **(B and C)** Percentage of NK cells within PBMCs of controls, pregnant women and postpartum in

776 cohort 1 **(B)** and cohort 2 **(C)**.

777

778 **Figure S2. CD38 expression in control, pregnant and postpartum women from cohort 1 and 2.**



779

780 **(A and B)** Manual gating for CD38 expression on NK cells in control, pregnant and postpartum women in

781 cohort 1 **(A)** and 2 **(B)**. The left panel represents the percent of CD38^{+} NK cells and the right panel represents

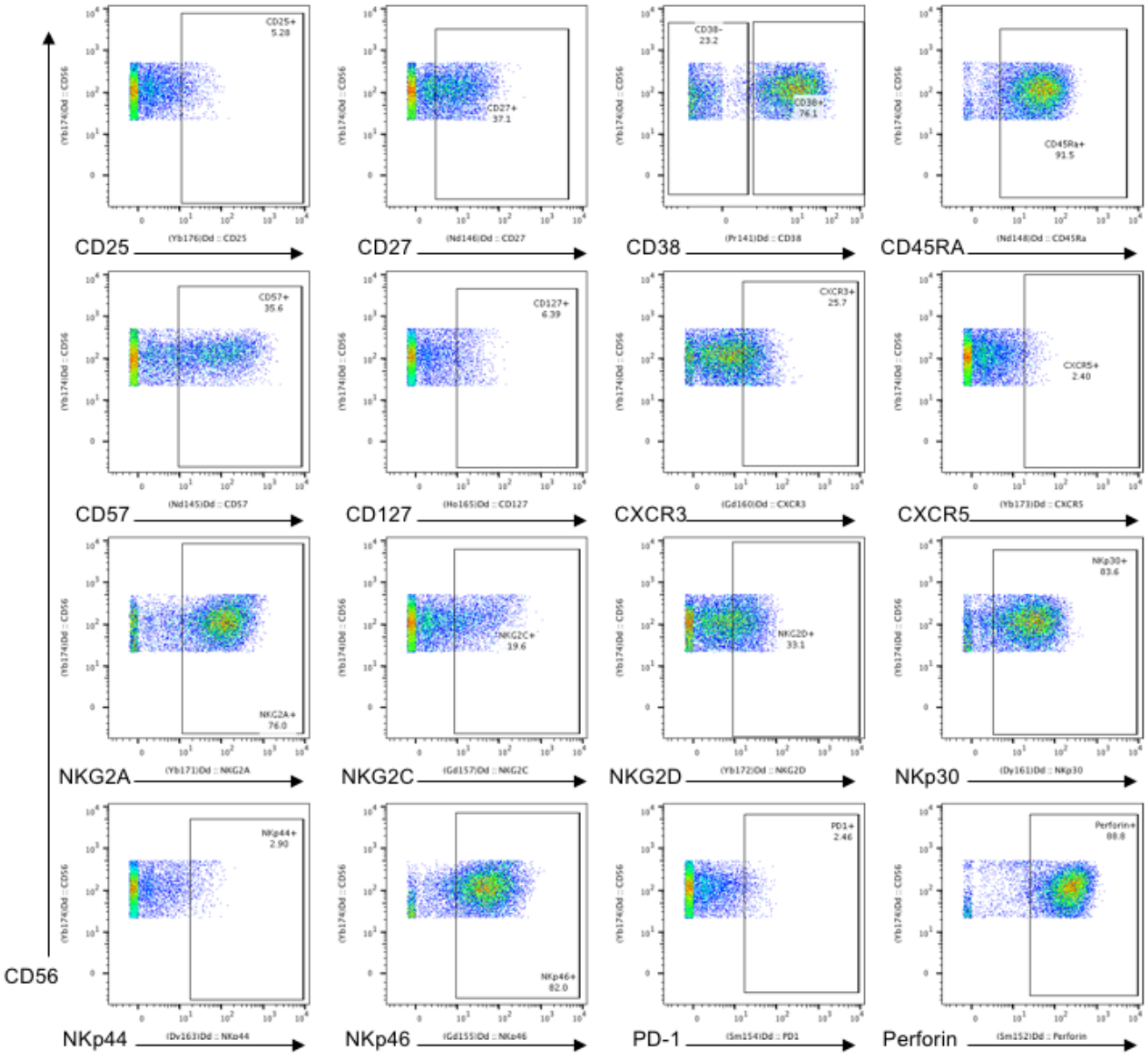
782 CD38 mean signal intensity. **(C to F)** NK cells from control and pregnant women (**C**, for cohort 1 and **E**,

783 for cohort 2), pregnant women and postpartum (**D**, for cohort 1 and **F**, for cohort 2) were analyzed and

784 expression of CD38 was measured and shown as density plots.

