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Activation priming and cytokine polyfunctionality modulate the enhanced functionality of low-affinity CD19 CAR T cells

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Abstract:
changes occurring in T-cells expressing low-affinity vs high-affinity CD19 CARs following stimulation with CD19-expressing cells. Our results show that CAT CAR T-cells exhibit enhanced activation to CD19 stimulation and a distinct transcriptomic and protein profile, with increased activation and cytokine polyfunctionality compared to FMC63 CAR T-cells. We demonstrate that the enhanced functionality of low-affinity CAT CAR T-cells is a consequence of an antigen-dependent priming induced by residual CD19-expressing B-cells present in the manufacture.

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Title

Activation priming and cytokine polyfunctionality modulate the enhanced functionality of low-affinity CD19 CAR T cells

Running title

Molecular profiling of low affinity CAT CAR T-cells

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Abstract

We recently described a low-affinity second-generation CD19 chimeric antigen receptor (CAR) CAT

that showed enhanced expansion, cytotoxicity, and anti-tumour efficacy compared to the high-

affinity

 (FMC63 based) CAR used in Tisagenlecleucel, in pre-clinical models. Furthermore, CAT

demonstrated an excellent toxicity profile, enhanced *in vivo* expansion, and long-term persistence in

a Phase I clinical

study. To understand the molecular mechanisms behind these properties of CAT CAR T-cells, we

performed a systematic *in vitro* characterization of the transcriptomic (RNA-seq) and protein

(CyTOF)

changes occurring in T-cells expressing low-affinity vs high-affinity CD19 CARs following stimulation

with CD19-expressing cells. Our results show that CAT CAR T-cells exhibit enhanced activation to

CD19 stimulation and a distinct transcriptomic and protein profile, with increased activation and

cytokine polyfunctionality compared to FMC63 CAR T-cells. We demonstrate that the enhanced

functionality of low-affinity CAT CAR T-cells is a consequence of an antigen-dependent priming

induced by residual CD19-expressing B-cells present in the manufacture.

Key points

- Low affinity CAT CAR T cells are characterized by a unique pattern of activation priming and

   cytokine polyfunctionality.

- The enhanced functionality of low-affinity CAT CAR T cells is a consequence of an antigen-

   dependent priming

62

63   **Regular Article**

64   **Introduction**

65   T-cells genetically engineered to express CD19 chimeric antigen receptors (CAR T-cells) have shown

66   remarkable efficacy in relapsed/refractory (r/r) B-cell malignancies leading to clinical licensing for r/r

67   B-cell acute lymphoblastic leukaemia (B-ALL) and Non-Hodgkin Lymphoma[1]. Despite this success,

68   several safety and efficacy hurdles remain[2]. CAR T-cells can trigger potent immune responses leading

69   to transient but potentially life-threatening inflammatory events, such as cytokine release syndrome

70   (CRS) and neurotoxicity[3,4]. Thus, the design of versatile CAR T-cells, capable of balancing safety and

71   efficacy, is contingent on our understanding of the molecular mechanisms underlying CAR T-cell

72   function. The engagement of CARs to their cognate antigens results in the activation of CAR T-cells

73   and promotes their rapid expansion as well as their differentiation into distinct T-cell subsets,

74   mediating tumour cytolysis (effector cells) and providing long-lasting protection (memory cells). As

75   tumour cell recognition by CAR T-cells relies on the binding of the CAR's single-chain variable

76   fragment (scFv) to its epitope, fine-tuning the affinity of CARs to their antigens has become a

77   strategy to modulate the strength of CAR T-cell responses[5].

78

79   The affinity of CARs is determined by their binding kinetics and the rates at which they associate to

80   and dissociate from their targets. The optimal affinity of a CAR is likely to vary depending on a

81   number of factors, including the CAR design, the CAR expression levels and the antigen density on

82   the target cells[6,7]. Chimeric immunoreceptors have an activation ceiling above which increasing the

83   binding affinity does not improve T-cell activation but can rather result in T-cell exhaustion[8]. In

84   contrast, by reducing CAR scFv affinity, the strength of the T-cell signal can be modulated, so that

85   CAR T-cells discriminate different levels of antigen expression. CARs exhibiting slower antigen-

86   association rates to ErbB2, EGFR and CD123 targets showed reduced activation in response to low

87   antigen concentrations, favouring differential targeting of tumour cells overexpressing the target *vs*

4

88   normal tissue expressing the same target at physiological levels[8-10]. We have recently described a

89   novel low-affinity CD19 CAR (CAT), with epitope, structure and stability similar to the widely used

90   FMC63, but characterized by faster rates of antigen dissociation, leading to an overall 40-fold

91   reduction of its affinity[11]. Pre-clinical testing of CAT CAR T-cells has revealed greater antigen-specific

92   cytotoxicity, higher proliferation both *in vitro* and *in vivo* and more potent *in vivo* anti-tumour

93   activity when compared to FMC63. In a Phase I clinical trial in patients with high-risk treatment-

94   refractory paediatric B-ALL, CAT CAR T-cells resulted in lower toxicity in terms of severe CRS and

95   displayed greater expansion than that reported for the FMC63 based tisagenlecleucel as well as

96   excellent persistence[11]. These results were recently confirmed in a multicenter Phase I trial in r/r

97   adult B-ALL[12].

98

99   The molecular mechanisms through which the fine-tuning of CARs affinity influences CAR T-cell

100   phenotypes and functions are largely unknown. The interaction between T-cell receptor (TCR) and

101   the peptide-MHC complex offers however some insight on how immunoreceptors' affinity can

102   dramatically influence T-cell functions[13]. Faster target off-rates in TCRs allow a single peptide-MHC

103   complex to serially trigger several TCRs, resulting in amplified and sustained T-cell activation[13].

104   Similar to TCRs, low-affinity CARs may lead to enhanced T-cell activation and decreased exhaustion.

105

106   Herein we perform a systematic *in vitro* characterization of the molecular and biochemical changes

107   occurring in CAR T-cells, comparing a low-affinity CD19 CAR (CAT) with a high-affinity one (FMC63).

108   By combining bulk RNA sequencing with single-cell mass cytometry analyses (cytometry by time of

109   flight, CyTOF), we show that the expression of the CAT CAR induces an antigen-dependent priming in

110   response to low concentrations of CD19-expressing B-cells found in the manufacture product. Upon

111   antigen stimulation, we identified distinct molecular features downstream of CAT CAR activation

112   responsible for enhancing CAT CAR T-cell responses.

113

114   **Methods**

115

116   ***Ex vivo* T-cell expansion**

117   Freshly isolated PBMCs and CD19-depleted PBMCs were cultured in TexMACS™ medium (Miltenyi

118   Biotec, Bergisch Gladbach, Germany), an optimised T-cell medium. To induce T-cell expansion,

119   CD3/CD28 beads (CTS™ Dynabeads™ CD3/CD28, Thermo Fisher Scientific) were added to cells in

120   MACS GMP Cell Differentiation Bags (Miltenyi Biotec) at a 1:3 lymphocyte:bead ratio. CD3/CD28

121   beads were magnetically removed from the culture on day 5 and CAR T-cells rested for 48 h before

122   proceeding to antigen stimulation.

