Targeting BTN2A1 enhances Vγ9Vδ2 T cell effector functions and triggers tumor cells pyroptosis

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Abstract

Vγ9Vδ2 T cells are potent but elusive cytotoxic effectors. Means to stimulate their function could lead to powerful new cancer immunotherapies. BTN2A1, a surface protein has recently been shown to bind the Vγ9 chain of the γδ TCR but its precise role in modulating Vγ9Vδ2 T cells functions remains unknown.

Here we show that 107G3B5, a monoclonal anti-BTN2A1 agonist antibody, significantly enhances Vγ9Vδ2 T cell functions against hematological or solid cell lines and against primary cells from adult acute lymphoblastic leukemia patients. New computer vision strategies applied to holotomographic microscopy videos show that 107G3B5 enhances the interaction between Vγ9Vδ2 T cells and target cells in a quantitative and qualitative manner. In addition, we provide evidence that Vγ9Vδ2 T cells activated by 107G3B5 induce caspase 3/7 activation in tumor cells, thereby triggering their death by pyroptosis.

We thus demonstrate that targeting BTN2A1 with 107G3B5 enhances the Vγ9Vδ2 T cell antitumor response by triggering the pyroptosis-induced immunogenic cell death.

Introduction

In contrast to αβ T cells, γδ T cell activation is MHC-unrestricted and relies on the detection of various host cell-derived molecules[1] or exogenous pathogens by both TCR and non-TCR receptors. γδ T cells represent the earliest source of IFNγ secretion in the tumor microenvironment[2] and recent transcriptome analyses of human tumors reveal that high γδ T infiltration has the best prognostic value in comparison to other immune subsets[3,4]. Vγ9Vδ2 T cells are the major subtype of blood γδ T cells and are activated by non-peptidic phosphorylated metabolites, called phosphoantigens (pAgs). These pAgs are produced by transformed or infected cells[5,6] via the human mevalonate pathway during the biosynthesis of isoprenoids[7].

A better understanding of the elusive mechanisms of action of Vγ9Vδ2 T cells will lead to new potential immunotherapies. In this context, the interaction between BTN3A and BTN2A1 at the plasma membrane, which enables the recognition of phosphoantigens (pAgs) by Vγ9Vδ2 T cells through an “inside-outside signaling” [8–10] is of particular interest. BTN3A1 cooperates with BTN2A1 for cell surface export[27] and binds to phosphoantigens (pAgs) through its intracellular B30.2 domain which also stabilizes its interaction with BTN2A1[11,12] with pAgs are acting as a “glue”. Most importantly, BTN2A1 is the direct ligand for the Vγ9 chain of the γδ TCR[13,14], representing a

49 fundamental new paradigm in Vγ9Vδ2 T cell biology. While it is clear that BTN2A1 is mandatory for
50 Vγ9Vδ2 T cell activation in tumoral context[10,13,14], its precise role in modulating the functions of
51 Vγ9Vδ2 T cells needs to be carefully investigated.

52 Cytotoxic functions of Vγ9Vδ2 T cells have been demonstrated in preclinical models of solid
53 cancers[15–19] and myeloid or lymphoid hematologic malignancies[20–23]. Therefore, potential
54 immunotherapies based on Vγ9Vδ2 T cells have been rapidly evaluated in clinical trials, including in
55 cancer patients with advanced solid or hematological cancers. Most studies used pharmacological
56 inducers of pAgs, such as aminobisphosphonates[24]. While these studies tended to show objective
57 responses, they were associated with limited efficacy results[25].

58 Low tumor immunogenicity and a cold tumor microenvironment are two of the main
59 features that occur in different cancer types and are responsible for resistance to immunotherapy.
60 One possibility is to transform 'cold' tumors into 'hot' tumors in particular by inducing immunogenic
61 tumor cell death [26]. Natural killer cells and cytotoxic T cells were thought to induce tumor cell death
62 mainly by non-inflammatory apoptosis, however, several studies have recently revealed that these
63 cells can suppress tumors by inducing immunogenic cell death including pyroptosis [27,28] . GSDME
64 (Gasdermin E) is activated by inflammatory caspases 3/7 and can form oligomers that insert into
65 membranes to form pores, thereby mediating pyroptotic cell death. The Induction of non-apoptotic
66 cell death has attracted much attention in the field of antitumor therapy, but until now the ability of
67 Vγ9Vδ2 T cells to induce pyroptosis or other immunogenic cell death remained unknown.

68 Here, we show that a unique agonist mAb 107G3B5 targeting BTN2A1 enhances the cytotoxic
69 activity of Vγ9Vδ2 T cells. We used several *in vitro* tumor models, including ALL in both allogeneic and
70 autologous settings, as well as solid tumor models. Newly developed computer vision powered by
71 artificial intelligence was used to analyze videos recorded with label-free holotomographic
72 microscopy this to demonstrate that 107G3B5 increased quantity and quality of Vγ9Vδ2 T cell
73 interactions with target cells. These cellular events were associated with a clustering of BTN2A1 and
74 its colocalization with BTN3A1, as demonstrated by confocal microscopy and FRET experiments.

75 Finally, we demonstrated that activation of Vγ9Vδ2 T cells using 107G3B7 induced pyroptosis, an
76 immunogenic cell death of cancer cells.
77

78 Results

79 BTN2A1 targeting with 107G3B5 markedly strengthens Vγ9Vδ2 T cell killing and cytokine 80 production against many cancer cell lines

81 First the expression of BTN2A was investigated at the transcriptomic level in normal and
82 tumor tissues (Supplementary Fig. 1a and b). BTN2A1 is ubiquitously expressed with a great
83 heterogeneity across tissues or tumor subtypes. BTN2A1 also shows a distinct expression pattern
84 compared to BTN2A2 and BTN3A1. Using publicly available scNA-Seq datasets of tissues of normal or
85 tumor origin, we observed that BTN2A1 is ubiquitously expressed in immune and non-immune cells
86 (Supplementary Fig. 1c). We next assessed BTN2A1 levels at the plasma membrane of tumor cells,
87 using an anti-BTN2A 7.48 antagonist mAb and 107G3B7 mAb (Supplementary Fig.2).