785

786 **Figure S3. Gating strategy for each marker on NK cells from cohort 1.**

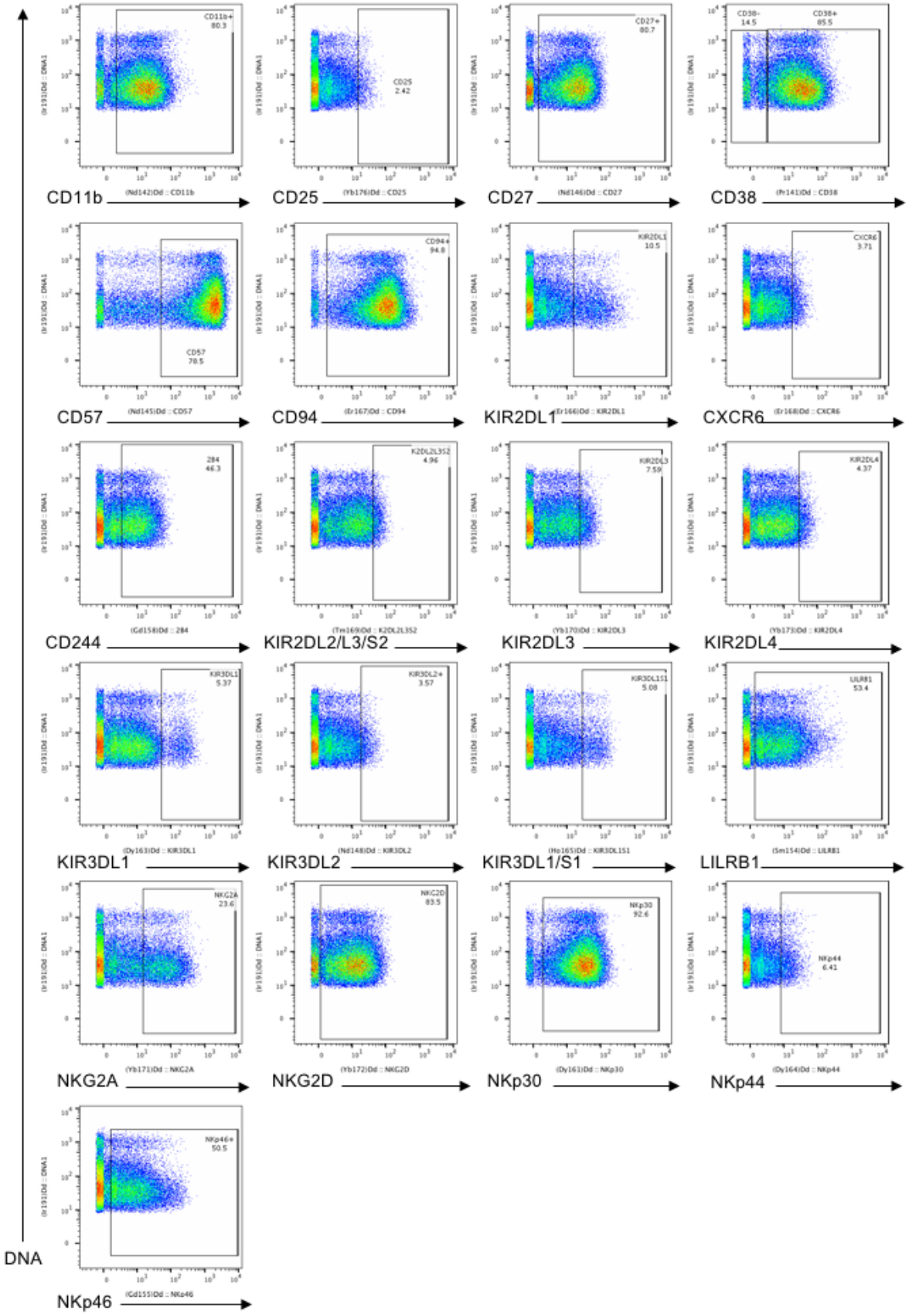


787

788 Representative plots for each marker are shown.

789

790 **Figure S4. Gating strategy for each marker on NK cells from cohort 2.**

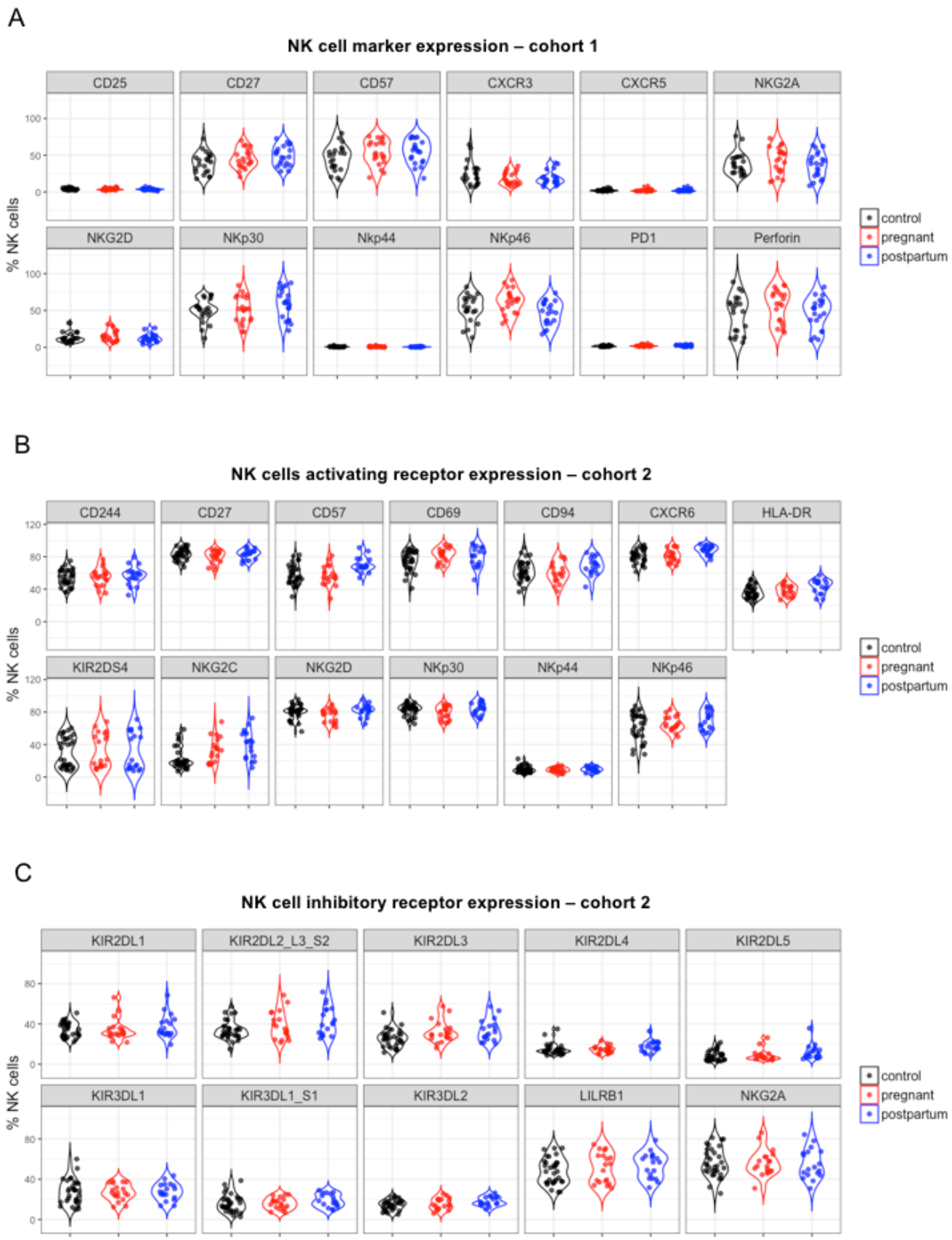


791

792 Representative plots for each marker are shown.

793 **Figure S5. Percentage of expression for each marker on NK cells from cohort 1 and 2 using**

