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4
5 **Myelodysplastic Syndrome associated *TET2* mutations affect NK cell function**
6 **and genome methylation**

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36

37 **Abstract:**

38 Myelodysplastic syndromes (MDS) are clonal hematopoietic disorders, representing high risk of
39 progression to acute myeloid leukaemia, and frequently associated to somatic mutations, notably in the
40 epigenetic regulator *TET2*. Natural Killer (NK) cells play a role in the anti-leukemic immune response via
41 their cytolytic activity.

42 Here we show that patients with MDS clones harbouring mutations in the *TET2* gene are characterised by
43 phenotypic defects in their circulating NK cells. Remarkably, NK cells and MDS clones from the same
44 patient share the *TET2* genotype, and the NK cells are characterised by increased methylation of genomic
45 DNA and reduced expression of Killer Immunoglobulin-like receptors (KIR), perforin, and TNF- α . *In*
46 *vitro* inhibition of *TET2* in NK cells of healthy donors reduces their cytotoxicity, supporting its critical
47 role in NK cell function. Conversely, NK cells from patients treated with azacytidine (#NCT02985190;
48 <https://clinicaltrials.gov/>) show increased KIR and cytolytic protein expression, and IFN- γ production.
49 Altogether, our findings show that, in addition to their oncogenic consequences in the myeloid cell
50 subsets, *TET2* mutations contribute to repressing NK-cell function in MDS patients.

51

52 **Introduction:**

53 Myelodysplastic Syndromes (MDS) are clonal myeloid malignancies characterized by ineffective
54 hematopoiesis and bone marrow (BM) failure leading to blood cytopenias and a high risk of
55 transformation to Acute Myeloid Leukaemia (AML)¹. MDS patients with high risk of AML progression
56 ("high-risk MDS" according to international prognostic scoring system) are treated with hypomethylating
57 agents (HMA) including azacitidine (AZA) and decitabine (DAC) which can increase overall survival and
58 reduce AML progression^{2,3}. Mutations in *TET2* and *IDH1/2* genes, involved in the epigenetic regulation
59 of gene transcription, are common genetic alterations found in MDS¹. Whereas *TET2* encodes for a
60 methylcytosine dioxygenase catalyzing the oxidation of the methyl group of 5-methylcytosine (5mC) to 5-
61 hydroxymethylcytosine (5-hmC), the isocitrate dehydrogenases (IDH) 1 and 2 control the production of
62 α -ketoglutarate (α KG), a metabolite required for TET2 activity⁴. Most *TET2* mutations described are loss
63 of function, and generally mutually exclusive with *IDH1/2* mutations. By contrast, *IDH1/2* mutations in
64 MDS lead to a new enzymatic activity that generates 2-hydroxyglutarate (2HG) in place of α KG and
65 inhibits TET2 activity⁵. Therefore, *TET2* and *IDH1/2* mutations lead to hypermethylation of the genomic
66 DNA in myeloid cells⁶. MDS patients carrying mutations in *TET2* are more likely to respond to HMA
67 treatment⁷, but mechanisms of action of HMA on MDS cells are still unclear and could involve BM
68 microenvironment, including immune (lymphocytes, myeloid-derived suppressive cells)⁸ and non-
69 immune partners (mesenchymal stromal cells)⁹.

70 Natural Killer (NK) lymphocytes are key players in the anti-leukemic response. NK cell targets malignant
71 cells by direct cytotoxicity through release of perforin, granzymes, engagement of death receptors, or
72 secreting cytokines like IFN- γ and TNF- α ^{10,11}. Their function is regulated by a balance between inhibitory
73 (KIR, NKG2A) and activating (NKp30, NKp46, DNAM-1, NKG2D) receptors. We and others have
74 shown defects in NK cell phenotype and function in AML and MDS patients^{12,13,14,15}, but mechanisms
75 leading to deficient NK cells are still unclear. Moreover, whether the enhanced sensitivity to HMA
76 treatment of MDS patients with *TET2/IDH^{MUT}* relies on the restoration of NK-cell activity is unknown.

77 In this study, we show MDS *TET2/IDH* mutations are carried by NK cells and associated with defects in
78 phenotype and function. Those perturbations rely on genome-wide hypermethylation of DNA, leading to
79 the reduced expression of key molecules for NK cell function. Finally, HMA treatment normalizes the
80 expression of NK cell related genes *in vitro* as well as *in vivo*. Altogether, our results established that
81 TET2 regulates NK-cell biology, and suggest that HMA treatments in *TET2/IDH* mutated patients could
82 also be beneficial by restoring the functionality of NK cells.

83 Results

84 *MDS/CMML patients with TET2/IDH mutations display a distinct NK cell*

85 *phenotype*

86 Blood NK cells from 33 MDS/CMML patients (myelodysplastic syndromes and chronic myelomonocytic
87 leukaemia, respectively, Supplementary Data 1) and 23 HD were phenotyped by flow-cytometry. Surface
88 expression of maturation markers (CD69, CD57, KLRG1), activating (NKp30, NKp46, DNAM-1,
89 NKG2D) and inhibitory (CD96, KIR2D, CD85j) receptors was investigated (Supplementary Table 1).

90 Small perturbations were observed on circulating NK cells of MDS/CMML individuals compared with
91 HD in the expression of some activating (DNAM-1, NKp46) and inhibitory (CD96) receptors.

92 Surprisingly, an almost 2-fold reduction of the killer immunoglobulin-like receptors (KIR) expression
93 was found on MDS patients NK cells compared to HD (31.2% and 54.8% respectively, $p < 0.01$,
94 Supplementary Table 1).

95 We asked whether patients with mutations affecting DNA methylation (i.e., mutations in *TET2*, *IDH1* or
96 *IDH2*) could exhibit specific NK-cell phenotype (Fig. 1, Supplementary Table 1). Interestingly, a deep
97 loss in the expression of KIRs with 2 Ig-like extracellular domains (KIR2D) was observed in patients
98 with *TET2/IDH* mutations compared to *TET2/IDH^{WT}* on blood ($n = 19$ vs 13 , median 11.9% vs. 56.5%,
99 $p < 0.001$, Fig. 1A) and BM NK cells ($n = 7$ vs 12 , median 24.2% vs 45.9% $p < 0.05$, Fig. 1B). In parallel,
100 the inhibitory receptor NKG2A was significantly increased on peripheral NK cells in *TET2/IDH^{MUT}*
101 patients compared to *TET2/IDH^{WT}* ($n = 15$ vs. 11 , 86.3% vs. 78.7%, $p = 0.025$, Fig. 1A, Supplementary
102 Table 2), underlying the balance between KIRs and NKG2A expression¹⁶. Of note, the KIR-positive T-
103 cell subset was not affected by the *TET2/IDH* mutational status in patients (Supplementary Fig. 1).

104 Importantly, censoring the few patients treated with either drugs to stimulate the erythro-thrombopoiesis
105 (EPO $n = 4$ out of 63 patients, TPO $n = 1$) or immunomodulating molecules (lenalidomide $n = 4$,
106 glucocorticoid $n = 4$) did not modify our observations demonstrating that treatments were not at the origin
107 of the decrease in KIR expression (Supplementary Fig. 2).

108 KIRs are encoded in a 15 genes locus with high variability in genes content across individuals¹⁷. To
109 investigate if *TET2/IDH* mutations could differentially affect *KIR* genes expression, the KIR locus was
110 genotyped for 22 MDS/CMML patients, and KIR surface expression was analyzed for genes highly
111 present with KIR2DL1, KIR2DL2/DL3, and KIR3DL1/DL2 specific mAbs (Fig. 1C-D, Supplementary
112 Fig. 3 and Table 3). We confirmed a specific reduction of KIR2DL1 (3.6% vs. 14.1%, $p<0.01$) and
113 KIR2DL2/DL3 (10.3% vs. 29.1%, $p<0.01$) on NK cells of *TET2/IDH^{MUT}* patients compared to WT (n=15
114 vs. n=11 respectively) (Fig. 1C-D, Supplementary Table 2). By contrast, KIR3DL1/DL2 expression was
115 not significantly modified by the *TET2/IDH* mutation status (Fig. 1D), despite the strong reduction
116 observed in some patients (Fig. 1C-D).

117 We next quantified perforin and granzyme B expression in NK cells of MDS/CMML patients as surrogate
118 markers of NK-cell cytolytic capacity. We observed a reduced percentage of perforin-positive NK cells in
119 *TET2/IDH^{MUT}* patients ($p<0.05$), whereas granzyme B expression was not affected (Fig. 1E). This loss of
120 intracellular perforin was correlated with reduced KIR (KIR2D and KIR3DL1/DS1) expression at the
121 NK-cell surface in *TET2/IDH^{MUT}* patients ($r=0.65$, $p=0.001$; Supplementary Fig. 4). Indeed, the loss of
122 perforin expression was mainly observed in KIR⁻ NK cells whereas KIR⁺ NK-cells were not affected
123 (Supplementary Fig. 5), suggesting a co-regulation of KIR and perforin expressions.

124 Then, we reasoned that TET2 function may be the main parameter modifying the NK cell phenotype.
125 Therefore, we analyzed the performance of KIR2D expression as a classifier of patients with or without
126 *TET2* mutations by calculating a receiver operation characteristic (ROC) curve using KIR2D expression
127 on circulating NK cells in *TET2/IDH^{WT}* (n=19) and *TET2^{MUT}* (n=17) patients. A maximum area under the
128 curve (AUC) equal to 0.89 predicted that a threshold of 25% of KIR2D⁺ NK cells allowed classifying
129 mutated and not-mutated patients with a specificity of 95% and a sensitivity of 77% ($p<0.0001$, Fig. 1F).
130 Studying KIR2D expression separately in *TET2^{MUT}* and *IDH1/2^{MUT}* patients, we observed that 4 out of 5
131 patients mutated for either *IDH1* or *IDH2* showed a comparable decrease of KIR2D expression than
132 *TET2^{MUT}* individuals (Fig. 1G). This observation confirmed that *IDH1/2* mutations could phenocopy
133 *TET2* mutations at least when considering NK cell phenotype.

134 Altogether, our results unraveled a strong relationship between the presence of mutations in *TET2* and
135 *IDH1/2* genes in MDS/CMML patients and the alterations in NK-cell phenotype. To gain further insights
136 into the molecular mechanisms accounting for this loss of NK cell function, we focused our study on
137 *TET2* mutated patients, excluding the *IDH* mutated patients from our cohort, and interrogated whether
138 NK and MDS cells could share *TET2* mutations.

139

140 ***TET2 mutations observed in MDS/CMML patients at diagnosis can be found in***
141 ***blood NK-cells***

142 All MDS/CMML patients recruited in this study were analyzed at diagnosis of the disease for the
143 presence of somatic mutations in 80 genes usually found mutated in myeloid malignancies
144 (Supplementary Table 4). As expected, *TET2*^{MUT} and *TET2/IDH*^{WT} patients could show mutations in
145 additional genes frequently observed in MDS/CMML¹, including *SRSF2* (29% and 3% respectively),
146 *SF3B1* (18% and 20% respectively) or *ASXL1* (18% and 17% respectively) (Supplementary Fig. 6 and
147 Supplementary Table 5). Of note, *TET2*^{MUT} patients presented a mean of 2 mutations in *TET2* (range 1-4
148 mutations).

