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PD-1 expression on NK cells can be related to cytokine stimulation and tissue residency

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ABSTRACT (*should be max 150, now 136 words*)

Although PD-1 was shown to be a hallmark of T cells exhaustion, controversial studies have been reported on the role of PD-1 on NK cells. Here, we found by flow cytometry and single cell RNA sequencing analysis that PD-1 can be expressed on MHC class I-deficient tumor-infiltrating NK cells *in vivo*. We also demonstrate distinct alterations in the phenotype of *PD-1*-deficient NK cells which in part could be attributed to a decrease in tumor-infiltrating NK cells in *PD-1*-deficient mice. NK cells from *PD-1*-deficient mice exhibited a more mature phenotype which might reduce their capacity to migrate and kill *in vivo*. Finally, our results demonstrate that PD-L1 molecules in membranes of *PD-1*-deficient NK cells migrate faster than in NK cells from wildtype mice, suggesting that PD-1 and PD-L1 form *cis* interactions with each other on NK cells.

50

51 INTRODUCTION

52 Natural killer (NK) cells are innate lymphoid cells (ILCs) that can kill tumour cells,
 53 stressed or virus-infected cells ¹⁻³. NK cell activation is dependent on signals from
 54 activating and inhibitory receptors as well as pro-inflammatory cytokines ⁴. Activating
 55 NK cell receptors can recognise stress-induced molecules, which induce
 56 phosphorylation events that may culminate in the release of cytotoxic granules and
 57 cytokines ⁵. Healthy cells are protected from killing by NK cells because of the
 58 expression of self-MHC class I molecules (MHC-I) on their surface which act as ligands
 59 for dominant inhibitory receptors ⁶. These receptors include killer-cell immunoglobulin-
 60 like receptors (KIRs) in humans, Ly49 molecules in mouse and NKG2A in both species
 61 ⁷. Engagement of inhibitory receptors results in recruitment of phosphates such as SHP-
 62 1, SHP-2 and SHIP-1, and dephosphorylation of signaling molecules which prevents
 63 NK cell-mediated killing.

64

65 NK cells express also non-MHC-I recognizing inhibitory receptors such as TIGIT,
 66 LAG-3, CTLA-4 and PD-1, molecules known as checkpoint receptors. Clinically,
 67 antibodies against CTLA-4 and PD-1 (or its ligand PD-L1) have been found to be
 68 relatively successful in therapy to certain forms of solid cancer ^{8,9}. Similar to KIR and
 69 Ly49 molecules, checkpoint receptors can recruit and activate phosphatases (DOI:
 70 10.1126/sciadv.aay4458). Several studies have identified subsets of NK cells expressing
 71 CTLA-4 ¹⁰ and PD-1 ¹¹⁻¹⁴ in various disease settings but also in healthy individuals ¹⁵.
 72 Furthermore, there is accumulating evidence that NK cells participate in the therapeutic
 73 effects of antibodies against PD-1 or PD-L1, especially against tumors with low MHC-I
 74 expression ^{11,12,16-21}.

75

76 Recently PD-1 expression was detected early in the development of some ILC subsets,
77 which was thought to play a role in the development of ILC responses and ILC subsets
78 could be depleted with anti-PD-1 antibody²². These data raise the question of if and
79 how PD1 is involved in NK cell development and education, and how a chronic lack of
80 PD1 may affect NK cell functions. In the present study, we examined the role of PD-1
81 in NK cell function using NK cells from *PD-1*-deficient mice as well as the potential
82 role of PD-1/PD-L1 interactions in controlling NK cell activity.

83

84

85

86 RESULTS

87 NK cell phenotype and population sizes are affected in *PD-1*^{-/-} mice compared to 88 wild-type mice

89 Although PD-1 plays an important role in the development of ILCs²², no studies to date
90 have examined the phenotype of NK cells from PD-1-deficient mice. Furthermore, lack
91 of PD-1 has been shown to affect T and B cell development, as well as maturation²³⁻²⁶.
92 When comparing the maturation status of NK cells from spleens of wildtype (WT) and
93 *PD-1*^{-/-} mice²⁷, NK cells from *PD-1*^{-/-} mice exhibited an increase in frequency of
94 mature phenotype (CD11b⁺CD27⁻) NK cells (Figure 1a). This appears to take place at
95 the expense of CD11b⁺CD27⁺ NK cells as this subset was reduced in *PD-1*-deficient
96 mice while the size of CD11b⁻CD27⁺ NK cell populations was not affected (Figure 1a).
97 In line with the fact that NK cells derived from *PD-1*-deficient mice exhibited a more
98 mature phenotype, the frequency of KLRG1⁺ NK cell subset²⁸ was also increased in
99 *PD-1*-deficient mice compared to WT mice (Figure 1b). In addition, the frequency of
100 CD62L, which is important for NK cell migration²⁹, was reduced in NK cells derived
101 from *PD-1*-deficient mice (Figure 1c).

102

103 It has recently been shown that PD-1 affects DNAM-1 expression on CD8 T cells³⁰.
104 We observed an increased frequency of DNAM-1^{high} NK cells as well as increased
105 expression levels of DNAM-1 in *PD-1*-deficient mice compared to WT mice (Figure
106 1d). This confirms that PD-1 can modulate DNAM-1 expression not only on CD8 T
107 cells but also on NK cells.

108

109 We further analyzed expression of the inhibitory receptors on the NK cells³¹, and
110 compared the repertoire of inhibitory molecules on NK cells from WT and *PD-1*-
111 deficient mice. We did not find any major differences in Ly49 and NKG2A expression

112 between these mice, apart from an increase in the NKG2A^{single} population on NK cells
 113 from *PD-I*^{-/-} mice (Figure 1e). The frequency of the activating Ly49D and Ly49H
 114 molecules was reduced in *PD-I*^{-/-} mice, and this appeared to be due to a reduction in the
 115 frequency of the Ly49D⁺Ly49H⁺ NK cell population (Figure 1f and g). The expression
 116 levels of other activating receptors for example NKG2D and CD244 were not
 117 significantly different between WT and *PD-I*-deficient mice (Supplemental Figure 1a-
 118 b).