794 **conventional gating.**

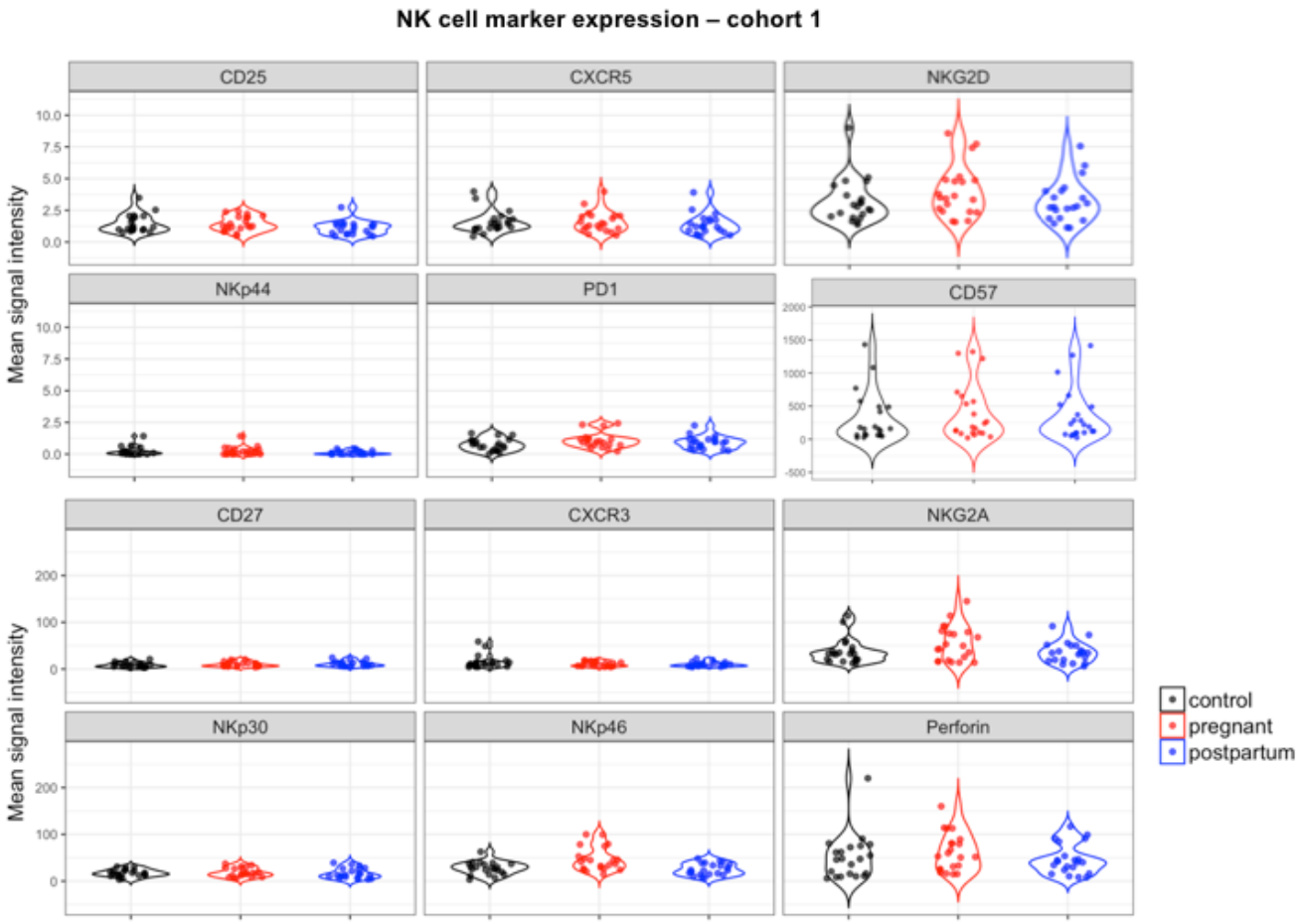


795

796 **(A to C)** NK cells from controls and pregnant women, as well as postpartum on cohort 1 and 2 were isolated
797 and stained using a 22- or 31 parameter antibody panel, respectively. Percentage of each marker on NK
798 cells was determined by conventional gating for cohort 1 **(A)** and 2 **(B and C)**.
799