88 Given the recently described key role of BTN2A1 in the activation of Vγ9Vδ2 T cells [10,13], we
89 hypothesized that the antibody-mediated agonistic activity of anti-BTN2A1 may enhance the
90 cytotoxic functions of Vγ9Vδ2 T cells. To test this hypothesis, we generated monoclonal antibodies
91 against the ectodomain of BTN2A1 by mouse immunization. Hybridoma culture supernatants
92 displaying the highest affinity for BTN2A1 underwent two rounds of selection for their ability to
93 enhance Vγ9Vδ2 T cell degranulation against Daudi cells (Fig. 1a). Out of 71 clones, only four clones
94 increased the proportion of CD107ab+ Vγ9Vδ2 T cells compared to control isotype. However,
95 sequencing of the VH/VL fragments revealed only one common unique sequence among these four
96 clones (data not shown). The anti-BTN2A1 encoded by this sequence was named 107G3B5. We then
97 sought to further characterize the agonistic activity of 107G3B5 on Vγ9Vδ2 T cell cytotoxicity.

98 As previously described[10], the relative abundance of BTN2A1 on cell lines revealed a wide
99 distribution in both hematological cell lines (Supplementary Fig. 2a,b) and solid cancer cell lines
100 (Supplementary Fig. 2c). We showed that BTN2A1 was also found at the membrane of PB blasts from

101 28 ALL patients at diagnosis (Supplementary Fig. 2c). Collectively, these data demonstrate that
102 BTN2A1 is ubiquitously expressed at the transcript and protein level, and that BTN2A1 is found on
103 the cell surface of different tumor origins, including primary tumor cells.
104 Apoptosis assays of hematological cancer cell lines were performed after 4-hours of coculture with
105 Vγ9Vδ2 T cells expanded from 3 HV. This revealed that 107G3B5 and zoledronate (ZOL) pretreatment
106 significantly increased Vγ9Vδ2 T cell-mediated apoptosis to a similar extent (Fig. 1b). Different
107 susceptibility profiles were found in anaplastic large cell lymphoma cell lines (Fig. 1c), ALL, non-
108 Hodgkin lymphoma cell lines (Fig. 1d) and acute myeloid cell lines (Fig. 1e). An increased activity of
109 107G3B5 was observed over time, indeed the killing of 6 hematological cancer cell lines was higher
110 after 6 and 10 hrs of co-culture (Fig. 1f and 1g). In addition, 107G3B5 improved both degranulation
111 and production of TNFα and IFNγ by Vγ9Vδ2 T cells after 4 hours of co-culture with hematological
112 cell lines (Supplementary Fig.3a and b).
113 To better describe the effects of 107G3B5 over time, holotomographic microscopy was used. This
114 measures the spatial distribution of the refractive index (RI) of the observed biological object, here
115 Vγ9Vδ2 T cells, which are small and dense in dry mass. As RI and dry mass content are linearly
116 related, Vγ9Vδ2 T cells were the brightest on the RI image. Cancer cells were larger, had a lower dry
117 mass density and therefore appeared darker on the RI image. Using Artificial Intelligence (AI) for
118 signal processing with advanced thresholding methods, the RI signal alone was used for cell
119 segmentation (Supplementary Fig. 4)[29]. This method has the significant advantage to be label-free
120 and is a valid approach to further characterize well-known apoptotic features such as: cell budding,
121 chromatin condensation, micronuclei, and apoptotic bodies[30]. We used SUP-T1 cells, a T-ALL model,
122 which is representative of the average response to 107G3B5 treatment regarding Vγ9Vδ2 T cells
123 activation and cytotoxicity (Supplementary Fig.3a and b and Fig.1g). Indeed, SUP-T1 cells co-cultured
124 with Vγ9Vδ2 T cells showed distinguishable Vγ9Vδ2 T cells activation leading to SUP-T1 killing upon
125 107G3B5 treatment. In parallel almost no apoptotic events were observed in the control condition
126 (Fig. 1h, Supplementary Movie 1 and 2). We also confirmed our results by demonstrating an increase