119

120 Lack of PD-1 has been associated with the accumulation of exhausted T cells²⁴. Lag3,
 121 CD39 and TIGIT are can be used as markers for T cell exhaustion²⁴. Comparing the
 122 NK cells from WT and *PD-I*^{-/-} mice, we observed only a small changes in the frequency
 123 of CD39⁺ NK cells in *PD-I*^{-/-} mice and the frequency of LAG3⁺ NK cells
 124 (Supplemental Figure 1c-d). In addition, the surface expression of PD-L1, the ligand for
 125 PD-1, was not significantly different between the two mouse strains (Supplemental
 126 Figure 1e). In addition, we did not observe any difference in the expression of GITR,
 127 CXCR3 or CXCR4 (Supplemental Figure 1f-h).

128

129 We were concerned that some of the phenotypic changes that we observed on *PD-I*-
 130 deficient NK cells might be due to perturbations caused by T cells lacking PD-1³².
 131 Therefore we compared NK cells from *PD-IxRAGI*^{-/-} and *RAGI*^{-/-} mice since both these
 132 mice have neither T nor B cells. Similarly to T and B cell-competent mice, NK cell
 133 maturation was still skewed in *PD-IxRAGI*^{-/-} mice with increased frequencies of
 134 CD11b⁺CD27⁻ and KLRG1⁺ NK cells compared to *RAGI*^{-/-} mice (Supplemental Figure
 135 2a-b). However, we no longer observed any significant difference in the frequency of
 136 CD62L⁺ NK cells between *RAGI*^{-/-} and *PD-IxRAGI*^{-/-} mice (Supplemental Figure 2c).

137 DNAM-1 expression levels were increased still on NK cells from *PD-1xRAGI*^{-/-} mice
 138 but unlike in T and B cell competent mice, the frequency of CD39 expressing NK cells
 139 was increased in the *PD-1xRAGI*^{-/-} mice (Supplemental Figure 2d and 2e). In contrast
 140 to the *PD-1*^{-/-} mice, analysis of the expression levels of inhibitory receptors revealed no
 141 longer any difference in frequency of the NKG2A^{single} NK cell population between
 142 *RAGI*^{-/-} and *PD-1xRAGI*^{-/-} mice (Supplemental Figure 2f).

143 While the frequency of Ly49D⁺ NK cells was reduced in *PD-1xRAGI*^{-/-} mice, there was
 144 no difference in Ly49H expression between *RAGI*^{-/-} and *PD-1xRAGI*^{-/-} mice. The
 145 reduction in the Ly49D population appeared to be occurring mostly in the
 146 Ly49D⁺Ly49H⁻ subset and not in the Ly49D⁺Ly49H⁺ population (Supplemental Figure
 147 2g and 2h).

148 In summary, we observed in mice lacking PD-1 increased NK cell maturation combined
 149 with higher DNAM-1, KLRG1 expression and reduced Ly49D expression.

150

151 Elimination of MHC-I-deficient cells is impaired in *PD-1*^{-/-} mice

152 Chronic loss of PD-1 could potentially affect not only the phenotype of NK cells as
 153 outlined above, but also their function. The recognition and elimination of cells
 154 expressing reduced MHC-I levels is a hallmark of NK cell function and education³³⁻³⁵.

155 We therefore examined the ability of *PD-1*-deficient and WT mice to eradicate MHC-
 156 I^{neg} spleen cells. We observed a significant reduction in the ability of *PD-1*^{-/-} mice to
 157 eliminate MHC-I^{neg} splenocytes compared to WT mice (Figure 2a). However this
 158 impairment was not at the level seen in *MHC-I*^{-/-} mice, suggesting that NK cells might
 159 be affected by non-MHC-I factors such as increased maturity of NK cell populations as
 160 described above.

161

162 It has been previously demonstrated that anti-PD-1 treatment increases NK cell
 163 elimination of MHC-I^{neg} PD-L1⁺ tumors¹². To determine if *PD-I*^{-/-} mice also had
 164 reduced eradication of a MHC-I^{neg} tumor with low expression of PD-L1¹², mice were
 165 injected with an LD₅₀ dose of TAP-deficient RMA-S lymphoma cells. While the
 166 survival rate of WT mice was 45%, only 30% of *PD-I*-deficient mice survived (Figure
 167 2b). Comparison of tumor infiltrating NK cells from WT and *PD-I*^{-/-} mice revealed a
 168 reduced frequency of tumor infiltration in *PD-I*-deficient mice (Figure 2c). PD-1 was
 169 heterogeneously expressed on NK cells infiltrating RMA-S in WT mice (Figure 2d),
 170 while splenic NK cells from the same mice exhibited little or no PD-1 expression
 171 (Figure 2e). While these findings are similar to previous studies¹², the frequency of PD-
 172 1 expression on the NK cells from our study were significantly lower¹².

173
 174 Next, we examined tumor infiltrating NK cells from a second MHC-I^{low} tumor cell line
 175 MTAP1A, which is a fibrosarcoma generated from the skin of a *Tap1*-deficient
 176 mouse³⁶. MTAP1A has low expression of PD-L1, and does not express PD-L2
 177 (Supplemental Figure 3). Here again, we found reduced infiltration of NK cells in *PD-I*^{-/-}
 178 mice but increased expression of PD-1 on tumor-infiltrating NK cells in WT mice
 179 compared to splenic NK cells (Fig. 2g-i and Supplemental Figure 4).

180
 181 In addition, PD-1⁺ tumor-infiltrating NK cells also displayed increased expression of
 182 KLRG1 compared to PD-1^{neg} NK cells (Figure 2f and j). This was observed for tumor-
 183 infiltrating NK cells in both RMA-S and MTAP1A, and suggested that PD-1-expressing
 184 NK cells might have a more mature phenotype.