800 **Figure S6. Mean Signal intensity of NK cell markers in control, pregnant and postpartum from**

801 **cohort 1 using conventional gating.**



802

803 NK cells from controls, pregnant, and postpartum women from cohort 1 were isolated and stained using a

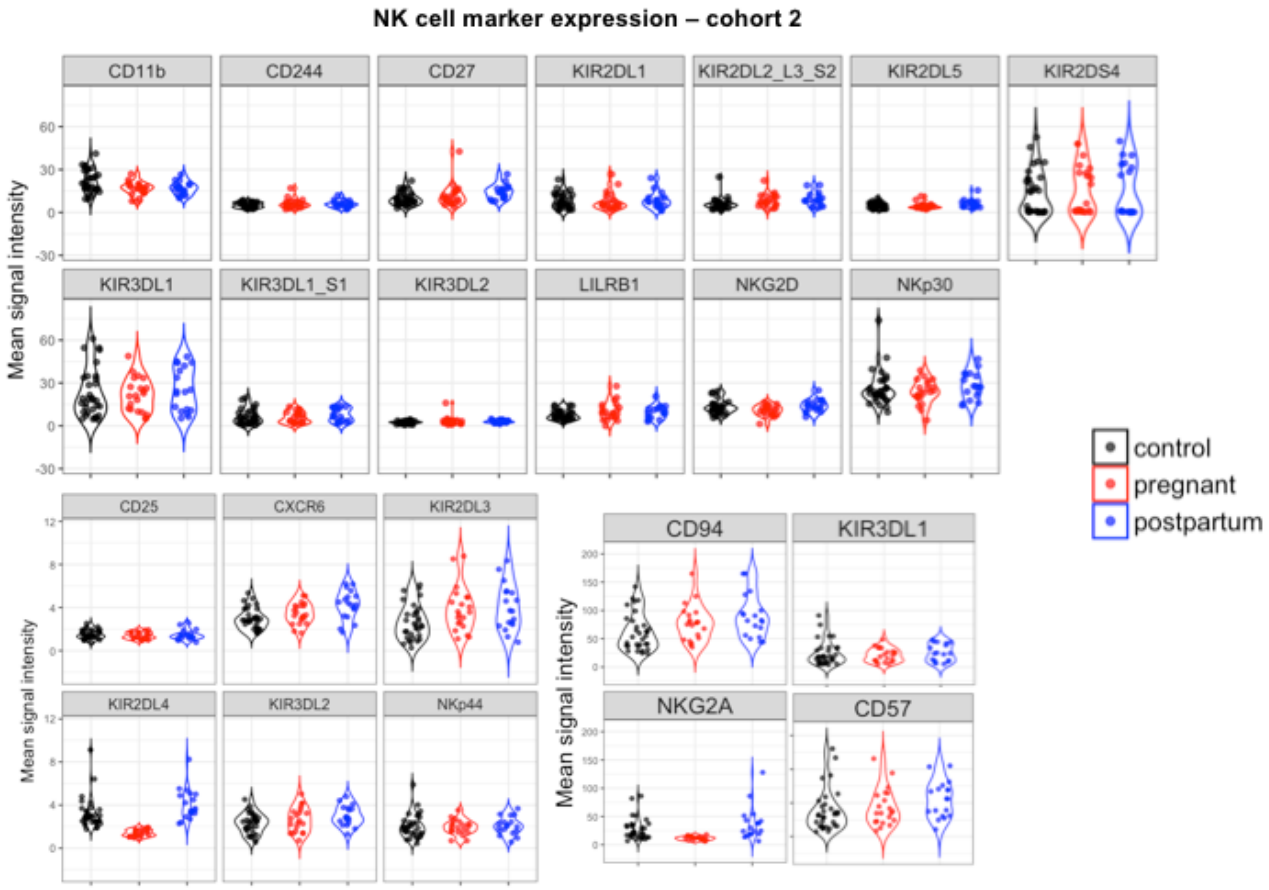
804 22-parameter antibody panel. Mean Signal Intensity for each marker was determined by conventional

805 gating.

