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1 **Natural Killer cells in the human lung tumor microenvironment display immune inhibitory**
2 **functions**

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42

43 **Abstract**

44 **Background:** Natural Killer (NK) cells play a crucial role in tumor immunosurveillance through
45 their cytotoxic effector functions and their capacity to interact with other immune cells to
46 build a coordinated antitumor immune response. Emerging data reveal NK cell dysfunction
47 within the tumor microenvironment through checkpoint inhibitory molecules associated with
48 a regulatory phenotype.

49 **Objective:** We aimed at analyzing the gene expression profile of intratumoral NK cells
50 compared to non-tumoral NK cells, and to characterize their inhibitory function in the tumor
51 microenvironment.

52 **Methods:** NK cells were sorted from human lung tumor tissue and compared to non-tumoral
53 distant lungs.

54 **Results:** In the current study, we identify a unique gene signature of NK cell dysfunction in
55 human Non-Small-Cell Lung Carcinoma (NSCLC). First, transcriptomic analysis reveals
56 significant changes related to migratory pattern with a down-regulation of *S1PR1* and *CX3CR1*
57 and over-expression of *CXCR5* and *CXCR6*. Second, *CTLA4* and *KLRC1* inhibitory molecules were
58 increased in intratumoral NK cells, and CTLA-4 blockade could partially restore MHC class II
59 level on dendritic cell that was impaired during the dendritic cells (DC)/NK cell cross-talk.
60 Finally, NK cell density impacts the positive prognostic value of CD8⁺ T cells in Non-Small-Cell
61 Lung Carcinoma.

62 **Conclusions:** These findings demonstrate novel molecular cues associated with NK cell
63 inhibitory functions in NSCLC.

64

65

66 **Key words**

67 Natural killer cells, non small-cell lung carcinoma, antitumor immune surveillance, gene
68 expression profile, CTLA-4, regulatory functions, clinical outcome, migration.

69 **Background**

70 Natural Killer (NK) cells are innate lymphoid cells (ILCs) representing the first line of defense
71 against infected or transformed cells. They are highly cytotoxic, express many activating and
72 inhibitory receptors and secrete cytokines and chemokines including TNF- α , IFN- γ , CCL3 and
73 GM-CSF, allowing them to attract and interact with other immune cells(1).

74 In the tumor context, NK cell activation is tightly regulated by their interaction with malignant
75 cells expressing various levels of NK receptor ligands. The current dogma is that NK cells act
76 early in the anti-tumor immune response by controlling tumor burden and stimulating
77 adaptive T cell immune responses(2) thereby curbing cancer cell metastasis(3). NK cells and T
78 cells cooperate to restrain tumor growth, highlighting a role for NK cells in shaping adaptive
79 anti-cancer responses. Indeed, patients with defective NK cell functions have been shown to
80 have a higher incidence of cancers(4), while decreased peripheral blood NK cell activity is
81 linked to increased carcinoma incidence(5).

82 Intratumoral NK cells display altered phenotype and functional impairment, relative to non-
83 tumoral NK cells in patients with lung cancer(6,7), prostate cancer(8), breast cancer(9),
84 hepatocellular carcinoma(10), and gastrointestinal stromal tumors(11). NK dysfunction has
85 been attributed to direct crosstalk between tumor cells and NK cells, activated platelets and
86 several soluble factors, such as myeloid derived suppressor cells (MDSCs), macrophage- and
87 tumor cell-derived transforming growth factor β (TGF- β), prostaglandin E2, indoleamine-2,3-
88 dioxygenase, adenosine, and interleukin-10 (IL-10)(4,7). In addition, loss of anti-tumor effects

89 in NK cells closely associated with aberrant fructose-1,6-biphosphatase (FBP1)-induced
90 inhibition of glycolysis, and reduced NK cell viability(12). This can be explained by persistent
91 stimulatory signaling and/or evading strategies used by tumor cells to escape NK cells,
92 including the downregulation of important NK cell-activating ligands and/or an
93 immunosuppressive tumor microenvironment (TME).

94 In Non-Small-Cell Lung Carcinoma (NSCLC), whereas tumor infiltrating CD8⁺ T cells, CD20⁺ B
95 cells and DC-LAMP⁺ mature dendritic cells (DC) strongly associate with a good clinical
96 outcome(13–15), NK cell density is not linked to a prognostic value(7,16,17). This discrepancy
97 can be explained by the fact that the TME is able to locally edit the phenotype of intratumoral
98 NK cells, leading to reduced expression of activating receptors, increased expression of the
99 inhibitory receptor natural killer group (NKG) 2A(7,18). NK cells may also acquire other
100 immune checkpoint molecules(19,20) such as PD-1, LAG3, TIGIT, TIM3, CD73(21), and CIS(22)
101 in certain tumor contexts. Preclinical studies show that NKG2A or TIGIT blockade enhances
102 anti-tumor immunity mediated by NK cells(23–25), demonstrating the importance of targeting
103 NK cells.

104 Microarray analysis of intratumoral, as compared to distant-lung tissue NK cells, reveal a
105 specific transcriptional signature suggesting modulation of NK cell activity within the TME(26).
106 However, the significance of this tumor-induced signature has not been investigated so far.
107 Here we demonstrate, for the first time, that human NK cells express CTLA-4 in the lung, which
108 is upregulated within the TME, and have a distinct expression of migratory receptors. Our data
109 also show that CTLA-4-expressing NK cells have a negative impact on dendritic cells within the
110 TME and impact the overall survival of CD8⁺T cells in NSCLC patients.

111

112 **Material and methods**

113 **Tissue samples from NSCLC patients**

114 Human primary NSCLC samples and non-tumoral distant tissues (situated at more than 10 cm
115 from the tumor) were obtained from non-treated patients the day of surgery at Institut
116 Mutualiste Montsouris (Paris), Hotel-Dieu Hospital (Paris) or Cochin Hospital (Paris), from two
117 prospective studies (Supplementary tables 1 and 4, respectively Cohort 1 discovery cohort,
118 and Cohort 2 validation cohort). A retrospective NSCLC cohort was also used in this study
119 (Cohort 3). Cohort 3 includes 539 untreated patients seen between 2001 and 2005 at the
120 Department of Thoracic Surgery of Hotel-Dieu Hospital (Paris, France)(27). The inclusion
121 criteria are histological subtypes squamous cell carcinoma (SCC) or lung adenocarcinoma
122 (ADC), all TNM stages, and associated with clinical data. Histopathologic features such as
123 histological subtypes, ADC grade, TNM stages are available for the majority of the patients.

124 All the patients gave an informed consent prior to inclusion. The study was conducted with
125 the agreement of the French ethic committee (number 2012 06 12 IRB00001072) in
126 application with the article L. 1121-1 of French law, according to the recommendations in the
127 Helsinki declaration.

128 **Preparation of human single-cell suspension**

129 Surgical samples were mechanically dilacerated and single cell suspensions obtained after
130 non-enzymatic disruption using the Cell Recovery Solution (Corning) for 1h at 4°C under
131 agitation and filtered through a 70 µm cell strainer (BD Biosciences). Cells were washed in
132 PBS+5%FCS+EDTA 0.5mM and mononuclear cells were purified using Lymphocyte Separation
133 Medium gradient (Eurobio, Les Ulis, France). The number of cells obtained was then

134 determined by manual counting on Kova slides (Kova International, Garden Grove, CA, USA)
135 for further use.

136 **NK cell sorting**

137 Mononuclear cells were incubated with Live/Dead Fixable Yellow Dead Cell Stain Kit
138 (ThermoFischer, Waltham, MA, USA), PE-conjugated anti-CD45 (mIgG1 κ , clone J.33, Beckman
139 Coulter), FITC conjugated anti-CD3 (BioLegend San Diego, CA, USA. mIgG1 κ , clone UCHT1),
140 APC-conjugated anti-CD56 (BD Biosciences, Allschwil, Suisse, mIgG2b κ , clone N-CAM16.2) and
141 AF700-conjugated anti-CD11c (BD Biosciences, mIgG1 κ , clone B-ly6) for 30 minutes at 4°C and
142 sorted with a FACS Aria III cell sorter (BD Biosciences). Sorted NK cells were defined as
143 CD45⁺CD3⁻CD56⁺ cells, DC as CD11c⁺ cells and purity after sorting was validated by flow
144 cytometry data using Diva software (BD Biosciences). For RNA analysis, sorted NK cells were
145 immediately collected in lysis buffer supplied in the RNA extraction kit (RNeasy Microkit –
146 Qiagen, MD, USA).

147 **Flow cytometry**

148 Cells were counted and stained using Live/Dead Fixable Yellow Dead Cell Stain Kit, CD45-PE or
149 FITC (Clone HI30, BioLegend), CD3-FITC (Clone UCHT1, ThermoFisher) or PerCP-Cy5.5 (Clone
150 SK7, BioLegend), CD56-APC or BV421 (Clone HCD56, BioLegend), NKp46-BV650 (9E2, BD
151 Biosciences), CX3CR1-APC (Clone 2A9-1, ThermoFisher), CD107a-APC-H7 (H4A3, BD
152 Biosciences), KLRC1-PE (Z199, Beckman Coulter), EOMES-Pe eFluor610 (Clone WD1928,
153 ThermoFisher), CXCR6-Pe-Cy7 (Clone SA051D1, BioLegend), BV450-conjugated anti-FoxP3
154 (Clone 259D/C7, BD Biosciences, mIgG1) and BV786-conjugated anti-CTLA-4 (Clone BNI3, BD
155 Biosciences, mIgG2 α).

156 Antibodies and cells were incubated in PBS, 10% FCS, 0.5 mM EDTA Medium for 30' at 4°C and
157 washed. Staining was acquired on Fortessa X20 (BD Biosciences) and analyzed using FlowJo
158 software.

159 **Microarray experiment and analysis**

160 Microarray analysis was performed from previously described experiment(26).

161 **RNA extraction from NK cells**

162 Total RNA was extracted with the RNEasy Micro Kit (Qiagen) according to manufacturer's
163 instructions. RNA quality and quantity were analyzed on a PicoChip (Total Eukaryote RNA
164 Assay Pico II Kit; Qiagen) by capillary electrophoresis (BioAnalyzer; Agilent).

165 **Reverse-Transcription and Preamplification**

166 Reverse-transcriptions were performed on 5ng of total RNA in a 20µL reaction volume with
167 the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (PN 4368814;
168 AppliedBiosystems™) according to the manufacturer's instructions.

169 12.5µL of cDNAs were pre-amplified with the Taqman® Preamp Master Mix Kit
170 (AppliedBiosystems™) in a 50µL reaction volume containing 25µL of Master Mix 2× and 12.5µL
171 of a pooled assay. Pooled assays combined equal volumes of each 20×Taqman® Gene
172 Expression Assay of interest including the endogenous gene *CDKN1B* diluted using 1×TE buffer
173 so that each assay is at a final concentration of 0.2×. A 14 cycles pre-amplification was
174 performed, as recommended by the manufacturer and pre-amplification products were 1:20
175 diluted in 1×TE buffer.

176 **Semi-quantitative real-time PCR**

177 Semi-quantitative real-time PCR was performed with faststart universal probe master mix
178 (Rox) 2× with 20×Taqman® Gene Expression Assay and 6.25µL of pre-amplified cDNA in a 25µL
179 total reaction volume in each well of a 96-well plate. *CDKN1B* endogenous gene was used as
180 recommended by the manufacturer in the preamp mastermix protocol. 7900HT Fast Real-
181 Time PCR System (AppliedBiosystems™) was used for the detection and semi-quantification of
182 gene expression.

183 TaqMan® Array Micro Fluidic Cards (Low-Density Arrays 384-wells format) were customized
184 with our genes of interest and performed with FastStart Universal Probe Master Mix (Rox) 2×
185 and pre-amplified cDNA in a 100µL total reaction volume on the 7900HT Fast Real-Time PCR
186 System (AppliedBiosystems™).

187 Quantitative real-time PCR results were analyzed with the dedicated SDS2.3 and RQManager
188 softwares (AppliedBiosystems™). For each probe and each sample, we normalized gene
189 expression with the *CDKN1B* endogenous gene expression (ΔCt) and calculated the $\Delta\Delta\text{Ct}$ and
190 the corresponding fold-change ($2^{-\Delta\Delta\text{Ct}}$) between the Tum-NK and the Non-Tum-NK samples for
191 each patient.

192 **Immunohistochemistry**

193 Tissues were deparaffinized and rehydrated by successive baths of Clearene and ethanol
194 gradient (100%, 90%, 70%, 50%). Antigen retrieval was performed with a Tris-EDTA pH8
195 solution in a preheated water bath (97°C, 30 minutes). Sections were cooled at room
196 temperature for 30 minutes and endogenous peroxidase was blocked with 3% hydrogen
197 peroxide (15 minutes). Thereafter, sections were incubated with Protein Block solution (Dako)
198 for 30 minutes and incubated with mouse anti-human NKp46 (clone 195314, R&D Systems,

199 5µg/mL) and/or goat anti-human CTLA-4 mAb (AF-386-PB, R&D Systems, 2.5 µg/mL) for 1 hour
200 at room temperature. Peroxidase-linked secondary antibody (ImmPress anti-goat HRP Vector)
201 and alkaline phosphatase-linked secondary antibody (Rabbit anti-mouse AP Rockland
202 Immunochemicals) were used for CTLA-4 and NKp46, respectively. AEC (3-amino-9-
203 ethylcarbazole) and SAP (Shrimp-Alkaline Phosphatase) substrate (Vector laboratories) were
204 used to detect specific staining. For immunofluorescence detection, PE-conjugated donkey
205 anti-goat (Jackson ImmunoResearch) and AF647-conjugated donkey anti-mouse (Jackson
206 ImmunoResearch) 1:100 diluted were used for CTLA-4 and NKp46, respectively. Mounting
207 medium containing DAPI was used (Prolong Gold Antifade Mountant with DAPI, Invitrogen).
208 Immunofluorescence was detected with AxioVert 200 microscope (Zeiss).

209 **NKp46 quantification and image quantification (cohort 3)**

210 NKp46 was stained by immunohistochemistry for 309 patients of the retrospective cohort
211 (cohort 3). Slides were then digitalized using a NanoZoomer scanner (Hamamatsu Photonics.
212 Hamamatsu, Japan) and NKp46 density was quantified (NK cell number per mm² tumoral
213 tissue) with Calopix software (Tribun healthcare, France).