185

186 **Single cell RNA-seq reveals tissue-specific transcriptional imprinting of tumor**
 187 **infiltrating NK cells**

188

189 Since it has been suggested that NK cells may express PD-1 through trogocytosis³⁷ and
190 since we observed differences in the phenotype of NK cells from WT and *PD-1*^{-/-} mice,
191 we performed single cell RNA-sequencing (scRNA-SEQ) using the SMART-SEQ2
192 platform³⁸ on tumor-infiltrating NK cells from mice inoculated with the MTAP1A
193 tumor. We chose MTAP1A over RMA-S since this tumor model gave consistently
194 higher frequency of PD-1-expressing NK cells. SMART-SEQ2 libraries of sorted NK
195 cells were generated from pooled tumors from either WT or PD-1-deficient mice
196 (Supplemental Figure 5a-b). These libraries were filtered and a combined analysis was
197 performed using Seurat v3^{39,40} for a total of 371 WT and 375 *PD-1*^{-/-} NK cells after
198 quality control (Supplemental Figure 5c). Outliers expressing very few or very many
199 genes were omitted, as were cells with a high frequency of apoptotic genes. Cells were
200 clustered and projected using UMAP, which delineated five clusters with both WT and
201 *PD-1*^{-/-} NK cells found in all clusters although *PD-1*-deficient cells were over-
202 represented in Clusters 3 and 4. (Figure 3a-c). Differentially expressed (DE) genes were
203 deciphered between all clusters and the top 10 genes per cluster shown by heatmap
204 (Supplemental Figure 6a). Selected genes were plotted using the Violin plot function
205 revealing significantly over-expressed genes in each cluster. Within clusters 3 and 4, we
206 could detect *Pdcd1* (PD-1) transcripts in both WT and *PD-1*^{-/-} NK cell populations,
207 suggesting an active upregulation of *Pdcd1* at the transcriptional level. (Figure 3d).
208 Detection of *Pdcd1* transcript in *PD-1*^{-/-} mice reflects that these mice do not have a
209 complete gene defect but rather a deletion spanning exon 3 and exon 4 of the *Pdcd1*
210 gene that prevents protein expression²⁶. Our analysis highlighted the heterogeneity of *in*
211 *vivo* NK responses with distinct patterns of *Prf1* and *Gzma*, *Gzmb* and *Gzmc* expression
212 (Figure 3e). Interestingly, expression of the tissue residency marker *Cd69* was closely
213 aligned with *Gzmc* detection (Figure 3e and g).

215 Clusters 3 and 4 were defined by a paucity of *Eomes* and *Irf8* whilst being enriched for
 216 expression of *Tnfrsf10* (TRAIL), *Cxcr6* and *Itgal* (CD49a) (Figure 3e-g). These clusters
 217 also had greater expression of *Amical* (JAML), *Ly6a*, *Il7r* and *Il21r* and lower levels of
 218 *Sell* (CD62L) (Figure 3g and Supplemental Figure 6b). Cells found in cluster 3 also had
 219 significantly enhanced *Lag3* levels suggesting this population may potentially harbor
 220 exhausted NK cells (Supplemental Figure 6b). Taken together these findings indicate
 221 that clusters 3 and 4 might represent a more mature/exhausted population and/or a
 222 tissue-resident-like subset of NK cells. Finally, we observed differential expression of
 223 transcripts for the inhibitory Ly49 genes *Klra1*, *Klra3*, *Klra7* and *Klra9* amongst the
 224 different clusters. In particular, *Klra3* (Ly49C) seemed to be present in cluster 3 but
 225 *Klra1* (Ly49A), *Klra7* (Ly49G2) and *Klra9* (*Ly49I*) seemed to be under-represented in
 226 the same cluster (Figure 3i). This indicated that different Ly49 subsets of NK cells in
 227 conjunction with PD-1 might play a role in the tumor environment as previously seen in
 228 other studies¹² (Figure 3i).

229

230 Comparison of all WT with all *PD-1*^{-/-} NK cells independent of cluster identity
 231 determined a total of 54 genes that were significantly altered between these two NK cell
 232 populations (Figure 3j and supplemental table I). Amongst those over represented in
 233 *PD-1*^{-/-} NK cells were transcripts for *Cd226* (encoding for DNAM-1), *Klrc1* (NKG2A)
 234 and *Klrg1*, which is very well in line with our flow cytometry data on spleen NK cells.
 235 Furthermore, we found that *PD-1*-deficient NK cells had altered levels of expression for
 236 *Cxcr6* and select members of both *Ly6* families, suggesting that NK cells from the *PD*-
 237 *I*^{-/-} mice had a more tissue resident phenotype. *Ifng* and *Ccl4* transcripts were also more
 238 abundant in NK cells from *PD-1*^{-/-} mice indicating an influence of PD-1 on *in vivo* NK
 239 cell responses (Figure 3j).

240

241 **PD-1 is induced on the surface of NK cells after stimulation with cytokines**

242 Since the expression profile of NK cells expressing PD-1 and CXCR6 suggested that
243 they may be connected, we stimulated DX5⁺-enriched NK cells from WT mice with a
244 combinatin of IL-12/15/18 cytokines for 96 hours, which has previously been show to
245 induce memory NK cells ⁴¹ as well as CXCR6 on the surface of NK cells ⁴². This
246 cytokine stimulation resulted in approximately 10% of WT NK cells expressing PD-1
247 (Figure 4a). Similar patterns of staining were seen in cytokine-stimulated NK cells from
248 *RAG1*^{-/-} mice (Figure 4b), which ruled out that expression of PD-1 might be on a T cell
249 subset with low CD3 expression, that T cells could induce PD-1 on NK cells or that PD-
250 1 expression was through trogocytosis from T cells.

251
252 We have previously demonstrated that we could define functional NK cell subsets based
253 on DNAM-1 and NKG2A expression ⁴³. Therefore, we here investigated whether PD-1
254 expression was confined to a specific NK cell subtype following cytokine stimulation.
255 We assessed the expression of PD-1 on DNAM-1⁺NKG2A⁺, DNAM-1⁺NKG2A⁻,
256 DNAM-1⁻NKG2A⁺ and DNAM-1⁻NKG2A⁻ NK cell subsets. Although we did not
257 observe any significant difference in the percentage of PD-1⁺ NK cells between these
258 subsets, DNAM-1⁺ NK cells had on average an increased percentage of PD-1
259 expressing NK cells following cytokine stimulation (Figure 4c).