123

124 **Lentiviral vector transduction**

125 Following overnight activation with CD3/CD28 beads, 0.5 x 10^6 beads-activated T-cells were

126 suspended in 0.5 ml of TexMACS, transduced to express CD19 CAR construct (FMC63 or CAT) with 1

127 ml of LV supernatant in RetroNectin® (Takara Bio, Kusatsu, Shiga, Japan)-coated 24-well plates and

128 spinoculated at 1,000 g for 40 min at room temperature. Generally, 2-10 x 10^6 beads-activated T-cells

129 per donor per construct were seeded for transduction.

130

131 **CAR T-cells co-cultures**

132 Briefly, beads-activated T-cells were incubated for 48 h in optimised T-cell medium before

133 stimulation. 0.1 x 10^6 UNTR or FMC63 or CAT beads-activated T-cells were seeded in 96-well plates in

134 complete TexMACS medium in a 1:1 ratio with irradiated (40 Gy) NALM6. Generally, 0.1-6 x 10^6

135 beads-activated T-cells per donor per condition (UNTR, FMC63 and CAT) were stimulated.

136 Unstimulated, beads-activated T-cells per each donor, condition and time point were kept in culture

137 under the same experimental conditions.

138

139 **Flow cytometry activated cell sorting and antibody staining**

140 All FACS experiments included Fluorescence minus one (FMO) and single-antibody stained BD[TM]

141 CompBeads (BD Biosciences) controls to set expression threshold and to calculate compensation,

142 respectively. Experiments were performed on a cell sorter FACSAria[TM] III (BD Biosciences) and on a

143 CytoFLEX analyzer (Beckman Coulter Inc., Brea, CA, USA) and analysed with FlowJo[TM] software

144 v10.6.1 (BD Biosciences) and Cytobank platform (www.cytobank.org). Details on the antibody panels

145 can be found in the Supplementary Methods.

146

147 **Mass cytometry analysis**

148 Following 24 h of stimulation, samples in all experimental conditions were treated with Brefeldin A

149 (BioLegend) (1:1,000) at 37°C for 4 h to favour intracellular cytokine accumulation and were fixed

150 with 1.6% of formaldehyde for 10 min at room temperature. Fixed samples were processed,

151 barcoded (Cell-ID[TM] 20-Plex Pd Barcoding Kit, Fluidigm) and stained as previously described[14]. Two

152 independent mass cytometry experiments were performed and the antibodies used are listed in

153 Supplementary Table 3. The samples were analyzed on a Helios mass cytometer (Fluidigm). EQ[TM]

154 Four Element Calibration Beads (Fluidigm) were added to cell suspensions immediately before

155 acquisition to guarantee inter-sample comparability.

156

157 **Statistical analyses**

158 Data are shown as mean $\pm$ standard error (se) and statistical analyses were performed in R

159 calculating Paired samples t-tests across experimental conditions unless otherwise stated.

160 EMD-related statistical analyses between selected experimental conditions and the results of

161 polyfunctionality analysis are reported in Supplementary Table 5.

162

163 **Data availability**

164 The RNA sequencing data and analyses are available at NCBI's Gene Expression Omnibus (GEO) data

165 repository   with   the   accession   code   GSE157584   and   in   GitHub

166 (https://github.com/EduardoGCCM/CATvsFMC63_Michelozzi).

167 Mass   cytometry   raw   and   processed   data   will   be   made   publicly   available   at

168 https://community.cytobank.org/.

169 **Results**

170 **Generation and quality assessments of low- (CAT) and high- (FMC63) affinity CD19 CARs from**

171 **healthy donors**

172 To dissect the molecular mechanisms behind the functional differences observed between low-

173 (CAT) and high- (FMC63) affinity CD19 CAR T-cells[11], we interrogated the transcriptional and protein

174 expression profiles of T-cells lentivirally (LV) transduced with CARs differing only in their scFv

8

175 (Supplementary Figure 1A). We performed bulk transcriptomic analyses (RNA-seq) to identify CAR T-

176 cell distinct gene expression signatures and mass cytometry analyses (CyTOF) to model differences

177 in their downstream signalling at a single-cell resolution[14]. RNA-seq and CyTOF readouts from

178 untransduced (UNTR) controls and T-cells LV transduced to express CAT or FMC63 CD19 CARs from

179 healthy donors (HD1-HD27, Supplementary Table 1) were compared at baseline and following

180 stimulation with CD19+ ALL cell line NALM6 (unstimulated and stimulated conditions, respectively),

181 as schematized in Figure 1A. We ruled out significant differences in the transduction of the two CARs

182 by fluorescence activated cell sorting (FACS), assessing the percentage of mCherry+ T-cells (LV

183 fluorescent reporter) across donors and experimental conditions. These ranged between 11.50 -

184 93.80% (median 39.1%) in FMC63 and 13.40 - 93.60% (median 36.25%) in CAT (Figure 1B, left) and

185 were thus comparable among individual HDs. In agreement, we found similar transgene expression

186 levels between the 2 CARs by measuring mCherry mean fluorescent intensity (MFI), a proxy for CARs

187 expression (Figure 1B, right) and by quantifying CARs surface expression levels (Figure 1C) and

188 number of integrated vector copies (VCN) (Supplementary Figure 1B). Finally, we assessed CAT and

189 FMC63 CAR T-cell products for their CD4:CD8 ratios and memory T-cell subsets composition, as

190 these characteristics can both affect CAR T-cells persistence and anti-tumour activity[15,16]. While no

191 difference in CD4:CD8 ratios was observed in the absence of antigen stimulation (Figure 1D), the

192 proportion of memory subsets differed in unstimulated FMC63 and CAT CAR T-cells, as measured by

193 FACS at 10 days post-transduction. CAT CAR T-cells exhibited a significant increase in the fraction of

194 central memory T-cells (T_{CM}, CD62+CD45RA-) as compared to both UNTR and FMC63 conditions

195 (Figure 1E, left). The increase in T_{CM} was largely at the expense of T effector memory cells ($T_{EM,}$ CD62-

196 CD45RA-) and effector memory re-expressing CD45RA ($T_{EMRA,}$ CD62-CD45RA+), whose proportion

197 was significantly reduced in CAT *vs* FMC63 CAR T-cells and in both CARs as compared to UNTR

198 control (Figure 1E, middle and right). These assessments confirmed that our CAR T-cell products

199 were suitable to investigate the molecular features of CAT and FMC63 CAR T-cells.