214 **CD8 staining of NSCLC validation cohort (cohort 2) and image quantification**

215 Serial 5-µm formalin-fixed paraffin-embedded NSCLC sections were stained using the Dako
216 Autostainer Plus. Heat-mediated antigen retrieval was performed using the EnVision FLEX
217 Target Retrieval Solutions (Agilent, Dako, CA. USA) at pH9 for 30 min on a PT-Link (Dako).
218 Immunodetection of CD8 expression was done using a mouse anti-human CD8 antibody
219 (Clone C8/144B. (Dako) at 1.6 µg/mL for 30 min in Dako REAL Antibody Diluent (Dako). Signal
220 intensity was improved with EnVision+ System-HRP-labelled Polymers anti-mouse (Dako) and

221 peroxidase was detected using diaminobenzidine (DAB)+ Substrate – Chromogen System
222 (Dako). Slides were then counterstained with Hematoxylin (Dako) and mounted with Glycergel
223 Mounting Medium (Dako). Slides were then digitalized using a NanoZoomer scanner
224 (Hamamatsu Photonics. Hamamatsu, Japan) and CD8 density was quantified with Halo
225 software (Indica Labs NM, USA).

226 **CD107a degranulation assay**

227 Tumor infiltrating lymphocytes (TILs) from NSCLC patients were cultured for 12h in the
228 presence of 100 U/ml IL-2 and incubated with K562 or P815 target cells at effector-target (E/T)
229 ratios of 10:1 during 4 h, in the presence or not of anti-CD16 Ab, with monensin and PE Cy5-
230 conjugated anti-CD107a (LAMP-1) mAb. Cells were then washed in PBS-FCS-EDTA and stained
231 for 20 min at 4°C with FITC-conjugated anti-CD3 and APC-conjugated anti-CD56 or control
232 conjugated isotypes.

233 **Co-cultures of dendritic and NK cells**

234 Tumor samples were processed as previously described (see “Preparation of human single-
235 cell suspension”). NK cells and DC cells were sorted using Singlet/Live/CD45⁺/CD3⁻/CD56⁺ and
236 Singlet/Live/CD45⁺/CD11c⁺, respectively. Purity after cell isolation. The purity of cell subsets
237 after cell sorting was assessed via flow cytometry prior to co-cultures and was between 94.6
238 to 100%, and between 98 to 99.6%, for NK cells and dendritic cells, respectively. Cells were
239 then plated in a 96-well U bottom plate (30 to 50 x 10³ cells/well) in RPMI 1640 Glutamax
240 (ThermoFisher) supplemented with human serum (Sigma-Aldrich. St-Louis. MO. USA) and
241 penicillin streptomycin (ThermoFisher) at 1:1 ratio of DC:NK. In the corresponding conditions,
242 lipopolysaccharide (LPS-EB – InVivoGen, San Diego, CA, USA) and blocking anti-CTLA-4
243 antibody (ThermoFisher; clone AS32) were added at 1 µg/mL and at 5 µg/ml, respectively. DC

244 activation was assessed after 48h or 72h of co-culture by flow cytometry. Cells were stained
245 using an APC Cy7-conjugated anti-HLA-DR (mIgG2 α k, clone L243, BioLegend) and an AF700-
246 conjugated anti-CD86 (mIgG1 κ , clone 2331, BD).

247 **CTLA-4 and cytokine quantification produced by NK cells**

248 Cell suspension from tumoral or non-tumoral tissues were obtained as described in
249 “Preparation of human single-cell suspension”. The cells were incubated overnight in a 96-
250 well plate in in RPMI 1640 Glutamax supplemented with 10% human serum and 1% penicillin
251 streptomycin, then the supernatant was kept at -80°C for CTLA-4 and cytokine dosages.
252 Soluble CTLA-4 was quantified using ELISA Kit (Abcam) in the supernatant of cell cultures,
253 following the manufacturer protocol. Cytokine production in the supernatant of cell cultures
254 was evaluated using Luminex Assay (Bio-Plex Pro Human Cytokine 27-plex Assay - BioRad),
255 following the manufacturer protocol.

256 **Statistical and data analyses**

257 Statistical analysis was performed using the R software version 3.6.0 and the packages
258 pheatmap and ggplot2. Gene enrichment analysis was achieved with ClueGO(28) app of
259 Cytoscape(29) with the Gene Ontology (GO) Biological processes database 2016 December.
260 Association between quantitative and qualitative variables was estimated with Mann-
261 Whitney U test. Association between quantitative variables was assessed using Pearson
262 correlation. Association between qualitative variables was assessed using Fisher Test. A p-
263 value <0.05 was considered statistically significant. The overall survival (OS) curves of patients
264 with low *versus* high (separated by median) density of NKp46⁺, CD8⁺ T, DC-LAMP⁺ DC or CD20⁺
265 B cells, cells were estimated by the Kaplan–Meier method and compared by the log-rank test,
266 and was performed using the script “*survfit*” on R studio software.

267 **Results**

268 **NK cells in the tumor microenvironment display a distinct transcriptomic signature**

269 To determine whether the gene expression signature of intratumoral NK cells is distinct in the
270 lung TME, a whole transcriptome analysis was previously performed on purified NK cells from
271 the tumor and matched distant tissue, in a discovery cohort of 12 NSCLC patients
272 **(supplementary Table S1)**(26). In the present study, we performed a more extensive analysis
273 of microarray data, and found a total of 968 genes differentially expressed between
274 intratumoral NK cells and non-tumoral NK cells, with 569 over-expressed and 399 under-
275 expressed genes, using the log2-fold change threshold of 1, with a p value inferior to 0.05
276 **(Figure 1A)** demonstrating that NK cells in the TME have a distinct transcriptomic signature.
277 **Figure 1B** illustrates expression levels of these up and down regulated genes.

278 A gene ontology analysis using Cluego revealed that differentially expressed genes were
279 involved in cell migration, regulation of response to stimulus, cell activation, defense response
280 and inflammatory response **(Figure 1C)**, supporting major functional changes of NK cells within
281 the TME. The under-expressed genes were associated with inflammatory response,
282 endocytosis, response to inorganic substance, blood coagulation and hemostasis whereas the
283 overexpressed ones were mainly associated with regulation of cell proliferation, cell activation
284 and migration **(Figure 1D)**. Among the complete list of the 968 most differentially expressed
285 genes **(Supplementary Tables S2 and S3)**, those strongly linked to NK cell functions in the TME,
286 i.e. migration, cell activation and regulation of immune responses and cytotoxicity, were
287 selected to analyze their expression in a validation cohort of 47 NSCLC patients
288 **(Supplementary Table S4)**.

289 The selected gene signature included *S1PR1*, *CX3CR1*, *CXCR5*, *CXCR6* and *CXCL13* (**Figure 2A**),
290 *CTLA4*, *FCRL4*, *IL22RA2* and *LILRP2* (**Figure 2B**) and *FAS*, *GZMA*, *GZMK*, *NCR2* and *KLRC1* (**Figure**
291 **2C**). Semi-quantitative polymerase chain reaction (qRT-PCR) analysis performed on NK cells
292 purified from tumor and matched non-tumor samples of the validation cohort confirmed the
293 significant down-regulation of *S1PR1* and *CX3CR1*, and the over-expression of *CXCR5*, *CXCR6*,
294 *CXCL13*, *CTLA4*, *FCRL4*, *IL22RA2*, *LILRP2*, *FAS*, *GZMA*, *GZMK*, *NCR2*, and *KLRC1* (**Figure 2A, B, C**).
295 Unsupervised hierarchical clustering of samples based on this gene expression signature
296 resulted in segregation of non-tumoral and tumoral NK cells into distinct groups (**Figure 2D**),
297 confirming the modulation of NK cell migration, activation and cytotoxic functions in the TME.
298 Unsupervised clustering based on expression variation of these genes in tumor NK cells
299 compared to non-tumor NK cells revealed two groups of patients (**Figure 2E**), with and an
300 enrichment in migratory capacities in group 1 and in cytotoxic molecules in group 2. The two
301 groups did not differ based on gender, tobacco, age, tumor size or presence of invaded lymph
302 nodes (**Supplementary Fig. S1**). However, we could notice an enrichment in stage 1 and 2
303 tumors and a trend for SCC histologic type enrichment in group 2 (**Supplementary Fig. S1**).

304 **A subset of intratumoral NK cells express CTLA-4**

305 Although CTLA-4 is mainly expressed on regulatory T cells (Tregs) and some activated
306 conventional T cell subsets (Tconv)(30) our analysis showed a strong upregulation of *CTLA4*
307 mRNA in intratumoral NK cells in a subset of patients (77 %) (**Figure 2B**). This expression was
308 then validated at the protein level by immunohistochemistry and flow cytometry analyses. We
309 observed the co-expression of CTLA-4 and NKp46 on intratumoral NK cells by
310 immunohistochemistry and immunofluorescence on lung tumor tissue sections (**Figure 3A, B**).

311 Of note, the expression of CTLA-4 by intratumoral NK cells was also observed in other solid
312 tumors including melanoma, breast cancer and renal cell carcinoma (**Supplementary Fig. S2**).
313 CTLA-4 expression was then compared by flow cytometry in NK, Tconv and Treg cells in several
314 patients (**Figure 3C**). CTLA-4 expression in Tregs within the tumor as well as in distant lung
315 non-tumoral tissue was confirmed both at the cell surface and intracellularly, whereas CTLA-
316 4 was only expressed intracellularly by a fraction of NK and Tconv (**Figure 3D**). In line with the
317 results obtained by quantitative PCR, intracellular CTLA-4 was highly overexpressed in
318 intratumoral NK cells in 11 out of 16 patients tested (69 %) (**Figure 3D**). We therefore
319 compared clinical data and survival of groups of patients with high and low CTLA-4⁺ NK cells
320 and found that there are significantly more patients with low CTLA-4⁺ NK cells with
321 adenocarcinoma histological type (75% vs 32%, p=0.02) and having stage I NSCLC (75% vs 40%,
322 p= 0.07). We also found an influence of the gender since more female are found in the group
323 of low CTLA-4⁺ NK cells patients (81% vs 42%, p=0.01). Other clinical parameters were similar
324 between patients with high and low CTLA-4⁺ NK cells (**Supplementary Fig. S3A**). We also found
325 no difference in the overall survival between the two groups of patients ((**Supplementary Fig.**
326 **S3B**).

327 **NK cells in the tumor microenvironment have a distinct phenotype and cytokine secretion** 328 **profile**

329 To better characterize the NK cells in the TME, we performed a flow cytometry analysis and
330 quantified cytokine production by sorted NK cells. We first observed that among CD3⁻CD56⁺
331 NK cells, most of NKp46⁺ cells co-express the transcription factor Eomes, which confirms that
332 the cells belong to the NK lineage (**Figure 4A, B**). We found a significant higher expression of
333 NK2GA and CXCR6 and lower expression of CX3CR1 in NK cells from the TME as compared to

334 NK cells from non-tumoral tissue, confirming at the protein level the results obtained with
335 gene expression analyses (**Figure 4A**). Interestingly, we found that intratumoral NK cells
336 display an activated phenotype, with high expression of CD69 and NKp44. However, no
337 difference was found for Fas, CD107a and Eomes when we compared NK cells from TME or
338 adjacent tissue (**Figure 4A**). We also confirmed the co-expression of CTLA-4 and NKp46 by NK
339 cells, and the co-expression of inhibitory CTLA-4 and NKG2A molecules on a subset of NK cells
340 (**Figure 4B**).

341 We found that NK cells from TME produced various levels of cytokines, with high levels of IL-
342 1 β , IL1RA, IL-6, IL-8, IL-9, IL-15, IL-17, TNF- α , IFN- γ and G-CSF and in similar amounts compared
343 to NK cells from adjacent tissue (**Figure 4C**).

344 **Intratumoral NK cells has reduced cytotoxicity and negatively regulate dendritic cell** 345 **maturation**

346 We had previously demonstrated that intratumoral NK had reduced capacity to degranulate
347 and to secrete IFN- γ after a coculture with autologous tumor cells(7). We also show that NK
348 cells display reduced cytolytic function in a redirected lysis assay, against P815 mastocytoma
349 cells (**Supplementary Fig. S4**).

350 To analyze a possible regulatory role of intratumoral NK cells, we cocultured purified NK cells
351 (CD3⁻CD56⁺) from fresh lung tumors and CD11c⁺ dendritic cells (DC) purified from the blood of
352 the same patients in the presence of LPS stimuli. When NK cell numbers permitted, we
353 analyzed DC maturation in the presence of a blocking anti-CTLA-4 monoclonal antibody (mAb).
354 After two to three days of coculture, DC maturation was assessed by flow cytometry (**Figure**
355 **5A, and gating strategy Supplementary Fig. S5**). Induction of MHC class II and CD86
356 expression on DCs was significantly reduced upon coculture with intratumoral NK cells (20 and

357 41% inhibition, respectively) (**Figure 5B**), and this was partially reverted by the addition of
358 anti-CTLA-4 mAb (**Figure 5C**). In addition, we didn't find any CTLA-4 protein in the supernatant
359 of intratumoral NK cells (data not shown). These data suggest that intratumoral NK can reduce
360 DC activation, through a mechanism that partially involves CTLA-4.

361 ***CTLA4* expression by intratumoral NK cells is highly correlated with *CXCR6*, *GZMK* and *KLRC1***

362 To understand the suppressive effect of intratumoral NK cells on DC maturation, we evaluated
363 the correlations between the expression of *CTLA4* and other genes in the signature. In tumors,
364 we found an inverse correlation with *S1PR1*, and a strong positive correlation with *CXCR6*,
365 *GZMK* and *KLRC1*, an inhibitory receptor on NK cells (**Figure 6**). This suggests that *CTLA4*-
366 expressing NK subsets may have a distinct migration and regulatory function profiles as
367 compared with other NK cells.

368 **Intratumoral NK cells negatively impact the clinical outcome of CD8⁺ T cells**

369 To investigate whether the presence of intratumoral NK cells could impact the clinical
370 outcome, the localization and density of tumor-infiltrating NK cells were determined by NKp46
371 quantification on a retrospective cohort of NSCLC patients(27). IHC staining revealed that
372 NKp46⁺ cells were mainly localized in the stroma and poorly infiltrated the tumor nest
373 (**Supplementary Fig. S6**).

374 The prognostic value of intratumoral NK cells was determined using median cutoff separation
375 of the groups. NK cell density was not linked to a prognosis in the entire cohort (**Figure 7A**), in
376 stage I and II (**Supplementary Fig. S7A**), tumor size (**Supplementary Fig. S7B**), histological
377 subgroups (**Supplementary Fig. S7C**), or chronic obstructive pulmonary disease (COPD)
378 patients (**Supplementary Fig. S7D**). Since some subsets of NK cells express inhibitory markers,
379 including *KLRC1* and *CTLA4*, we hypothesized that the presence of inhibitory NK cells could

380 impact on the prognostic value of CD8⁺ T cells. As previously reported, high CD8⁺ T cell density
381 strongly correlated to a good clinical outcome(14) (**Figure 7B**). Consequently, the prognostic
382 impact of NK cells differed when considering patient groups with high or low CD8⁺ T cell
383 infiltration. In patients with low numbers of CD8⁺ T cells, high NK cell density improved the
384 clinical outcome, whereas in patients with high CD8⁺ T cell density, NK cells negatively
385 impacted the overall survival (**Figure 7C**), supporting the inhibitory role of intratumoral NK
386 cells. This prognostic effect of NK cells is specific of T cells, since NK cell densities did not
387 impact the clinical impact of B cells and mature DC-LAMP⁺ dendritic cells (**Supplemental Figure**
388 **S8**).