260
261 Since the intratumoral NK cells from *PD-1*^{-/-} mice exhibited a trend towards expressing
262 more IFN γ , we examined the intracellular levels of IFN γ in the IL-12/15/18 cytokine
263 stimulated NK cells. We found increased levels of intracellular IFN γ in the *PD-1*^{-/-} NK
264 cells compared to NK cells from WT mice (Figure 4d), confirming that lack of PD-1
265 might predispose NK cells to increased IFN γ expression. Furthermore, we also found
266 that CXCR6 levels were increased on the surface of NK cells from *PD-1*^{-/-} mice

267 following cytokine stimulation, suggesting a role for PD-1 in controlling the expression
268 CXCR6 (Fig 4e).

269 **PD-1 can form *cis* interactions with PD-L1 on NK cells**

270 The tumor cells used in our experiments had little or no PD-L1 expression. Therefore,
271 our observations that the PD-1 expressing cells is increased on intratumoral NK cells
272 and IL-12/15/18 stimulated NK cells might mean that PD-L1 could interact with PD-1
273 on NK cells both in *cis* or *trans*. It has been shown previously that inhibitory MHC-
274 class I binding molecules on NK cells could form *cis*-interactions with their ligands
275 ^{44,45}. Furthermore, PD-1 and PD-L1 have recently been shown to form *cis*-interactions
276 in artificial lipid structures and in antigen-presenting cells (APCs) ⁴⁶. We therefore
277 assessed whether the movement of PD-L1 was restricted in the presence of PD-1 and
278 determined PD-L1 diffusion on the membranes of NK cells lacking PD-1 compared to
279 WT NK cells using fluorescence correlation spectroscopy (FCS), a method that detects
280 diffusion of molecules and has previously been used to measure the diffusion of
281 receptors in the membrane of NK cells ^{45,47}. FCS measurements were performed on the
282 cell membrane, and a series of autocorrelation curves were generated and fitted to the
283 2D diffusion FCS curve fitting equation. Representative autocorrelation curves with 2D
284 curve fit are shown in Figure 5a. Interestingly, PD-L1 diffused significantly faster on
285 the membrane of NK cells lacking PD-1, compared to PD-1⁺ NK cells from WT mice
286 (Figure 5a and b). Furthermore, we observed a trend for high levels of PD-L1
287 molecules per μm^2 on the surface of NK cells lacking PD-1 (Figure 5c). Since molecule
288 crowding factor is ruled out on PD-1⁺ NK cells, the slow diffusion of PD-L1 molecules
289 on cell membrane can be due to specific interactions or clustering. To investigate
290 whether PD-1 and PD-L1 form clusters on the surface of NK cells, the brightness of
291 PD-L1 was quantified that is measured in terms of counts per molecule, diffusing within
292 the observation volume. We observed a tendency towards larger clusters, as the

293 brightness of PD-L1 on PD-1 positive NK cells was higher compared to *PD-1*^{-/-} NK
 294 cells (Figure 5d). These data suggest that the PD-L1 on PD-1⁺ NK cells clusters with
 295 PD-1, indicating *cis* interactions on the membrane of NK cells. In conclusion, the PD-
 296 L1 diffusion faster without any hinders on *PD-1*^{-/-} NK cells whereas in presence of PD-
 297 1 on cell membrane PD-L1 diffuse slower, this suggest that PD-L1 might be clustering
 298 in *cis* with PD-1 on the cell membrane (Figure 5e).

299

300 Three-dimensional molecular models of the full-length extracellular domains of PD-1
 301 and PD-L1 reveal that their structural features easily allow for the formation of *cis*-
 302 interactions. Indeed, a model of the stalk region of PD-1 (comprising the stretch of
 303 residues R147-V170) in extended conformation demonstrates that its length is sufficient
 304 to allow both *cis*- and *trans*-interactions with the N-terminal domain of PD-L1 (Figure
 305 6). Our molecular models thus suggest a binding in which PD-1 “tip-toes” to reach PD-
 306 L1 with an extended stalk, while keeping the same PD-1/PD-L1 “cheek-to-cheek”
 307 interface found in previous crystal structures (Figure 6).

308

309

310 **DISCUSSION**

311 Expression of PD-1 on NK cells has been observed in many human and mouse
 312 studies^{11,12,14,16,32,48-50}. However, some recent studies suggested that NK cells do not
 313 express PD-1 and expression may due to artifact of flow cytometry staining or through
 314 interactions with PD-L1 and the NK cells acquiring PD-1 via trogocytosis³⁷. However
 315 in the present study we could find transcript and surface expression of PD-1 in tumor
 316 infiltrating NK cells, as well as upon IL-12/15/18 stimulation of NK cells in culture.
 317 Furthermore, we also found that PD-1 was induced on tumor-infiltrating NK cells even
 318 though the tumors used in our study expressed little or no PD-L1. NK cells from mice
 319 lacking PD-1 displayed phenotypic differences compared to NK cells from WT mice,
 320 suggesting that background low levels of PD-1 might still play a role in NK cell
 321 homeostasis or in NK cell development. In particular, NK cells from *PD-1*-deficient
 322 mice exhibited increased maturation as well as increase in expression of CD226.

323
 324 We found that *PD-1*-deficient mice were poor at rejecting MHC-I^{-/-} cells and had low
 325 NK cell infiltration into tumors expressing low levels of MHC-I *in vivo*. In part, this
 326 might be due to the increased maturity of NK cells in *PD-1*-deficient mice, but the
 327 reduced frequency of tumor infiltrating NK cells could also be due to (i) reduced
 328 CD62L found on the PD-1^{-/-} NK cells or (ii) reduced survival once these NK cells
 329 encounter tumor cells. It is unclear if the increased NK cell maturation is a direct effect
 330 on NK cells since we observe very little or no PD-1 on NK cells in circulation.
 331 However, others have shown that lack of PD-1 on dendritic cells leads to increased IL-
 332 12 and TNF production by dendritic cells⁵¹. Thus lack of PD-1 on DCs could indirectly
 333 affect NK cell maturation. Furthermore, absence of PD-1 on T and B cells could affect
 334 NK cells indirectly as well^{52,53}. For this reason, we crossed *PD-1*-deficient mice to
 335 mice deficient in *RAG1*. In the *PD-1xRAG1*^{-/-} mice, we still had more mature NK cells

336 and increased expression of DNAM-1 suggesting that the *PD-1*^{-/-} T and B cells had little
 337 effect on the NK cell phenotype. *PD-1*^{-/-} NK cells stimulated with IL-12/15/18 had
 338 increased numbers of IFN γ -producing cells suggesting that increased IL-12 from
 339 accessory cells in *PD-1*^{-/-} mice⁵¹ might already prime NK cells to make more IFN γ .
 340 Chronic infection and IL-18 expression have previously been associated with higher
 341 expression of PD-1 on NK cells^{32,50,54,55}. Even though PD-1 expression on T cells has
 342 been associated with exhaustion, it is more likely that it is also a marker for activation
 343 and that its expression controls T cells from being overly activated^{24,56}. Thus, PD-1
 344 expression on NK cells might play a similar role within the frame of NK cell activation.