149 Thus, we analyzed whether MDS-associated mutations were present in NK cells. PBMC of 10 MDS
150 patients diagnosed with mutations in *TET2* among other genes were sorted for NK cells. Nine out of 10
151 patients showed some mutations in NK cells shared with the blood or BM white mononuclear cell
152 (WMC) samples at diagnosis, including in *TET2* and other genes (Supplementary Table 6). The variant
153 allele frequencies (VAF) for *TET2* mutations were not significantly different in NK cells (median=11%,
154 range 0 – 54%) compared to WMC (median=32%, range 2 - 49%, Fig. 2A). By contrast, consistent with
155 their unchanged phenotype previously observed, MDS-associated mutations were rarely found in T cells
156 (Supplementary Table 6, Supplementary Fig. 7A), and with a VAF below 7% when present (Fig. 2A).
157 Importantly, whereas 7 patients presented a strict concordance in the mutation profiles between analyses

158 on WMC and NK cells (Fig. 2B and Supplementary Table 6), 3 patients (MUT19, MUT22, MUT31)
159 showed mutations in NK cells unobserved in WMC (Fig. 2B).
160 Furthermore, we observed a strong correlation between the expression of KIR2D receptor and the VAF of
161 *TET2* mutations in NK cells (Fig. 2C, $R^2=0.88$, $p<0.0001$). In the 10 patients analyzed by NGS, those
162 with a percentage of KIR2D⁺ NK-cells below 25% showed a *TET2* mutation VAF higher than 20% in the
163 WMC at diagnosis and higher or equal to 34% in the NK-cell subset. To confirm this observation, we
164 selected 5 patients with sufficient amount of KIR2D⁺ and KIR2D⁻ NK cells and performed a NGS
165 analysis on cells sorted according to the expression of the KIR2D and KIR3DL1/DL2 (hereafter designed
166 as KIR⁺ or KIR⁻ NK cells). Importantly, 4 out of 5 patients showed a high percentage of KIR2D⁺ cells,
167 superior to the threshold of 25% we have calculated previously (Fig. 1F). As expected, patients showed
168 higher *TET2*^{MUT} VAF in the KIR⁻ (median= 18.2%, range 6.1 – 42.1%) than the KIR⁺ cells (median= 5%,
169 range 0 – 48%, $p<0.01$, Fig. 2D, Supplementary Fig. 7B). In addition, KIR⁻ NK cell subset showed *TET2*
170 mutations leading to non-functional TET2 proteins in all patients tested. In detail, patients MUT08,
171 MUT19 and MUT23 presented frameshift mutations, leading to premature stop-codon, with VAFs
172 ranging from 22% to 37% in KIR⁻ cells, as compared to a range from 2% to 15% in the KIR⁺ cells.
173 Patients MUT24 and MUT32 showed missense mutations in the TET2 catalytic domain, with VAFs
174 ranging from 11% to 39% in the KIR⁻ cells as compared to 1% to 7% in the KIR⁺ cells. Moreover, two
175 patients (MUT19 and MUT23) showed a shared nonsense mutation at position 548 only present in the
176 KIR⁻ NK cells. Moreover, mutations in other genes were also enriched in the KIR⁻ population
177 (Supplementary Table 6). Altogether, these observations suggested that *TET2* mutations can be found in
178 NK cells and are related to reduced KIR expression in *TET2*^{MUT} MDS/CMML patients.

179

180 ***TET2 directly binds to and regulates KIR locus expression in NK cells***

181 To evaluate the role of TET2 in regulating KIR expression, we first analyzed the type and the positioning
182 of the *TET2* mutations found in MDS/CMML patients with a reduced percentage of KIR⁺ NK cells

183 (below 25 %) at diagnosis (Fig. 3A). This analysis revealed that all but one patient showed either
184 nonsense or frameshift mutations leading to a premature stop-codon and the absence of a functional TET2
185 protein, extending the observation made in KIR⁻ NK cells (Supplementary Table 6). The only exception
186 was the patient MUT30 who presented a missense mutation p.R1261H (c.G3782A substitution). This
187 mutation has already been described in the catalogue of somatic mutations in cancer (COSMIC)
188 database¹⁸ with a functional consequence prediction (Functional Analysis through Hidden Markov
189 Models FATHMM) score equal to 0.99¹⁹, forerunning a highly significant functional impact on the TET2
190 protein.

191 Therefore, we asked whether the inhibition of the enzymatic function of TET2 in NK cells could also lead
192 to a significant reduction of the KIR2D proteins expression. We treated NK cells from healthy individuals
193 with dimethylxalyl glycine (DMOG), an α -ketoglutarate (α -KG) analog which inhibits α -KG-dependent
194 enzymes including TET2²⁰. In line with our results in *TET2*^{MUT} MDS patients, TET2 inhibition in DMOG
195 treatment significantly reduced the percentage of KIR2D⁺ NK cells (p<0.001, Fig. 3B), suggesting a role
196 for TET2 in modulating the KIR expression on NK cells or the KIR2D⁺ subset proliferation.

197 We then sought to determine whether TET2 had a direct impact on *KIR* genes expression. We performed
198 chromatin immunoprecipitation (ChIP) followed by real time qPCR analysis on sorted NK cells from
199 control PBMC with a TET2-specific monoclonal antibody (mAb), and evaluated the presence of the
200 proximal promoter regions of *KIR2DL1* and *KIR2DL2/3* genes. We also tested TET2 binding site onto a
201 predicted *cis*-regulatory element (CRE) localized upstream of the KIR locus (chr19:54,688,519-
202 54,702,287)²¹. We found that tested KIR promoters and CRE sequences were enriched in TET2-specific
203 ChIP fragments (Fig. 3C).

204 To substantiate the role of TET2 in driving KIR expression, we generated a luciferase reporter vector
205 containing 210 (-147; +63) nucleotides of the predicted KIR2DL1 promoter associated with TET2 ChIP
206 assay experiment. Co-transfection of this reporter with a TET2 overexpression vector into HEK293T cell
207 line is associated with an increase of luciferase activity increased by 2.3-fold compared to the empty
208 vector (Fig. 3D). To corroborate this data, the TET2 expressing Jurkat lymphoid cell line was transfected

209 with the KIR2DL1-luciferase reporter vector and then treated with ascorbic acid (L-AA), a catalyst of the
 210 TET2 hydroxylation activity of 5-methylcytosine in DNA and an already-known enhancer of the *KIR*
 211 locus demethylation^{22,23}. We observed a significant increase of the luciferase signal by 2-fold in the
 212 treated condition compared to control, demonstrating that L-AA enhanced TET2 binding and activity onto
 213 the *KIR* promoter sequence (Fig. 3E).

214 Based on these observations, we anticipated that NK cells with low KIR expression and carrying *TET2*
 215 mutations could show perturbations in the methylation profile of the *KIR* locus. Therefore, we performed
 216 reduced representation bisulfite sequencing (RRBS) analysis on NK-cells sorted from *TET2*^{WT} and
 217 *TET2*^{MUT} MDS/CMML patients (n=1 and n=4 respectively, Fig. 3F-H) to assess their DNA methylation
 218 pattern. Three *TET2*^{MUT} MDS/CMML patients were chosen based on a KIR2D expression on NK cells
 219 below 25%, and a *TET2* mutation VAF superior to 40% in the cell bulk at diagnosis (*TET2*^{MUT} KIR^{LOW}
 220 patients: MUT22, MUT27, MUT33). One *TET2*^{MUT} patient chosen because of a KIR2D expression on NK
 221 cells at 90% (*TET2*^{MUT} KIR^{HIGH} patient: MUT14) and one *TET2*^{WT} patient (*TET2*^{WT} KIR^{HIGH} patient:
 222 WT13) were used as controls.

223 The analysis of the CpG islands in the CRE sequence and the *KIR2DL1* gene showed increased
 224 methylation in the three *TET2*^{MUT} KIR^{LOW} patients compared to the *TET2*^{MUT} KIR^{HIGH} and the *TET2*^{WT}
 225 ones (Fig. 3G-H).

226 Altogether, these findings demonstrate the targeting of the *KIR* locus by TET2 and strongly suggest that
 227 loss of TET2 leads to the hypermethylation of the *KIR* locus in NK cells.

228

229 ***TET2 mutations lead to hypermethylation of key genes for cytotoxicity and*** 230 ***cytokine release by NK cell***

231 The analysis of DNA methylation in TET2-deficient mouse models showed widespread DNA
 232 hypermethylation, including in enhancer elements²⁴. RRBS analyses of NK cells from *TET2*^{MUT} KIR^{LOW}
 233 MDS/CMML patients confirmed that genomic DNA was more methylated in NK cells with a high VAF

234 of $TET2^{MUT}$ and a reduced KIR2D cell surface expression compared to $TET2^{WT}$ NK cells (Fig. 4A). We
235 then asked whether *TET2* mutations in NK cells could interfere with the methylation and expression of
236 genes relevant for NK cell function. RRBS analyses of NK cells from $TET2^{MUT}$ KIR^{LOW} MDS/CMML
237 patients showed a significant hypermethylation compared to KIR^{HIGH} patients with 11,594
238 hypermethylated CpG sites among the 15,827 differentially methylated sites (methylation difference \geq
239 20%, $p \leq 0.05$) (Fig. 4B, upper panel, Supplementary Data 2). Among the 97 genes found
240 hypermethylated in $TET2^{MUT}$ KIR^{LOW} patients compared to KIR^{HIGH} patients (Fig. 4B, lower panel,
241 Supplementary Data 3), methylation of *TYROBP* (coding for DAP12) and *TNF* was predicted to
242 significantly inhibit the NK cell mediated cytotoxicity pathway as defined by the KEGG database
243 ($p < 0.05$, KEGG pathway hsa04650)²⁵. Reports suggested variations in methylation by TET2 of the CpG
244 loci according to their position regarding the gene body and the respective regulatory elements^{24,26,27}. We
245 analyzed the percentage of 5hmC in the gene bodies and 10kb upstream or downstream on the whole
246 genome²⁶. Then, mean of methylation has been calculated for genes involved in pathways of interest for
247 NK cell biology. Percentage of 5hmC trends up in $TET2^{MUT}$ KIR^{LOW} NK cells compared to KIR^{HIGH}
248 samples in coding and flanking regions of genes implicated in the cytokine-cytokine receptor interactions
249 (KEGG pathway reference hsa04060), the JAK-STAT signalling pathway (hsa04630), the TNF signalling
250 pathway (hsa04668) and the NK cell mediated cytotoxicity (hsa04650) (Fig. 4C), indicating a decrease of
251 these cell functions. Interestingly, the increase of methylation affected the gene bodies as well as their
252 flanking regions, suggesting a differential role for TET2 in NK cells as compared to myeloid cells in
253 which TET2 mainly regulates enhancers^{24,27}.

254 We then looked at the methylation of *IFNG*, *TNF* and *PRF1*, three genes determinant in NK cell anti-
255 leukemic activity²⁸. RRBS analysis showed hypermethylated CpG islands in the three loci in the
256 $TET2^{MUT}$ KIR^{LOW} patients compared to $TET2^{MUT}$ KIR^{HIGH} or the $TET2^{WT}$ patients, suggesting a reduced
257 transcription of these genes (Fig. 5A).

258 To evaluate the functional relevance of DNA methylation in controlling the regulatory regions of these
259 genes, a luciferase reporter assay was performed in the HEK293T cell line (Fig. 5B). Regions of interest,

260 showing differences in their methylation profile between $TET2^{MUT}KIR^{LOW}$ and $TET2^{MUT}KIR^{HIGH}$ NK cells,
261 were cloned into a CpG-free vector upstream of the luciferase gene, without minimal promoter to
262 determine the functional activity of the promoter. The constructs were then methylated *in vitro*
263 specifically onto the insert sequences. Methylation resulted in significant decreased luciferase activity
264 compared to the unmodified construct, suggesting that the promoter function of the region of interest is
265 dampened upon DNA methylation (Fig. 5B). In parallel, the decreased expression of *TNF* and *INFG* in
266 presence of *TET2* mutations was confirmed by qRT-PCR in KIR2D⁻ NK cell sorted from $TET2/IDH^{WT}$
267 and $TET2^{MUT}$ patients (Fig. 5C).