389 **CTLA-4 expression by intratumoral NK cells is correlated to CD8⁺ T cell density**

390 Considering the expression pattern and suppressive role of CTLA-4 in tumor NK cells, we
391 investigated the dynamics of *CTLA4* in tumor NK cells in the prospective cohort described in
392 table 2 (cohort 2). We found a positive correlation between the density of CD8⁺ T cells and the
393 level of *CTLA4* gene expression by intratumoral NK cells (**Figure 7D**), and a positive correlation
394 between the percentage of CTLA-4⁺ NK cells and the total percentage of CD3⁺ T cells
395 (**Supplementary Fig S9A**). Finally, we also found a positive correlation between the gene
396 expression level of *CTLA-4* and CD4 or CD8, both in adenocarcinoma and squamous cell
397 carcinoma (**supplementary figure S9B**), using gene expression profiling interactive analysis
398 (GEPIA2) tool (<http://gepia2.cancer.pku.cn/>). Of note, total NK cell densities did not correlate
399 with numbers of CD8⁺ T cells, B cells or DCs (**Supplementary Table S5**), suggesting that the
400 possible inhibitory effect of NK cells was restricted to the subset of CTLA-4 expressing NK cells.

401

402

403 Discussion

404 In previous studies, we found that intratumoral NK cells exhibit altered phenotypes and
405 functions(7) characterized by a specific gene expression signature(26). Here we extended this
406 study with an in-depth transcriptomic analysis of NK cells in human NSCLC comparing the
407 signature of intratumoral NK cells with distant non-tumoral lung NK cells. Our results show a
408 distinct transcriptomic signature of NK cells in the lung TME suggesting that the lung TME may
409 induce suppressive NK cells, that could play a role in the regulation of anti-tumor adaptive
410 immunity.

411 Our signature is enriched with the chemokine receptors *CXCR5*, *CXCR6* and a show a strong
412 downregulation of *S1PR1* and *CX3CR1*. The density of NK cells in the TME is lower than in
413 distant tissue(7) and NK cells in the TME express more *CXCR6* and less *CX3CR1*. This specific
414 migratory signature could explain the exclusion of NK cells from the tumor core. Indeed,
415 several studies show the involvement of *CX3CR1* and fractalkine - the latter being expressed
416 by tumor cells - in the recruitment and cytotoxicity of NK cells against tumors(31), and have
417 shown TGF- β -mediated down-regulation of *CX3CR1*(32). In addition, *CX3CR1*-deficient mice
418 have a defective anti-tumor response(33). On the other hand, a population of NK cells
419 expressing *CXCR6* is characterized by a more immature phenotype, producing fewer cytotoxic
420 mediators and pro-inflammatory cytokines(34). The mechanism that explain this migratory
421 profile found in intratumoral NK cells is unknown, but could be due to a preferential migration
422 of cells that display this specific signature, to a local modification induced by the TME or a
423 combination of both.

424 Intratumoral NK cells are also enriched with a number of inhibitory receptors most notable of
425 which is CTLA-4, a well-known immune checkpoint molecule expressed by effector T cells after

426 TCR activation. It regulates T-cell activation by out-competing the co-stimulatory molecule
427 CD28 by binding its partners CD80 and CD86 with a higher avidity(30). However, data on the
428 expression and function of CTLA-4 in NK cells are scarce and poorly investigated. In studies by
429 Chiossone et al.(35) and Terme et al.(36), *CTLA4* transcripts were detected by whole-genome
430 microarray analysis of mouse NK cells, indicating that CTLA-4 may be expressed by NK cells. In
431 a recent study, CTLA-4 was found in CD69⁺CD103⁺ tissue resident NK cell subsets in human
432 lungs(37), and CTLA-4 expression is shown to be up-regulated in activated murine(38) and
433 human(39) NK cells. However, there is no evidence demonstrating CTLA-4 expression by NK
434 cells in the context of human lung tumors. We found that a subpopulation of intratumoral NK
435 cells have intracellular CTLA-4, as previously observed in a murine model of lung tumors,
436 showing CTLA-4 gene expression by intratumoral NK cells (38). Our immunohistochemistry
437 studies reveal that CTLA-4 is co-expressed with NKp46 also in other solid tumors suggesting
438 that its expression by NK cells is not restricted to lung tumors. Several isoforms of CTLA-4 have
439 been characterized(40). By RT-PCR, we found all the isoforms in NK cells purified from NSCLC
440 tumors (data not shown). Interestingly, *CTLA4* expression strongly correlated with that of
441 another inhibitory receptor *KLRC1*, specifically in the tumor tissue and co-expression of both
442 inhibitory receptors was confirmed in intratumoral NK cells, suggesting that, in the TME, NK
443 cells acquire inhibitory receptors making them less efficient effector cells.

444 The mechanism of action of CTLA-4 is still not completely clear. In co-culture experiments, we
445 found that tumor NK cells reduced DC maturation, and this was partially reversed by the
446 addition of CTLA-4 blocking antibodies. A possible effect of secreted CTLA-4 was excluded
447 since we did not detect soluble CTLA-4 in the NK cell supernatants. The precise mechanism of
448 this inhibition is unclear, but it could involve CTLA-4 -by maintaining its expression at the
449 surface level in the presence of the anti CTLA-4 Ab, as it has been suggested (41) - and other

450 yet unidentified molecules secreted or expressed by tumor-experienced NK cells, blocking the
451 maturation or the recruitment of DC. Indeed, instances of NK cells suppressive DCs have been
452 reported in previous studies: upregulation of PD-L1 on NK cells and PD-1 on DC led to impaired
453 DC maturation and low CD8⁺ T cell priming(42), while the impaired NK cell viability caused by
454 tumor released PGE2 led to reduced recruitment of cDC1 in the TME(43).

455 While intratumoral NK cells exhibit altered functions, their density within the TME does not
456 associate with improved clinical outcome, contrary to CD8⁺ T cells that are linked to good
457 overall survival (OS). Surprisingly, we found that high density of NK cells associates with a good
458 OS only in patients with low infiltration of CD8⁺ cells whereas it conferred a poor outcome in
459 patients with high CD8 density. This highlights a role for the tumor immune microenvironment
460 in re-programming NK cells. Accordingly, we found a positive correlation between higher CD8⁺
461 T cell infiltration with CTLA-4 expression by NK cells suggesting that immune-active tumors
462 also harbor suppressive NK cells. Of note, high NK cells density was also linked to good clinical
463 outcome in COPD patients(27).

464 Despite the fact that NK cells have been widely implicated in anti-tumoral immune responses
465 in various tumor models, several studies highlight their potential inhibitory/regulatory role.
466 Regulatory NK cells produce IL-10 and/or express the immune checkpoint molecule CD73 and
467 inhibit autologous CD4⁺ T cell proliferation(21,44,45). Activated NK cells express granzyme K
468 and NCR2(46,47) and kill autologous activated CD4⁺ T cells by a mechanism involving granzyme
469 K(48). We found that NK cells from the TME have a phenotype of activated cells - expressing
470 CD69 and NCR2 molecules – and we found a strong correlation between *CTLA4* and *GZMK*
471 expression, suggesting that tumor-experienced CTLA-4⁺ NK cells could kill CD4⁺ T cells.
472 However, we did not find any decrease in T cell populations in tumors enriched in CTLA-4⁺ NK

473 cells. The inhibitory function of intratumoral NK cells was also reported by Crome et al(49),
474 who defined in high grade serous ovarian tumors a population of regulatory CD56⁺CD3⁻ cells
475 that suppressed TIL expansion, displayed low cytotoxic activity and produced IL-9 and IL-22
476 cytokines. This population resembles partially the one we describe in our study, based on
477 cytokine profile and phenotypic characteristics. Finally, in advanced NSCLC, a regulatory role
478 of NKp46⁺ NK cells has been reported, showing that low rates of circulating NKp46⁺ NK cells
479 significantly associated with better OS(50).

480 **Conclusions**

481 Our findings on the regulatory function of NK cells in the lung TME have several therapeutic
482 implications. First, the characterization of intratumoral NK cells reveals a specific migration
483 profile potentially restricting their entry into the TME. It would be of great interest to target
484 chemokine receptors on NK cells to enable them to enter tumor tissues. Second, our results
485 show that NK cells acquire inhibitory functions within the TME, the reversion of which will
486 enable NK cells to activate other immune cells and exert anti-tumoral cytotoxic functions.
487 During the past 10 years, antibodies targeting CTLA-4 and PD1/ PD-L1, have entered clinical
488 trials to reverse T cell exhaustion and restore the anti-tumor capacity of T cells, with proven
489 efficacy in patients with various types of advanced cancers, including NSCLC(51). In addition,
490 several clinical trials based on NK cell checkpoints are ongoing, targeting KIR, TIGIT, LAG-3,
491 TIM-3 and KLRC1(19). In this context, both CD8⁺ T cells and NK cells have been shown
492 necessary for the therapeutic effectiveness of combination IL-2 and CTLA-4 blockade
493 immunotherapy in B16 melanoma(52). Our study provides data supporting the pertinence of
494 targeting the inhibitory activity of NK cells within the TME.

495 In conclusion, our results support the notion that the TME contains regulatory NK cells, that

496 represents a new escape mechanism.

497

498 **List of abbreviations**

499 CCL3 : C-C Motif Chemokine Ligand 3

500 cDC1 : conventional dendritic cell 1

501 CIS : cytokine-inducible SH2-containing protein

502 COPD : chronic obstructive pulmonary disease

503 CTLA-4 : cytotoxic T-lymphocyte-associated protein 4

504 CX3CR1 : CX3C chemokine receptor 1

505 CXCL13 : C-X-C motif ligand 13

506 CXCR5 : C-X-C chemokine receptor type 5

507 CXCR6 : C-X-C chemokine receptor type 6

508 FAS : FS7-associated cell surface antigen

509 FCRL4 : Fc receptor-like protein 4

510 GM-CSF : Granulocyte Macrophage Colony-Stimulating Factor

511 GZMA : Granzyme A-encoding gene

512 GZMK : Granzyme K-encoding gene

513 IL22RA2 : Interleukin 22 Receptor Subunit Alpha 2

514 KLRC1 : Killer Cell Lectin Like Receptor C1

515 LAG3 : Lymphocyte-activation gene 3

516 LILRP2 : Leukocyte immunoglobulin-like receptor pseudogene 2

517 NCR2 : Natural Cytotoxicity Triggering Receptor 2

518 NKG2A : Natural Killer Group 2 Natural Killer Group 2 member A

519 PD-(L)1 : Programmed death (ligand) 1
520 PGE2 : prostaglandin E2
521 S1PR1 : Sphingosine-1-phosphate receptor 1
522 TIGIT : T cell immunoreceptor with Ig and ITIM domains
523 TIM3 : T cell immunoglobulin and mucin domain-containing protein 3

524

525 **Declarations**

526 **Ethics approval and consent to participate**

527 All the patients gave an informed consent prior to inclusion. The study was conducted with
528 the agreement of the French ethic comity (number 2012 06 12 IRB00001072) in application
529 with the article L. 1121-1 of French law, according to the recommendations in the Helsinki
530 declaration.

531 **Consent for publication**

532 Not applicable

533 **Availability of data and materials**

534 The datasets used and/or analyzed during the current study are available from the
535 corresponding author on reasonable request.

536 **Competing interests**

537 The authors declare that they have no competing interests.

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542 **Authors' contributions**

543 PEJ, JR, MGB, CT, SM, NJ, CG, JG and MADD performed the experiments. MM, FP, and GB
544 performed bioinformatic analysis. PEJ, JR, CT, MGB and IC analyzed the data. PV, LF, AL, DD
545 and MA provided clinical samples and pathological data. IC designed and supervised the study.
546 PE, JR, MGB and IC wrote the manuscript. AV, MCDN and LZ revised the manuscript.

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698 **Figure legends**

699 **Figure 1. Gene expression differential analysis of tumoral versus non-tumoral NK cells, in** 700 **the discovery cohort (cohort 1)**

701 **(A)** Volcano plot presenting differentially expressed genes between tumoral NK and non-
702 tumoral NK cells. X axis displays log₂ Fold changes between the two groups and Y axis the -
703 log₁₀(p.value). Differentially expressed genes (highlighted in cyan) between tumoral and non-
704 tumoral samples were characterized by fold changes superior/inferior to 2 and with a
705 significant p.value (<0.05). Genes of interest are colored in orange. **(B)** Heatmap of
706 differentially expressed genes between tumoral and non-tumoral groups, organized by
707 hierarchical clustering (after normalization of expression values). **(C)** Gene enrichment
708 analysis for all the differentially expressed genes, using ClueGo application (Cytoscape
709 software). **(D)** Gene enrichment analysis for down- (left panel) and up- (right panel) regulated
710 biological processes. Color intensity of dots is proportional to the adjusted p values and size
711 corresponds to the number of differentially expressed genes in the discovery cohort.

712 Horizontal axis represents overlap between differentially expressed genes and genes in the
713 biological processes.

714 **Figure 2. Specific gene variation between tumoral vs non-tumoral NK cells in the validation**
715 **cohort (cohort 2)**

716 NK cells were sorted from non-tumoral distant tissue (Non-Tum NK – black dots) and from
717 tumor (Tum NK – red dots) for 47 patients and total RNA were extracted and analyzed for the
718 expression of 14 genes involved in NK migration **(A)**, cell activation and regulation of immune
719 responses **(B)** and cytotoxic functions **(C)** by quantitative PCR. Each dot represents a duplicate
720 measurement of the gene expression in one individual. The mean values are indicated by blue
721 dashes. Statistical differences were assessed by the Wilcoxon non-parametric-test method
722 with Graphpad software. **(D)** Heatmap of delta delta CT for tumoral and non-tumoral samples
723 of the 14 genes of interest. Hierarchical clustering identified two groups. Expression values
724 were standardized **(E)** Heatmap of fold changes of delta delta CT values between tumoral and
725 non-tumoral for the 14 genes of interest. Patient information are represented on top for each
726 sample.

727 **Figure 3. CTLA-4 protein expression in tumor infiltrating NK cells**

728 **(A, B)** Identification of CTLA-4 expressing NKp46⁺ cells in NSCLC patients by
729 immunofluorescence **(A)** or immunohistochemistry **(B)** double staining.

730 **(C, D)** CTLA-4 protein expression was analyzed in NK cells (CD3⁻ CD56⁺), conventional T cells
731 (CD3⁺ CD56⁻ Foxp3⁻) and regulatory T cells (CD3⁺ CD56⁻ Foxp3⁺) by flow cytometry after
732 intracellular (Intra.) or cell surface staining of cells from tumoral (Tum) or non-tumoral (Non
733 Tum) tissue of NSCLC patients. Cells from blood were also analyzed for some patients.