345

346 A recent study has also called into question whether PD-1 is actually expressed at all on
 347 NK cells³⁷. Our results are in agreement with some of these findings, including the low
 348 expression of PD-1 on NK cells under normal conditions. However, in contrast to the
 349 study of Judge *et al* which used IL-2-stimulated NK cells to investigate PD-1
 350 expression on NK cells³⁷, we established here that the combination of IL-12, IL-15 and
 351 IL-18 leads to PD-1 expression, which is in line with previous publications^{13,57,58}.
 352 Furthermore, we did not see much expression of PD-1 on splenocytes but we could see
 353 that there was clear expression of PD-1 on tumor infiltrating particularly since we used
 354 *PD-1*-deficient mice as controls. When comparing our two tumor models, PD-1
 355 expression on NK cells seemed to be highest when infiltrating the MTAP1A tumors
 356 rather than RMA-S. The MTAP1A tumor was generated by painting the skin with
 357 methylcholanthrene whereas RMA-S is lymphoma. Therefore, the tumor
 358 microenvironment (TME) within these tumors might determine the level of expression
 359 of PD-1. For example, fibrotic tumors have been associated with TGF- β ⁵⁹ and TGF- β
 360 itself can induce PD-1 expression on T cells⁶⁰. This suggests that the microenvironment
 361 surrounding NK cells could lead to PD-1 expression. Metzger *et al.* have also suggested

362 that false positives can be obtained by anti-PD-1 antibodies binding to nuclear antigen
 363 in dying cells⁶¹. In our studies, we have compared our staining of WT NK cells with
 364 NK cells from *PD-1^{-/-}* mice and did not see non-specific binding using the anti-PD-1
 365 antibody clone RMP1-14 nor clone 29F.1A12. We did not use the J43 clone in our
 366 studies as we had previously found some non-specific binding with this clone. This
 367 suggested that at least in our hands, our observed PD-1 expression was not due to cross-
 368 reactivity with another antigen. Finally, since PD-1 is expressed on other tumor-
 369 infiltrating cells, there is still the possibility that PD-1 may be transferred by
 370 trogocytosis from surrounding cells to NK cells via PD-L1. We cannot rule this out but
 371 since we see PD-1 expression by mRNA from NK cells, this probably suggests that the
 372 observed increase in PD-1 expressing NK cells is at least in part through induction of
 373 PD-1.

374
 375 NK cells play an important role in clearance of tumor cells, and impairment of NK cell
 376 functions results in an increased risk for the development of cancer. Both tumor-
 377 infiltrating NK cells (TINKs) and tumor-associated NK cells (TANKs) have been
 378 described⁶², but their function and expression profiles have yet to be defined. Our single
 379 cell gene expression data reveal that NK cells within the tumor microenvironment
 380 (TME) separate into five distinct clusters. Many DE genes of our intra-tumoral NK cells
 381 have been previously described in tissue resident NK cells in different organs including
 382 liver, lung, lymph node and placenta. The high expression of tissue residency markers
 383 in NK cells within the TME could indicate that these NK cells are tumor tissue resident.
 384 Whether these NK cells infiltrate tumors (TINKs) to eliminate them, or whether they
 385 associate with tumor cells (TANKs) and facilitate pro-angiogenic properties, remains
 386 difficult to assess. In many tumors, TINKs exhibit a profoundly altered phenotype with
 387 defects in degranulation and IFN γ expression⁶³. It is still unclear whether PD-1 on NK

cells leads to exhaustion and functional impairment, or if expression of PD-1 restricts NK cell activation to prevent the exhausted phenotype, as has been suggested for T cells^{24,48}.

Previous work has established the bidirectional signaling of PD-1 and PD-L1. *Cis* interactions between PD-1 and PD-L1 on antigen presenting cells have been shown to decrease availability of PD-L1 for *trans* binding to PD-1 on T cells, and both *cis* and *trans* interactions are susceptible to antibody blockade⁴⁶. In the current study, we have shown that *cis* interaction between PD-1 and PD-L1 and a potential sequestration of available PD-1 for *trans* signaling also occurs on NK cells. We show that in the absence of PD-1, the diffusion rate of PD-L1 is significantly increased, while the size of PD-L1 clusters is decreased, indicating that PD-L1 forms clusters with PD-1 on the same membrane, thus limiting the movement of PD-L1 and potentially also that of PD-1. This suggests that the levels of PD-L1 on NK cells can determine their response to PD-1 signaling imposed by PD-L1⁺ cells inside the tumor microenvironment. We further provide a model for how PD-1 and PD-L1 interact in *trans* and in *cis*, where the same amino acid residues are involved in these interactions.

The binary PD-1/PD-L1 complex was crystallized both for human PD-L1 and murine PD-1⁶⁴ and for human PD-L1 and human PD-1⁶⁵. In both cases, protein-protein binding occurs via “cheek to cheek interaction” of Ig domains of PD-1 and PD-L1, and this was almost identical in the two structures. We hypothesize that the long flexible stalk of PD-1 allows both *cis* and *trans* interaction, where PD-1 “tip-toes” to reach PD-L1 with an extended stalk, while keeping the same PD-1/PD-L1 “cheek-to-cheek” interface found in the crystal structures. The stalk region of PD-1 (residues R147-V170) was modelled in extended conformation to demonstrate that its length is sufficient to

allow both *cis*- and *trans*-interaction with the N-terminal domain of PD-L1. High sequence homology between murine and human proteins (77% for the PD-L1 and 64% for PD-1) and conservation of the residues forming intermolecular hydrogen bonds suggest that the *cis* and *trans*-interaction for the PD-1 and PD-L1 could be possible for the human cells as well. Indeed, *cis* binding of human PD-1 and human PD-L1 has recently been demonstrated ⁴⁶.