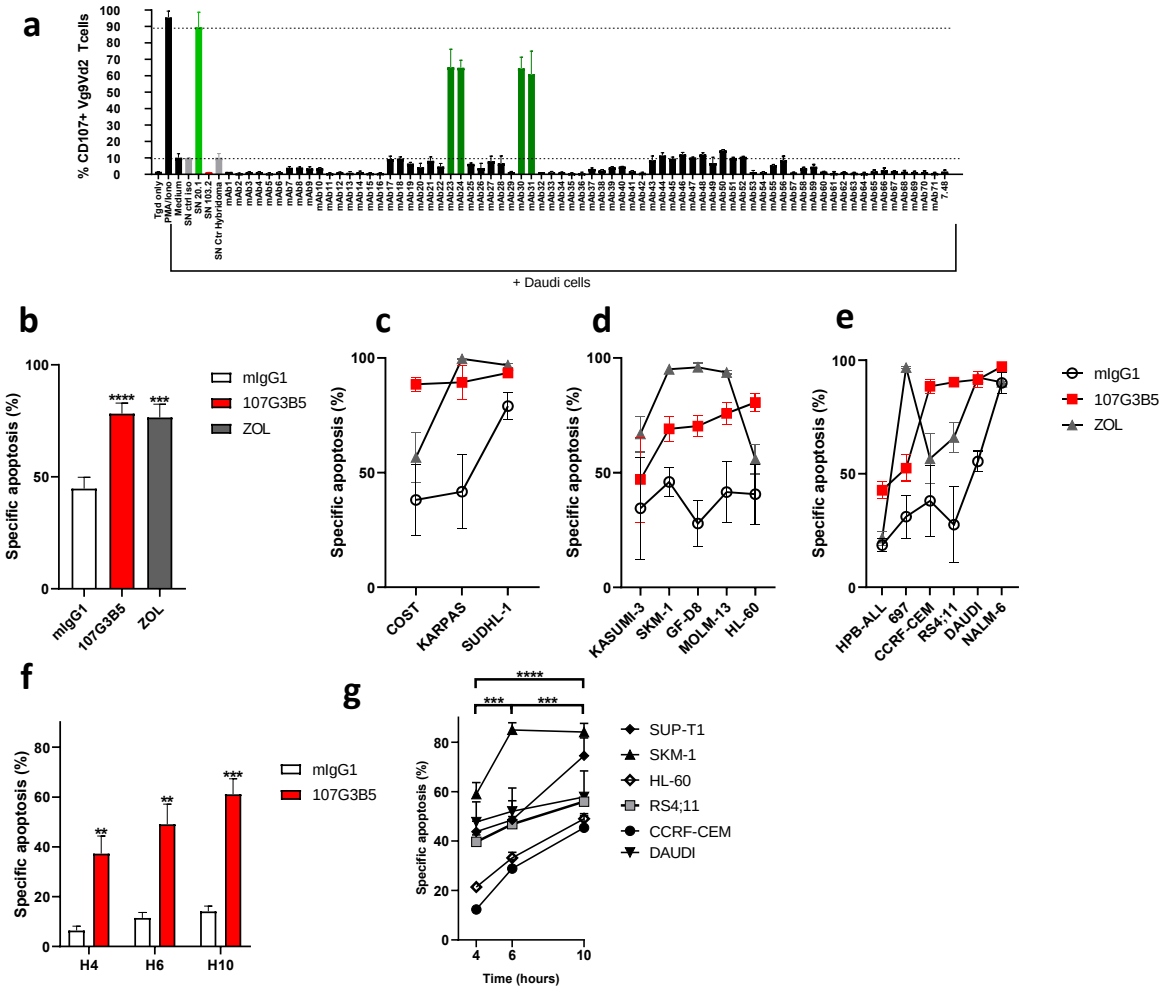
127 of the lethal fraction over time (Fig. 1i). This was associated with an increased production of IFNγ

128 (Fig. 1j). Thus, 107G3B5 improved the killing of a wide range of hematological cell lines using a

129 holotomographic microscopy setting. This broad effect is probably due to the ubiquitous expression

130 of BTN2A1 on various type of tumor cells (Supplementary Fig.2)[10], which allows Vγ9Vδ2 T enhanced

131 degranulation and Th1 cytokine production upon 107G3B5 treatment .