806

807 **Figure S7. Mean Signal intensity of NK cell markers in control, pregnant and postpartum from**

808 **cohort 2 using conventional gating.**



809

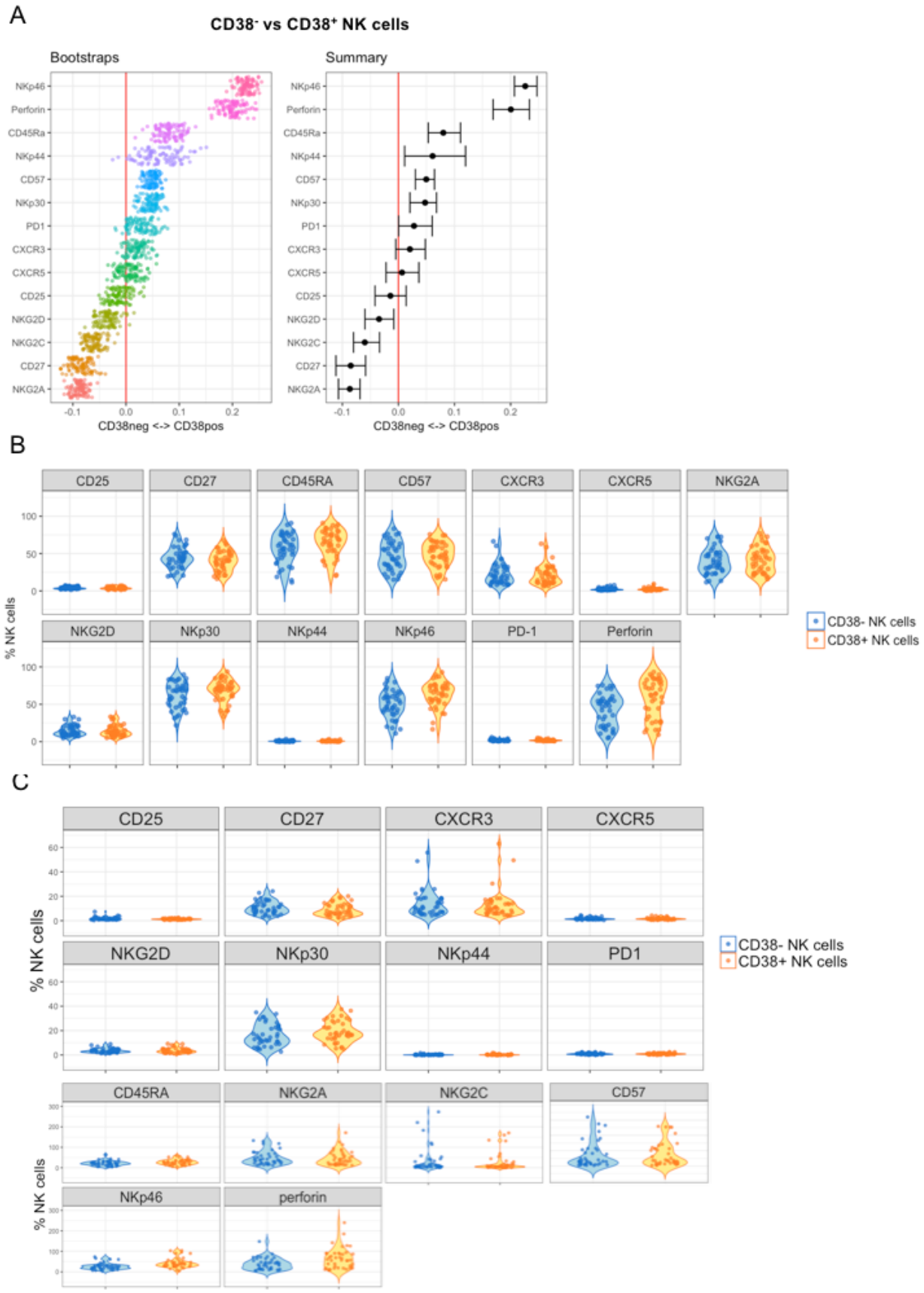
810 NK cells from controls, pregnant, and postparum women in cohort 2 were isolated and stained using a 31-

811 parameter antibody panel. Mean Signal Intensity for each marker was determined by conventional gating.