734 Representative images of intracellular CTLA-4 staining are shown in **(C)** and the summary of
735 analyzes are shown in **(D)**. Statistical analyses were performed by Wilcoxon method with the
736 Graphpad software. ns: not significant.

737 **Figure 4. Intratumoral NK cells phenotype and cytokine secretion**

738 **(A)** Eomes, NKG2A, CD69, NKp44, CD107a, Fas, CXCR6, CX3CR1 and S1PR1 protein expression
739 was analyzed in NK cells (CD3⁻CD56⁺) by flow cytometry after cell surface staining of cells from
740 non-tumoral (Non-tum) or tumoral (Tum) tissue of NSCLC patients. Percentages or GeoMean
741 are presented. **(B)** Co-staining for NKp46/Eomes, NKp46/CTLA-4 and CTLA-4/NKG2A are
742 shown on CD3⁻CD56⁺ intratumoral NK cells. **(C)** Quantification of cytokines produced by NK
743 cells sorted from tumoral or non-tumoral tissue. Statistical analyses were performed by
744 Wilcoxon method with the Graphpad software. ns: not significant.

745 **Figure 5. Intratumoral NK cells reduces DC maturation**

746 **(A, B)** MHC-II and CD86 surface expression was analyzed by flow cytometry on LPS-treated
747 CD11c⁺ DC after 2-3 days of co-culture with CD3⁻CD56⁺ intratumoral NK cells. Experimental
748 design of the co-culture experiment is shown in **(A)**. **(B)** Expression of CD86 or MHC-II
749 expression on LPS-treated DC alone (DC alone) or in co-culture with CD56⁺ cells (DC + CD56⁺
750 cells) was analyzed. Data are represented as a ratio of mean fluorescence intensity (MFI) of
751 CD86 or MHC-II expression in DC alone (blue dots) *versus* DC + CD56⁺ cells co-culture (red dots).
752 **(C)** The ratio of MHC-II surface protein expression on LPS-treated DC were analyzed by flow
753 cytometry after 3-4 days of culture of DC cells alone (blue dots) or in co-culture with CD56⁺
754 cells (red dots) and in the presence of CTLA-4 blocking antibody (green triangles). Statistical
755 analyses were performed by the Wilcoxon non-parametric test with the Graph-pad software.
756 ns: not significant.

757 **Figure 6. Correlation between CTLA-4 expression and gene signature expression in tumor vs**
758 **non-tumoral NK cells**

759 Sorted NK cells from non-tumoral distant tissue (Non-Tum NK) and tumoral (Tum NK) for 47
760 patients were extracted and total RNA was analyzed. CTLA-4 mRNA expression was then
761 compared to Ct values of the 14 genes previously identified. The correlation between both
762 values was assessed in non-tumoral and tumoral NK cells using Pearson correlation test with
763 the Graph-pad software.

764 **Figure 7. Clinical impact of NKp46⁺ cells in NSCLC patients**

765 **(A, B)** Patients from the retrospective cohort (cohort 3) were splitted into 2 groups according
766 to the density of intratumoral NKp46⁺ cells **(A)** or CD8⁺ cells **(B)**. Separation was done by
767 median and their overall survival was analyzed. **(C)** Patients with low density (CD8⁺ cells^{Low}) or
768 high density (CD8⁺ cells^{High}) of CD8⁺ cells were splitted into 2 groups according to their number
769 of intratumoral Nkp46⁺ cells and the overall survival were done in each group. Separation was
770 done by median. **(D, E)** CD8 density of patients from the validation cohort (cohort 2) was
771 assessed by immunohistochemistry on paraffin-embedded slides and the correlation with
772 CTLA-4 mRNA expression in NK cells was calculated using Pearson correlation test with the
773 Graph-pad software **(D)**. **(E)** Patients from validation cohort (cohort 2) were split in 2 groups
774 according to the density of intratumoral CD8⁺ cells (using median) and the expression of CTLA-
775 4 mRNA was analyzed in each group. Statistical analyses were performed by the Mann-
776 Whitney non-parametric test with the Graph-pad software.

777

778

Supplemental information

779 **Supplemental Table S1. Discovery cohort (cohort 1).**

780 Pathological staging and histological types of the tumors were determined according to the 2009
781 TNM staging system and World Health Organization. PY, packs per year; ADC,
782 adenocarcinoma; SCC, squamous cell carcinoma.

783 **Table S2. Top 56 up-regulated genes**

784 **Table S3. Top 56 down-regulated genes**

785 **Table S4. Validation cohort (Cohort 2)**

786 Pathological staging and histological types of the tumors were determined according to the 2009
787 TNM staging system and World Health Organization. PY, packs per year; ADC,
788 adenocarcinoma; SCC, squamous cell carcinoma

789 **Table S5. Correlation between intra-tumoral NK cells and B, mature DC or CD8⁺ T cell** 790 **density**

791 Coefficients of correlation of NK cell density with B, mature DC or CD8⁺ T cells density.
792 Spearman correlations (*r²) were calculated with the Kendal method using R studio software.

793 **Fig S1. Association between clinical parameters and groups 1 and 2 defined by** 794 **unsupervised clustering.**

795 Bar plot representing the repartition of patients among assigned groups defined in figure 2E.
796 Colors indicate the different categories for each clinical parameter.

797 **Fig.S2. Multicolor immunofluorescence of formalin-fixed paraffin-embedded tumoral** 798 **tissues from patients with melanoma. breast or renal cancer.**

799 The double-staining of NKp46 (Red) and CTLA-4 (White) was performed in intra-tumoral
800 tissues of several cancer types. Nucleus was stained with Dapi (Blue).

801 **Fig.S3. Association between clinical parameters and CTLA-4 expression by intratumoral**
802 **NK cells and clinical impact of CTLA-4 expressing NK cells.**

803 **(A)** Bar plot representing the repartition of patients with high and low CTLA-4 expressing NK.
804 Colors indicate the different categories for each clinical parameter. **(B)** Overall survival
805 between patients with high and low CTLA-4 expressing NK cells.

806 **Fig S4. Reduced cytotoxicity in redirected lysis assay**

807 Cytotoxicity against K562 or P815 mastocytoma was determined by degranulation assay, using
808 CD107a labeling and FACS analysis. IL-2-stimulated PBMC from blood of NSCLC patients,
809 or IL-2-stimulated tumor infiltrating lymphocytes (TIL) were used as effector cells, at a ratio
810 E:T of 10:1, in the presence or not of anti-CD16 mAb for redirected lysis against P815 cells.
811 The percentages indicated give the proportion of CD107 positive PBMC after gating on CD3⁻
812 CD56⁺ NK cells.

813 **Fig. S5. Gating strategy**

814 FACS analysis was done by selecting the live cells by morphology (FSC/SSC), removal of
815 doublets (FSC-A/FSC-H and SSC-A/SSC-H) and live cells (Live/Dead- cells). NK cells were
816 then characterized as CD3⁻CD56⁺ among the CD45⁺ cells and DC cells as CD11c⁺, among the
817 CD45⁺ cells.

818 **Fig.S6. Example of NKp46 staining by IHC in the retrospective cohort (cohort 3).**

819 **Fig.S7. Clinical impact of NKp46⁺ cell density according to the stage, the tumor size or**
820 **the histology of the tumors, in the retrospective cohort (cohort 3).**

821 (A - D) Patients from the retrospective cohort (cohort 3) were splitted into 2 groups according
822 to density of intra-tumoral NKp46⁺ cells. Separation was done by median and their overall
823 survival was analyzed according to (A) the stage (stage I and II), (B) the size, (C) the histology
824 (ADC = adenocarcinoma, SCC = squamous cell carcinoma) of the tumor, or (D) the Chronic
825 Obstructive Pulmonary Disease (COPD) status of the patient.

826 **Fig.S8. Clinical impact of NKp46⁺ cells according to B or dendritic cells infiltration in**
827 **the retrospective cohort (cohort 3).**

828 Patients with low density or high density of B cells (B cells^{Low} and B cells^{High}, respectively –
829 separated by median, and determined as surface of CD20 B-cell follicles in square millimeter
830 per tumor surface) or with low density or high density of mature DC-LAMP expressing
831 dendritic cells (DC cells^{Low} and DC cells^{High}, respectively – separated by median, and
832 determined as the numbers of DC-LAMP⁺ cells per tumor surface) were splitted into 2 groups
833 according to their number of intra-tumoral Nkp46⁺ cells and the overall survival were analyzed
834 in each group.

835 **Fig S9. Correlation between CTLA-4 and T cells in NSCLC**

836 (A) Correlation between the percentages of CTLA-4⁺ NK cells and the percentage of CD3⁺ T
837 cells in the tumor was analyzed, based on flow cytometry data. The pearson coefficient of
838 correlation and the p-value are represented. (B) The correlation between *CTLA4* and *CD4* or
839 between *CTLA4* and *CD8* gene expression, measured as transcripts per million (TPM), was
840 analyzed in lung adenocarcinoma (LUAD), and lung squamous cell carcinoma (LUSC), with
841 gene expression profiling interactive analysis (GEPIA2) tool (<http://gepia2.cancer.pku.cn/>), in
842 TCGA database. The pearson coefficient of correlation between *CTLA4* and *CD4* or *CD8*
843 expression and the p-value are represented for each tumor type.

844

845 **Supplemental table 1: Clinical characteristics of NSCLC patients of the discovery cohort**

Patient	Age	Sex	Tobacco (PY)	Histology	Stage (2009)
P1	61	M	45	ADC	IB
P2	62	F	50	ADC	IB
P3	59	F	>10	ADC	IB
P4	72	F	60	ADC	IB
P5	78	F	50	ADC	IB
P6	60	M	50	ADC	IIA
P7	61	M	50	ADC	IIIA
P8	71	F	40	SCC	IB
P9	57	M	80	SCC	IB
P10	79	M	50	SCC	IIB
P11	80	M	40	SCC	IIB
P12	84	M	>10	SCC	IIIA

846 Pathological staging and histological types of the tumors were determined according to the 2009

847 TNM staging system and World Health Organization. PY: packs per year. ADC:

848 Adenocarcinoma; SCC: Squamous Cell Carcinoma.

849

850 **Supplemental table 2: List of the genes that are over-expressed (more than 4-fold**
851 **change) in intra-tumoral compared to non-tumoral NK cells**

Gene name	Log2 FC expression (T vs NT)	adj p-value
CXCL13	5.01	9.8e-04
EPCAM	4.26	4.9e -04
SOST	4.21	9.8e -04
NCR2	4.17	1.5e -03
SPP1	3.97	4.9e-04
FNDC4	3.77	4.9e-03
SIX4	3.72	2.4e-03
LILRP2	3.63	1.5e-03
PLS3	3.59	4.9e-03
APOD	3.53	2.1e-02
FCRL4	3.40	9.8e-04
P4HA2	3.32	4.9e-04
XAGE1A	3.29	9.3e-03
PHOSPHO2-KLHL23	3.25	4.9e-04
GEM	3.21	4.9e-04
CHRNA5	3.20	4.9e-04
ITGA1	3.16	1.5e-03
TM4SF1	3.13	4.9e-04
CNN3	3.09	9.8e-04
CHRNA3	3.09	4.9e-04

KRT6C	2.93	9.3e-03
CEACAM5	2.89	9.3e-03
CDH17	2.82	2.1e-02
IGLL5	2.81	4.9e-03
PRRG4	2.81	9.8e-04
SFTA3	2.78	1.2e-02
CEACAM6	2.73	1.2e-02
CAMK4	2.72	9.8e-04
TRAT1	2.67	4.9e-04
ELF3	2.64	1.2e-02
KRT6A	2.60	4.2e-02
CYR61	2.59	2.4e-03
AGR2	2.57	3.4e-03
ITM2C	2.56	4.9e-03
NDFIP2	2.54	1.6e-02
IRX3	2.53	4.2e-02
SCNN1A	2.50	2.7e-02
CTLA4	2.50	4.9e-04
CRYAB	2.50	9.8e-04
KIAA1644	2.49	4.9e-04
BAG3	2.48	4.9e-04
LAPTM4B	2.48	4.9e-04
CLDN1	2.47	3.4e-03
KRT18P55	2.47	4.9e-04

SERPINH1	2.47	3.4e-03
SLC6A14	2.46	1.2e-02
CEACAM3	2.45	2.1e-02
TMPRSS3	2.44	4.9e-03
PLOD2	2.40	9.8e-04
ANLN	2.38	2.1e-02
PIK3R6	2.35	2.4e-03
TPSAB1	2.34	4.2e-02
GSG2	2.34	2.4e-03
SERPINE1	2.34	2.4e-03
TMEM200A	2.33	6.8e-03

852

853

854 **Supplemental table 3: List of the genes that are under-expressed (more than 4-fold**
855 **change) in intra-tumoral compared to non-tumoral NK cells**

Gene name	Log2 FC expression (T vs NT)	adj p-val
FABP4	-4.03	4.9e-04
PPARG	-3.05	4.9e-04
SCGB1A1	-2.88	4.9e-04
CX3CR1	-2.66	4.9e-04
AKR1CL1	-2.54	4.9e-04
PLEKHG3	-2.35	4.9e-04
TLL1	-2.05	4.9e-04
RGS9	-2.05	4.9e-04
GPR141	-1.95	4.9e-04
GLDN	-4.10	9.8e-04
STAC	-3.39	9.8e-04
MARCO	-3.09	9.8e-04
PHYHD1	-2.49	9.8e-04
PLBD1	-2.31	9.8e-04
VSIG4	-2.24	9.8e-04
MSR1	-2.12	9.8e-04
ZNF69	-2.11	9.8e-04
LRP1	-1.95	9.8e-04
TM7SF4	-2.83	1.5e-03
PODN	-3.16	2.4e-03

LIPC	-2.77	2.4e-03
B3GAT1	-2.05	2.4e-03
LPL	-3.64	3.4e-03
KRT72	-2.73	3.4e-03
SPIRE2	-2.57	3.4e-03
HBB	-2.21	3.4e-03
NEIL1	-2.17	3.4e-03
PLXDC2	-2.08	3.4e-03
AOC3	-2.34	3.9e-03
CD6	-2.52	4.9e-03
COLEC12	-2.39	4.9e-03
CPVL	-1.96	6.8e-03
CLEC9A	-3.07	9.3e-03
SERPINB2	-2.88	9.3e-03
RBP4	-2.40	9.3e-03
FEZ1	-2.02	9.3e-03
ADCY9	-2.48	1.2e-02
S100A12	-2.23	1.2e-02
SFTPC	-2.19	1.2e-02
C1QA	-2.03	1.2e-02
MT1G	-4.22	1.6e-02
CYP27A1	-2.67	1.6e-02
NCF2	-2.41	1.6e-02
ASMT	-2.24	1.6e-02