420

A recent study has shown that NK cells up-regulate PD-L1 in response to IFN- γ and that NK cells from AML patients show increased expression of PD-L1 ⁶⁶. PD-L1⁺ cells in the TME negatively regulate PD-1⁺ effector cells, but at the same time, PD-L1 on T and NK cells might inhibit survival of PD-1⁺ APCs ⁶⁷. In addition to binding PD-1 in *cis*, PD-L1 can also bind to CD80 on the same membrane, which may repress both PD-1 and CTLA-4 signaling while favouring the CD28 axis ⁶⁸. These multi-faceted binding patterns in *trans* and *cis* may contribute to the fine tuning of the immune response within the TME, and may be the cause for the differences observed when treating cancer patients with anti-PD-1 vs. anti-PD-L1 blocking antibodies ⁶⁹.

430

Antibody immunotherapy against PD-1 or its ligands has emerged as one of the breakthrough immunotherapies in the clinics ⁷⁰. PD-1 was expressed on NK cells in a number of clinical studies examining patients with different cancers ^{11,14,15,49}. NK cells expressing PD-1 in human cancers appear to be confined to more mature NK cells ¹⁵. However no data exists on the effects of anti-PD-1/PD-L1 therapy has on NK cell function and development. Within the mouse NK cells, we did not find to date a specific NK cell population/subset that expressed PD-1. However it may well be that anti-PD-1 therapy does not only affect adaptive cells and also innate cells that would have potential knock-on effects on adaptive immune-responses ⁷¹.

440

441

442 **MATERIAL AND METHODS (977 words)**

443 **Mice**

444 C57BL/6, *PDCD-1*(PD-1)^{-/-} (generously provided Dr. Tasuku Honjo, Kyoto University,
445 Kyoto, Japan)²⁶, *RAG1*^{-/-72} and *PD-1*^{-/-}x*RAG1*^{-/-} (PD-1xRAG1^{-/-})⁷³, *H-2K^bxH-2D^b*^{-/-}
446 (MHC-I^{-/-})⁷⁴ mice on the C57BL/6 background were housed under specific pathogen
447 free conditions at the Department of Microbiology, Tumor and Cell Biology and Astrid
448 Fagraeus Laboratories, Karolinska Institutet, Stockholm. All procedures were
449 performed under both institutional and national guidelines (Ethical numbers from
450 Stockholm County Council N147/15). Sex and aged match mice were used for all
451 experiments. Mice were chosen randomly for control or treated groups.

452

453 **Tumors**

454 MHC-I-deficient lymphomas RMA-S (*TAP2*-deficient), and *TAP1*-deficient MCA
455 fibrosarcoma (clone MTAP1A) have been previously described^{6,36}. RMA-S cells were
456 inoculated at the LD₅₀ dose of 10⁵ s.c. in the flank of mice. MTAP1A was inoculated at
457 a dose of 10⁵ cells/mouse. Tumor growth was measured every two days and mice were
458 sacrificed when the tumor reached 10³ mm.

459

460 **NK cell purification and culture**

461 Single-cell suspension from spleens was depleted of erythrocytes, and NK cells were
462 positively sorted using anti-DX5⁺ magnetic beads or by negative sorting using MACS
463 separation, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch
464 Gladbach, Germany). Cells were resuspended in complete medium (αMEM; 10 mM
465 HEPES, 2 × 10⁻⁵ M 2-ME, 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin) with
466 with 100 ng/ml mouse IL-12 (PeproTech), IL-15 (Immunotools) and 100 ng mouse IL-
467 18 (MBL International, Woburn, MA, USA) for 4 days. For isolation of NK cell

subsets, NK cells were isolated as above and then sorted on MoFlo XDP cell sorter (Beckman Coulter, Brea, CA, USA).

In vivo rejection assay

Splenocytes from B6 or MHC-I^{-/-} mice were labeled with 0.5 μM CFSE (target cells) or 0.5 μM CellTrace Violet (control cells; Thermo Fisher Scientific Life Sciences) for 10 min. Target and control cells were washed, then mixed and $1-3 \times 10^6$ cells coinjected intravenously via the tail vein into B6, PD-1^{-/-} mice or MHC-I^{-/-} mice as controls for NK cell-mediated killing. The injection mix was analyzed by flow cytometry for reference. Two days later, the spleens were harvested and erythrocytes depleted, and the relative percentages of target and control cells were measured by flow cytometry³¹. Rejection was estimated as the relative survival of target or cells, calculated as: % remaining target cells of labeled cells/% target cells in inoculate or % remaining control cells of labeled cells/% control cells in inoculate.

Antibodies and Flow Cytometry

Antibodies used in the study were purchased from BD, Biolegend or eBioscience. Clones for the different antibodies were: anti-NK1.1 (clone PK136 Biolegend), -Nkp46 (29A1.4), -PD-1 (RMP1-14 and 29F.1A12), -NKG2A (20d5 Biolegend), Ly49A (YEI/48 BD) Biolegend), Ly49C (5E6), Ly49D (4E5), Ly49G2 (4D11), Ly49H (3D10 eBioscience), Ly49I (YLI-90, eBioscience) -CD11b (clone M1/70, BioLegend), -CD127 (clone A7R34, BioLegend), -GITR (DTA-1, Biolegend), -CD244 (m2B4(B6)458.1), -TIGIT (GIGD7, eBioscience), CD39 (Duha59, Biolegend), -KLRG1 (clone MAFA, Biolegend), -CD226 (10E5, Biolegend), -GR1 (GR1, Biolegend), -CXCR3 (CXCR3-173 eBioscience), -CXCR4 (clone L276F12 Biolegend)

493 -CD274 (clone MIH1, Biolegend). The 4LO3311 (Ly49C) hybridoma was a kind gift
494 from Suzanne Lemieux.

495 Flow cytometry was performed on CyAN ADP LX 9-colour flow cytometer (Beckman
496 Coulter, Pasadena, CA) or LSRII (Becton Dickinson). Data were analyzed using
497 FlowJo software (Tree Star Inc, OR).