268 Altogether, these results showed that key molecules for NK-cell function were under the control of the
269 TET2 demethylation pathway, and that their transcription was reduced in $TET2^{MUT}$ NK cells.
270 Consequently, we asked whether TET2 inhibition could impair NK cell function. NK cells from HD were
271 treated *in vitro* with DMOG and then co-cultured with the K562 cell line. As expected, K562 killing by
272 DMOG-treated NK-cells was significantly reduced demonstrating that the NK-cell cytotoxicity pathway
273 was, at least in part, under the control of TET2 ($p < 0.05$, Fig. 5D).

274

275 ***Hypomethylating agents rescue KIR expression on MDS NK cells***

276 To evaluate whether demethylation could restore KIR expression on NK cells, PBMC from $TET2^{MUT}$
277 patients were cultured *in vitro* in presence of IL-2 and with or without AZA or DAC for 5 days. Then,
278 KIR2D expression on NK cells was assessed by flow cytometry (Fig. 6A). Whereas AZA showed only a
279 trend, DAC significantly restored the KIR expression on treated NK cells ($p = 0.07$ and $p < 0.001$
280 respectively). Further, L-AA was used either alone or in combination with DAC to treat PBMC of
281 $TET2^{MUT}$ MDS/CMML patients. L-AA associated with DAC significantly increased KIR expression on
282 NK cells compared with DAC alone ($p < 0.05$). Of note, NK cells from HD and $TET2^{WT}$ patients also
283 increased KIR expression in response to HMA with or without AA (Supplementary Fig.8).

284 To confirm *in vivo* our observations, NK cells from MDS patients recruited in the clinical trial
285 NCT02985190 were analyzed for their capacity to produce IFN- γ and to restore their phenotype, before
286 and after 3 or 6 cycles of treatment with AZA. NCT02985190 clinical trial is a phase II study of efficacy
287 and tolerance of AZA in MDS patients with concomitant systemic auto-immune and inflammatory
288 disorders (SAID), for which the primary outcome measure was the overall response rate of MDS and
289 SAID²⁹. Functional experiments showed an increase in the production of INF- γ by NK cells stimulated *in*
290 *vitro* by a mixture of PMA and ionomycin in patients after 3 cycles of treatment with AZA (n=6, Fig.
291 6B). Moreover, KIR2D, perforin and granzyme B expressions, quantified by flow-cytometry, were
292 significantly increased in this group of patients after 6 cycles of treatment (n=9, Fig.6C). Importantly,
293 despite the absence of significance due to the small number of patients, this effect of AZA on NK cell
294 phenotype and function was similar in *TET2^{WT}* and *TET2^{MUT}* patients.

295 Taken as a whole, these findings showed that demethylation mediated by TET2 is pivotal in regulating
296 NK cell biology, including the expression of KIR and functional proteins, and, more importantly, that
297 treatment by HMA could normalize the hypermethylated profile harboured by NK cells in MDS patients.

298 **Discussion**

299 In this work, we described the epigenetic, phenotypic, and functional consequences of *TET2* mutations for
300 NK cells in MDS patients. We could demonstrate that mutations observed in MDS cancer cells, including
301 in *TET2* and *IDH1/2* genes, are also generally observed in NK cells. Consequently, *TET2* mutations in
302 NK cells are associated with perturbations in methylation of the genomic DNA inducing a reduced
303 expression of KIRs, locus on which TET2 directly binds, and of other key molecules for NK cell function
304 including perforin and TNF- α . The reduced KIR expression and cytotoxicity of NK cells of healthy
305 donors after TET2 inhibition confirmed its requirement for an efficient physiological NK cell function.
306 Finally, NK cell phenotype can be restored *in vitro* and *in vivo* after treatment with ascorbic acid and
307 HMA.

308 The fact that NK cell defects observed in MDS can be partly due to the oncogenic mutations at the origin
309 of the disease could suggest that both events (i.e. mutations in MDS cells and NK cells) may synergize to
310 sustain the emergence of myeloid malignancies. Abnormalities in NK cell phenotype and function could
311 reduce the antitumor immuno-surveillance efficacy and allow the survival of potential pre-leukemic
312 progenitors. Notably, NK-cell perturbations in MDS patients including reduction of activating NK
313 receptors, such as NKp30, DNAM-1 and NKG2D, and impaired cytotoxicity, have already been
314 reported^{12,15,30}. KIR genotype has also been associated with clinical outcome³¹. Our data suggest that these
315 observations should be reinterpreted based on patient mutation profiles, especially for mutations
316 associated with *TET2* or *IDH1/2* genes. In line with this comment, dissecting the specific role of TET2 in
317 NK cells by contrast to other mutated genes commonly found in MDS or clonal hematopoiesis will
318 require further investigations, which could be decisive in our comprehension of the disease and its
319 treatments. Whether NK-cell function could be associated to a particular mutation landscape may have
320 important consequences in developing NK-cell-based immunotherapeutic intervention. In that context,
321 using autologous NK cells or attempting to rescue NK cell function in MDS patients may be impeded.
322 Combining such therapeutic strategies with the use of HMA could provide a relevant option.

323 Loss-of-function somatic mutations in *TET2* are strongly associated with haematological malignancies,
324 including myeloid, T- and B-cell malignancies and chronic lymphoproliferative disorders of NK cells
325 (CLPD-NK)^{32,33,34}. Germline mutations in *TET2* have been also associated with lymphoma^{35,36}. Here, we
326 found that *TET2* mutation in NK cells from MDS patients, with or without additional mutated genes, does
327 not seem to affect NK cell from the oncogenic point of view (i.e. NK cells were not leukemic) but alters
328 their phenotype and function. This raises the question of the requirement of other mutated genes, in
329 addition to *TET2*, in order to induce malignant hemopathies and how these mutation profiles can be
330 specific to lymphoid or myeloid malignancies.

331 Intriguingly, we noticed that *TET2* mutations in NK cells trended to increase methylation together on the
332 gene body and its flanking regions, at least for genes involved in NK cell function. *TET2* has been
333 reported as preferentially targeting regulatory enhancers in hematopoietic and myeloid cells, notably to
334 facilitate the recruitment of transcription factors^{24,27}. However, gene bodies and other genomic regions
335 can be also, although in a less efficient manner, targeted by *TET2*²⁴. In addition, our results could suggest
336 that key genes for NK cell function, including *KIR* and *PRF1*, are regulated in a coordinated manner by
337 *TET2* and more generally DNA methylation. If it is confirmed, this hypothesis would point out *TET2* as a
338 master regulator in NK cell development and education. It remains to be investigated whether and how
339 genomic DNA methylation by *TET2* is differentially regulated in NK cells, compared to myeloid subsets,
340 due to differences in chromatin modification and accessibility or in the timing of *TET2* expression during
341 NK-cell lymphopoiesis.

342 From the mechanistic point of view, our results are reminiscent of the work by Wu *et al* describing a role
343 for the ascorbic acid in promoting *KIR* locus demethylation through the mobilization of Runx3, *TET2*
344 and *TET3*²². Interestingly, it has been recently shown that *TET2*^{KO} HSC treated with ascorbic acid could
345 increase the 5hmC levels suggesting a role for TET proteins other than *TET2* in this rescuing²⁶. Herein,
346 we showed that the addition of ascorbic acid onto NK cells allow the restoration of *KIR* expression.
347 Altogether, these data support the hypothesis that *TET3* could compensate for *TET2* mutations in NK
348 cells after treatment with ascorbic acid.

349 Clonal hematopoiesis (CH) is frequently presented as a precursor state for haematological malignancies,
350 upstream of a continuum including MDS and then AML³⁷. Arends *et al* recently demonstrated that *TET2*
351 mutations observed in individuals with CH could be found not only in myeloid lineage but also in B- and
352 NK-cell³⁸. Our results support the finding that CH infuses into the complete tree of the hematopoiesis,
353 including lymphopoiesis, and could also participate to a reduced NK cell immunity in elderly. It remains
354 to discover the mechanisms by which *TET2* mutations alter the production of new lymphocytes in the
355 elderly without significantly reducing the lymphocyte number, and with which consequences for the
356 immune response. High-throughput experimental strategies, including multiomics approaches combining
357 RNAseq and ATACseq at the single cell level, applied not only to patients' samples but also to *TET2*-
358 edited HSC²⁶ or iPSCs³⁶, may help to solve this question.

359 Altogether, our findings showed that *TET2* is a key epigenetic regulator of NK-cells, and that *TET2*
360 mutations associated with MDS can alter their phenotype and function. In that context, HMA treatments
361 could normalize the hypermethylated profile harboured by NK cells in MDS patients, and may participate
362 to the emergence of a NK cell-mediated anti-tumour immunosurveillance.

363 **Methods**

364 ***Patient cohorts and Samples***

365 The first cohort was composed of 20 sternal bone marrow (BM) aspiration and 58 peripheral blood
366 mononuclear cell (PBMC) samples that were collected from 63 MDS/CMML patients. Patients' samples
367 were obtained with written informed consent in the Haematology Departments of the Saint Louis
368 Hospital, Paris, France (Supplementary Data 1 and Table 7). MDS and CMML diseases were defined
369 according to the World Health Organization 2016 criteria³⁹, and classified according to the Revised
370 International Prognostic Scoring System (IPSS-R)⁴⁰. Low-Risk (LR) disease was defined as IPSS-R very-
371 low, low or intermediate whereas High-Risk (HR) disease designated as IPSS-R high and very-high.
372 Disease evolution was assessed according to 2006 International Working Group criteria⁴¹. Among the 63
373 patients recruited, a few received treatments consisting in either Darbepoietin (EPO, n=4) or Eltrombopag
374 (TPO, n=1) without obvious effect on immune cells, or the immunomodulating drugs Lenalidomid (n=4)
375 or the glucocorticoid Prednisone (n=4). The second cohort was composed by MDS patients recruited in
376 the clinical trial #NCT02985190 (sponsored by Groupe Francophone des Myélodysplasies [GFM]) and
377 repeatedly treated with azacitidine at 75mg/m²/j subcutaneously daily for 7 days every 4 weeks²⁹
378 (Supplementary Table 8). In addition, PBMC samples from 23 healthy individuals (HD) were collected
379 through the EFS (Etablissement Français du Sang) (median age = 46 [27-66]). The study was approved by
380 the Ethical Board Ile-de-France X and conducted in accordance with Helsinki's declaration. Recruited
381 patients gave their written informed consent for participating.
382 PBMCs were purified from BM and blood samples by using a ficoll-based gradient density method¹³,
383 and stored in liquid nitrogen to be further analysed.

384

385 ***Patient KIR genotyping***

386 Generic *KIR* typing was performed using a *KIR* multiplex PCR-SSP method as previously described⁴².

387 The presence or absence of *KIR2DL1*, *2DL2*, *2DL3*, *2DL5*, *3DL1*, *2DS1*, *2DS2*, *2DS3*, *2DS4/1D*, *2DS5*

388 and *3DS1* genes were assigned. *KIR* genotypes were determined based on the presence or the absence of
389 activating *KIR*. Thus, an AA *KIR* haplotype was defined by the presence of only *KIR2DS4* or its deleted
390 variant (1D) as activating *KIR* gene, and a B+ (AB, BB) *KIR* haplotype by the presence of several
391 activating *KIR* genes. Centromeric (AA, AB, BB) and telomeric (AA, AB, BB) *KIR* motifs were defined
392 considering *KIR2DL2/3/S2* and *KIR3DL1/S1/2DS1/2DS4* genes for centromeric and telomeric motifs
393 respectively⁴³ (Supplementary Table 3).

394

395 ***Cell lines***

396 HEK293T and Jurkat cells lines (ATCC) were maintained either with DMEM or RPMI1640 medium
397 containing 10% of FBS, penicillin/streptomycin antibiotics and L-glutamine.