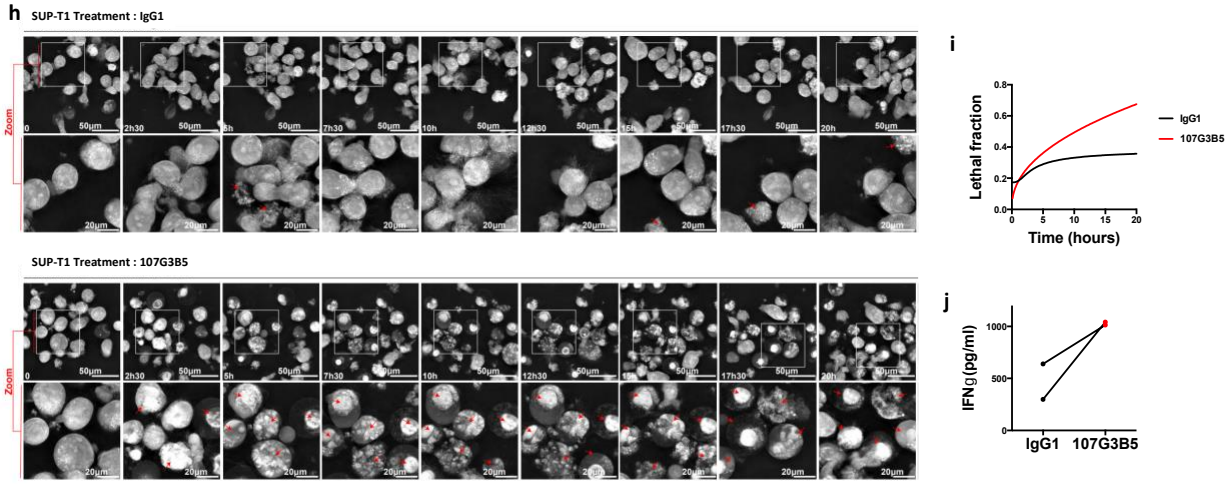


Fig. 1. Anti-BTN2A1 107G3B5 enhances Vγ9Vδ2 T cell-mediated killing of a broad range of hematological cancer cell lines. (**a**) Vg9Vd2 T cells expanded from healthy donor PBMCs (n=3) were co-cultured with Daudi cells (E: T ratio 1:1) in the presence of 50 µL of the indicated anti-BTN2A hybridoma supernatants and %CD107ab+ cells were assessed after 4h by flow cytometry. (**b**) Apoptosis of 15 hematological cancer cell lines, including 3 ALCL cell lines (**c**), 7 ALL and NHL cell lines (**d**), and 5 AML cell lines (**e**) was evaluated by flow cytometry analysis of caspase 3/7 cleavage after 4-hour coculture with Vγ9Vδ2 T cells from 3 HV (E:T ratio 5:1) in the presence of an isotype control or anti-BTN2A 107G3B5 mAb (10µg/mL). Target cells preincubated over-night (O/N) with zoledronate (45 µM) were used as positive control.
(**f**,**g**) Apoptosis of 6 hematological cancer cell lines was monitored by flow cytometry analysis of caspase 3/7 cleavage after 4, 6 and 10-hours of coculture with Vγ9Vδ2 T cells from 3 HV (E:T ratio 2:1) in the presence of an isotype control or anti-BTN2A 107G3B5 mAb (10µg/mL). (**h**) Sequence of snapshots depicting γδ T cells (E) coculture with SUPT1 (T; ratio T/E = 1/2) treated with 10ug/ml of mIgG1 control isotype (upper panel) or anti-BTN2A1 107G3B5 (down panel) within 20hours. The red arrows indicate apoptotic cells. (**i**) Quantification of lethal fraction. (**j**) IFNγ concentration in the culture supernatants at the end of the acquisition. Means ± s.e.m. pooled from one experiment (**b**,**c**,**d**,**e**) or two experiments (**f**,**g**) are depicted. Statistical significance was established using one-way Anova with Tukey's post-test (**b**), Friedman test with Dunn's post-test (**c**,**d**,**e**) or two-way Anova with Tukey's post-test (**f**,**g**), . *p< 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

107G3B5 improves allogeneic and autologous Vγ9Vδ2 T cell functions against primary lymphoblastic blasts

The anti-tumor functions of Vγ9Vδ2 T cells have been extensively studied in a variety range of hematological malignancies [22,23,31–33] but have been less explored in ALL[20,34–36]. Given the promising results obtained with 107G3B5 in ALL cell lines, we next sought to investigate whether 107G3B5 may enhance the functions of allogeneic Vγ9Vδ2 T cells against primary ALL blasts. Compared to the control condition, 107G3B5 increased Vγ9Vδ2 T cell-mediated apoptosis of 17 primary ALL blasts (Fig. 2a). As previously found in hematological cell lines, 107G3B5 enhanced the degranulation and Th1

140 cytokine production capacity of Vγ9Vδ2 T cells in co-culture with primary ALL blasts (Fig. 2b). This
141 agonistic effect is increasing over time (Fig. 2c, and d). Additionally, 107G3B5 treatment induced
142 sustained IFNγ secretion by allogeneic Vγ9Vδ2 T cells co-cultured with 3 primary lymphoblasts (Fig.
143 2e).

144 The antileukemic response of autologous Vγ9Vδ2 T cells has been poorly studied in ALL[35] and
145 to our knowledge, their expansion capacities after stimulation with pAg were unknown. Using ZOL-
146 stimulation combined with IL-2 plus IL-15 for 14 days, we expanded Vγ9Vδ2 T cells from PBMCs of 17
147 ALL patients. After 14 days of culture, Vγ9Vδ2 T cells from ALL patients were able to expand, with a
148 median fold-increase of 124.8 [0.0-101 211] (Fig. 2g). In one patient, Vγ9Vδ2 T cell expansion allowed
149 further testing of their cytotoxic capacity, which was improved after treatment of the expanded cells
150 with 107G3B5 (Fig. 2h). As in allogeneic assays, 107G3B5 increased degranulation and cytokine
151 secretion of Vγ9Vδ2 T cells expanded from 4 ALL patients (Fig. 2i).

152 Taken together, these findings consolidate the previous results obtained on cell lines, we show that
153 Vγ9Vδ2 T cells from ALL patients proliferate in response to 107G3B5 treatment and also enhance
154 both allogeneic and autologous Vγ9Vδ2 T cell functions against primary ALL cells.