MT1H	-1.99	1.6e-02
MT1M	-2.69	2.1e-02
SIGLEC1	-2.26	2.1e-02
PRSS23	-2.00	2.1e-02
TLR8	-1.94	2.7e-02
APOC1	-2.79	3.4e-02
PDZD4	-2.00	3.4e-02
P2RY13	-2.17	4.2e-02
AKR1E2	-2.05	4.2e-02

856

857

858 **Supplemental table 4: Clinical characteristics of NSCLC patients of the validation cohort**

Patients	Gender	Age	Tobacco (PY)	Histological Type	TNM	Stade
P1	M	71	30	LCNEC	pT2aN2Mx	IIIA
P2	F	68	30	SCC	pT2bN0	IIA
P3	M	63	80	SCC	pT3N1Mx	IIIA
P4	F	64	20	ADC	pT2aN2	IIIA
P5	F	68	0	ADC	pT1bN0	IA
P6	F	62	N/A	ADC	pT1bN0	IA
P7	M	79	50	SCC	pT2aN0	IB
P8	F	76	40	ADC	pT2aN0Mx	IB
P9	F	57	30	SCC	ypT2aN0	IB
P10	F	70	50	SCC	pT2aN0	IB
P11	F	61	15	ADC	pT2aN1Mx	IIB
P12	F	80	0	ADC	pT2aN2Mx	IIIA
P13	F	75	25	ADC	pT3N0	IIB
P14	F	65	80	SCC	pT2aN0	IB
P15	M	78	N/A	LCNEC	pT3N0Mx	IIB
P16	F	73	50	SCC	pT2aN0	IB
P17	M	78	45	SCC	pT2aN1	IIB
P18	F	51	28	ADC	pT2aN0	IB
P19	M	66	50	SCC	pT2aN0	IB
P20	M	72	17	SCC	pT2bN1	IIB
P21	M	77	60	ADC	pT2aPL2N0	IB

P22	M	67	80	SCC	pT1bN0	IA
P23	F	58	30	ADC	pT2aN0	IB
P24	F	59	20	ADC	pT2aN0	IB
P25	M	66	50	ADC	pT2aPL1N0Mx	IB
P26	M	45	30	SCC	pT3N1Mx	IIIA
P27	M	63	50	ADC	pT1aN0	IA
P28	M	65	50	SCC	pT2aN0	IB
P29	F	30	N/A	ADC	pT2aPL2N2	IIIA
P30	F	84	0	ADC	pT2b(PL3)N0	IIA
P31	M	73	30	SCC	pT3N1Mx	IIA
P32	F	58	20	SCC	pT3N2	IIIA
P33	M	62	40	ADC-LCNEC	pT1aN1	IIA
P34	F	84	0	ADC	pT2aN0	IB
P35	M	82	20	SCC	pT2aN0Mx	IB
P36	F	76	18	ADC	pT2b(PL2)N1	IIB
P37	F	57	25	ADC	pT2aN0Mx	IB
P38	M	63	50	SCC	pT3N1	IIIA
P39	M	63	N/A	ADC	pT2aN0	IB
P40	F	57	10	ADC	pT2aN0	IB
P41	F	N/A	N/A	ADC	pT2bN1	IIB
P42	F	69	0	ADC	pT2aN0	IB
P43	M	57	37	SCC	pT2bN1	IIB
P44	M	71	75	SCC	pT3N0Mx	IIB
P45	M	69	50	SCC	pT2bN0Mx	IB

P46	F	76	50	SCC	pT2bNx	IIA
P47	F	80	N/A	SCC	pT3N0Mx	IIB

859 SCC squamous cell carcinoma, ADC adenocarcinoma, LCNEC large cell neuroendocrine
860 carcinoma
861

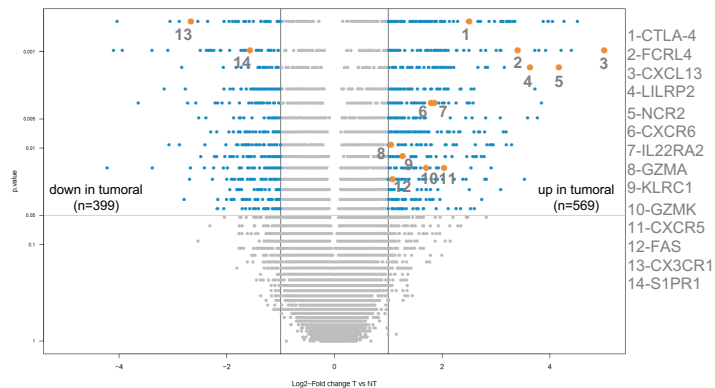
862 **Supplemental table 5: Correlation between intra-tumoral NK cells and B, mature DC or**
863 **CD8⁺ T cell density**

	NK cells	B cells	Mature DC	CD8 ⁺ T cells
NK cells	1*	0.08*	0.15*	0.025*

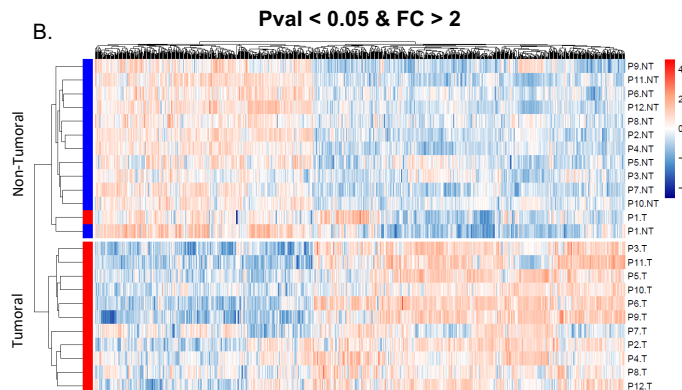
864 ***spearman correlation coefficient**

865

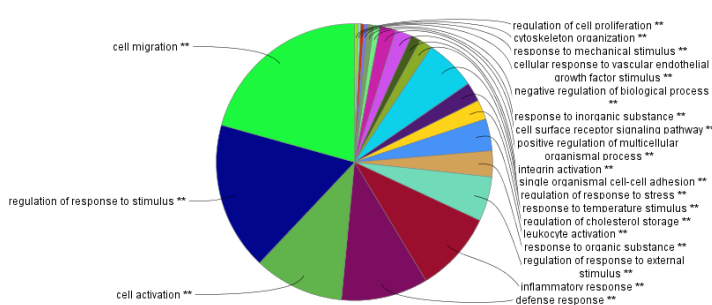
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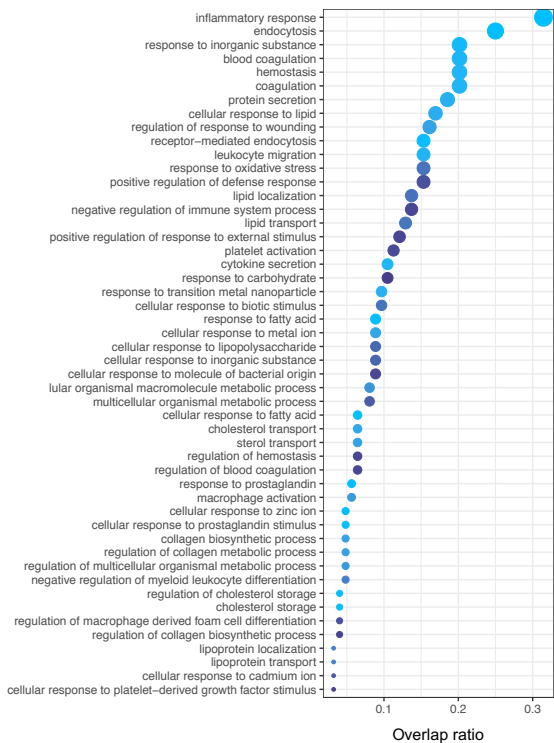


C.



D.

Down-regulated pathways



Up-regulated pathways

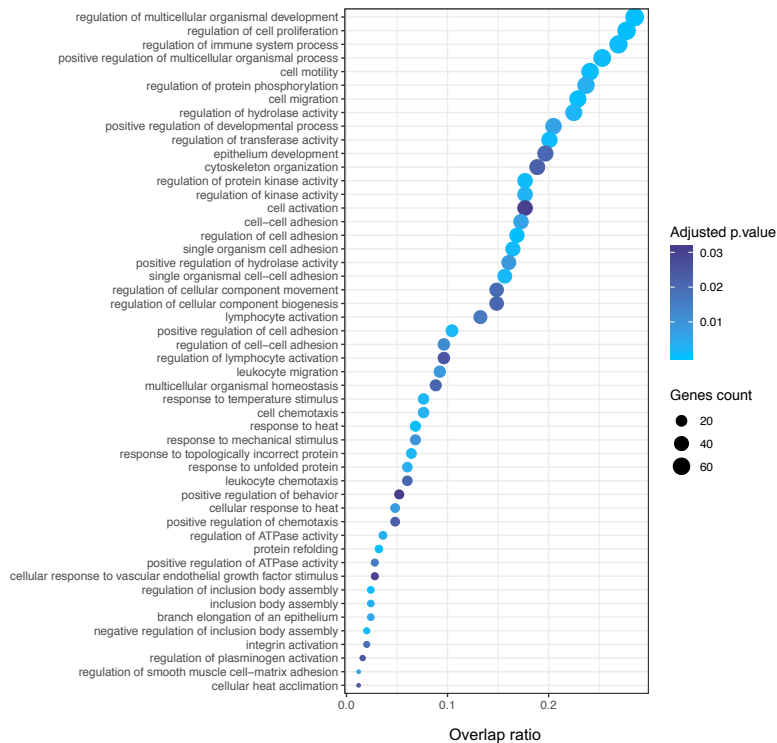


Fig. 1

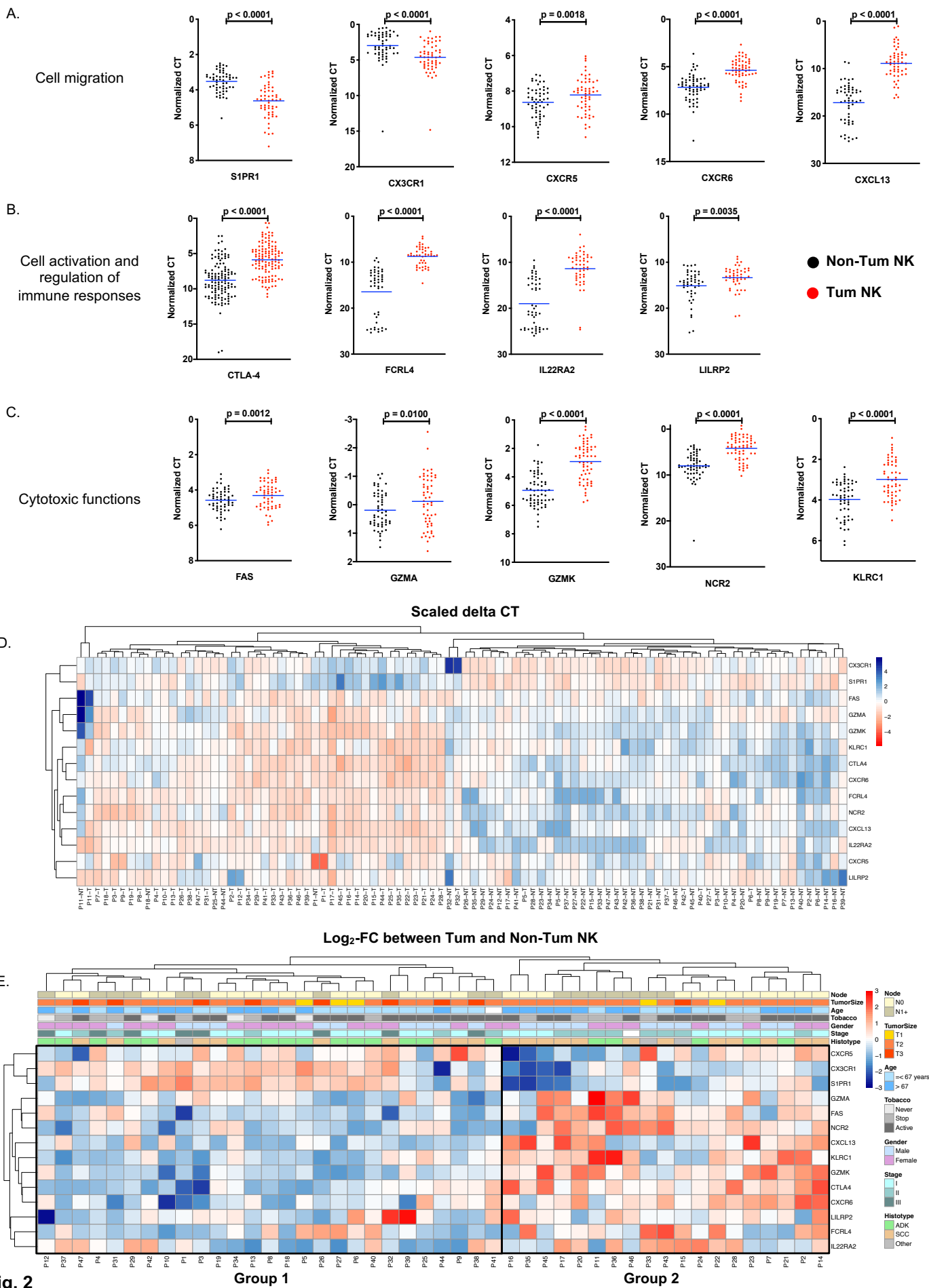
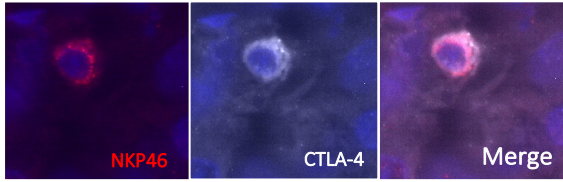
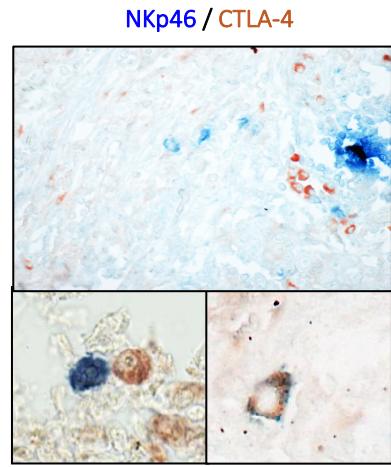


Fig. 2

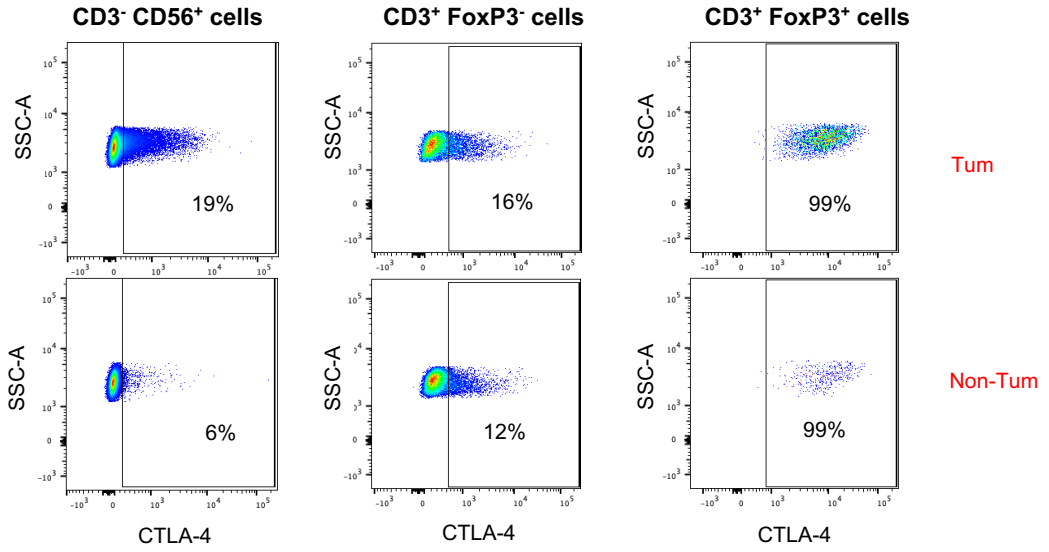
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B.