398 For the measurement of target lysis, K562 cells were first cultured overnight in RPMI1640 without
399 phenol red (Gibco) enriched with 10% FBS, non-essential amino acids (NEAA, Gibco), L-glutamine
400 (Life Technologies) and Sodium Pyruvate (Eurobio). Prior killing assay, K562 cells were diluted at
401 1.10^6 /mL in culture medium supplemented with 2.5mM Probenecid (Invitrogen) and stained with calcein-
402 AM (Invitrogen) for 30min. Then, excess of calcein was washed out.

403 For *in vitro* cell treatment, PBMCs were resuspended in RPMI 1640 supplemented with 10% Human
404 Serum (EFS, Etablissement Français du Sang), 1% Penicillin-Streptomycin (Dutscher), L-glutamine (Life
405 Technologies), Sodium Pyruvate (EuroBio) and HEPES (EuroBio).

406

407 ***Flow Cytometry Analysis***

408 Frozen sternal BM aspiration and PBMCs were stained for 30min on ice¹³, with the fixable viability dye
409 eFluor 506 and monoclonal antibodies (mAb) listed in Supplementary Table 9. Of note, mAb used in this
410 work to detect *KIR* molecules on cell surface were able to identify *KIR2DL1*, *KIR2DL2/DL3*, *KIR2DL4*,
411 *KIR3DL1/DL2*, *KIR3DL1/DS1*, *KIR2DL5* and *KIR2DS4* molecules. Cells were fixed in PBS with 2% of
412 paraformaldehyde (PFA), acquired on a BD Fortessa X20 (flow cytometry core facility of Saint Louis

413 Research Institute, Paris, France) (Supplementary Fig. 9). For intracellular staining, Foxp3 Transcription
414 factor buffer set (eBioscience 00-5523-00) was used and data were acquired on a BD Canto II or Cytex
415 Aurora flow cytometer. Data was analyzed using FlowJo v10.7 software or Cytex SpectroFlo v3.0.3.

416

417 ***NK and T-cells isolation for Mutational Analysis and reduced representation***

418 ***bisulfite sequencing (RRBS)***

419 After thawing, PBMCs were first labeled in PBS 30min at 4°C with the fixable viability dye eFluor 506
420 (eBioscience) and then stained with antibodies specific for surface markers to distinguish NK cell subsets
421 (at the whole or based on the surface expression of KIR2D and KIR3DL1/DL2 molecules) and T-cells,
422 and directly sorted using BD Aria III system (Supplementary Table 9 and Supplementary Fig. 10). Dry
423 pellets have been stored after cells sorting for DNA extraction. DNA was purified according to
424 manufacturer (DNeasy KIT, Qiagen) and quantify by Qubit (Thermofisher) for NGS and RRBS studies.
425 Of notes, clinical evaluation of MDS mutations were performed in the haematology laboratory, in St-
426 Louis hospital, Paris, on WMC obtained using gradient density separation on either BM or blood samples.
427 A panel of 80 genes commonly analyzed in myeloid malignancies (Supplementary Table 4) was
428 examined using next-generation sequencing on an Illumina platform (Illumina, San Diego, CA, USA).
429 Libraries were prepared from 200 ng of DNA using a custom probes panel for capture of all coding
430 sequences (SureSelectXT Target Enrichment System, Agilent, Santa Clara, CA, USA). Sequencing data
431 were analyzed for variant calling using an in-house pipeline. High-probability pathogenic mutations were
432 retained based on the available databases for SNPs, somatic mutations in cancer, prediction algorithm and
433 frequencies of variant reads. Overall, a mean coverage of 851 reads/amplicon was achieved allowing
434 reliable detection of low burden mutations. Lollipplot has been realized using the St Jude protein paint
435 software available online at (<https://proteinpaint.stjude.org/>) (Fig. 3A)⁴⁴. Major domains of TET2 protein
436 were determined based on the work by Hu et al⁴⁵. The mutational landscape was established using

437 cBioPortal Cancer Genomics Portal OncoPrinter software available online at
 438 (<https://www.cbioportal.org/>) (**Supplementary Fig. 4**).
 439 RRBS was performed by Diagenode (Seraing, Belgium) on dry samples of NK- and T-cell sorted from 5
 440 MDS patients according to the manufacturer procedure. RRBS data are available on the Gene Expression
 441 Omnibus platform under the accession code GSE183020
 442 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE183020>). 100ng of purified genomic DNA
 443 were used, including pre-RRBS quality control, enzymatic digestion and bisulfite conversion. Sequencing
 444 was performed in paired-end mode 50bp (PE50), quality control of sequencing reads was performed using
 445 FastQC, and reads alignment against human reference genome hg38/GRCh38 using Bismark v0.20.0
 446 (available at <https://www.bioinformatics.babraham.ac.uk/projects/bismark/>) according to Diagenode
 447 protocol. Differential gene and site methylation analyses were performed by Genoplice Technology (Paris
 448 Biotech Santé, Paris, France). To assess the frequency of methylated CpG sites across all genes of a
 449 pathway of interest, gene lists were extracted from KEGG pathway website²⁵. The position of each CpG
 450 site was calculated relatively to the transcript start and end positions as follows:

$$451 \quad CG_{relativeposition} = \frac{CG_{position} - transcript\ start}{transcript\ end - transcript\ start}$$

452 Considering 10kb upstream and downstream sites. Next, frequency of methylation was calculated and
 453 aggregated across all genes of a given pathway for each sample.

454 Of note, all sequencing data were analysed using the human reference genome hg38/GRCh38.

455

456 ***Chromatin Immuno-Precipitation (ChIP)-qPCR assay***

457 ChIP assay was performed with the Active Motif magnetic kit following the manufactory protocol.

458 Briefly, after sonication the sheared chromatin was incubated overnight with TET2 (Diagenode,
 459 C15410255), control H3 (Abcam, AB1791) and H3K18 (Abcam, AB1191) specific mAb and the isotype
 460 control (Diagenode, C15410206). ChIP-enriched DNA was analyzed by qPCR with SYBR Green Master
 461 Mix (Thermofisher). Primers are listed in Supplementary Table 10.

462

463 ***Transfection and Luciferase assay***

464 HEK293T cell lines (ATCC) were transfected by polyethylenimine (PEI, Sigma) following previous
465 publication⁴⁶.

466 The plasmid pcDNA3-TET2 overexpressing the full-length mouse TET2 gene as the empty pcDNA3
467 vector were obtained from Addgene (Plasmid #60939 and #45346). All constructs have been verified by
468 DNA sequencing.

469 One region of the predicted *KIR2DL1* promoter (KIRprom -147+60, Supplementary Fig. 11) was cloned
470 in the pT109-tkLUC plasmid. 0.6µg promoter vector together with the 2.7µg TET2 plasmids were co-
471 transfected in HEK293T cells with 0.06µg of pRL-CMV Vector (#E2261 Promega) in presence of PEI
472 solution.

473 In parallel, Jurkat cells (ATCC) were transfected with 0.26µg of the KIR promoter plasmid and 0.026µg
474 of pRL-CMV vector by Nucleofactor Kit V (Lonza). Jurkat transfected cells were then cultured at
475 1.10^6 /mL and treated with 500µg of L-ascorbic acid (L-AA, Sigma) for 16h before analysis⁴⁷. The
476 luciferase activity was measured after 24h according to manufacturer's instructions (Dual-Luciferase
477 Reporter Assay, Promega).

478 To investigate the role of the methylation in controlling the functional NK role, putative promoter
479 regions, differentially methylated between KIR^{HIGH} and KIR^{LOW}, were cloned into pCpGfree basic vector
480 (Invivogen) for testing promoter activity. To determine the effect of methylation on the activity of the
481 region, *in vitro* methylation of the constructs was performed with M.SssI (NEB). 0.6µg promoter vector,
482 "native" (unmethylated) or *in vitro* methylated, were co-transfected with 0.06µg of pRL-CMV Vector
483 (#E2261 Promega) in presence of PEI solution. After 24h the Lucia and Firefly read-out was generated
484 with a CLARIOstar^{Plus} (BMG LABTECH).

485

486 ***Microfluidic Multiplex qPCR***

487 Multiplex qPCR analysis (Biomark, Fluidigm) was performed on 100 cells. NK cell as the whole and
488 KIR⁺ (KIR2D or KIR3DL1/DS1 positive) or KIR⁻ (KIR2D or KIR3DL1/DS1 negative) NK cell subsets
489 were sorted into 5 μ L of reverse transcription/pre-amplification mix, prior to multiplex qRT-PCR
490 following manufacturer's protocol⁴⁸. Briefly, the mix contained 2X Reaction mix and Superscript III
491 (CellDirect One-Step qRT-PCR kit, Invitrogen) and 0,2X Taqman assay specific probes (Life
492 technologies). Targeted cDNA pre-amplification was performed for 19 cycles and the pre-amplified
493 product was diluted 1:5 in TE buffer before processing with Dynamic Array protocol according to the
494 manufacturer's instructions (Fluidigm). All the probes used for this analysis are described in
495 Supplementary Table 11. Mean expressions for *RPL27* and *GAPDH* house-keeping genes were used for
496 signal normalization.

497

498 ***In vitro cell treatment and functional assay***

499 PBMC were treated in cell culture medium (i.e., RPMI 1640 supplemented with 10% foetal bovine
500 serum) with 0.5 μ M or 1 μ M of AZA, DAC, Dimethyloxaloylglycine (DMOG) (Sigma Aldrich), alone or
501 in combination with 125 μ M Ascorbic Acid. Interleukin-2 (IL-2) (Miltenyi Biotec) was added to a final
502 concentration of 100UI/mL. DMSO condition was used as control. The treatments were maintained for 5
503 days with a half change of medium after 48h (IL2 \pm drugs or DMSO). Then, cells were collected and NK
504 cells were analyzed for their phenotype (Supplementary Table 9 and Supplementary Fig. 9).

505 After treatment with DMOG, PBMCs were collected, enumerated and co-cultured with calcein-labelled
506 K562 cells in 96-well U bottom plates in medium supplemented with Probenecid at 10:1 effector:target
507 ratio. Killing was quantified after 4 hours of incubation at 37°C by measuring calcein-release into
508 supernatant. Specific killing was calculated as below: (Measured fluorescence of K562+PBMC well –
509 Spontaneous Fluorescence)/(Maximum fluorescence – Spontaneous fluorescence)*100.

510 For the *ex vivo* IFN- γ quantification, enriched NK cells from MDS patients enrolled in the NCT02985190
511 clinical trial²⁹ were cultured with PMA-Ionomycin (SIGMA, 50ng/mL and 500ng/mL respectively) for 5h

512 at 37°C. Brefeldin A (Sigma) was added at a final concentration of 10µg/ml after 1h of incubation. The
513 percentage of IFN-γ positive cells was estimated by flow cytometry in the CD3⁻CD56⁺ NK cells.
514 Spontaneous release was detected in the absence of target cells.

515

516 *Statistical Analysis*

517 Data are shown as median ± interquartile range, if not otherwise specified. Cytometry analysis were
518 extracted from FlowJo v10.7 software and analyzed with Graph Pad Prism v8.0 software. Unpaired
519 statistical analyses were calculated with the nonparametric Mann Whitney test. Paired statistics analyses
520 were calculated with the nonparametric Wilcoxon matched pairs signed rank test. The Kolmogorov-
521 Smirnov D test was used to calculate differences between treated/transfected and control situations.
522 Pearson coefficient (r) was calculated to evaluate significant correlation. Receiver operation characteristic
523 (ROC) curve was realized based on TET2 mutation status and percentage of KIR2D⁺ NK cells. Area
524 under the curve, confidence interval, p-value and likelihood ratio were calculated. A Friedman test
525 followed by a Dunn's test were used to calculate differences in experiments with multiple conditions.
526 p<0.05 was considered as significant.