200

Low-affinity CD19 CAT CARs display higher activation priming during CAR T cell manufacture

We next performed bulk RNA-seq of FACS-sorted UNTR T-cells (CD3+) and FMC63 or CAT CAR T-cells (CD3+mCherry+) with or without a 24 h stimulation with NALM6. Transcriptomics confirmed that mCherry was an accurate proxy for CAR transgene expression levels, as evidenced by the significant positive correlation between the MFI of mCherry by FACS and the normalized RNA-seq counts aligning to the scFv region of each of the 2 CARs (Supplementary Figure 2A). Similarly, the proportion of CD4 and CD8 T-cells detected by FACS was in line with the *CD4* and *CD8* mRNA levels (Supplementary Figure 2B, C). Principal component analysis (PCA) on the 500 most variable expressed genes (top 100 genes shown in Supplementary Figure 2D), distributed samples according to a T-cell activation gradient (PC1, from UNTR to CAR activated samples) (Figure 2A). The majority of variance in gene expression across experimental conditions was explained by CD19-mediated CAR activation. As expected, UNTR T-cells not expressing any CARs were largely unaffected by antigen stimulation (Figure 2A). Similarly, PCA on protein expression from CyTOF, based on earth mover's distance (EMD) scores (a sensitive measure of multivariate changes in protein levels)[17], in the same experimental conditions and timepoints than the gene expression, followed a similar gradient of sample activation, shifting from UNTR samples to antigen stimulated CAR T-cells on PC1 (Figure 2B and Supplementary Figure 3A). The most variable genes upregulated upon stimulation with NALM6 in both FMC63 and CAT CAR T-cells included genes involved in T-cell activation (*IL2RA*, *GZMB*) and proliferation (*PCNA*, *LDHA*), which are expressed at very low levels in control T-cells (Supplementary Figure 2D). Consistent with this, the highest protein expression variation was from markers of T-cell activation (CD25, NFAT1, HLA-DR) and proliferation (pRB) (Supplementary Figure 3A). In both RNA and protein analyses, unstimulated CAR T-cells had an intermediate RNA/protein expression profile between UNTR and stimulated CAR T-cells. This suggests that CAR expression on its own, in absence of antigen stimulation, induces basal T-cell activation (Figure 2A-B).

226   To gain further insights into the intermediate activation state observed in unstimulated CAR T-cells,

227   we fuzzy clustered individual samples by their gene expression, to resolve intermediate cell states

228   and trajectories (Figure 2C)[18]. We identified two clusters, highly enriched for either inactive T-cells

229   (cluster 1, which includes UNTR samples) or antigen-activated CAR T-cells (cluster 2, which includes

230   stimulated CAR T samples) (Figure 2C). Notably, the probability of unstimulated CAT samples of

231   belonging to cluster 2 (activated CAR T-cells) was substantially higher than that of unstimulated

232   FMC63 (4/6 HDs in CAT *vs* 0/6 HDs inFMC63), further evidencing the functional proximity between

233   unstimulated CAT and activated CAR T-cells. Using gene set enrichment analyses (GSEA) on the

234   Hallmark collection, we confirmed that while antigen stimulated CAT and FMC63 CAR T-cells have

235   similar enrichment for most of the gene sets involved in immune functions and cell proliferation, in

236   the absence of antigen stimulation CAT CAR T-cells are uniquely enriched for T-cell activation

237   pathways (Supplementary Figure 3B).

238   Next, we compared the transcriptome of FMC63 and CAT CAR T-cells, in absence of antigen

239   stimulation. Following differential gene expression (DGE) analysis, we found that only 10 genes were

240   significantly DE between these two conditions (FDR < 0.1, Supplementary Table 2), 9 of which were

241   upregulated in CAT *vs* FMC63 CAR T-cells. Among those, we found genes involved in cytotoxicity

242   (*GNLY*, *GZMK*) and markers of T-cell activation such as MHC class II molecules (MHCII) (*HLA-DRA* and

243   *HLA-DPA*) (Figure 2D)*.* This supports our previous observation of gene expression in unstimulated

244   CAT resembling more that of antigen activated CAR T-cells than gene expression in unstimulated

245   FMC63 (Figure 2C). Mass cytometry analyses using a panel of antibodies against markers of T-cell

246   activation (Supplementary Table 3), confirmed that while unstimulated FMC63 and CAT CAR T-cells

247   have similar EMD scores for many of the proteins investigated (Supplementary Table 5a),

248   unstimulated CAT CAR T-cells have significantly stronger activation priming with higher expression of

249   markers of T-cell activation (HLA-DR, CD25 and NFAT1), pro-inflammatory (Granzyme B, Perforin B)

250   and stimulatory/activation-related (GM-CSF, IL-17A) cytokines and increased phosphorylation of the

11

251 TCR/CAR CD3ζ chain (pZAP70) and MTOR downstream effector (pS6) (Figure 2E and Supplementary

252 Figure 3C).

253 Altogether, these results show that unstimulated CAT CAR T-cells, prior to antigenic stimulation,

254 have more pronounced T-cell activation priming than FMC63 CAR T-cells.

255

256 **CD19 stimulation of low-affinity CAT CAR T-cells results in a distinct transcriptomic and protein**

257 **profile with increased activation/proliferation over high-affinity FMC63 CAR T-cells**

258 While antigen-independent CAR activation, also known as tonic signalling, has been often associated

259 with CAR T-cell accelerated differentiation and exhaustion[19-21], recent data show that the induction

260 of CAR T-cell priming can lead to CAR T enhanced anti-tumour functions *in vivo*[22,23]. We wanted to

261 assess the molecular impact of the "activation priming" observed in CAT CAR T-cells on their

262 molecular response to antigen stimulation. As the superior cytotoxicity of CAT CAR T-cells over

263 FMC63 CAR T-cells has been previously shown in functional assays[11], we focused on characterizing

264 their distinct molecular profiles upon exposure to antigenic stimulation. Upon stimulation, we found

265 a slight but statistically significant reduction of CD4:CD8 ratio in CAT as compared to FMC63 (Figure

266 3A), largely attributable to a relative decrease of the CD8+ fraction (and increase of CD4+) in FMC63

267 CAR T-cells (Supplementary Figure 4A). Interestingly, when looking at the memory T-cell subsets

268 composition, we found that CAT CAR T-cells continued to exhibit a higher proportion of T_{CM} as

269 compared to FMC63 (Figure 3B), while no differences were observed in the expression of exhaustion

270 markers (PD1, TIM3 and LAG3) between the two CAR constructs (Figure 3C).

271 Importantly, the stronger basal activation observed in CAT CAR T-cells did not prevent an even

272 stronger molecular response when exposed to CD19-expressing NALM6, as shown by the increase in

273 the expression of proliferation and cytotoxic/stimulatory markers, relatively to the unstimulated

274 constructs (Supplementary Figure 5 and 6). Consistent with this, we identified 51 DE genes, 35 of

275 which were upregulated in CAT compared to FMC63 (Figure 3D and Supplementary Table 4). CD19

12

276   stimulation in CAT CAR T-cells also led to significantly augmented expression of immune

277   stimulatory/proliferation cytokines (*IFNG, CSF2, CXCL8*), and IFN-γ responsive genes (*CIITA*) (Figure

278   3D and Supplementary Figure 4B). Conversely, CAT CAR T-cells displayed significantly decreased

279   expression of the genes encoding for CRIF1 (*GADD45GIP1*), an inhibitor of cell cycle progression, and

280   for FOXP3, the Treg-associated transcription factor known to be only transiently expressed in the

281   initial stages of Th1 response and rapidly downregulated afterwards[24,25] (Figure 3D). These results

282   suggest that following antigenic stimulation CAT CAR T-cells show a different transcriptomic profile

283   resulting in stronger activation and proliferation than stimulated FMC63. In addition, CAT CAR T-cells

284   showed increased expression of the *TNFSF4* gene (Figure 3D), encoding for the ligand of the T-cell

285   co-stimulatory receptor OX40 (OX40L). While OX40L is mainly expressed by antigen presenting cells

286   to promote T-cell activation, it is also expressed in activating T-cells, where it leads to a homotypic

287   OX40L-OX40 signalling axis promoting T-cell longevity and memory differentiation[26]. Further, we also

288   noted the increased expression of the chemoattractants *CCL4* and *CCL3L1* and genes involved in cell