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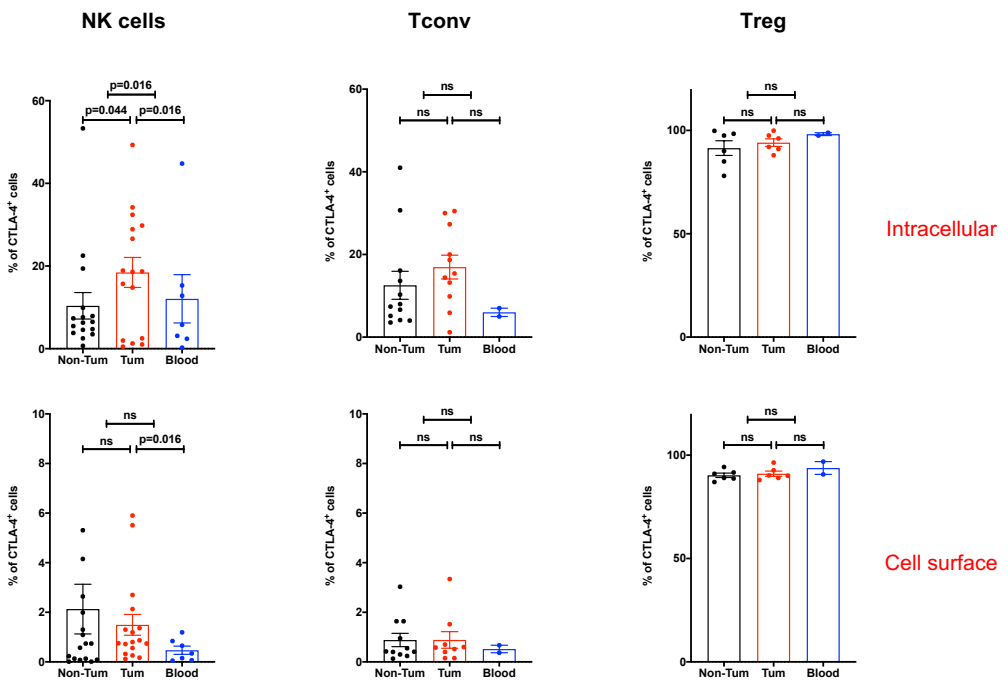
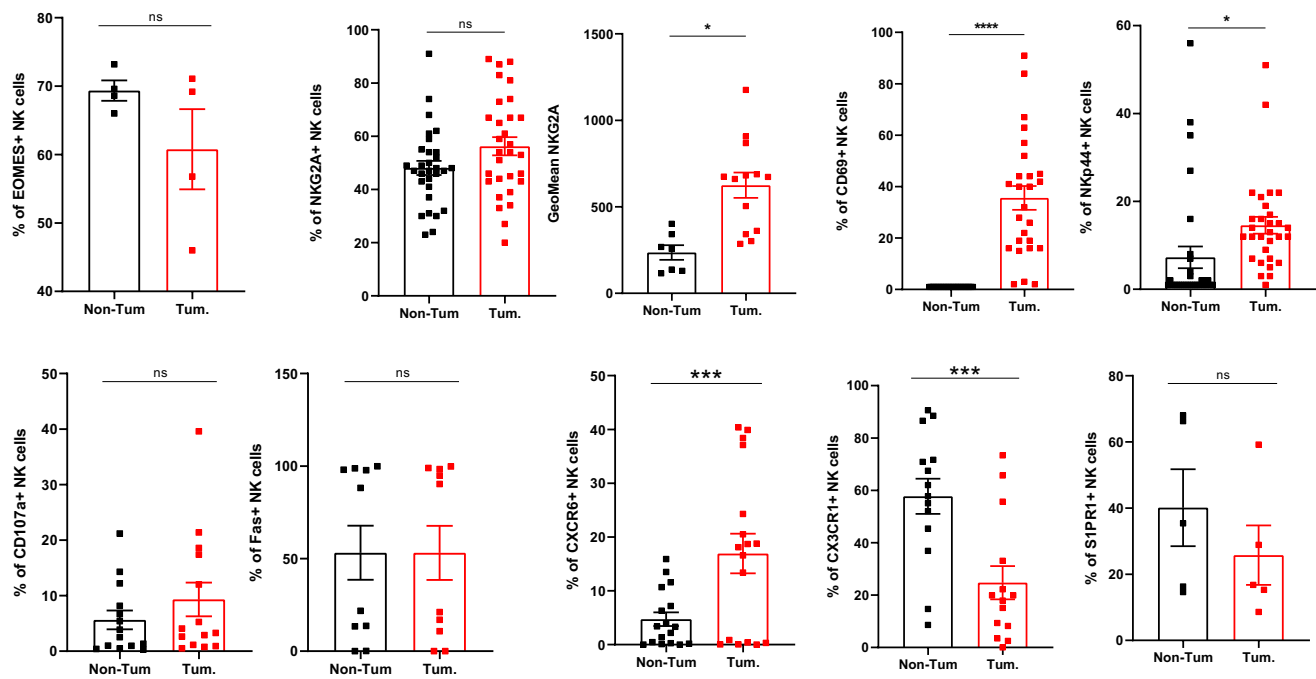
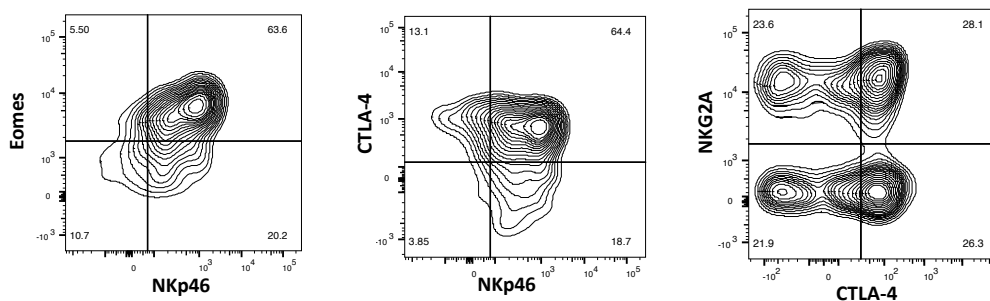