527

528

529

530 Data Availability

531 Reduced representation bisulfite sequencing (RRBS) data that support the findings of this study have
532 been deposited in the Gene Expression Omnibus data repository with the accession number GSE183020
533 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE183020>). The *in vitro* data generated in this
534 study are available in the Supplementary information. Sequences of oligonucleotides used for the KIR
535 genotyping (PCR-SSP) are limited to non-commercial and research use and are available upon request to
536 Dr K. Gendzekhadze (kgendzek@coh.org), City Of Hope Medical Foundation, 1500 Duarte Rd, Duarte,
537 CA 91010. Source data are provided with this paper as a Source Data file.

538

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639

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651 **Author contributions**

652 M.B., V.B. and N.D. designed, analyzed and interpreted all experiments, and wrote the manuscript. M.B.
653 V.B., F.G., and E.L. performed experiments and analyzed the data. L-P.Z. managed the collection of the
654 patients' samples and clinical information. B.S. analyzed the RRBS data. G.H. and J.V. performed
655 preliminary feasibility experiments. F. R-L. provided reagents and analyzed data. K.G. and C.R.
656 performed KIR genotyping. L.L., R.K. and E.C. performed next-generation sequencing and analyzed
657 mutation profile data. L-P.Z., M.S., P.F and L.A. recruited the patients for the study. A.M., O.F. and P.F.
658 supervised the NCT02985190 clinical trial. A.C, M.E., K.B., A.T., P.F. and L.A. contributed to the
659 scientific orientation of the study and critically reviewed the manuscript. L.A. designed the patients'
660 cohort suitable for the study. N.D. supervised the study.

661 **Competing interests**

662 The authors declare no competing interests.

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669 **Figures**

670 **Fig. 1: *TET2/IDH* mutations in MDS/CMML patients lead to the reduction of KIR and Perforin**
 671 **expression in NK cells.**

672 (a) Percentages of blood NK cells expressing activating (NKp30, NKp46, DNAM-1, NKG2D) and
 673 inhibitory (CD96, CD85j, KIR2D, NKG2A) receptors, and maturation/activation markers (KLRG1,
 674 CD69, CD57) were measured by flow cytometry in *TET2/IDH*^{WT} (n=13, n=11 for NKG2A) and
 675 *TET2/IDH*^{MUT} (n=19, n=15 for NKG2A) MDS/CMML patients. Statistics were calculated with the
 676 nonparametric Mann-Whitney test, two-sided, KIR2D *** p=0.0005, NKG2A * p= 0.0246. (b)
 677 Percentages of KIR2D⁺ BM NK cells measured by flow cytometry in *TET2/IDH*^{WT} (n=12) and
 678 *TET2/IDH*^{MUT} (n=7) MDS/CMML patients. Statistics were calculated with the nonparametric Mann-
 679 Whitney test two sided * p=0.013. (c-d) Specific expression of KIR2DL1, KIR2DL2/DL3 and
 680 KIR3DL1/DL2 in blood NK cells detected by flow cytometry in *TET2/IDH*^{WT} (n=11) and *TET2/IDH*^{MUT}
 681 (n=15) MDS/CMML patients. One representative example is shown in (C). Statistics were calculated with
 682 the nonparametric Mann-Whitney test, KIR2DL1 ** p=0.0092, two sided KIR2DL2/DL3 ** p= 0.0077.
 683 (e) Intracellular perforin and granzyme B expression in blood NK cells measured by flow cytometry in
 684 *TET2/IDH*^{WT} (n=19) and *TET2/IDH*^{MUT} (n=22) MDS/CMML patients. Statistics were calculated with the
 685 nonparametric Mann-Whitney test * p=0.0337 (f) Receiver operating characteristic (ROC) curve
 686 depicting the relationship of true *TET2* mutation presence (sensitivity) and false *TET2* mutation presence
 687 (100%-specificity) for a KIR2D expression threshold at 25% in blood NK cells (p<0.0001) quantified by
 688 flow cytometry in *TET2/IDH*^{WT} (n=19) and *TET2*^{MUT} (n=17) MDS/CMML patients. (g) KIR2D
 689 expression on blood NK cells of the *TET2/IDH*^{WT} (n=19), *TET2*^{MUT} (n=17) and *IDH*^{MUT} (n=5) patients
 690 showed in the ROC curve. The horizontal black bar indicates the threshold at 25% KIR2D⁺ NK cells.
 691 Statistics were calculated with the nonparametric Mann-Whitney test, *TET2/IDH*^{WT} vs *TET2*^{MUT}: ****
 692 p<0.0001; *TET2/IDH*^{WT} vs *IDH*^{MUT}: * p=0.0152. For all the analysis, data are presented as medians and
 693 interquartile ranges. Source data are provided as a Source Data file.

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Fig. 2: *TET2* mutations identified in bulk cells of MDS patients at diagnosis are also observed in NK cells and correlate with *KIR2D* expression.

(a) The percentage of Variant Allele Frequency (VAF) for the mutations of *TET2* observed in the white mononuclear cells (WMC) and in sorted NK/T cells at diagnosis of *TET2*^{MUT} MDS/CMML patients (n=10) was evaluated by NGS analysis (with a range from 1 to 4 mutations per patient). Data are represented as box-and-whisker plot (minimum VAF, 25% percentile, median, 75% percentile and maximum VAF respectively for WMC: 0%, 11.25%, 32.5%, 42.5%, 49%; for NK cells: 0%, 3.5%, 14%, 35.5%, 54%; and for T cells: 0%, 0%, 0%, 0%, 7%). Nonparametric two-sided Wilcoxon matched pairs signed rank test was used to determine statistical significance. **** p<0.0001 (b) VAF (%) of the different mutations detected in the WMC at diagnosis (blue) and in sorted NK cells (red) in 4 patients (MUT08, MUT22, MUT19, MUT31; see **Supplementary Table 6** for more information). (c) Correlation curve between the percentage of *KIR2D*⁺ NK cells and the *TET2* VAF (%) in blood NK cells from the MDS/CMML patients analyzed in (A). Linear regression was calculated, r=0.88, p<0.0001. (d) The VAF percentage in the WMC and in sorted *KIR*⁺ and *KIR*⁻ NK cells at diagnosis from *TET2*^{MUT} MDS/CMML patients (n=5, range from 1 to 4 mutations per patient) was evaluated by NGS analysis. Data are represented as box-and-whisker plot (minimum VAF, 25% percentile, median, 75% percentile and maximum VAF respectively for *KIR*⁻ NK cells: 6.1%, 8.3%, 18.2%, 36.5%, 42.1%; and *KIR*⁺ NK cells: 0%, 1.2%, 7.3%, 13%, 48%). Statistics were calculated with two-sided nonparametric Wilcoxon matched pairs signed rank test. * p=0.0186. Source data are provided as a Source Data file.

716 **Fig. 3: TET2 regulates KIR expression on NK cells through its binding onto and regulating**
 717 **methylation of the KIR locus.**

718 (a) *TET2* mutational landscape in the 13 *TET2*^{MUT} patients with KIR2D expression below 25% established
 719 with the St Jude protein paint software (<https://proteinpaint.stjude.org/>). Mutations were classified as
 720 Missense (blue), Frameshift (red) and Nonsense (orange). Numbers associated to each mutation designed
 721 patients (**Supplementary Data 1**). (b) Percentage of KIR2D⁺ NK cells after 5 days of *in vitro* treatment
 722 with 1 μ M DMOG (n=10). DMSO alone was used as control. One dot represents one PBMC sample.
 723 Nonparametric two-sided Wilcoxon matched pairs signed rank test was used to determine statistical
 724 significance. **p= 0.0059. Data are presented as median and interquartile range. (c) Fold enrichment of
 725 sequences specific for the Cis-regulatory element (CRE), *KIR2DL1* promoter and *KIR2DL2/3* promoter
 726 analyzed in sorted NK cells by CHIP-qPCR with TET2, H3 or H3K18 specific mAbs or IgG isotype
 727 control. Means \pm SD are shown (n=3). Each dot represents one independent experiment. (d) Luciferase
 728 activity in HEK293T cells co-transfected with a *TET2* full length plasmid or an empty plasmid as control,
 729 and the luciferase-reporter construct containing the region (-147+60) of the *KIR2DL1* promoter. Means \pm
 730 SD are shown (n=4). Each dot represents one independent experiment. Nonparametric two-sided
 731 Wilcoxon matched-pairs signed rank test was used to determine statistical significance. *p= 0.0286 (e)
 732 Jurkat cells transfected with the *KIR2DL1* promoter-luciferase reporter plasmid were treated with 500 μ M
 733 of L-AA for 16h and analyzed by detecting luminescence signal. Means \pm SD are shown (n=4). Each dot
 734 represents one independent experiment. Nonparametric two-sided Wilcoxon matched pairs signed rank
 735 test was used to determine statistical significance. *p= 0.0286 (f) RRBS DNA methylation profiles of the
 736 extended KIR locus of NK cells with low or high KIR2D expression. Each graph represents the DNA
 737 methylation profile of sorted NK cells from blood samples of 5 patients; vertical bars represent the
 738 percentage of DNA methylation at CpG position. CRE and *KIR* genes were highlighted in green and grey
 739 respectively. (g-h) IGV (Integrative Genomics Viewer) view of CpG read signals corresponding to DNA
 740 methylation in NK cell, based on the high (*TET2*^{WT/MUT}KIR^{HIGH}, in blue bars) and low (*TET2*^{MUT}KIR^{LOW},
 741 in red bars) KIR expression. Genome profiles at the CRE region (g) and the *KIR2DL1* gene (h) loci

742 showed variation in the DNA methylation pattern between the two groups of patient NK cells. Red
743 peaks/boxes show significant differences in the DNA methylation levels at particular CpG positions of
744 these specific loci. Source data are provided as a Source Data file.
745

746 **Fig. 4: Loss of TET2 leads to DNA hypermethylation and decrease key gene expression for NK cells**
747 **function.**

748 (a) Circos plots showing whole-genome CpG methylation status in patient MUT14 characterised by the
749 absence of *TET2* mutations in NK cells and a high expression of KIR2D, and in patient MUT22 with a
750 VAF of 50% for the *TET2* mutation [NM_001127208:exon11:c,4669_4672del:p.V1557fs] and a very low
751 expression of KIR2D in NK cells. (b) Volcano plots and heatmaps showed the overall increase of global
752 DNA methylation levels reported after RRBS analysis, in the *TET2*^{MUT}KIR^{LOW} (n=3) vs.
753 *TET2*^{WT/MUT}KIR^{HIGH} (n=2) NK cells. Heatmaps depicted supervised clustering of the significantly
754 modified sites and genes between patients' subgroups. Blue dots/bars show the hypomethylated
755 CpG/sites whereas red dots/bars show the hypermethylated ones (methylation difference $\geq 20\%$,
756 unadjusted p-value ≤ 0.05). Top panel shows the differentially methylated CpG sites. Bottom panel shows
757 the differentially methylated genes. (c) GO-enrichment analysis on the differentially methylated genes in
758 the *TET2*^{MUT}KIR^{LOW} and *TET2*^{WT/MUT}KIR^{HIGH} NK cells. Percentages of methylated CpG sites were
759 calculated in gene bodies and 10kb upstream or downstream of the gene of interest in *TET2*^{MUT}KIR^{LOW}
760 and *TET2*^{WT/MUT}KIR^{HIGH} NK cells (in red and blue respectively) and aggregated across all genes of a
761 given KEGG pathway for each sample. Pathways of interest shown are the cytokine-cytokine receptor
762 interactions (KEGG reference hsa04060), the JAK-STAT signalling pathway (hsa04630), the TNF
763 signalling pathway (hsa04668) and the NK cell mediated cytotoxicity pathway (hsa04650). Source data
764 are provided as a Source Data file.