289   migration (*FLT1*, *DOCK5*) and focal adhesion (*COL6A3*) in stimulated CAT CAR T-cells (Supplementary

290   Figure 4B).

291   These observations were further substantiated at the protein level. CAT CAR T-cells exhibited a

292   marked increase in the expression (as measured by EMD score) of markers of T cell activation (CD25)

293   (Figure 3E). Moreover, the augmented gene expression of the MHCII trans-activator CIITA observed

294   in CAT, resulted in a corresponding increase of HLA-DR protein (Figure 3E)[27,28]. When measuring the

295   CAR T-cell intracellular signalling, CAT CAR T-cells showed enhanced phosphorylation of the effectors

296   of the TCR/CAR CD3ζ chain (pZAP70, pp38) and increased expression of their downstream

297   transcription factors (NFAT1, pCREB, FOXP3). CAT CAR T-cells also exhibited significant upregulation

298   of the Target of Rapamycin Complex 1 (mTORC1) downstream effectors (pS6 and pRB), both

299   involved in cell proliferation and protein translation, in line with the previously reported CAT CAR T

300   cells increased proliferative capacity over FMC63[11].

301 Our analysis shows that the increased "activation priming" observed in CAT CAR T-cells over FMC63

302 resulted in an even stronger T-cell activation gene expression and signaling profile when CAT CAR T-

303 cells were exposed to antigenic stimulation. These observations are in line with the CAT CAR T cells

304 enhanced cytotoxic functional properties previously reported[11].

305 **The enhanced functionality of low-affinity CD19 CAR T-cells is associated with cytokine**

306 **polyfunctionality upon antigen stimulation**

307 We next assessed CAR T-cell functional phenotypes by measuring intracellular cytokines levels in

308 individual cells by mass cytometry. The overall protein intensities measured by EMD scores indicated

309 an increased expression of effector cytokines (Granzyme B , IFN-γ and TNF-α) and of immune

310 stimulatory molecules (GM-CSF and IL-2) in CAT CAR T as compared to FMC63 (Figure 4A). No

311 upregulation was observed for Th2/immune-modulatory cytokines (IL-4, IL-5 and TGF-ß) and for

312 Perforin B (Supplementary Table 5b).

313

314 Next, we investigated the pattern of cytokine co-expression in CAT and FMC63 CAR T-cell responses.

315 The ability of a single T-cell to express simultaneously more than one cytokine (polyfunctionality) has

316 been linked to productive immune responses[29,30] and more recently described as a distinctive

317 feature of CAR T-cells associated to their potency and anti-tumour efficacy [31-33]. We measured the

318 frequency at which the eight cytokines included in our analysis were co-expressed in single CAR T-

319 cells, thus providing a comprehensive profile of their cytokine polyfunctionality. Upon stimulation

320 with NALM6, 15.02% of FMC63 and 29.60% of CAT CAR T-cells were polyfunctional (expressing two

321 or more cytokines per cell) (Supplementary Table 5n).

322 Not only the frequency of polyfunctional CAR T-cells, but also the number of cytokines co-expressed

323 was higher in CAT than in FMC63, with a stastically significant increase in their mean

324 polyfunctionality (Figure 4B) and a marked increment in the proportion of cells expressing

325 combinations of three or more cytokines (2.28% in FMC63 *vs* 7.89% in CAT) (Figure 4C and

14

326   Supplementary Table 5o). The polyfunctional profiles of stimulated CAT CAR T-cell products were

327   dominated by combinations of cytokines involving the effector molecules IFN-g, TNF-α, IL-2 and

328   Granzyme B (Figure 4D).

329   **Low-affinity CD19 CAT CAR activation priming is associated with and driven by residual CD19-**

330   **expressing B-cells**

331   To investigate whether the mechanism behind the activation priming observed in unstimulated CAT

332   CAR T-cells was antigen-dependent or -independent, we checked if residual CD19+ B-lymphocytes

333   were detectable in the CAR T-cell product and could serve as a potential source of antigen specific

334   activation.

335

336   We monitored the proportion of CD19+ B-lymphocytes in culture at different timepoints. While B-

337   cells were detectable at day 0 in all samples (5.96% of cells on average), at day 8 (5 day stimulation

338   with CD3/CD28 beads + 3 day rest) they could only be detected in the UNTR condition (2.33% of cells

339   on average), and had been completely depleted from both CAR constructs, as shown by FlowSOM

340   analysis of mass cytometry data, clustering single-cells by cell types[34], and the relative frequencies

341   (Figure 5A and Supplementary Figure 7A). We next applied the experimental setup described in

342   Figure 1A, only now including as additional experimental condition CAR T-cells generated from

343   CD19-depleted PBMCs. We confirmed effective CD19 depletion by flow cytometry, with an average

344   of residual B cells of 0.041% upon depletion as compared to 5.96% with the standard protocol

345   (Supplementary Figure 7B). The transduction levels obtained in the CD19-depleted CAT and FMC63

346   CAR T-cells were comparable based on the expression of the fluorescent reporter (mCherry) and the

347   VCN (Supplementary Figure 7C-D). The baseline CD4:CD8 ratios only showed a slight increase in CAT

348   as compared to FMC63 CAR T cells (Supplementary Figure 7E). Of note, CD19-depletion impacted on

349   the T memory subsets composition, with CAT CAR T-cells no longer displaying any statistical

350   difference when compared to the UNTR control, while FMC63 CAR T-cells still showing a significant

351   increase in the proportion of T_{EM} when compared to the UNTR control (Figure 5B).

352   Mass cytometry analyses revealed that the residual B cells in the CAR T-cell manufacture were

353   responsible for the activation priming observed in unstimulated CAT and FMC63 CAR T-cells

354   (Supplementary Figure 8, 9). While CD19 depletion led to a general decrease in the activation

355   priming previously observed in unstimulated CAR T-cells, this reduction was more pronounced in

356   CAT (Supplementary Figure 8) than in FMC63 (Supplementary Figure 9). As a result, upon B cell

357   depletion we no longer detected statistically significant differences in the expression of T-cell

358   activation markers (HLA-DR, CD25 and NFAT1), pro-inflammatory cytokines (Granzyme B, Perforin B)

359   and CAR-downstream signaling molecules (pZAP70 and pS6) between CAT and FMC63 (Figure 5C).

360   Differential protein expression between CD19-depleted CAT and FMC63 CAR T-cells was only

361   observed for pRB and CD69 (Supplementary Figure 7F). Both CAT and FMC63 CAR T cells exhibited

362   increased expression of activation and cytotoxic markers with respect to the UNTR controls they are

363   normalized to, indicating similar levels of antigen-independent activation (Figure 5C).

364   When later exposed to NALM6, both CD19-depleted CAT and FMC63 were able to activate, as shown

365   by the upregulation of the expression of cytotoxic markers and cytokines compared to their

366   unstimulated counterparts (Supplementary Figure 10, 11). No differences in the CD4:CD8 ratios and

367   in the expression of exhaustion markers were observed between the two stimulated CAR conditions

368   upon CD19-depletion (Supplementary Figure 7G, H). Most importantly, upon antigenic stimulation,

369   CD19-depleted CAT CAR T-cells activated a molecular response with no stastically significant

370   differences when compared to FMC63 (Figure 5D and Supplementary Figure 7I), except for an

371   increased expression of IL-2 (Supplementary Figure 7I). The increased cytokine polyfunctionality

372   observed in CAT *vs* FMC63 CAR T-cells in standard manufacture condition (Figure 4) was no longer

373   observed in CD19-depleted manufacture condition (Figure 5E).