Fig. 3

A



B



C

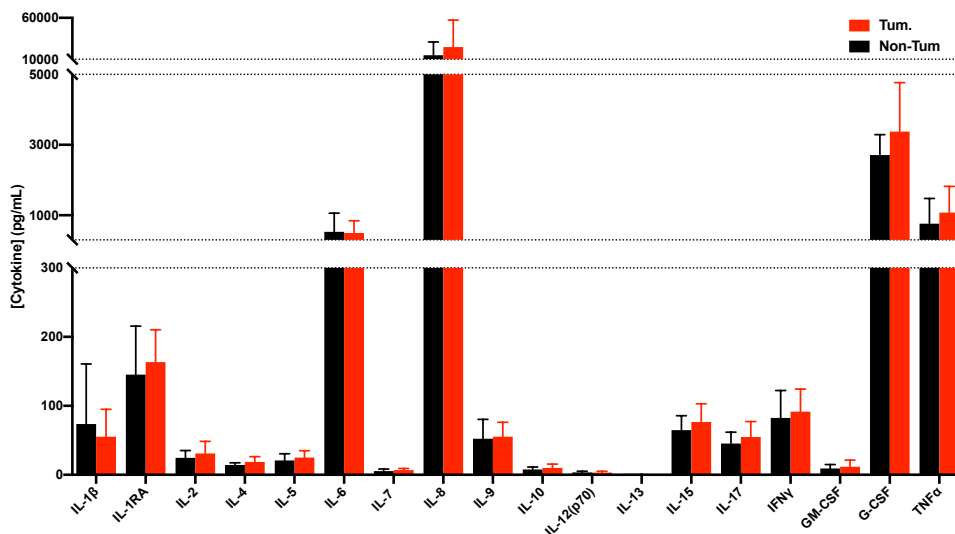


Fig. 4

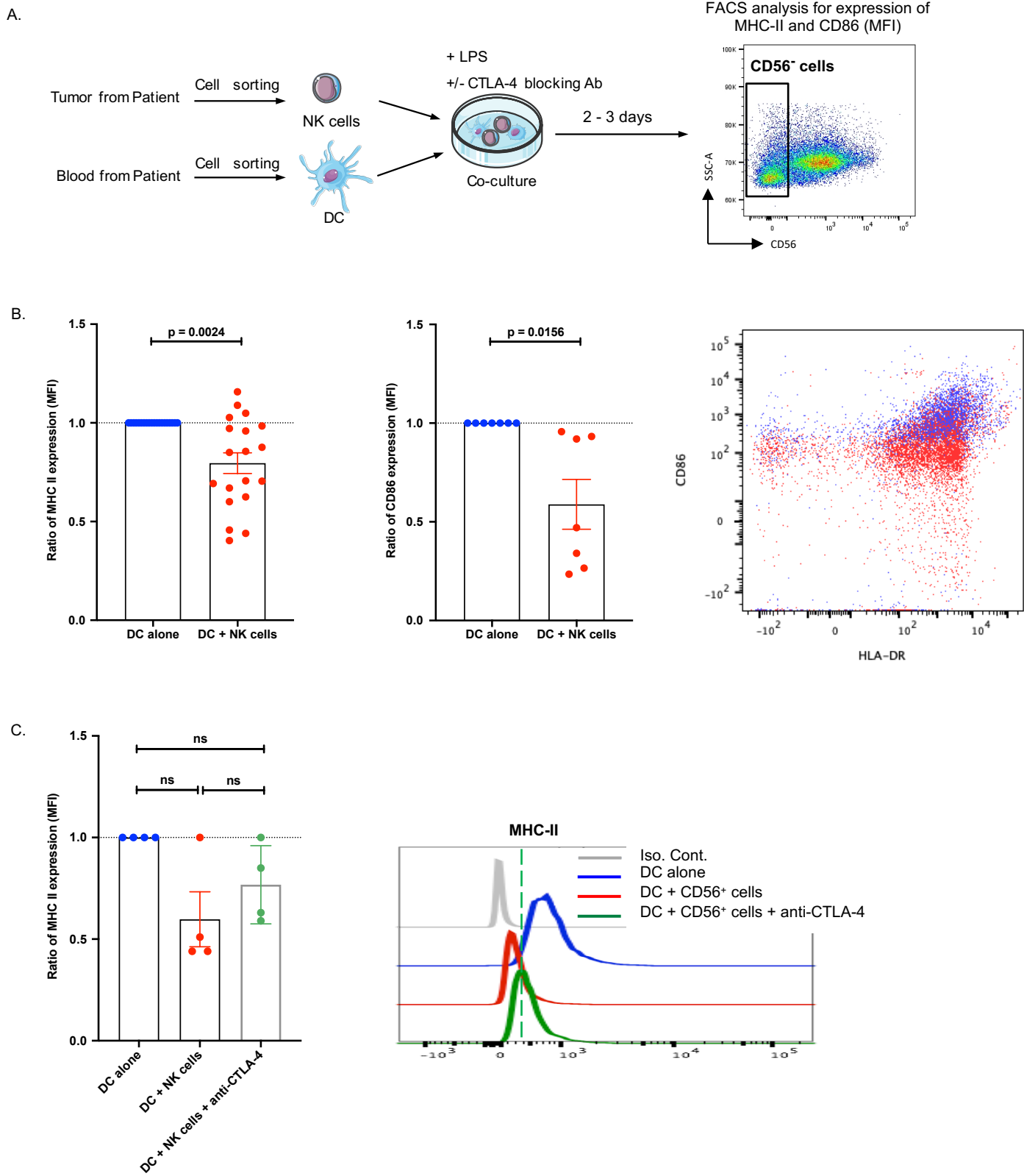


Fig. 5

Non-Tum NK

Tum NK

Non-Tum NK

Tum NK

Non-Tum NK

Tum NK

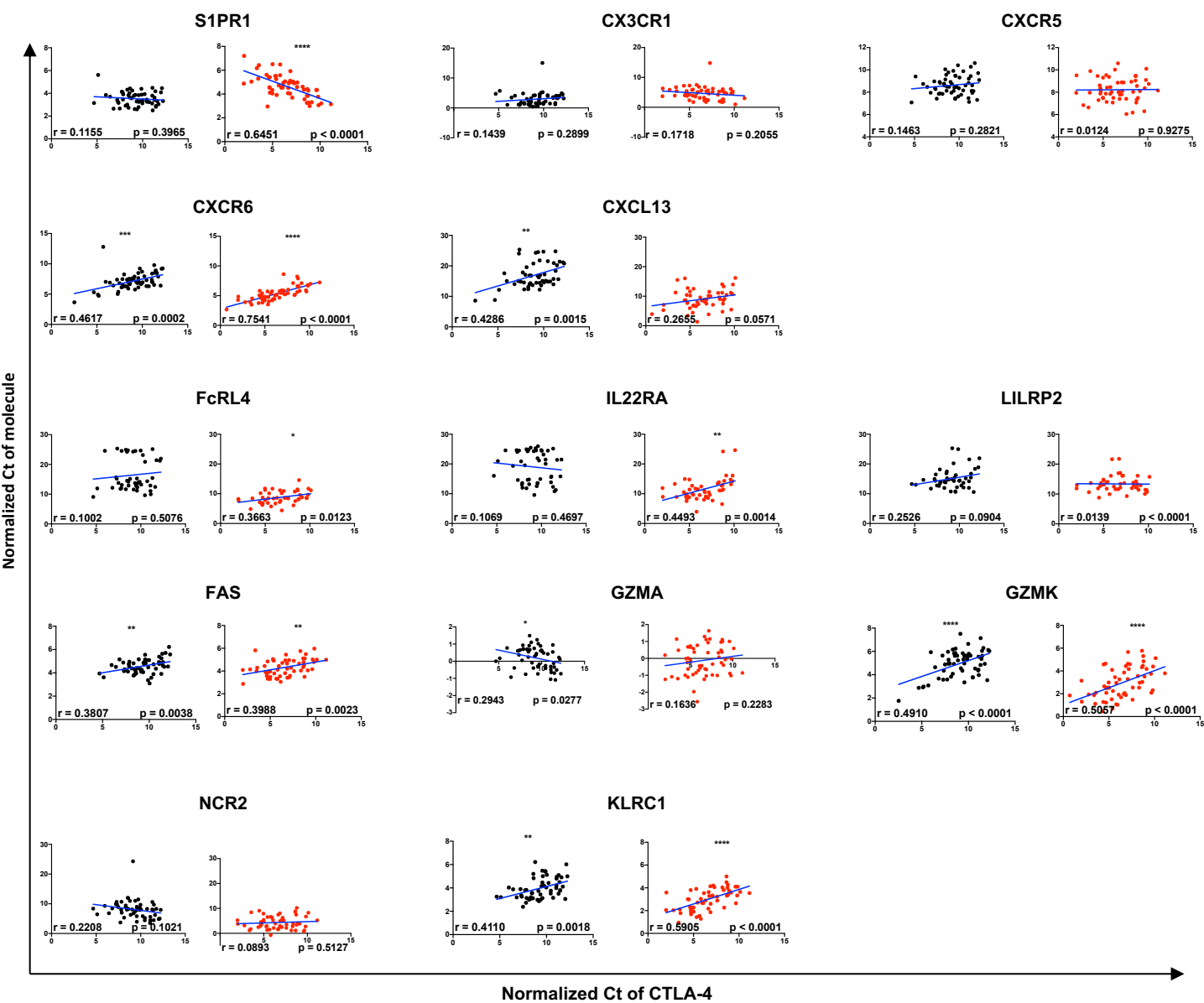


Fig. 6

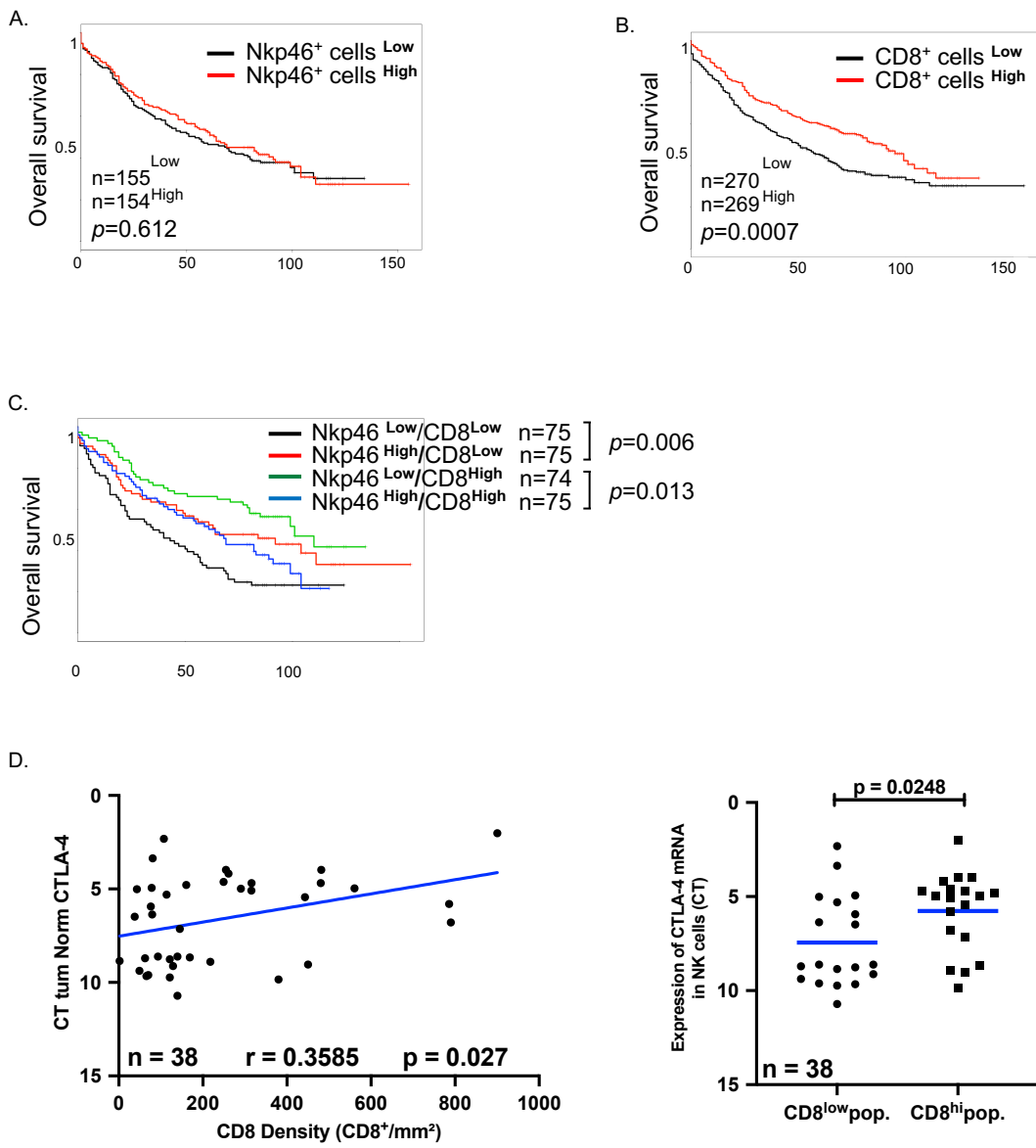
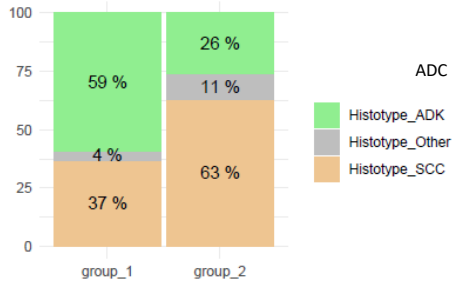
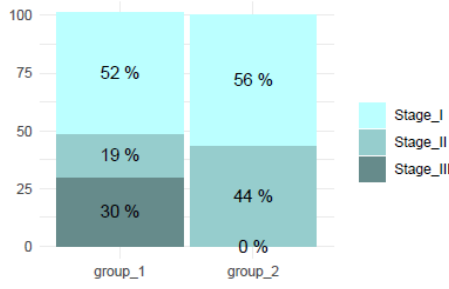


Fig. 7

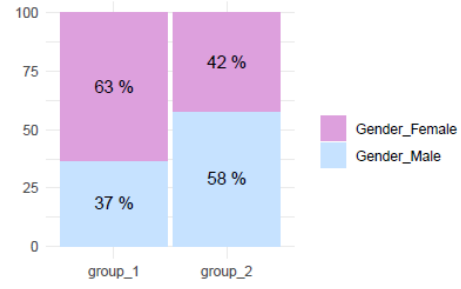
Histotype
fisher.test p = 0.0672



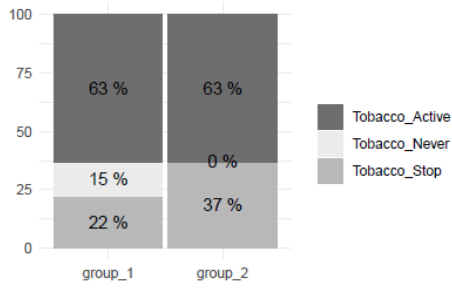
Stage
fisher.test p = 0.017



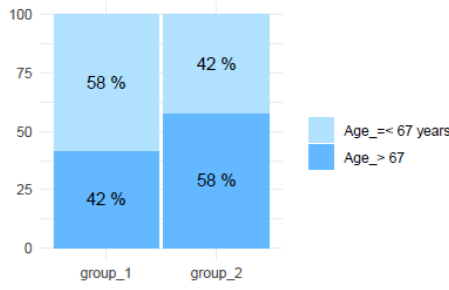
Gender
fisher.test p = 0.231



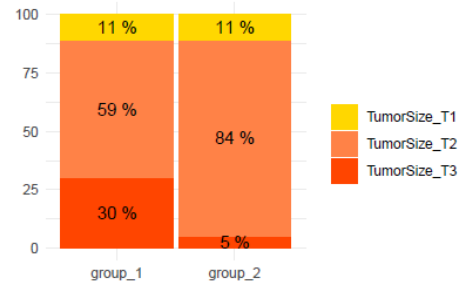
Tobacco
fisher.test p = 0.197



Age
fisher.test p = 0.373



TumorSize
fisher.test p = 0.105



Node
fisher.test p = 1

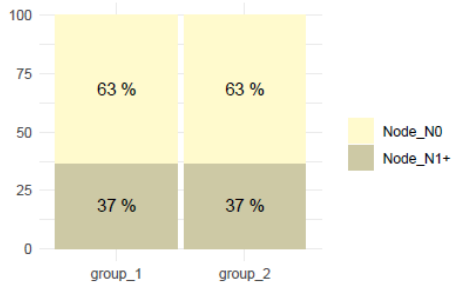


Fig.S1

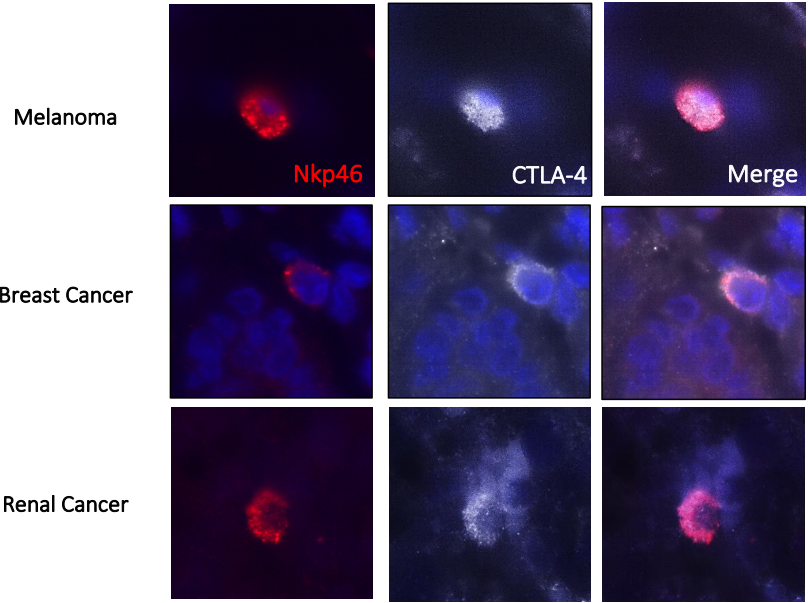
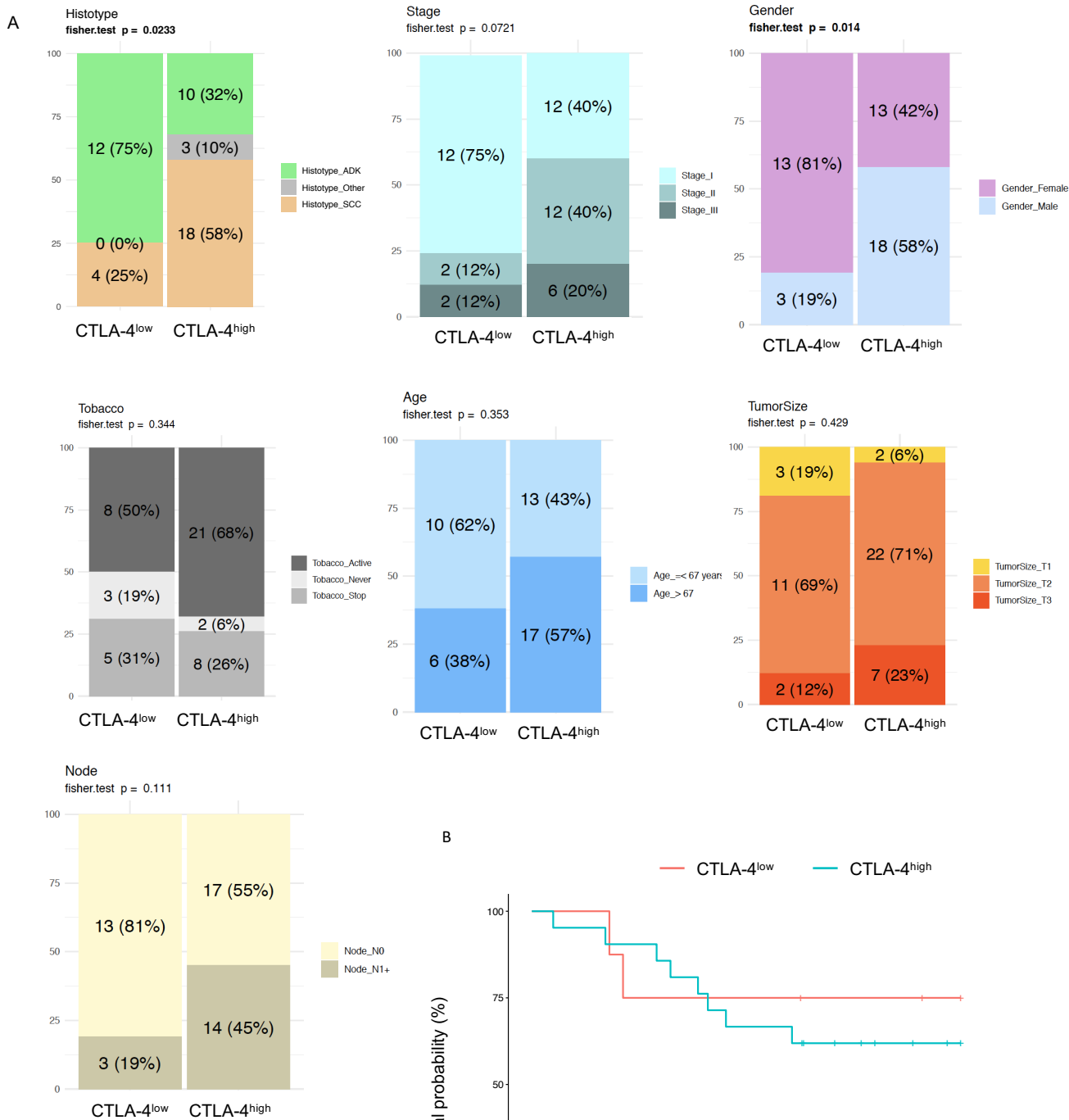