765

766 **Fig. 5: Hypermethylation of particular CpG sites repress NK cell genes expression and function.**

767 **(a)** Methylation profiles, established after RRBS analyses, depicted with IVG at the *IFNG* (upper panel),

768 *TNF* (middle panel) and *PRFI* (lower panel) loci, were shown in NK cells from patients segregated on the

769 high ($TET2^{WT/MUT}KIR^{HIGH}$, in blue bars) and low ($TET2^{MUT}KIR^{LOW}$, in red bars) KIR expression. Red

770 peaks/boxes show significant differences in the DNA methylation levels of these specific loci. **(b)** The

771 regulatory activity of specific CpG sites was analysed in the HEK293T cell line transfected with a

772 luciferase-reporter plasmid including methylated or non-methylated genes' regulatory regions. Relative

773 luciferase activities of the *in vitro* methylated regions were compared to their non-methylated counterpart

774 (FC= Methylated/Putative Promoter). Means \pm SD are shown (n=4). Data were analysed using the one-

775 way Friedman test followed by a Dunn's test. *p=0.0433 **(c)** Quantification of the *KIR2DL1*, *TNF* and

776 *IFNG* transcripts by RT-qPCR on KIR2D⁻ NK cells sorted from $TET2/IDH^{WT}$ (n=11, in blue) and

777 $TET2^{MUT}$ (n=10, in red) patients. Medians and interquartiles are shown. Statistics were calculated with the

778 two-sided Mann-Whitney test, **p=0.0036. **(d)** Fold changes of NK cells killing activity against the NK-

779 sensitive cell line K562 previously treated for 5 days with 1 μ M DMOG (n=10). Each dot represents one

780 independent experiment. DMSO alone was used as control. Means \pm SD are shown. Data were analysed

781 using the one-way Friedman test followed by a Dunn's test. *p=0.0417. Source data are provided as a

782 Source Data file.

783

784

785 **Fig. 6: Hypomethylating agents normalize the NK cell phenotype of MDS patients.**

786 (a) Evaluation of KIR2D surface expression on NK cells of *TET2/IDH^{MUT}* patients after treatment with
787 azacitidine (AZA, n=12), decitabine (DAC, n=12), acid ascorbic (AA, n=7), and DAC+AA (n=7).
788 Nonparametric two-sided Wilcoxon matched pairs signed rank test was used to determine statistical
789 significance, DAC: *** p=0.001, DAC+AA: * p=0.0156. (b) NK cells were isolated from patients PBMC
790 before and after 3 cycles of treatment with AZA, and cultured overnight at 100U/ml of IL-2.
791 Subsequently cells were cultured with PMA-Ionomycin for 6h. The frequency of responding cells in
792 terms of IFN- γ were assessed by flow cytometry (n=7). (c) KIR2D, perforin and granzyme B expression
793 was measured by flow cytometry in blood of MDS patients (n=9) before (in blue) and after (in red) 6
794 cycles of AZA treatment. Nonparametric two-sided Wilcoxon matched pairs signed rank test was used to
795 determine statistical significance, KIR2D: * p=0.0195, Perforin: * p=0.0273, Granzyme B: * p=0.0273.
796 Data are presented as medians and interquartile ranges. Source data are provided as a Source Data file.
797

figure 1

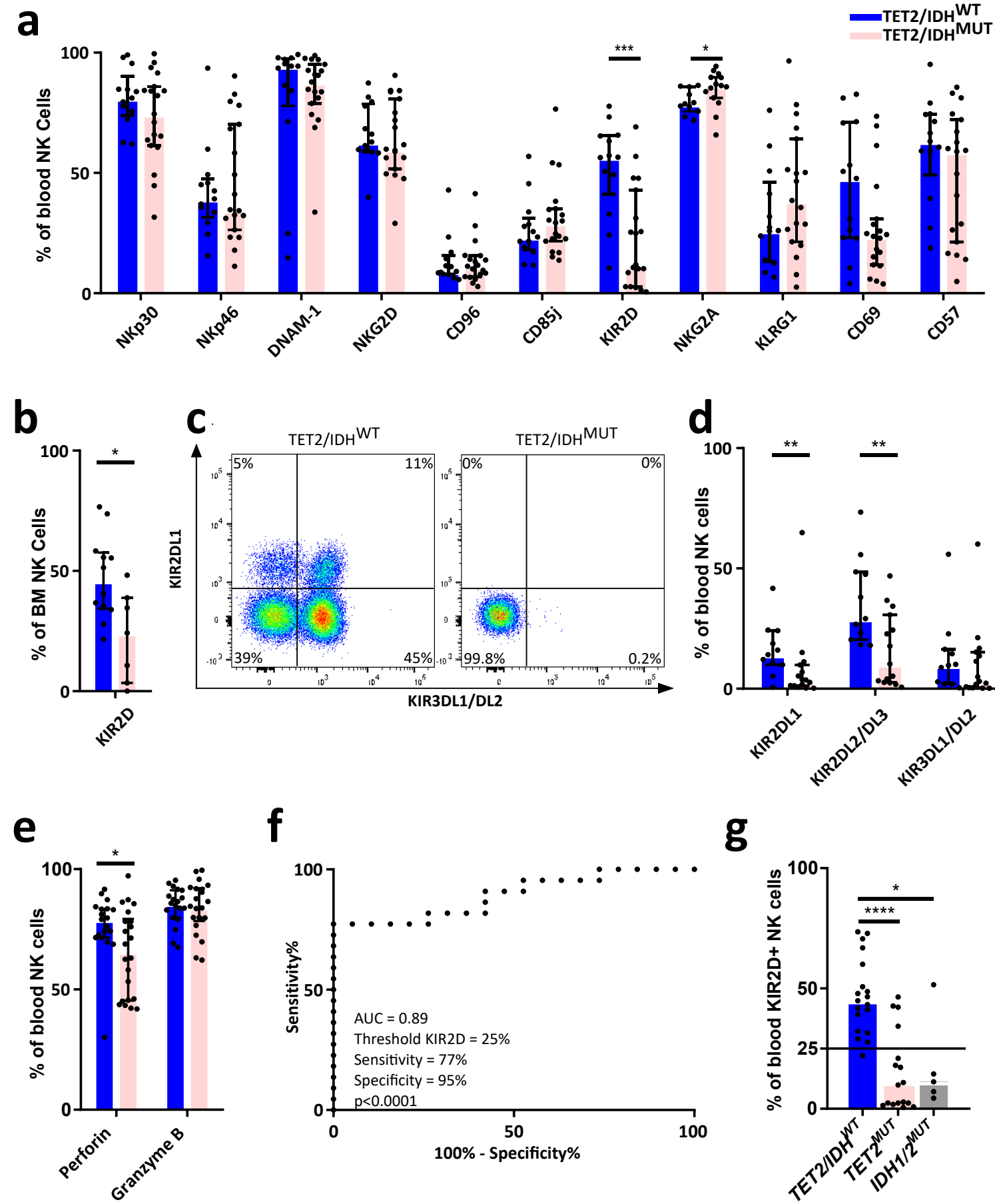


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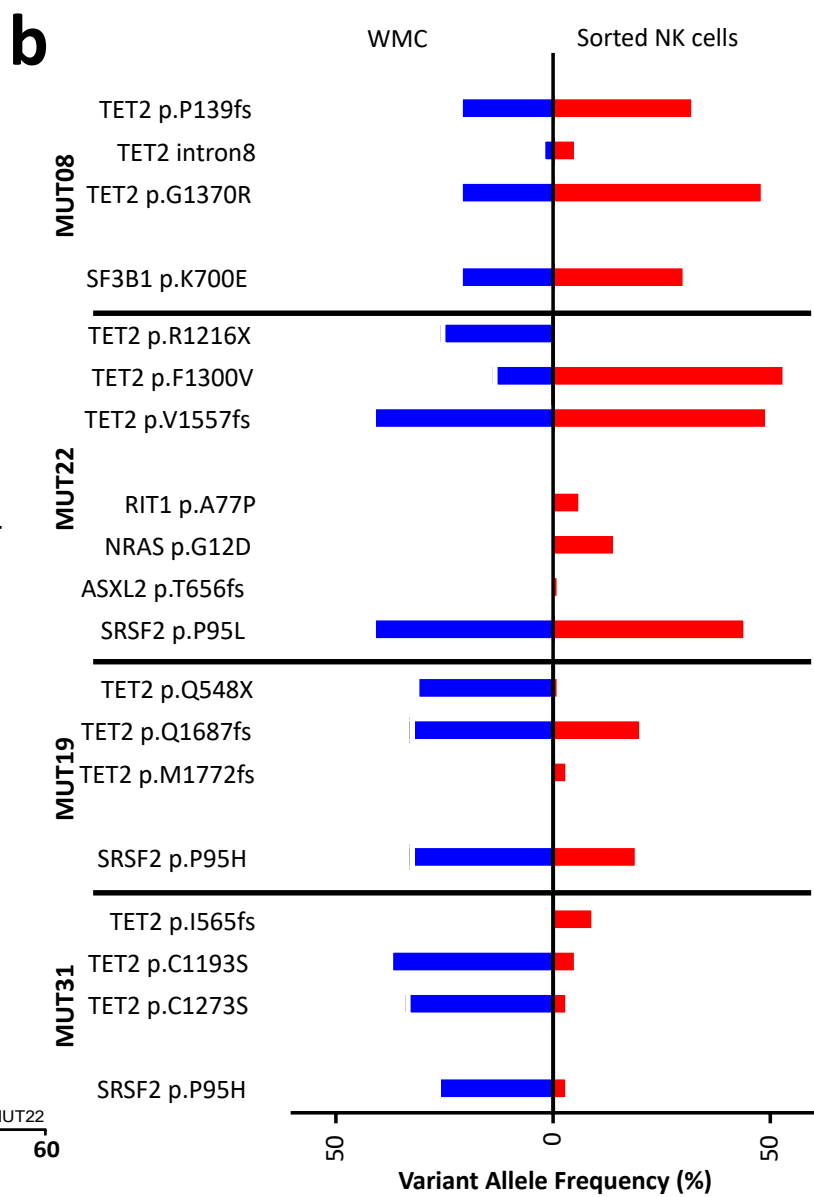
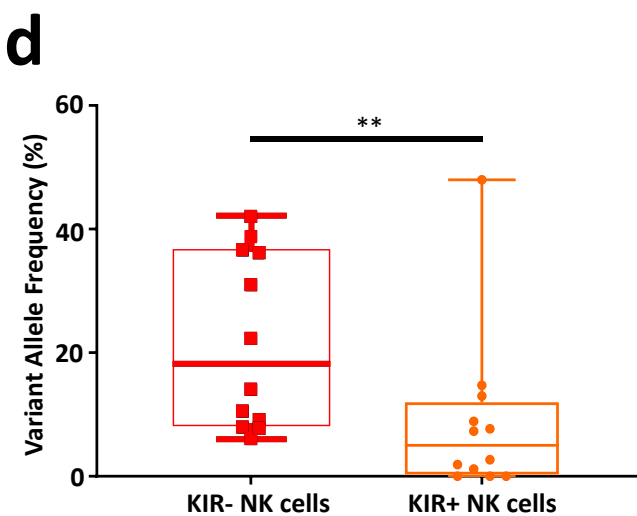
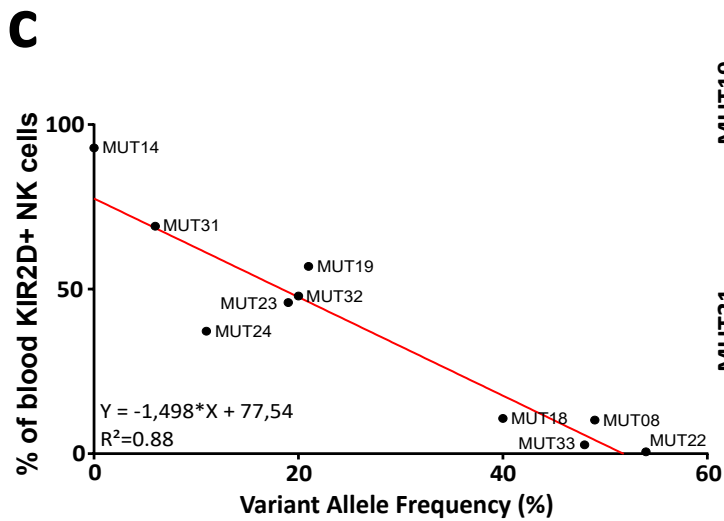
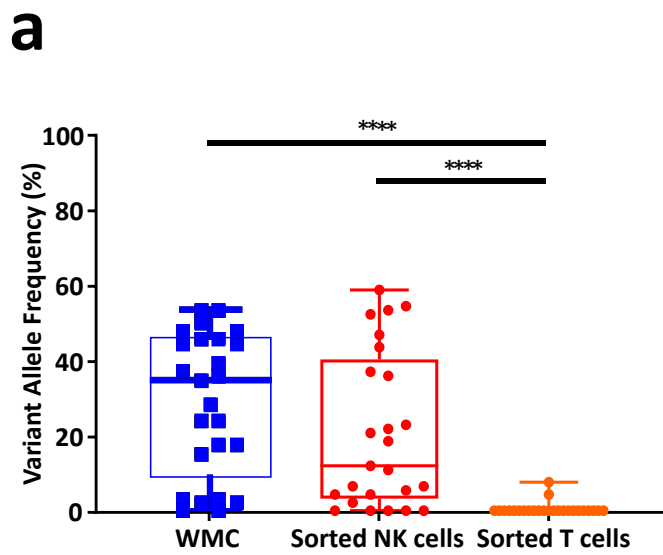
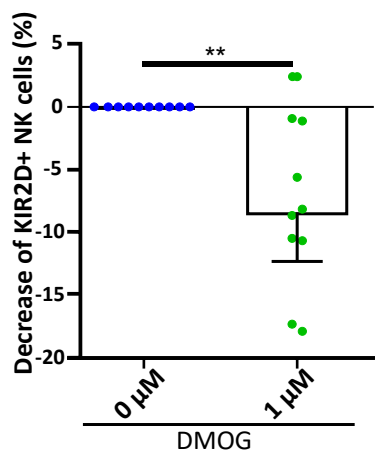