374

16

375 Altogether these results demonstrate that residual B-cells in the CAR T-cell manufacture can

376 mediate an antigen dependent activation priming, which is more pronounced in low affinity CAT CAR

377 T-cells when compared to FMC63 CAR T-cells. Such activation priming contributes to boosting CAT

378 CAR T-cell response, as CAT CAR T-cells generated from CD19-depleted PBMCs not only do not

379 display increased activation priming but also do not exhibit increased molecular responsed to

380 antigenic stimulation with NALM6.

381 **Discussion**

382 Modulating CAR T-cell affinity may enable us to enhance anti-tumour response and long-term

383 tumour surveillance, while minimizing CAR T-cell related toxicity. We thus investigated the

384 transcriptomic and proteomic phenotype of CD19 CAT CAR T-cells, compared with the widely used

385 FMC63, to begin unravelling the molecular mechanisms behind the observed preclinical and clinical

386 differences between these two CD19 CARs[11].

387 We found that CAT CARs induce stronger activation responses than FMC63, which could be

388 explained by the faster target off-rate of low-affinity CARs. Faster dissociation requires fewer targets

389 to serially trigger a larger number of CARs and amplify anti-tumoural response[13]. This would align

390 with the proposed model of temporal and spatial summation of T-cell activation, in which signals

391 from serially triggered immunoreceptors can be accumulated and integrated overtime to reach the

392 threshold required for T-cell activation[35]. This model may also explain why low levels of residual

393 donor B cells in the manufacture can induce stronger activation priming in CAT CAR T cells than in

394 FMC63.

395 Our transcriptomic and protein profiling revealed that unstimulated CAT CAR T-cells are functionally

396 closer to antigen activated CAR T-cells than FMC63 CAR T-cells, with a number of upregulated

397 activation genes (*HLA-DBP1*, *HLA-DRA* etc) and proteins (HLA-DR, CD25 and NFAT1). Despite these

398 genes/proteins being commonly associated to T-cell activation, their basal expression is also

399 increased in memory T-cells as compared to $T_{NAÏVE}$ cells, as memory T-cells are characterized by an

17

400   open chromatin conformation favouring the access of transcription factors to immune response

401   genes, thus ensuring a pool of readily available mRNAs that can be rapidly translated following

402   stimulation[36]. This hypothesis is in agreement with the increased proportion of T_{CM} observed in CAT

403   CAR T cells as compared to FMC63. However, further studies are needed to elucidate the molecular

404   signallings underlying the preferential differentiation towards T_{CM} in low-affinity CAT CAR T-cells.

405

406   By performing B cell depletion prior to CAR T-cell manufacture, we demonstrate that residual B-cells

407   in the CAR T-cell product are responsible for the activation priming observed in CAR T-cells, which

408   was more pronounced in CAT when compared to FMC63, and preferentially induce T_{CM}

409   differentiation of CAT CAR T-cells. This is consistent with the hypothesis that CAT serial triggering

410   may amplify T-cell activation from lower antigen levels[13] and points to a boosting role of low dose

411   CD19-priming during CAR T-cell manufacture. The preferential T_{CM} phenotype in CAT CAR T-cells

412   might be due to kinetic differences in clearance of residual B cells or to different downstream

413   signalling after antigenic stimulation with low- vs high-affinity CAR T-cells. Further work elucidating

414   these mechanisms is needed. Recent results have shown that antigen-independent induction of CAR

415   T-cell priming, by either 4-1BB-based tonic signalling[22] or by low-dose of hypomethylating agents[23],

416   can lead to enhanced CAR T anti-tumour functions *in vivo* and it is regulated by the recruitment of

417   LCK or THEMIS-SHP1 phosphatase into the CAR synapse[37]. Our results indicate that low dose

418   antigen-specific priming can also promote CAR T-cell functionality in a CAR construct specific manner

419   with an enhanced effect in low-affinity CAR T-cells.

420

421   When stimulated with CD19-expressing NALM6 cell line, CAT CAR T-cells have a distinct

422   transcriptomic and protein response to CD19 antigenic stimulation from FMC63 CAR T-cells with

423   increased expression of proliferation, activation and cytotoxic markers at both RNA and protein

424   levels. *In vivo* clonal kinetics analyses have shown that single CD19 CAR T-cells with higher

425　expression of cytotoxic related genes in the manufacture product, many of which in common with

426　ours (including *IFNG*, *HLA-DRA*, *CCL4*), gave rise to superior *in vivo* expansion and survival,

427　significantly contributing to later timepoints after adoptive transfer in patients[19,38]. Analysis of gene

428　expression also revealed the upregulation of chemoattractive cytokines CCL4 and CCL3L1 in CAT,

429　which induce T-cell homotypic interactions and promote the reciprocal exchange of self-reinforcing

430　signals such as OX40L,[26,39] which are upregulated in CAT as well. Single-cell transcriptomic studies

431　have revealed that subsets of CAR T with elevated expression of CCL3 and CCL4 are associated to

432　longer persistence in vivo[38] and achievement of complete remission[40]. Mass Cytometry analyses

433　revealed a CAT CAR T enhanced activation protein profile, as measured by the increased expression

434　of T-cell activation markers (CD25, HLA-DR) and CAR downstream signaling effectors (pZAP70, pp38,

435　NFAT1, pCREB, FOXP3). Furthermore, CAT CAR T-cells showed increased mTORC signaling (pS6 and

436　pRB), which is commonly associated to cell proliferation and protein translation. Altogether, CAT

437　CAR T-cells distinct gene expression and protein profiles are very much in line and likely responsible

438　for the enhanced proliferative responses that CAT CAR T-cells exhibited *in vitro*, in *in vivo* murine

439　models and in patients, as previously reported by Ghorashian et al.[11].

440

441　Upon stimulation, CAT CAR T-cells were characterized by a unique polyfunctional pattern of cytokine

442　expression, with a marked increase in the frequency of single CAR T-cells expressing >=3 cytokines

443　when compared to FMC63. CAT polyfunctional profile was dominated by combinations of effector

444　cytokines, consistent with their potent anti-tumour activity. It has been suggested that the ability of

445　CAR T-cells to produce multiple cytokines in response to antigen exposure is associated with

446　improved anti-tumour responses *in vivo*[31] and cytokine polyfunctionality has been recently proposed

447　as a criteria to predict CAR T-cell potency [33]. The increased cytokine polyfunctionality observed in

448　CAT CAR T-cells contributes to explain their increased cytotoxic potential previously observed *in vitro*

449　*settings* and in *in vivo* murine models[11].

19

450

451   In conclusion, we describe the molecular mechanisms underlying the low-affinity CAT CAR T-cell

452   functional phenotype. We provide evidence that the potent and long-term anti-tumour responses

453   observed with low-affinity CAT CAR T-cells[11] reflect a distinct pattern of both activation priming and

454   cytokine polyfunctionality. We show that low-affinity CAT CAR T-cells are preferentially primed by

455   low concentration of CD19-expressing B-cells present in the manufacture and such priming is

456   instrumental to their higher cytotoxic response upon stimulation. Although our observations are

457   limited to one low-affinity CAR, future work extending this characterization to a panel of low-affinity

458   CARs, may reveal whether these findings are generalisable. In future work, we will focus on

459   elucidating the mechanism by which residual B cells in the starting material induce

460   differential effects on low vs high affinity CAR T-cells and whether there is a dose-

461   dependent relationship between residual CD19+ B cells and CAR T-cell functionality. This will

462   have important implications for CAR T-cell manufacturing protocols.