812

813 **Figure S8. Comparison of CD38⁻ and CD38⁺ NK cell expression of each marker in control and**

814 **pregnant women from cohort 1 using logistic regression model and conventional gating.**

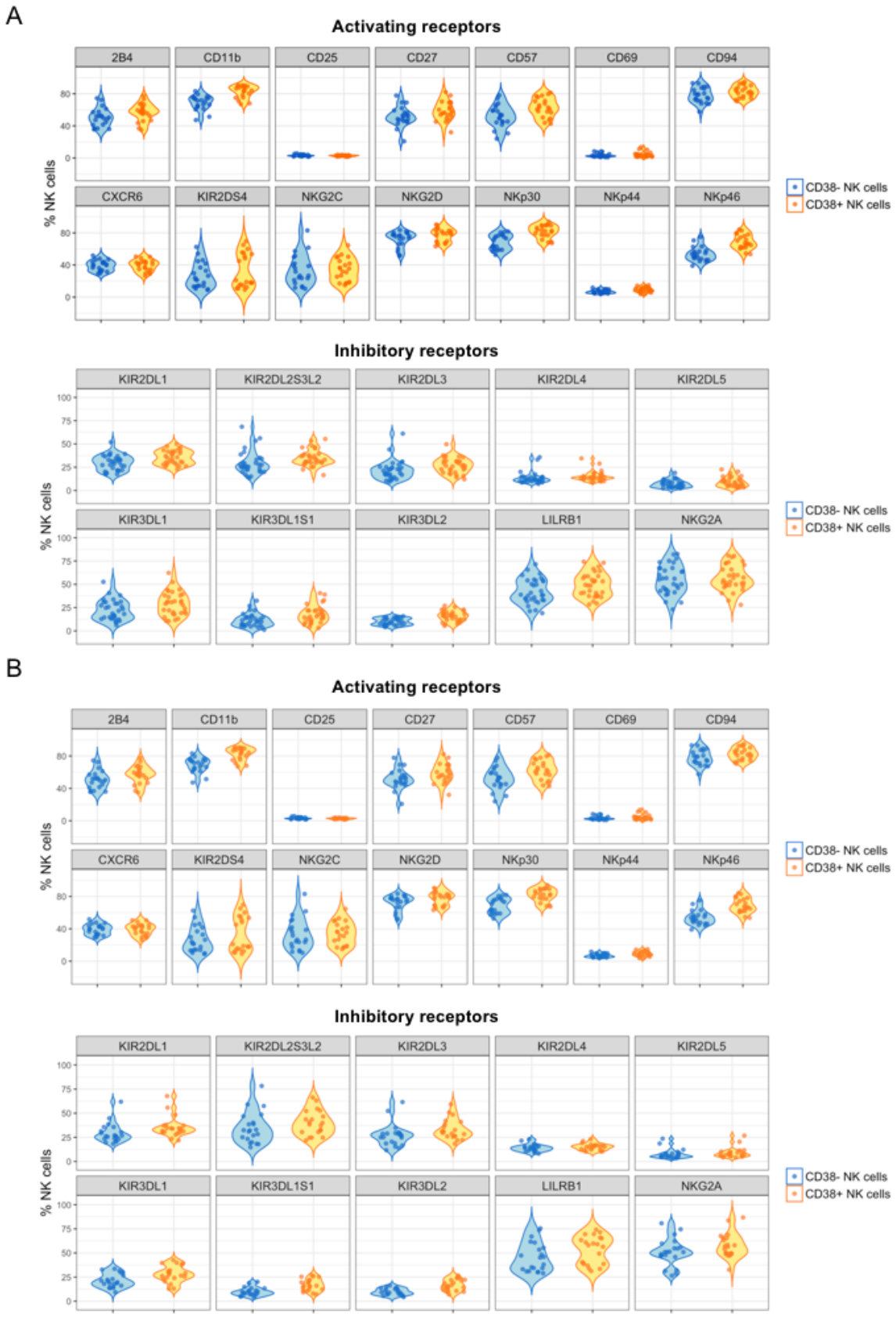


815

816 **(A to C)** PBMCs from control and pregnant women in cohort 1 were stained using specific NK cell
817 antibodies without stimulation and acquired through a mass cytometer. Data from control and pregnant
818 women were pooled and analyzed using a logistic regression model **(A)** showing whether each NK cell
819 marker is predictive of $CD38^-$ or $CD38^+$ NK cells. Pooled data were also analyzed by conventional gating
820 to determine the percentage of **(B)** and mean signal expression **(C)** for each marker on $CD38^-$ or $CD38^+$ NK
821 cells.
822

823 **Figure S9. Comparison of activating and inhibitory receptor expression on CD38⁻ and CD38⁺ NK**