498

499 **Molecular modelling of cis- and trans-interactions between PD-1 and PD-L1**

500 Three-dimensional molecular models of the full-length extracellular regions of murine
501 or human PD-1/PD-L1 complexes (PD-L1 residues 19-239 and PD-1 residues 21-170)
502 were created based on the crystal structure of the chimeric complex of human PD-L1
503 and murine PD-1 (pdb code3BIK)⁶⁴. To our knowledge, no crystal structure of murine
504 PD-L1 has been determined yet, although several crystal structures of human PD-L1 are
505 available^{64,75}. The crystal structure of human PD-L1 revealed that it consists of two Ig
506 domains linked by a 10 residues-long stalk region. The sequence identity between
507 murine and human PD-L1 is 77%, which means that their 3D structures may be very
508 similar. Indeed, the model of murine PD-L1 created using SwissModel⁷⁶ is very similar
509 to human PD-L1. Replacement of human PD-L1 with its murine orthologue in the 3BIK
510 structure allowed us to generate a full-length model of the murine PD-1/PD-L1
511 complex. Conversely, replacement of murine PD-1 with the human orthologue allowed
512 us to create a three-dimensional model of the full-length human PD-1/PD-L1 complex.
513 The stalk regions of PD-1 (residues 147-170) and PD-L1 (residues 229-239) were
514 modelled in an arbitrary extended conformation using the program Coot⁷⁷ followed by
515 model regularization to improve the geometry of the peptide chain and remove all
516 possible sterical clashes.

517

518 **SMART-SEQ2**

519 scRNA-Seq was performed in 384-well format. The tumors were isolated, rapidly
520 processed, stained for a panel of surface markers and single cell sorted within
521 approximately 90 minutes of organ harvest. In total 382 NK cells were sorted directly
522 into 2 µl lysis buffer using a BD Influx from pooled tumors from either 3 WT and 3 PD-
523 1^{-/-} KO mouse respectively. SMART-Seq2 libraries were prepared using the method
524 described in Picelli et al.³⁸ by the Eukaryotic Single Cell Genomics national facility at
525 SciLife Laboratory, Stockholm.

526

527 Digital gene expression matrices were preprocessed and filtered using the Seurat v3.0 R
528 package (<https://github.com/satijalab/seurat>). Outlier cells were first identified based on
529 3 metrics (library size, number of expressed genes, etc). Low abundance genes were
530 removed by removing all genes that were expressed in less than 3 cells. The raw counts
531 were normalized and transformed using the ‘LogNormalize’ function of Seurat. Highly
532 variable genes were detected using the proposed workflow of the Seurat R package.
533 Unsupervised clustering of the cells was performed and visualized in two-dimensional
534 scatterplots via Uniform Manifold Projection (UMAP) function using the Seurat R
535 package.

536

537 **Microscopy and FCS analysis**

538 *Diffusion of PD1 and PDL1 on cell surface*

539 Zeiss 510 microscope with a Confocor 3 system (Carl Zeiss Microimaging GmbH), C-
540 Apochromat 40x/1.2 NA water objective was used for Fluorescence Correlation
541 Spectroscopy (FCS) measurements⁷⁸. Diffusion of interested molecules were measured
542 using fluorescent labelled antibodies and FCS measurements were calibrated by
543 measuring Alexa-488 and Alexa-647 dyes in solution at different power scale
544 concentration whose diffusion coefficient is known. For cell preparation, spleens were

545 isolated from from *RAGI*^{-/-} and *PD-1xRAGI*^{-/-} mice. From single cell suspension of
 546 splenocytes of mice, NK cells were isolated by MACS NK cell isolation kit mouse II
 547 (Miltenyi Biotech Norden AB, Sweden). NK cells were stained for PD-1-Alexa flour
 548 488 and PD-L1-Alexa flour 647, and microscopic chambers were coated with poly-L-
 549 lysine , so the cells are made to attach to the glass surface ^{45,79}. All the FCS
 550 measurements on cells were made on the cell surface for the diffusion of PD-1 and PD-
 551 L1.

552

553 *FCS analysis*

554 FCS Data was analyzed using MATLAB based written algorithm to have graphical user
 555 interface (GUI) for fitting. GUI permits to assume the initial fit coefficient like N-
 556 number of molecules, Tau D-Diffusion time for the molecule to diffuse within the focal
 557 volume, triplet state of the molecules. Different fit models and time fit domain was
 558 considered for free dyes and cells. Where 3D diffusion model fit was chosen for free
 559 dyes with time domain fit 0.5 μsecond to 0.1 msecond and 2D diffusion model fit for
 560 cells with time fit between 1 millisecond to 5 second.

561

562 **Statistical analyses**

563 All statistical analysis was performed using GraphPad Prism software (La Jolla, CA).

564

565

566 **Data availability:** Smart-Seq2 data will be made available at upon acceptance. Other
567 data is available from the corresponding author upon reasonable request.

568

569 **Author Contributions:** Conceptualization: A.K.W., N.K., C.T., B.J.C.; methodology:
570 A.K.W., N.K., C.T., S.B.S., P.R., T.S., A.A., K.K., B.J.C; Data collection: A.K.W.,
571 N.K., C.T., K.v.d.V., S.B.S., D.O., E.L.G., N.C., S.T., T.S., B.J.C;; Analysis and
572 interpretation: A.K.W., N.K., C.T., K.v.d.V., S.B.S., D.O., E.L.G., N.C., S.T., P.R., T.S.,
573 A.A., K.K., B.J.C.; writing - original draft preparation, A.K.W., C.T. T.S., B.J.C.;
574 critical revision of the article: A.K.W., N.K., A.A., K.K., B.J.C., visualization,
575 A.K.W.,C.T., S.B.S., T.S., B.J.C; funding acquisition, K.K. and B.J.C.; All authors have
576 read and agreed to the published version of the manuscript.