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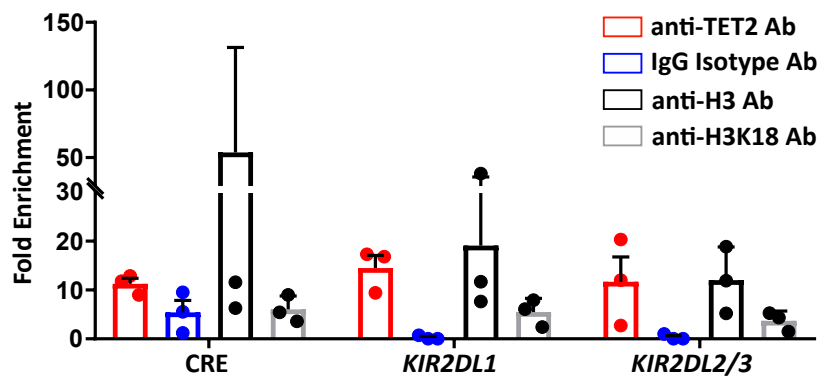
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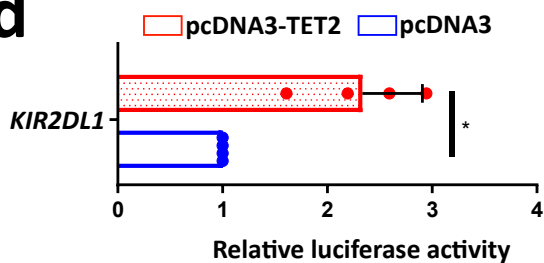
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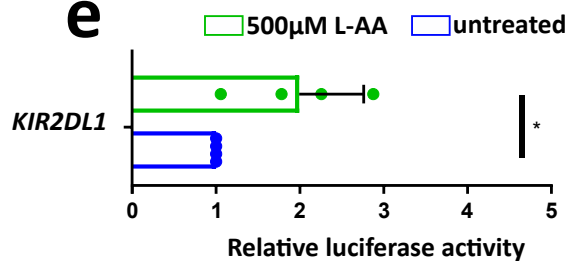
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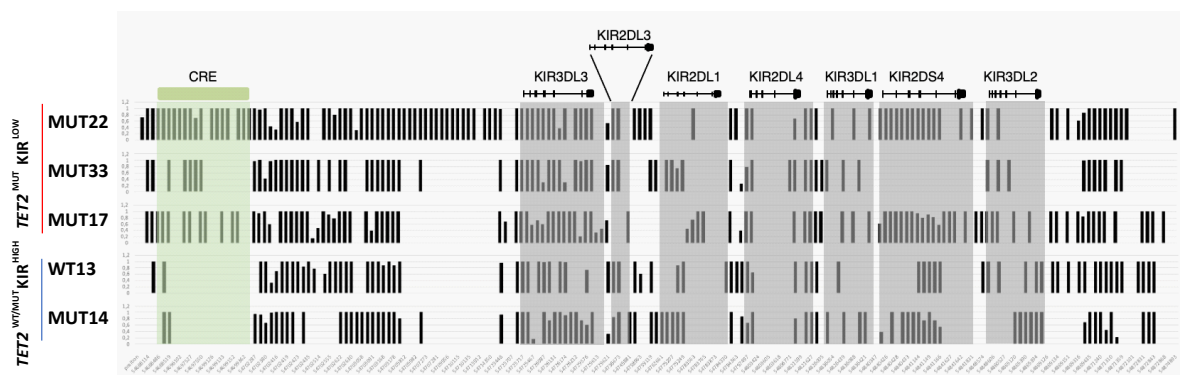
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Cis-Regulatory Element (CRE)

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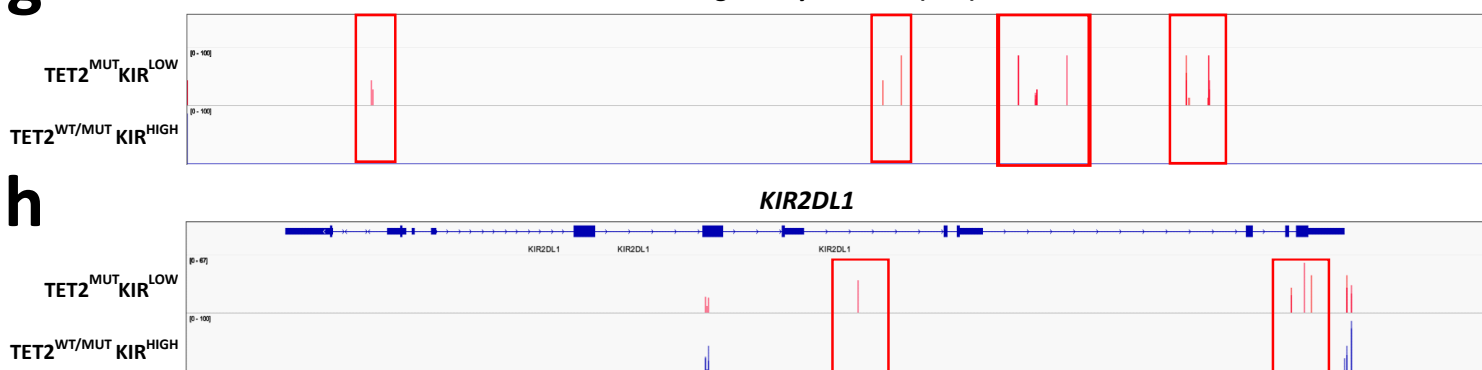
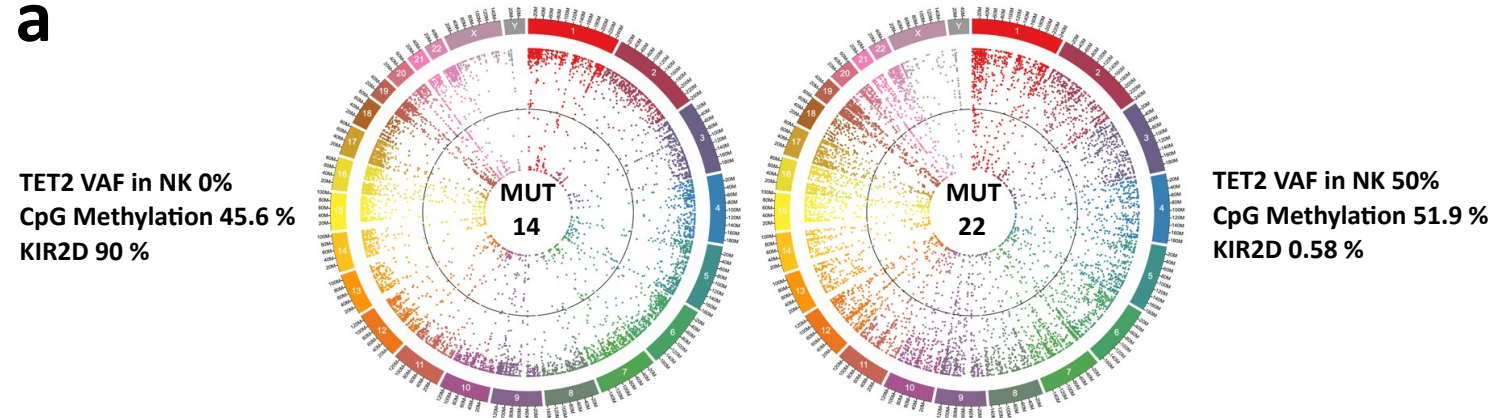
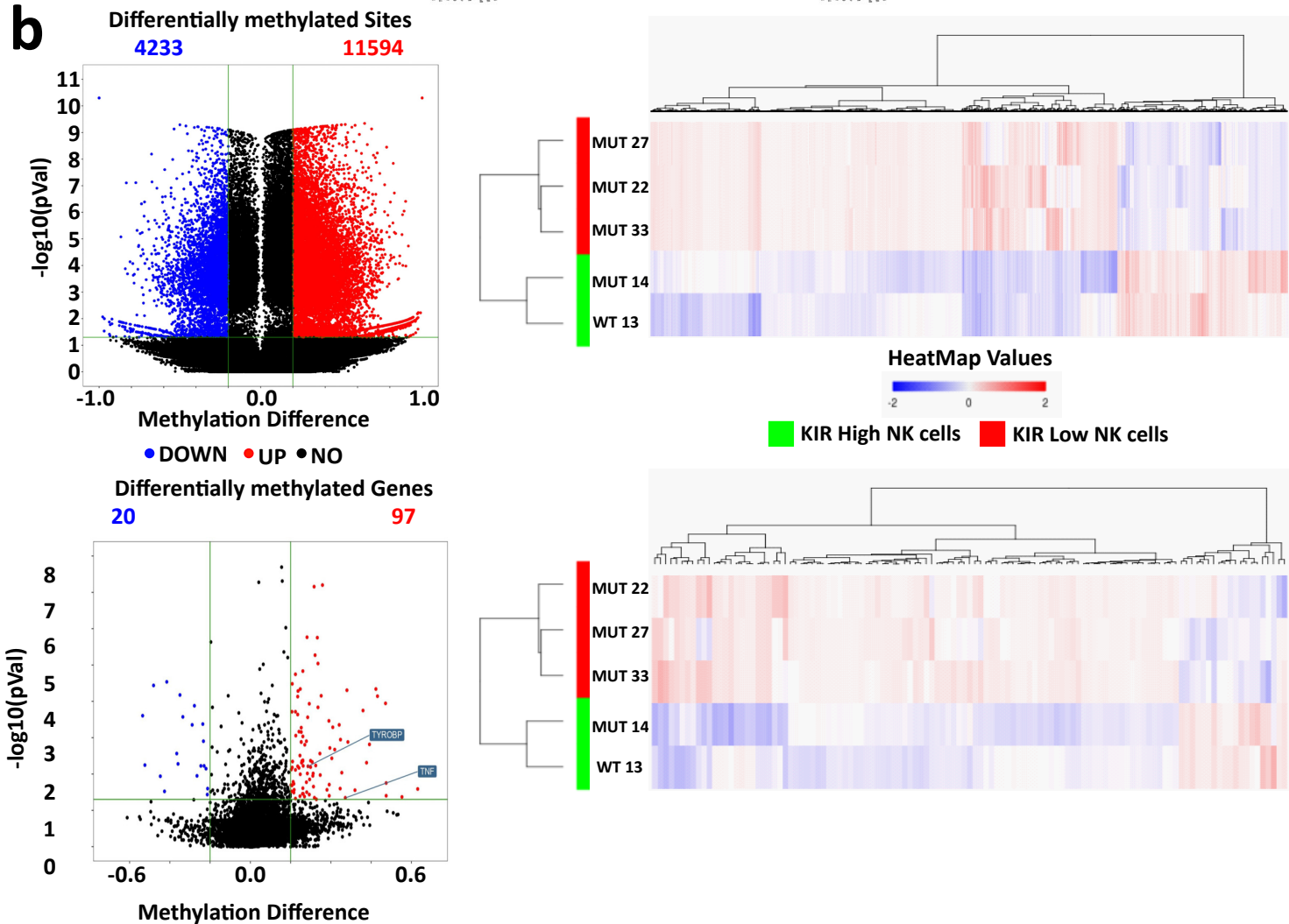