824 **cells from cohort 2 in control or pregnant women by conventional gating.**

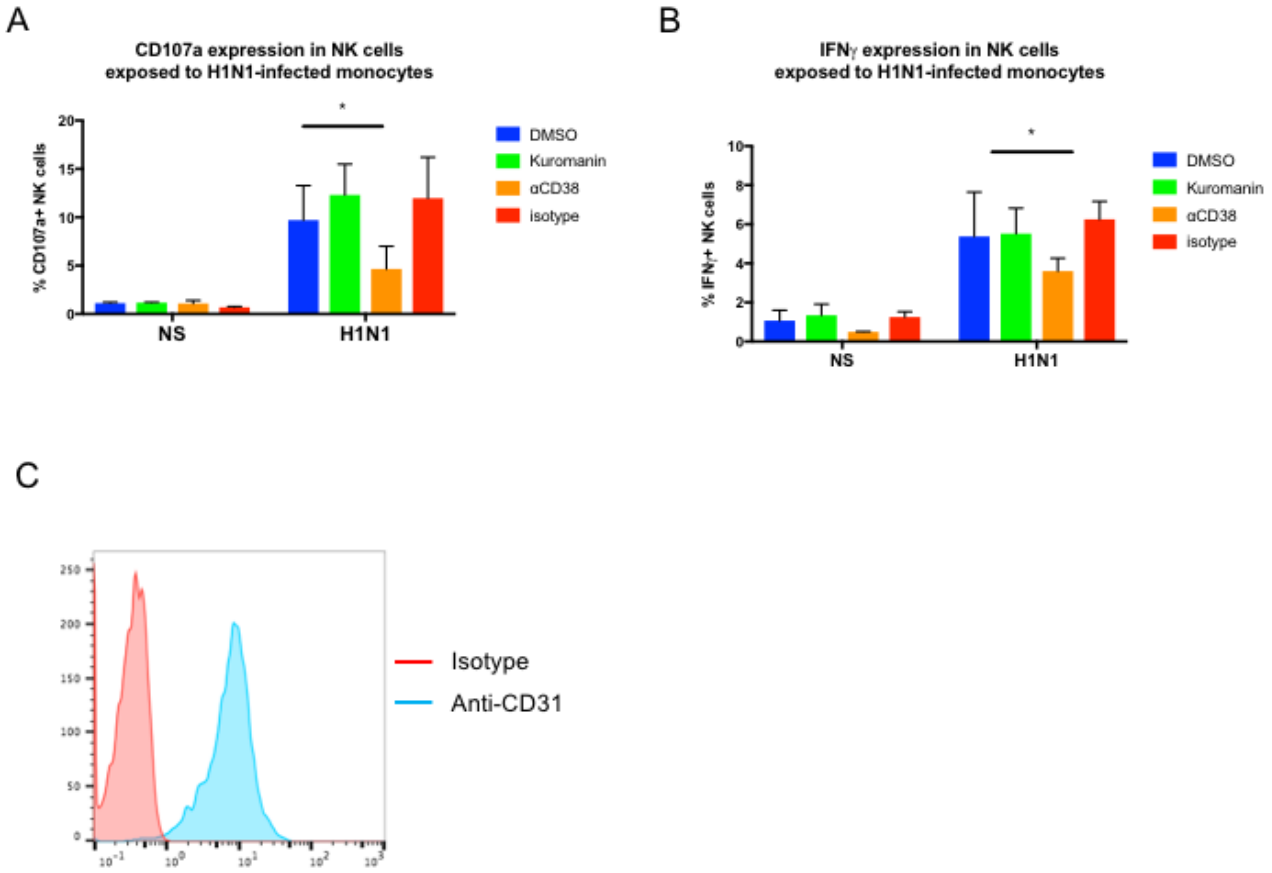


825

826 **(A and B)** PBMCs from control and pregnant women were stained using specific NK cell antibodies
827 without stimulation and acquired through a mass cytometer. **(A)** Percentage of expression of activating and
828 inhibitory receptors on $CD38^-$ and $CD38^+$ NK cells from control. **(B)** Percentage of expression of activating
829 and inhibitory receptors on $CD38^-$ and $CD38^+$ NK cells from pregnant women.
830

831 **Figure S10. Effect of the CD38 enzyme inhibitor, kuromanin, on the NK cell response to H1N1-**

832 **infected monocytes.**



833

834 NK cells and monocytes from healthy blood donors (N=6) were isolated and monocytes were infected by

835 H1N1 virus. Autologous NK cells were exposed to infected monocytes in presence of isotype or CD38

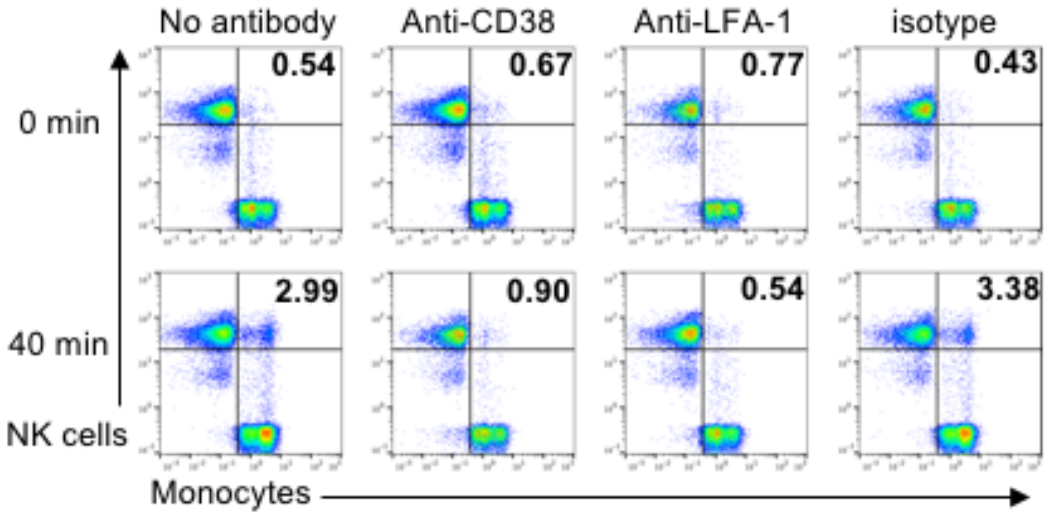
836 blocking antibody, or kuromanin. CD107a **(A)** and IFN-γ **(B)** expression was then measured by flow