463   Overall, our work has important implications for the future design of versatile CAR T-cells

464   manufacture protocols, capable of boosting efficacy and long-term persistence.

465

476

477   **Authorship**

478 Contributions: I.M.M. designed, performed and analyzed experiments, performed bioinformatic

479 analyses of mass cytometry experiments and contributed to writing the manuscript. E.G-C

480 performed bioinformatic analyses of transcriptomic data and wrote the relative bioinformatic

481 supplementary information. R.V.C.P. performed experiments and bioinformatic analyses of mass

482 cytometry and transcriptomic data. F.C-R provided data analysis tools and contributed to

483 bioinformatic analyses of mass cytometry experiments. P.P-C. performed transcriptomic

484 bioinformatic analyses data checks and contributed to writing the bioinformatic supplementary

485 information. J. S., M.S., S.W.W, A.Gu. and E.K. performed experiments. A.E. provided support to cell

486 sorting. J.F. provided analytical pipelines and useful discussion for the analysis and normalization of

487 mass cytometry data. S.G. and M.P. provided CAR constructs. C.J.T. provided expertise in mass

488 cytometry and reagents. P.J.A. provided reagents and expertise and contributed to writing the

489 manuscript. S.C. supervised the bioinformatic analyses and contributed to writing the manuscript.

490 A.G. designed and supervised the project, performed and analyzed experiments and wrote the

491 manuscript. All authors provided critical feedback and helped shape the research, analysis and

492 manuscript.

493 Conflict of Interest: S.G. received speaker's honoraria from Novartis and patents and royalties from

494 UCLB. M.A.P. owns stock in and is in part employed by Autolus Therapeutics, that has licensed CAT

495 CAR. P.J.A. has received Royalties for a patent related to CAT CAR from Autolus and receives

496 research funding from Autolus. The remaining authors declare no competing interests.

497

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References

1.   Miliotou AN, Papadopoulou LC. CAR T-cell Therapy: A New Era in Cancer Immunotherapy. *Curr Pharm Biotechnol*. 2018;19(1):5-18.

2.   Pehlivan KC, Duncan BB, Lee DW. CAR-T Cell Therapy for Acute Lymphoblastic Leukemia: Transforming the Treatment of Relapsed and Refractory Disease. *Curr Hematol Malig Rep*. 2018;13(5):396-406.

3.   Lee DW, Gardner R, Porter DL, et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood*. 2014;124(2):188-195.

4.   Gust J, Hay KA, Hanafi LA, et al. Endothelial Activation and Blood-Brain Barrier Disruption in Neurotoxicity after Adoptive Immunotherapy with CD19 CAR-T Cells. *Cancer Discov*. 2017;7(12):1404-1419.

5.   Watanabe K, Kuramitsu S, Posey AD, Jr., June CH. Expanding the Therapeutic Window for CAR T Cell Therapy in Solid Tumors: The Knowns and Unknowns of CAR T Cell Biology. *Front Immunol*. 2018;9:2486.

6.   Kowolik CM, Topp MS, Gonzalez S, et al. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Res*. 2006;66(22):10995-11004.

7.   Singh AP, Zheng X, Lin-Schmidt X, et al. Development of a quantitative relationship between CAR-affinity, antigen abundance, tumor cell depletion and CAR-T cell expansion using a multiscale systems PK-PD model. *MAbs*. 2020;12(1):1688616.

8.   Chmielewski M, Hombach A, Heuser C, Adams GP, Abken H. T cell activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not increase T cell activation against antigen-positive target cells but decreases selectivity. *J Immunol*. 2004;173(12):7647-7653.

9.   Caruso HG, Hurton LV, Najjar A, et al. Tuning Sensitivity of CAR to EGFR Density Limits Recognition of Normal Tissue While Maintaining Potent Antitumor Activity. *Cancer Res*. 2015;75(17):3505-3518.

10.   Liu X, Jiang S, Fang C, et al. Affinity-Tuned ErbB2 or EGFR Chimeric Antigen Receptor T Cells Exhibit an Increased Therapeutic Index against Tumors in Mice. *Cancer Res*. 2015;75(17):3596-3607.

11.   Ghorashian S, Kramer AM, Onuoha S, et al. Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR. *Nat Med*. 2019;25(9):1408-1414.

12.   Roddie C, Dias J, O'Reilly MA, et al. Durable Responses and Low Toxicity After Fast Off-Rate CD19 Chimeric Antigen Receptor-T Therapy in Adults With Relapsed or Refractory B-Cell Acute Lymphoblastic Leukemia. *J Clin Oncol*. 2021:JCO2100917.

13.   Valitutti S, Muller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature*. 1995;375(6527):148-151.

14.   Michelozzi IM, Sufi J, Adejumo TA, Amrolia PJ, Tape CJ, Giustacchini A. High-dimensional functional phenotyping of preclinical human CAR T cells using mass cytometry. *STAR Protoc*. 2022;3(1):101174.

15.   Wang X, Popplewell LL, Wagner JR, et al. Phase 1 studies of central memory-derived CD19 CAR T-cell therapy following autologous HSCT in patients with B-cell NHL. *Blood*. 2016;127(24):2980-2990.

16.   Wang X, Wong CW, Urak R, et al. Comparison of naive and central memory derived CD8(+) effector cell engraftment fitness and function following adoptive transfer. *Oncoimmunology*. 2016;5(1):e1072671.

549 17. Orlova DY, Zimmerman N, Meehan S, et al. Earth Mover's Distance (EMD): A True
550 Metric for Comparing Biomarker Expression Levels in Cell Populations. *PLoS One*.
551 2016;11(3):e0151859.

552 18. Fuzzy Analysis (Program FANNY). Finding Groups in Data; 1990:164-198.

553 19. Gomes-Silva D, Mukherjee M, Srinivasan M, et al. Tonic 4-1BB Costimulation in
554 Chimeric Antigen Receptors Impedes T Cell Survival and Is Vector-Dependent. *Cell Rep*.
555 2017;21(1):17-26.

556 20. Frigault MJ, Lee J, Basil MC, et al. Identification of chimeric antigen receptors that
557 mediate constitutive or inducible proliferation of T cells. *Cancer Immunol Res*.
558 2015;3(4):356-367.

559 21. Long AH, Haso WM, Shern JF, et al. 4-1BB costimulation ameliorates T cell
560 exhaustion induced by tonic signaling of chimeric antigen receptors. *Nat Med*.
561 2015;21(6):581-590.

562 22. Singh N, Frey NV, Engels B, et al. Antigen-independent activation enhances the
563 efficacy of 4-1BB-costimulated CD22 CAR T cells. *Nat Med*. 2021;27(5):842-850.

564 23. Wang Y, Tong C, Dai H, et al. Low-dose decitabine priming endows CAR T cells
565 with enhanced and persistent antitumour potential via epigenetic reprogramming. *Nat
566 Commun*. 2021;12(1):409.