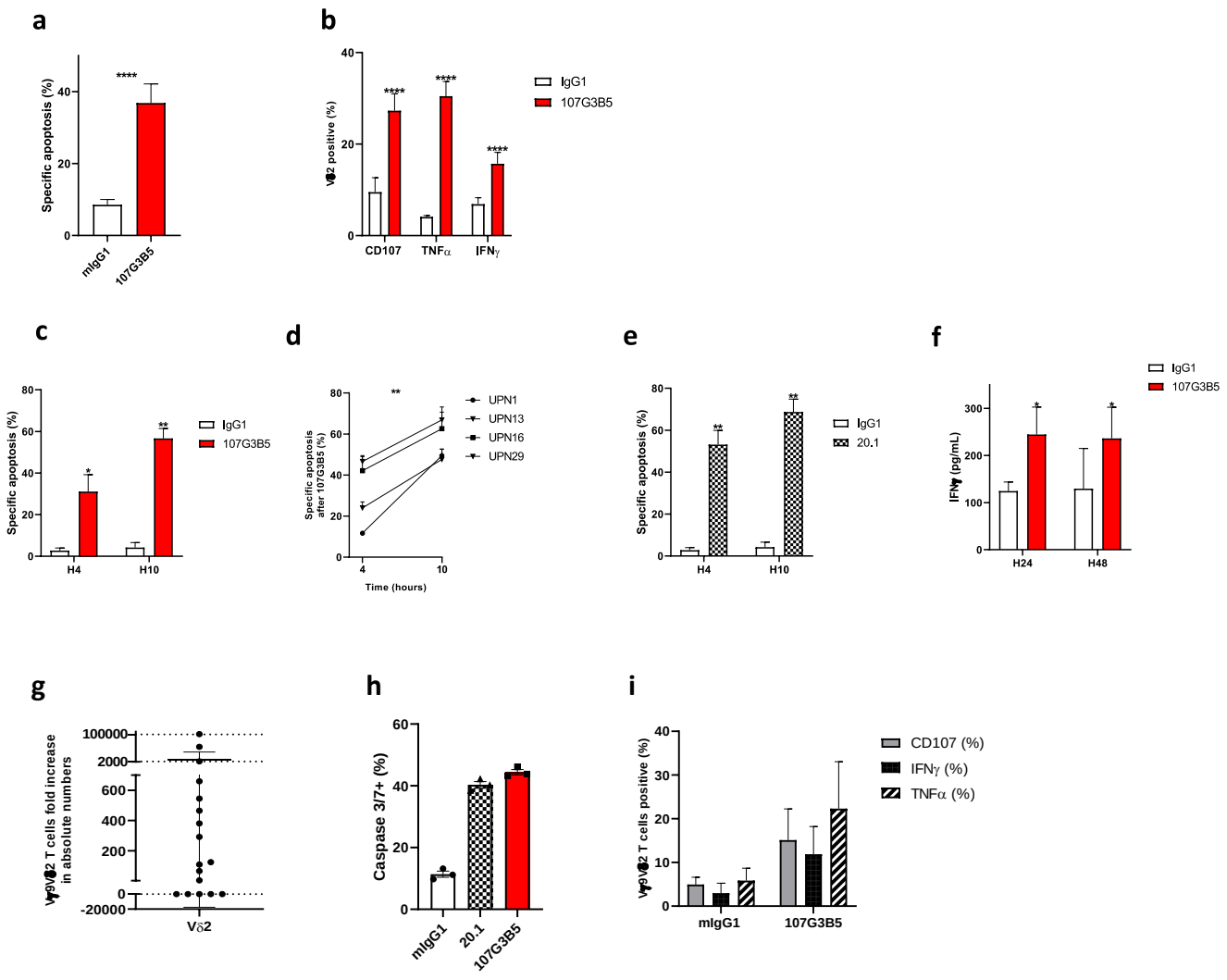

a
Specific apoptosis (%)
mIgG1
107G3B5
b
Vδ2 positive (%)
CD107
TNFα
IFNγ
IgG1
107G3B5
c
Specific apoptosis (%)
H4
H10
IgG1
107G3B5
d
Specific apoptosis after 107G3B5 (%)
Time (hours)
UPN1
UPN13
UPN16
UPN29
e
Specific apoptosis (%)
H4
H10
IgG1
20.1
f
IFNγ (pg/mL)
H24
H48
IgG1
107G3B5
g
Vγ9Vδ2 T cells fold increase in absolute numbers
Vδ2
h
Caspase 3/7+ (%)
mIgG1
20.1
107G3B5
i
Vγ9Vδ2 T cells positive (%)
mIgG1
107G3B5
CD107 (%)
IFNγ (%)
TNFα (%)

Fig. 2 107G3B5 improves allogeneic and autologous effector response of Vγ9Vδ2 T cells against primary lymphoblastic blasts. a Apoptosis of 17 primary ALL blasts was assessed by analysis of caspase 3/7 cleavage after 4-hour coculture with Vγ9Vδ2 T cells from 3 HV (E:T ratio 15:1) with either isotype control or 107G3B5 (10µg/mL). (**b)** Effector functions of Vγ9Vδ2 T cells (HV, n=2 or 3) were assessed after a 4 hours co-culture with 17 primary ALL blasts (E:T ratio 1:1) in the presence of 107G3B5 or its isotype control (1µg/mL). Pooled from 6 independent experiments (**a**,**b**,**c**). (**c**-**e**) Apoptosis of 4 primary ALL blasts was monitored by analysis of caspase 3/7 cleavage after 4 and 10-hours coculture with Vγ9Vδ2 T cells from 3 HV (E:T ratio 10:1) in the presence of an isotype control, 107G3B5 or anti-BTN3A 20.1 agonist mAb (10µg/mL). (**f**) IFNγ concentration in supernatants of 24 and 48-hour coculture of Vγ9Vδ2 T cells from 6 HV with 3 primary ALL blasts either with isotype control or 107G3B5 (10µg/mL). (**g**) PBMCs from ALL patients at diagnosis (n = 17) were cultured with ZOL for 14 days. (**h**) Apoptosis of primary ALL blasts (n=1) was evaluated by analysis of caspase 3/7 cleavage after a 4-hour coculture with autologous Vγ9Vδ2 T cells (E:T ratio 10:1), in the presence of isotype control, 107G3B5 or anti-BTN3A 20.1 agonist mAb (10µg/mL). The experiment was performed in triplicate. **i** Effector functions of Vγ9Vδ2 T cells (n=4) were assessed after a 4 hours co-culture with autologous primary ALL blasts (E:T ratio 1:1) in the presence of 107G3B5 (10µg/mL) or its isotype control. The statistical significance was established using a paired T-test (**a**), Wilcoxon test (**b**), two-way anova test (**c**,**d**,**e**,**f**) *p< 0.05; **p < 0.01; ****p < 0.0001