577

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579

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856 **FIGURE LEGENDS**

857 **Figure 1. Phenotype of NK cells from WT and PD-1^{-/-} mice.** (a) Expression of
858 CD11b and CD27 on NK cells from WT (open boxplots) and PD-1^{-/-} (shaded boxplots)
859 mice (*p<0.01 Mann-Whitney test, n=18-20 mice) (b) Expression of KLRG1 on NK
860 cells from WT and PD-1^{-/-} mice (*p<0.01 Mann-Whitney test, n=18-20 mice). (c)
861 Expression of CD62L on NK cells from WT and PD-1^{-/-} mice. (d) Expression of
862 DNAM-1 on NK cells from WT and PD-1^{-/-} mice, bar graphs represent percent
863 expressing cells and the mean fluorescent intensity of expression (*p<0.01 Mann-
864 Whitney test, n=18-20 mice. (e) Expression of inhibitory Ly49 molecules and NKG2A
865 on NK cells from WT and PD-1^{-/-} mice (*p<0.01 Mann Whitney n=18-20 mice). (f)
866 Expression of activating Ly49 molecules on NK cells from WT and PD-1^{-/-} mice.
867 (*p<0.01 Mann-Whitney test, n=18-20 mice). (g) Expression of Ly49D and Ly49H
868 populations on NK cells from WT and PD-1^{-/-} mice (*p<0.01 Mann-Whitney test, n=18-
869 20 mice).

870

871 **Figure 2. PD-1-deficient mice exhibit poor rejection of MHC-I-deficient cells.** (a)
872 WT and MHC-I^{-/-} splenocytes labelled with CFSE were injected and the rejection ratio
873 measured in WT, PD-1^{-/-} and MHC-I^{-/-} mice ***p<0.001 (ANOVA three separate
874 experiments total of 6-8 mice). (b) Rejection of RMA-S cells injected s.c. WT and PD-
875 I^{-/-} mice were given an LD₅₀ dose of RMA-S cells (10⁵ cells) and the survival rate of
876 mice was measured (survival measured using log rank test, three separate experiments
877 with n= 15-16 mice). (c) Percent intratumoral NK cells amongst the lymphocyte
878 population in mice receiving RMA-S. (d-e) Expression of PD-1 on intratumoral NK
879 cells in RMA-S treated mice compared to expression on splenocytes (**p<0.001 Mann-
880 Whitney test). (f) Expression of PD-1 on intratumoral KLRG1⁺ and KLRG1⁻ NK cell
881 populations (p<0.05 paired t-test). (g) Frequency of intratumoral NK cells amongst

lymphocytes in mice receiving MTAP1A (**p<0.01 Mann-Whitney test). (h-i) Expression of PD-1 on intratumoral NK cells in MTAP1A treated mice compared to expression on splenocytes (**p<0.01 Mann-Whitney test, n=12-15 mice). (j) Expression of PD-1 on intratumoral KLRG1⁺ and KLRG1⁻ NK cell populations in MTAP1A tumors (**p<0.01 paired t-test n=8).

Figure 3. Single cell analysis of intratumoral NK cells. (a, b) UMAP projection of 746 tumor-infiltrating NK cells (371 WT cells, 375 PD-1^{-/-} cells). (c) Percentage of each cluster derived from either WT or PD-1^{-/-} NK cells. (d-i) Violin plots for several genes enriched across various clusters. (j) Violin plots depicting expression of several genes differentially expressed between WT and PD-1^{-/-} NK cells.

Figure 4. Induction of PD-1 on NK cells following cytokines stimulation. (a) PD-1 expression on WT NK cells (white histogram) following stimulation with IL-12/15/18 for 4 days, expression on PD-1^{-/-} NK cells is shown as comparison (grey histogram). (b) Expression of PD-1 on NK cells from RAG1^{-/-} mouse following cytokine stimulation. (c) Expression of PD-1 on DNAM-1 and NKG2A populations following cytokine stimulation (from four experiments. (d) Intracellular levels of IFN γ following cytokine stimulation of NK cells from (dashed line) WT mice and (shaded line) PD-1^{-/-} mice (representative plot from three experiments). (e) Expression of CXCR6 on the surface following cytokine stimulation of NK cells from (dashed line) WT mice and (shaded line) PD-1^{-/-} mice.

Figure 5. Movement of PD-L1 in WT and PD-1^{-/-} NK cells. (a) Representative FCS auto correlation curves of PD-L1 on PD-1 positive (*right panel*) and PD-1^{-/-} NK cells (*left panel*), decline part of the curve indicates the rate of diffusion on cell membrane.

908 FCS readouts of PD-L1 molecule on PD-1 positive and negative NK cells. (b) The
 909 diffusion rate of PD-L1, (c) the density of PD-L1 and (d) the counts per molecule
 910 (CPM) of PD-L1 were measured on individual PD-1⁺ NK cells from *RAG1*^{-/-} mice and
 911 NK cells from *PD-1xRAG1*^{-/-} mice. (b) The diffusion rate of PD-L1 is faster in the
 912 absence of PD-1. (c) The density of PD-L1 was higher on *PD-1xRAG1*^{-/-} NK cells,
 913 while (d) the CPM, indicates the size of the cluster measured based on the brightness or
 914 number of molecules per entity, PD-L1 clusters was higher when PD-1 was present. (e)
 915 Model for PD-L1 movement in the membrane in the presence and absence of PD-1.
 916 Comparison between two unpaired groups of 12 NK cells by Mann Whitney test, ***
 917 $p < 0.001$.

918

919 **Figure 6. Molecular Modelling of PD-1-PD-L1 *cis*-interaction.** The 24 amino
 920 residues long stalk region of PD-1 is long and flexible enough to allow for both *trans*-
 921 and *cis*-interaction between PD-1 and PD-L1. (a) *Trans*-interaction between PD-L1 on
 922 tumor cells and PD-1 on NK cells. (b) *Cis*-interaction between PD-1 and PD-L1 on NK
 923 cells. The interactions and mode of binding between the N-terminal part of PD-L1 and
 924 PD-1 could be highly similar, as found in the crystal structure of the human PD-1/PD-
 925 L1 complex⁶⁵. The stalk-region of PD-1 (residues R147-V170) was modelled in an
 926 arbitrary extended conformation to show that its length is sufficient to allow for *cis*-
 927 interaction with the N-terminal domain of PD-L1.

928

929

a

PD1+ NK cells

PD-1^{-/-} NK cell

b

c

d

e

a

b