567 24. Allan SE, Crome SQ, Crellin NK, et al. Activation-induced FOXP3 in human T
568 effector cells does not suppress proliferation or cytokine production. *Int Immunol*.
569 2007;19(4):345-354.

570 25. Ran Q, Hao P, Xiao Y, et al. CRIF1 interacting with CDK2 regulates bone marrow
571 microenvironment-induced G0/G1 arrest of leukemia cells. *PLoS One*. 2014;9(2):e85328.

572 26. Soroosh P, Ine S, Sugamura K, Ishii N. OX40-OX40 ligand interaction through T
573 cell-T cell contact contributes to CD4 T cell longevity. *J Immunol*. 2006;176(10):5975-5987.

574 27. Holling TM, van der Stoep N, Quinten E, van den Elsen PJ. Activated human T cells
575 accomplish MHC class II expression through T cell-specific occupation of class II
576 transactivator promoter III. *J Immunol*. 2002;168(2):763-770.

577 28. Gourley TS, Chang CH. Cutting edge: the class II transactivator prevents activation-
578 induced cell death by inhibiting Fas ligand gene expression. *J Immunol*. 2001;166(5):2917-
579 2921.

580 29. Betts MR, Nason MC, West SM, et al. HIV nonprogressors preferentially maintain
581 highly functional HIV-specific CD8+ T cells. *Blood*. 2006;107(12):4781-4789.

582 30. Caccamo N, Guggino G, Joosten SA, et al. Multifunctional CD4(+) T cells correlate
583 with active Mycobacterium tuberculosis infection. *Eur J Immunol*. 2010;40(8):2211-2220.

584 31. Rossi J, Paczkowski P, Shen YW, et al. Preinfusion polyfunctional anti-CD19
585 chimeric antigen receptor T cells are associated with clinical outcomes in NHL. *Blood*.
586 2018;132(8):804-814.

587 32. Xhangolli I, Dura B, Lee G, Kim D, Xiao Y, Fan R. Single-cell Analysis of CAR-T
588 Cell Activation Reveals A Mixed TH1/TH2 Response Independent of Differentiation.
589 *Genomics Proteomics Bioinformatics*. 2019;17(2):129-139.

590 33. Spiegel JY, Patel S, Muffly L, et al. CAR T cells with dual targeting of CD19 and
591 CD22 in adult patients with recurrent or refractory B cell malignancies: a phase 1 trial. *Nat
592 Med*. 2021;27(8):1419-1431.

593 34. Van Gassen S, Callebaut B, Van Helden MJ, et al. FlowSOM: Using self-organizing
594 maps for visualization and interpretation of cytometry data. *Cytometry A*. 2015;87(7):636-
595 645.

596 35. Rachmilewitz J, Lanzavecchia A. A temporal and spatial summation model for T-cell
597 activation: signal integration and antigen decoding. *Trends Immunol*. 2002;23(12):592-595.

598   36.   Weng NP, Araki Y, Subedi K. The molecular basis of the memory T cell response:
599   differential gene expression and its epigenetic regulation. *Nat Rev Immunol*. 2012;12(4):306-
600   315.
601   37.   Sun C, Shou P, Du H, et al. THEMIS-SHP1 Recruitment by 4-1BB Tunes LCK-
602   Mediated Priming of Chimeric Antigen Receptor-Redirected T Cells. *Cancer Cell*.
603   2020;37(2):216-225 e216.
604   38.   Sheih A, Voillet V, Hanafi LA, et al. Clonal kinetics and single-cell transcriptional
605   profiling of CAR-T cells in patients undergoing CD19 CAR-T immunotherapy. *Nat
606   Commun*. 2020;11(1):219.
607   39.   Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN.
608   Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-
609   dendritic cell interaction. *Nature*. 2006;440(7086):890-895.
610   40.   Bai Z, Lundh S, Kim D, et al. Single-cell multiomics dissection of basal and antigen-
611   specific activation states of CD19-targeted CAR T cells. *J Immunother Cancer*. 2021;9(5).
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Figure legends

Figure 1: Generation and phenotypic characterisation of CAR T-cells from HD-PBMCs.

A Experimental workflow. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from HDs and

LV transduced to express CD19 CAR construct (FMC63 or CAT) following overnight activation with

CD3/CD28 beads. Six days after transduction, CAR T-cells were cultured without (unstimulated) or

with target cells (NALM6) at 1:1 ratio (stimulated). Unstimulated and stimulated cells were analysed

by flow cytometry and sorted for RNA-sequencing 24 hours post-stimulation. Mass cytometry

analysis was performed on unstimulated and stimulated cells at 24 h post-stimulation. Activated

UNTR T-cells were used as a control throughout the experiment.

B (left) Spaghetti plots showing transduction levels of CAR T-cells as percentage of mCherry+ (in

CD3+) and (right) as MFI of mCherry in unstimulated transduced T-cells measured by FACS 7 days

post-transduction. Lines connect results from individual donors (n= 12 HDs, n = 3 independent

experiments).

C Spaghetti plot showing the percentage of surface CAR expression (in CD3+) in unstimulated

transduced T-cells measured by FACS 10 days post-transduction. Lines connect results from

individual donors (n = 4 HDs, n = 1 independent experiment).

630 **D** Variation (log2 fold change) of CD4 and CD8 proportion in unstimulated UNTR T-cells and FMC63

631 and CAT CAR T-cells measured by FACS 7 days post-transduction. The dotted horizontal line (0)

632 represents the conditions in which CD4=CD8. Data represent mean ± se (n = 12 HDs, n = 3

633 independent experiments).

634 **E** (left) Barplots showing the percentage of TCM (CD45RA-CD62L+), (middle) TEM (CD45RA-CD62L-)

635 and (right) TEMRA (CD45RA+CD62L-) in unstimulated CD3+ UNTR T-cells and FMC63 and CAT CAR T-

636 cells measured by FACS 10 days post-transduction. Data represent mean ± se (n = 7 HDs, n = 2

637 independent experiments).

638 **B-E** Statistical significance was calculated by Paired t-test. *P < 0.05, **P < 0.01, ****P < 0.0001.

639 Each experimental condition is indicated by a specific colour code (UNTR light grey, FMC63 light

640 blue, CAT orange).

641

642 **Figure 2: RNA-seq and mass cytometry analyses of unstimulated untransduced and CAR-**

643 **transduced T-cells.**

644 **A** PCA of the top 500 variable genes from RNA-seq analysis across all the experimental conditions (n

645 = 6 HDs, n = 2 independent experiments).

646 **B** PCA of mass cytometry EMD scores computed at 24 h post-stimulation in CD3+ cells across all the

647 experimental conditions (n = 4 HDs, n = 1 independent experiment).

648 **C** Fuzzy clustering analysis of RNA-seq data across all experimental conditions (n = 6 HDs, n = 2

649 independent experiments).

650 **D** (top) Volcano plot showing DE genes between unstimulated FMC63 and CAT CAR T-cells. The

651 dashed horizontal line represents the statistical significance threshold (FDR < 0.1). (bottom) The

652 barplots show the expression of selected DE genes (FDR < 0.1) in unstimulated untransduced and

653 transduced T-cells. Data represent mean ± se (n = 6 HDs, n = 2 independent experiments).