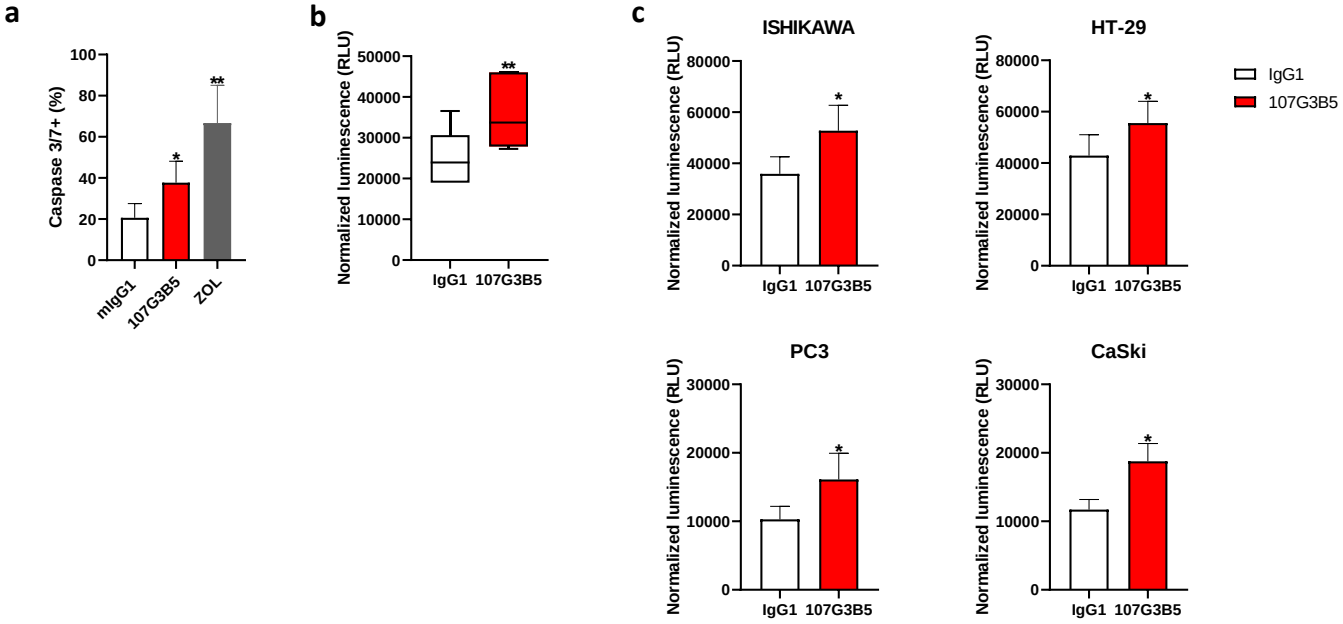
155 **107G3B5 sensitizes Vγ9Vδ2 T cell cytotoxicity in 3D tumor spheroid models**

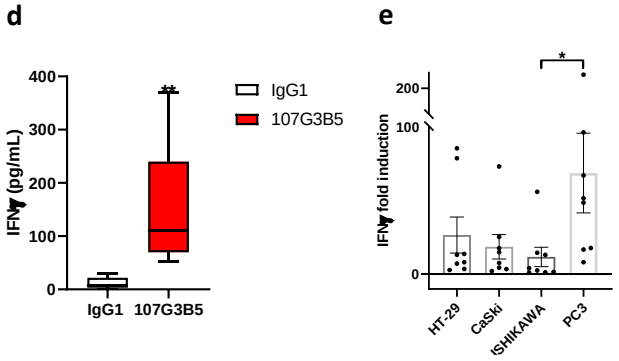
156 Next, we evaluated the activity of 107G3B5 in a solid tumor setting. Using cell lines of different tumor
157 origins, we found that 107G3B5 significantly increased Vγ9Vδ2 T cell-mediated killing compared to
158 the control condition (Fig. 3a). To assess 107G3B5 activity in a relevant solid tumor model, we further
159 investigated the effect of 107G3B5 on Vγ9Vδ2 T cell effector functions using 3D spheroid assays and
160 demonstrated that 107G3B5 increased significantly Vγ9Vδ2 T cell-mediated apoptosis of Ishikawa,
161 HT-29, CaSki and PC-3 spheroids (Fig. 3b and c).

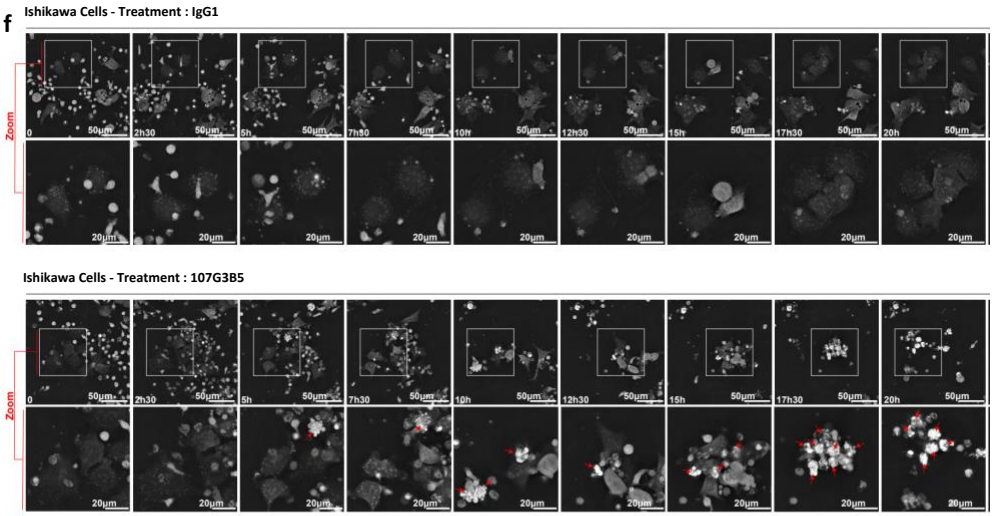
162 Live-cell fluorescence images of PC-3 spheroids co-cultured with Vγ9Vδ2 T cells from 6 HV showed a
163 more pronounced decrease in calcein area over time after treatment with 107G3B5 compared to the
164 isotype control condition (Supplementary Fig. 5a, b and c). After 24 hours, calcein area was
165 significantly reduced in PC-3 spheroids after 107G3B5 treatment compared to the control condition
166 (Supplementary Fig. 5b).