figure 4

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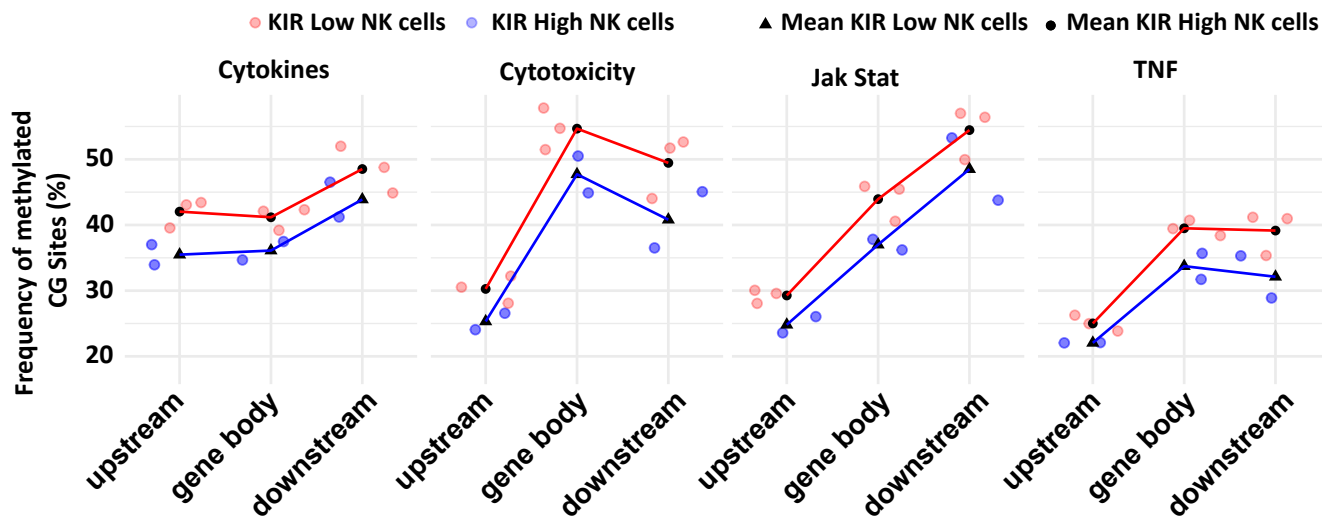
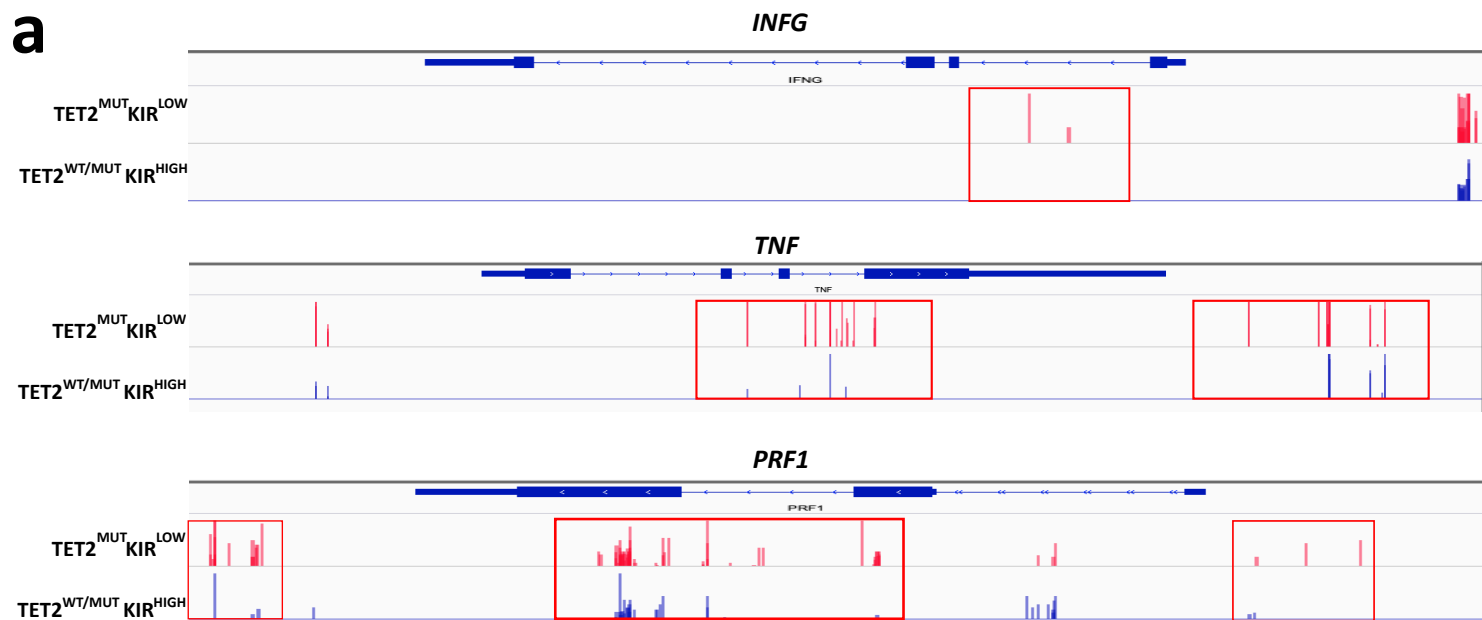
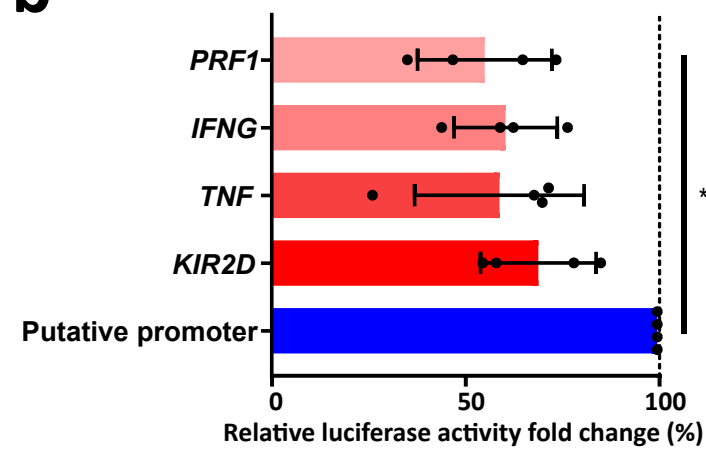


figure 5

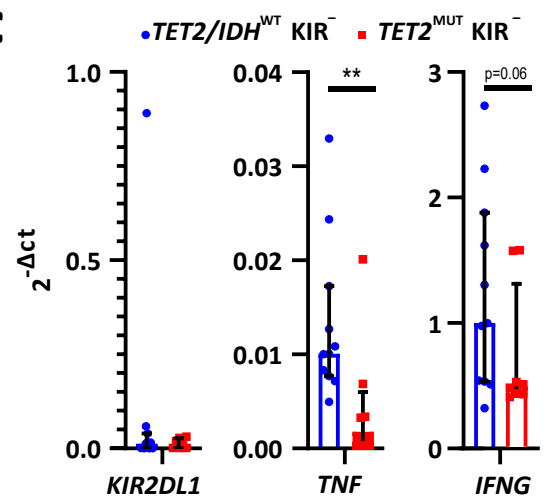
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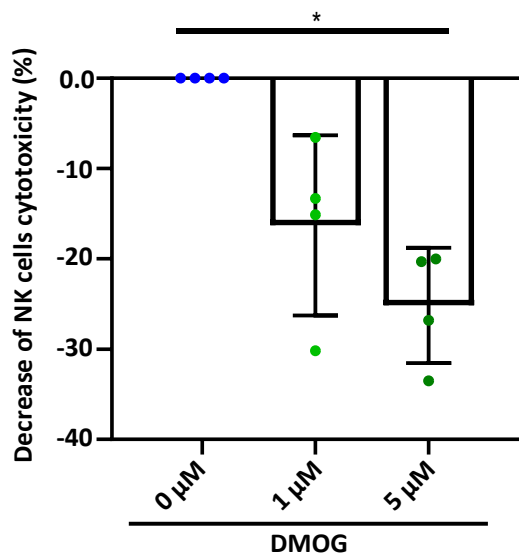
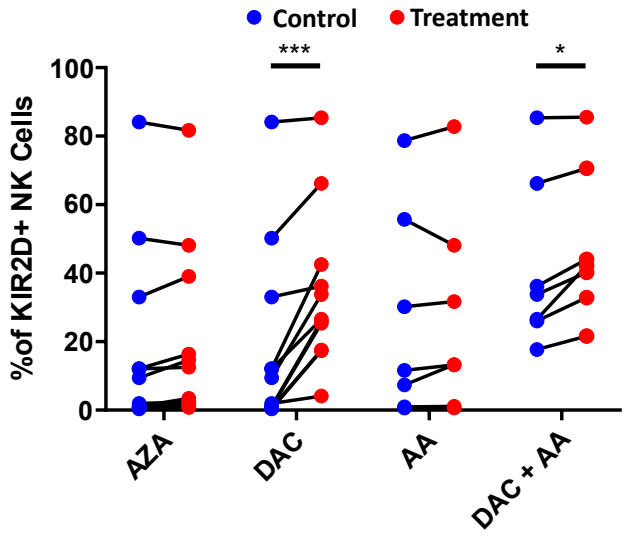
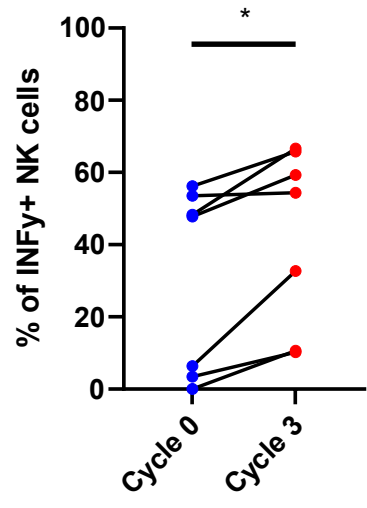


figure 6

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