654 **E** The barplots show the expression of mass cytometry EMD scores for Granzyme B, Perforin B, HLA-

655 DR, CD25, NFAT1, pZAP70 and pS6 in unstimulated CAR T-cells at 24 h upon stimulation. The data

25

656   shown are normalized to stimulated CD3+ UNTR T-cells. The dotted horizontal line (0) represents the

657   expression of a specific marker in unstimulated CD3+ UNTR T-cells. Data represent mean ± se (n = 7

658   HDs, n = 2 independent experiments). Statistical significance was calculated by Paired t-test. *P <

659   0.05, **P < 0.01.

660   **A-E** Each experimental condition is indicated by a specific colour code (Unstimulated conditions =

661   UNTR light grey, FMC63 light blue, CAT orange; stimulated conditions = UNTR grey, FMC63 blue, CAT

662   red).

663

664   **Figure 3: Phenotypic and molecular characterisation of stimulated CAR-transduced T-cells.**

665   **A** Variation (log2 fold change) of CD4 and CD8 proportion in stimulated UNTR T-cells and FMC63 and

666   CAT CAR T-cells measured by FACS (n = 12 HDs, n = 3 independent experiments). The dotted

667   horizontal line (0) represents the conditions in which CD4=CD8.

668   **B** Barplot showing the percentage of TCM (CD45RA-CD62L+) in stimulated CD3+ UNTR T-cells and

669   FMC63 and CAT CAR T-cells measured by FACS 96h post-antigen stimulation (n = 7 HDs, n = 2

670   independent experiments).

671   **C** Barplots showing the expression of T-cell exhaustion markers (PD1, TIM3 and LAG3) as MFI in

672   stimulated CD3+ UNTR T-cells and FMC63 and CAT CAR T-cells measured by FACS 96h post-antigen

673   stimulation (n = 4 HDs, n = 1 independent experiment).

674   **D** (left) Volcano plot showing DE genes between FMC63 and CAT CAR T-cells upon NALM6 co-

675   culture. The dashed horizontal line represents the statistical significance threshold (FDR < 0.1).

676   (right) The barplots show the expression of selected DE genes (FDR < 0.1) in stimulated UNTR T-cells

677   and in CAR T-cells (n = 6 HDs, n = 2 independent experiments).

678   **E** The barplots show the expression of mass cytometry EMD scores for CD25, HLA-DR, NFAT1,

679   FOXP3, pZAP70, pS6, pp38, pCREB, pRB and CD4 in stimulated CAR T-cells at 24 h upon stimulation.

680   The data shown are normalized to stimulated CD3+ UNTR T-cells. The dotted horizontal line (0)

681   represents the expression of a specific marker in stimulated CD3+ UNTR T-cells. (n = 7 HDs, n = 2

682   independent experiments).

683   **A-E** Barplots show mean ± se. Each experimental condition is indicated by a specific colour code

684   (UNTR grey, FMC63 blue, CAT red).

685   **A, B, C, E** Statistical significance was calculated by Paired t-test. *P < 0.05, **P < 0.01, ***P < 0.001,

686   ****P < 0.0001.

687

688   **Figure 4: Cytokine polyfunctionality in stimulated CAR-transduced T-cells.**

689   **A** The barplots show the expression of mass cytometry EMD scores for effector (Granzyme B, IFN-γ,

690   TNF-α) and stimulatory (GM-CSF, IL-2) cytokines in stimulated CAR T-cells. The data shown are

691   normalized to stimulated CD3+ UNTR T-cells. The dotted horizontal line (0) represents the

692   expression of a specific marker in stimulated CD3+ UNTR T-cells. (n = 7 HDs, n = 2 independent

693   experiments).

694   **B** Barplots showing the mean cytokine polyfunctionality in stimulated CAR T-cells, normalized to

695   stimulated CD3+ UNTR T -cells. The dotted horizontal line (1) represents the mean polyfunctionality

696   in stimulated CD3+ UNTR T-cells. (n = 7 HDs, n = 2 independent experiments).

697   **C** The stacked barplots show the percentage of stimulated CAR T cells (CD3+mCherry+) expressing 1

698   to 4 or >=5 cytokines/cell as measured by mass cytometry.

699   **D** The circos plots show all the combinations of the 8 cytokines analysed in stimulated FMC63 (left)

700   and CAT (right) CAR T-cells by mass cytometry. The numbers indicate patterns of cytokine co-

701   expression (from 1 to 4 or >=5 cytokines/cell). A specific colour code has been assigned to each

702   cytokine.

703   **A-B** Data represent mean ± se. Statistical significance was calculated by Paired t-test. *P < 0.05, ***P

704   < 0.001.

705   Each experimental condition is indicated by a specific colour code (FMC63 blue, CAT red).

706

707 **Figure 5: Molecular characterisation of CAR T-cells generated from CD19-depleted PBMCs.**

708 **A** (left) UMAP representation of the 4 cell populations (CD8, CD4, CD3 low/neg, CD19) identified by

709 FlowSOM analysis in 4 representative unstimulated samples analysed by mass cytometry at 24 h

710 post-stimulation (n = 4 HDs, n = 1 independent experiment). The cell types are indicated by different

711 colours. (right) Percentage of residual B-cells detected by mass cytometry in unstimulated samples

712 at 24 h post-stimulation. (n = 7 HDs, n = 2 independent experiments).

713 **B** (left) Barplots showing the percentage of TCM (CD45RA-CD62L+) and (right) TEM (CD45RA-CD62L-)

714 in unstimulated CD19-depleted CD3+ UNTR T-cells and FMC63 and CAT CAR T-cells measured by

715 FACS (n = 4 HDs, n = 1 independent experiment).

716 **C** The barplots show the expression of mass cytometry EMD scores for Granzyme B, Perforin B, HLA-

717 DR, CD25, NFAT1, pZAP70 and pS6 in unstimulated CD19-depleted CAR T-cells at 24 h upon

718 stimulation. The data shown are normalized to stimulated CD3+ UNTR T-cells. The dotted horizontal

719 line (0) represents the expression of a specific marker in unstimulated CD3+ CD19-depleted UNTR T-

720 cells. (n = 3 HDs, n = 1 independent experiment).

721 **D** The barplots show the expression of mass cytometry EMD scores for CD25, HLA-DR, NFAT1,

722 pZAP70, pS6, pp38, pRB and CD4 in stimulated CD19-depleted CAR T-cells at 24 h upon stimulation.

723 The data shown are normalized to stimulated CD3+ UNTR T-cells. The dotted horizontal line (0)

724 represents the expression of a specific marker in stimulated CD19-depleted CD3+ UNTR T-cells. (n =

725 3 HDs, n = 1 independent experiment).

726 **E** Barplots showing the mean polyfunctionality in CD19-depleted CAR T-cells at 24 h upon

727 stimulation. The data shown are normalized to stimulated CD3+ UNTR T-cells. The dotted horizontal

728 line (1) represents the mean polyfunctionality in stimulated CD3+ CD19-depleted UNTR T-cells. (n = 3

729 HDs, n = 1 independent experiment).

730 **A-E** Each experimental condition is indicated by a specific colour code (Unstimulated conditions =

731 UNTR light grey, FMC63 light blue, CAT orange; stimulated conditions = FMC63 blue, CAT red).

732   Barplots show mean ± se. Statistical significance was calculated by Paired t-test. *P < 0.05, **P <

733   0.01.

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5