167 Analysis of the 24-hour coculture supernatant revealed a significant increase in IFNγ secretion after
168 107G3B5 treatment compared to the control condition (Fig. 3d). Consistent with previous findings,
169 the PC-3 cell line appeared to be highly susceptible to Vγ9Vδ2 T cells after treatment with 107G3B5,

170 as Vγ9Vδ2 T cells cocultured with PC-3 spheroids produced higher levels of IFNγ compared to other
171 solid tumor cells lines (Fig. 3e).
172 We next used holotomographic microscopy to visualize the impact of 107G3B5 on the efficiency of
173 Vγ9Vδ2 T cells to induce apoptosis in Ishikawa solid tumor cells. Consistent with previous results, we
174 observed a rapid accumulation of apoptotic cells over time under 107G3B5 treatment, whereas no
175 apoptotic feature was distinguishable in the control condition (Fig. 3f, Supplementary Movie 3 and
176 4). The dead cells fraction was strongly increased upon 107G3B5 treatment (Fig. 3g) and was
177 correlated with higher IFNγ production (Fig. 3h).


a
Caspase 3/7+ (%)
mIgG1
107G3B5
ZOL
b
Normalized luminescence (RLU)
IgG1
107G3B5
c
ISHIKAWA
HT-29
PC3
CaSki
IgG1
107G3B5


d
IFNγ (pg/mL)
IgG1
107G3B5
e
IFNγ fold induction
HT-29
CaSki
ISHIKAWA
PC3


f
Ishikawa Cells - Treatment : IgG1
Ishikawa Cells - Treatment : 107G3B5
Zoom

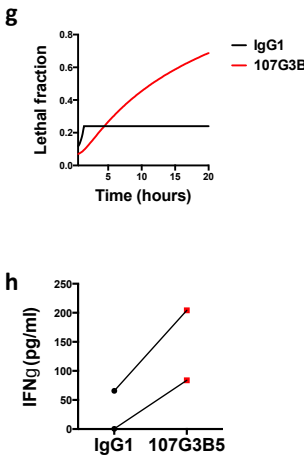

g
Lethal fraction
Time (hours)
IgG1
107G3B
h
IFNg (pg/ml)
IgG1
107G3B5

Fig. 3. Anti BTN2A1 107G3B5 increases interactions between target cells and Vγ9Vδ2 T cells. (**a**) Apoptosis of 2D solid cancer cell lines (MCF-7, HT-29, DoTc2, PC-3, CaSki, PANC-1) was assessedby flow cytometry analysis of caspase 3/7 cleavage after 10-hour coculture with Vγ9Vδ2 T cells from 5 or 6 HV (E:T ratio 5:1) in presence of an isotype control, 107G3B5 mAb (10µg/mL). (**b, c**) Caspase 3 and 7 activity of tumor spheroids (Ishikawa, HT-29, PC-3 and CaSki) after 24-hrs coculture with Vγ9Vδ2 T cells from 6 HV (E:T ratio 5:1) in the presence of an isotype control, 107G3B5 mAb (10µg/mL). Luminescence is normalized by subtracting spontaneous caspase 3/7 activity for each spheroid. Box-plots represent mean luminescence of the four tumor spheroids (**c**). (**d,e**) IFNγ concentration in supernatants of 24-hour coculture of Vγ9Vδ2 T cells (HV, n=8) with 3D spheroids from Ishikawa, HT-29, PC-3 and CaSki cell lines (E:T ratio 15:1) with either isotype control or 107G3B5 mAb (10µg/mL). Box-plots represent the IFNγ concentration assayed in the four tumor spheroids relative to control condition **(e)**. (**f**) Sequence of snapshots depicting γδ T cells cocultured with Ishikawa (E:T ratio 1:3) treated with 10µg/mL of mIgG1 control isotype (toppanel) or anti-BTN2A1 107G3B5 (bottompanel) during 20hours. The red arrows indicate apoptotic cells. (**g**) Quantification of the lethal fraction in HTM videos **h** IFNγ concentration in the culture supernatants at the end of the acquisition. Experiments were performed in biological (**b,c**) or technical duplicate (**d,e**).The statistical significance was established using one-way Anova with Tukey's post-test (**a**), using Wilcoxon test (**b,c,d**) or Kruskal-Wallis test with Dunn's post-

178 **107G3B5 increases interactions between target cells and Vγ9Vδ2 T cells**

179 To further characterize the mechanisms of action of 107G3B5, we assessed its impact on the
180 interactions between target cells and Vγ9Vδ2 T cells. To this end, we used holotomographic
181 microscopy, a live-imaging technique that enables the close monitoring of both target and effector
182 cells at high resolution [37]. To obtain relevant findings, we used both a hematological malignancy
183 model; SUP-T1 and a solid cancer model, Ishikawa. Upon 107G3B5 treatment, we observed an
184 increased proportion of Vγ9Vδ2 T cells in contact with a target cell in both cell lines (Fig. 4a).
185 Consistently there was an increase in the target cell area covered by Vγ9Vδ2 T cells (Fig. 4b) and the
186 average minimum distance between Vγ9Vδ2 and target cells. Conversely the movement of Vγ9Vδ2
187 cells was significantly decreased (Fig. 4c and d). These results strongly suggest that 107G3B5
188 promotes contacts between Vγ9Vδ2 T cells and target cells and stabilizes their interactions.
189 Interestingly, different behaviors have been reported when T cells encounter antigen on the surface
190 of APC (antigen-presenting cells) such as cessation of movement and the formation of a stable
191 immunological synapse or continuous rapid movement from APC to APC [38]. Accordingly, molecular
192 interactions between Vγ9Vδ2 T cells and target cells are sufficient to induce the formation of an
193 immunological synapse, but effector functions require the full engagement of the γδ TCR by a potent
194 agonist[39].Overall, we have provided new insights into the mechanism of Vγ9Vδ2 T cell functionality