Fig.S2



B

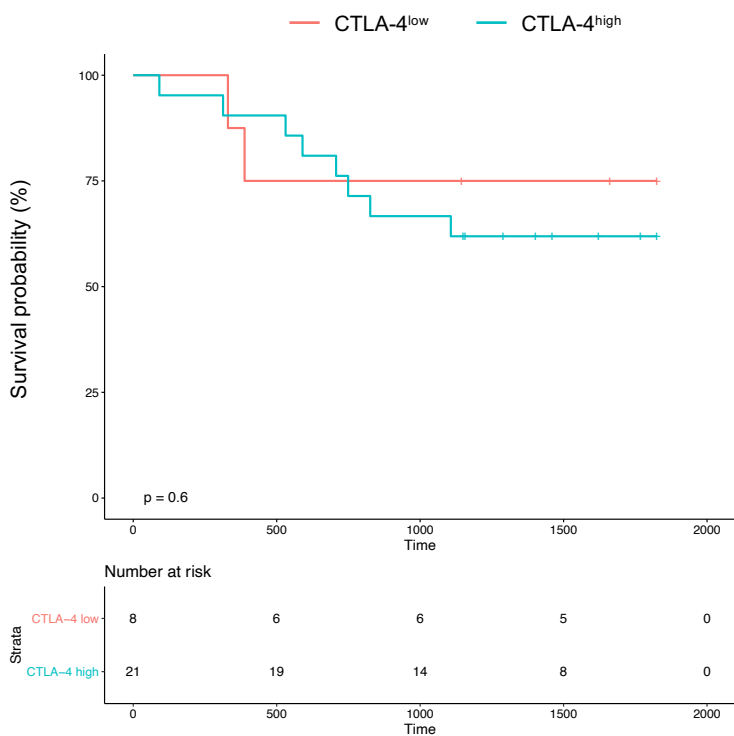


Fig.S3

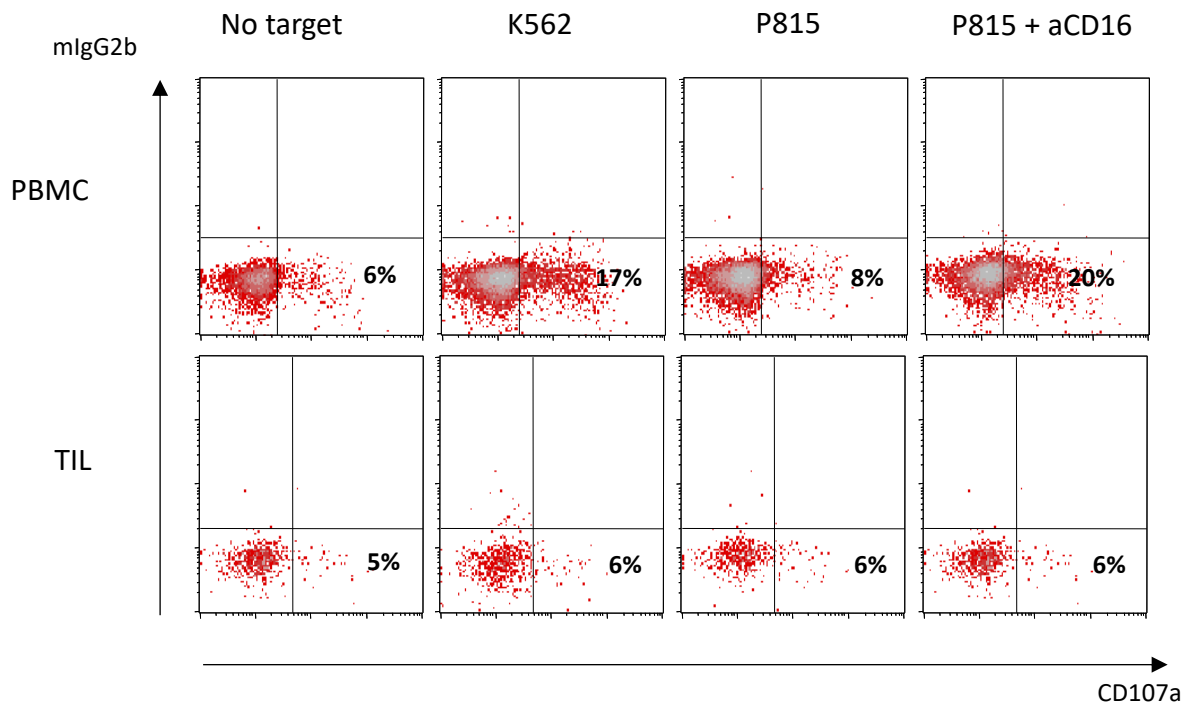
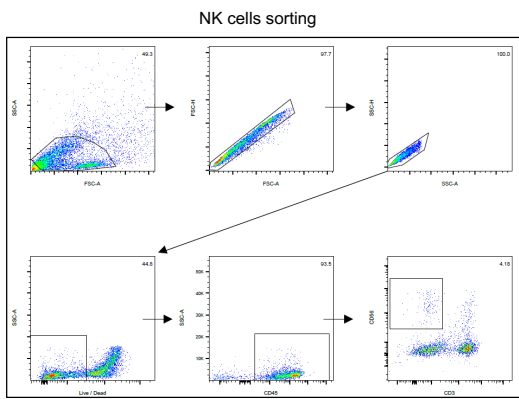
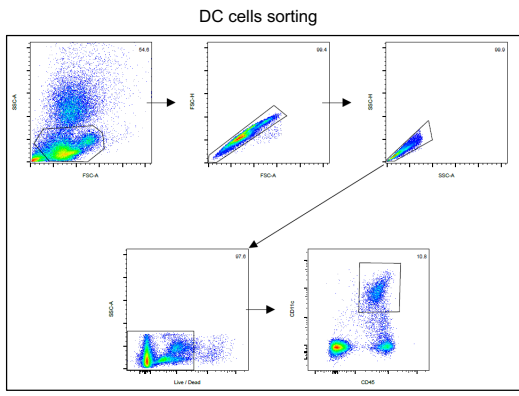


Fig.S4

Tumor from Patient



Blood from Patient



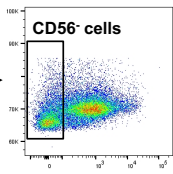
CD56⁺ cells

DC (CD11c⁺)

+ LPS
+/- CTLA-4 blocking Ab

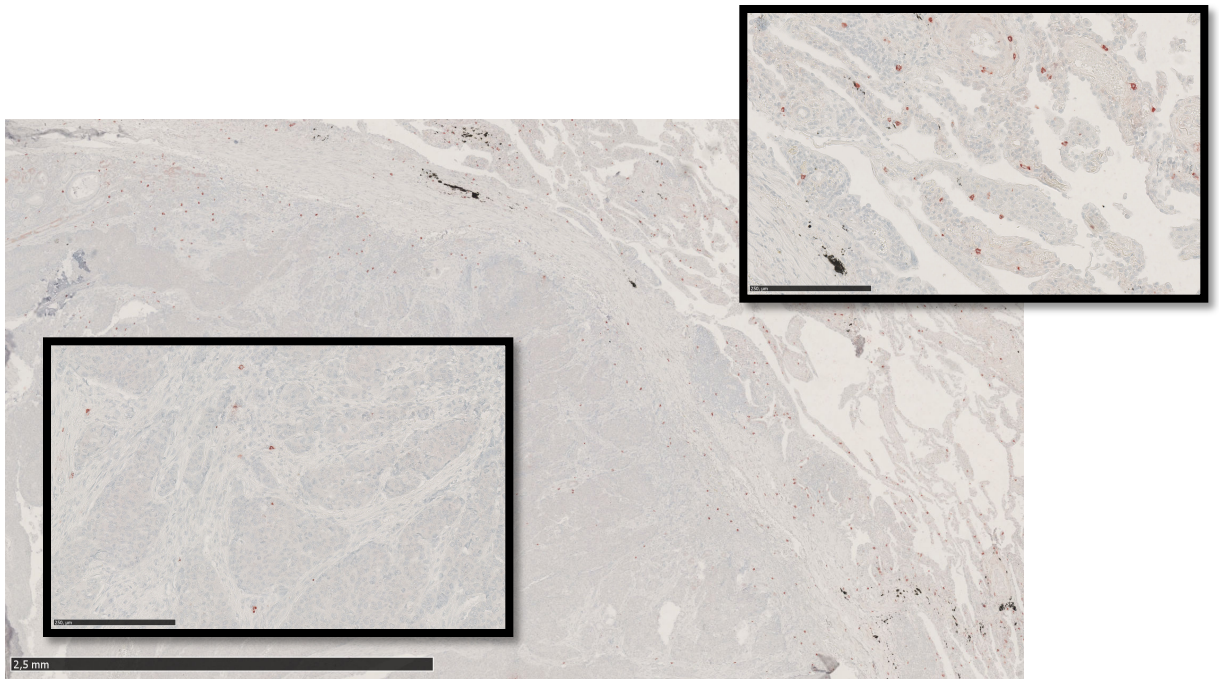
Co-culture

2 - 3 days



FACS analysis for expression of MHC-II and CD86 (MFI)

Fig.S5



Nkp46 staining

Fig.S6

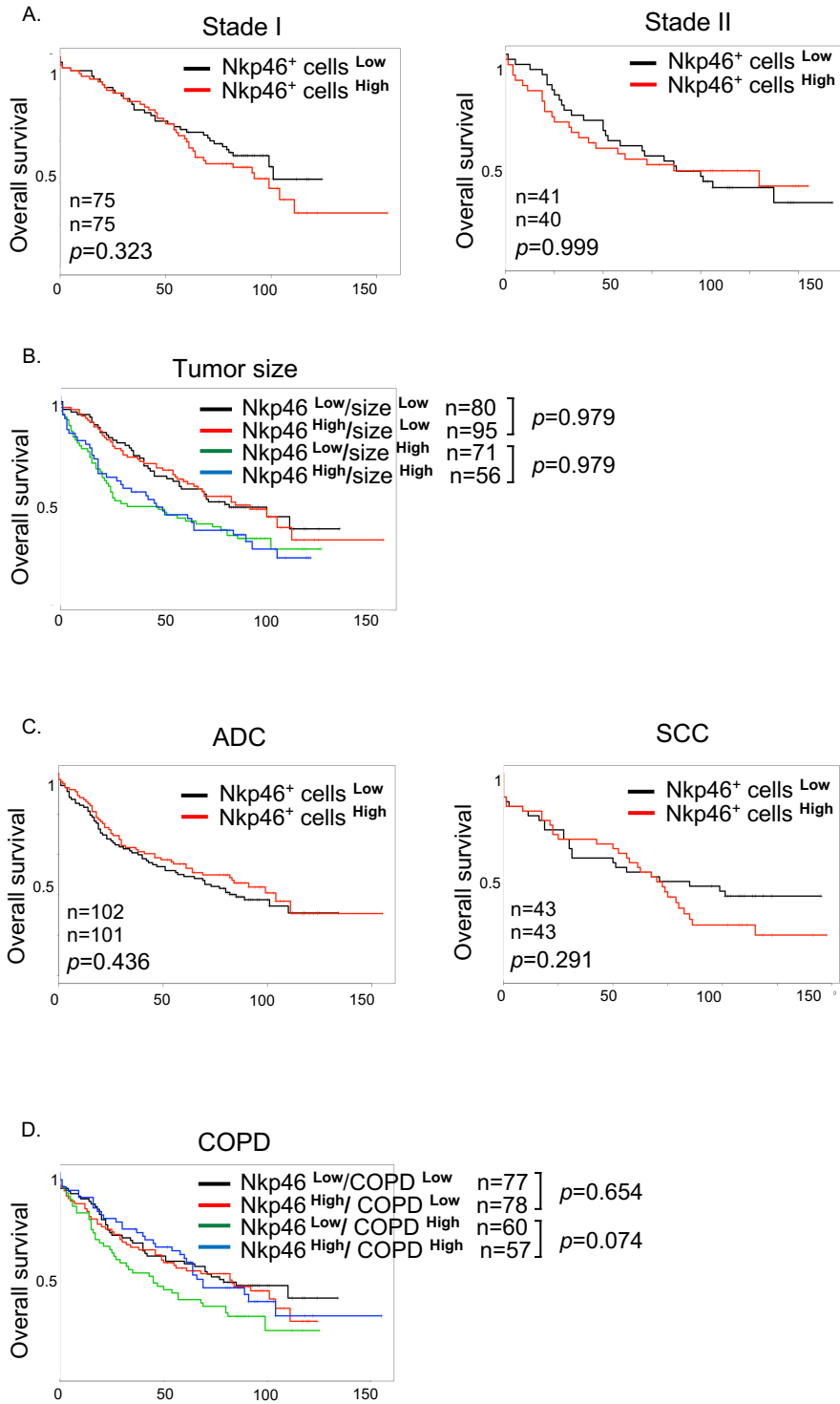


Fig.S7

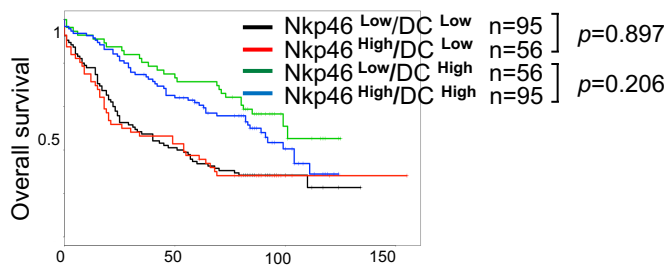
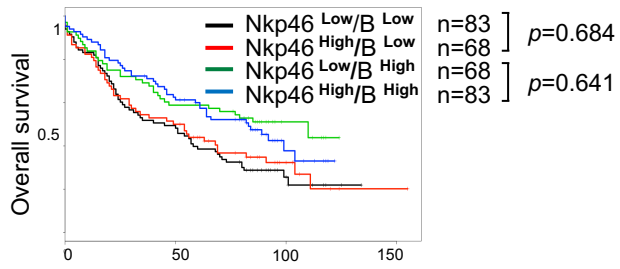
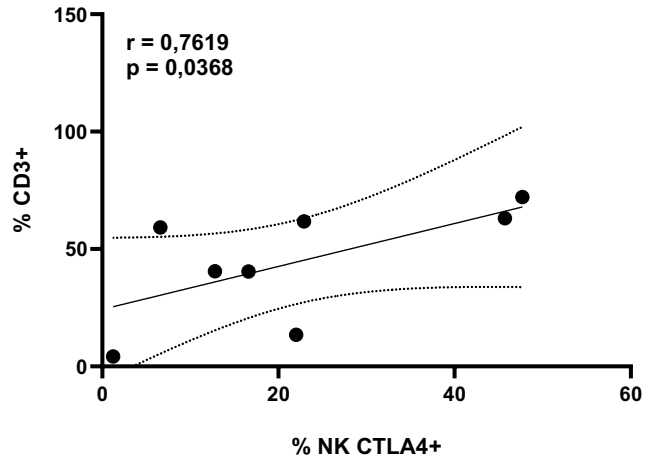


Fig.S8

A



B