837 cytometry. **(C)** CD31 measured expression by flow cytometry in non-infected monocytes. *P<0.05

838 (Wilcoxon matched-paired test to compare $CD38^-$ and $CD38^+$ NK cells).

839

840 **Figure S11. CD38 contributes to NK cell-influenza-infected monocytes conjugation.**



841

842 NK cells and monocytes were isolated from healthy donors (N=6). NK cells and monocytes were stained
843 with CFSE and Cell Trace Violet, respectively. Monocytes were infected by H1N1 virus. NK cells were
844 incubated with a CD38 or LFA-1 blocking antibody, an isotype control antibody or no antibody. NK cells
845 were then exposed to H1N1-infected monocytes for 0 or 40 min. NK cell-monocyte conjugation was
846 determined by the accumulation of the double positive $CFSE^{+}$CellTrace $Violet^{+}$ population by flow
847 cytometry. Representative plots from an individual are depicted.

848

849 **Table S1.** Demographics for cohort 1.
850

Characteristic	Pregnant, n = 21	Control, n=21
Age, years (median)	*30.0 (21 to 42)*	*27.2 (19 to 43)*
White, n (%)	*4 (19)*	11 (52)
Asian, n (%)	*4 (19)*	*5 (24)*
Hispanic, n (%)	*9 (43)*	*3 (14)*
Race, other, n (%)	*4 (19)*	*1 (5)*
Trimester 2, n (%)	*10 (48)*	
Trimester 3, n (%)	*11 (52)*	

851
852
853
854 **Table S2** Demographics for Cohort 2
855

Characteristic	Pregnant, n = 21	Control, n=32
Age, years (median)	*29.2 (19 to 40)*	*36.8 (19 to 44)*
White, n (%)	*4 (19)*	20 (62)
Asian, n (%)	*4 (19)*	*6 (18)*
Hispanic, n (%)	*9 (43)*	*5 (15)*
Race, other, n (%)	*4 (19)*	*1(3)*
Trimester 2, n (%)	*10 (48)*	
Trimester 3, n (%)	*11 (52)*	

856
857

858 **Table S3.** Antibody panel for mass cytometry in cohort 1.
859

Isotope	Antigen	Clone
115In	CD20	2H7
141Pr	CD38	HIT2
142Nd	CD3	UCHT1
145Nd	CD57	HCD57
146Nd	CD27	O323
148Nd	CD45Ra	HI100
149Sm	CD19	HIB19
150Nd	CD14	M5E2
152Sm	Perforin	B-D48
154Sm	PD1	EH12.2H7
155Gd	NKp46	9E2/NKp46
157Gd	NKG2C	134591
159Tb	CD16	3G8
160Gd	CXCR3	G025H7
161Dy	NKp30	P30-15
163Dy	NKp44	P44-8
165Ho	CD127	A019D5
171Yb	NKG2A	Z199
172Yb	NKG2D	1D11
173Yb	CXCR5	51505
174Yb	CD56	NCAM16.2
176Yb	CD25	M-A251
191lr	DNA1	
193lr	DNA2	
195Pt	Cisplatin	

860

861

862 **Table S4.** Antibody panel for mass cytometry in cohort 2.
863

Isotope	Antigen	Clone
115Ln	CD20	2H7
141Pr	CD38	HIT2
142Nd	CD11b	ICRF44
145Nd	CD57	HCD57
146Nd	CD27	O323
147Sm	CD127	A019D5
149Sm	CD16	3G8
150Nd	CD14	M5E2
151Eu	CD19	HIB19
152Sm	CD15	W6D3
153Eu	KIR2DS4	179315
154Gd	LILRB1	GHI/75
155Gd	NKp46	9E2/NKp46
156Gd	CD3	UCHT1
157Gd	NKG2C	134591
158Gd	CD244	2-69
159Tb	CD33	WM53
161Dy	NKp30	P30-15
163Dy	KIR3DL1	DX9
164Dy	NKp44	P44-8
165Ho	KIR3DL1_S1	REA168
166Er	KIR2DL1	143211
167Er	CD94	DX22
168Er	CXCR6	56811
169Tm	KIR2DL2_L3_S2	DX27
170Yb	KIR2DL3	180701
171Yb	NKG2A	Z199
172Yb	NKG2D	1D11
173Yb	KIR2DL4	mAB 33
174Yb	CD56	NCAM16.2
176Yb	CD25	M-A251
191lr	DNA1	
193lr	DNA2	
195Pt	Cisplatin	

864