195 in different tumor cell lines. 107G3B5 improved the quantity and the quality of interactions between

196 target cells and Vγ9Vδ2 T cells over time, with subsequent increased cytotoxicity.

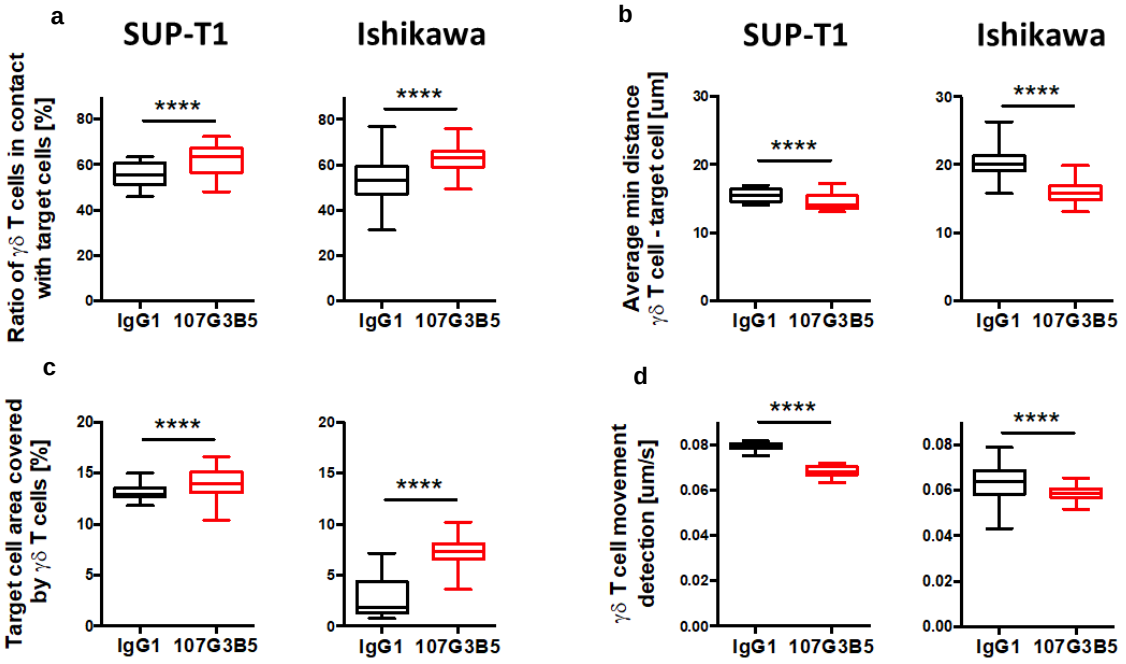


Fig. 4 107G3B5 induces a strong increase in Vγ9Vδ2 TCR binding which is associated with BTN2A1 clustering (**a**) The ratio of γδ T cells in contact with target cells, (**b**) target cell area covered by γδ T cells in percentage, (**c**) the average minimal distance between γδ T cells centroid and target cells centroid and (**d**) the movement of γδ T cells in um/s were analyzed using a holotomography microscope. These four parameters were analyzed in γδ T cells (Effector: E) coculture with SUPT1 (Target : T ; ratio T/E = 1/2) or Ishikawa (ratio 1/3) treated with 10ug/ml of mIgG1 control isotype (in black) or anti-BTN2A1 107G3B5 (in red). Acquisitions were performed each 9min for 20 hours. The boxplots represent for each condition the cumulative results of the acquisition between 4 and 20hours. The statistical significance was established using paired T test . ****$p < 0.0001$

197 **107G3B5 enhances binding of a recombinant Vγ9Vδ2 TCR on HEK293T, SUPT-T1 and Ishikawa cell**

198 **lines**

199 To gain insight into the underlying molecular mechanisms we assessed whether 107G3B5 can directly

200 affect the binding of a recombinant Vγ9Vδ2 TCR to target cells. First, using HEK293T, SUP-T1 and

201 Ishikawa cell lines, we demonstrated by flow cytometry that 107G3B5 significantly increased Vγ9Vδ2

202 TCR binding to target cells (Fig. 5a and b). Second, we confirmed this result by confocal microscopy

203 experiments using the SUP-T1 cell line. We observed that the binding of Vγ9Vδ2 TCR did not occur
204 diffusely but in specific areas of the target cells creating clusters of fluorescence (Fig. 5c). Indeed, we
205 observed both mini and microclusters. Using a manual analysis, enabling quantification of the
206 number of miniclusters, we found that 107G3B5 induced a significant increase in the number of
207 Vγ9Vδ2 TCR miniclusters on the target cell surface (Fig. 5d). Consistent with our results, confocal
208 microscopy experiments also showed that the 107G3B5 antibody induced BTN2A1 clustering
209 (Supplementary Fig. 7). Interestingly, treatment of target cells using the potent Vγ9Vδ2 activator
210 HMBPP induced only microclusters of Vγ9Vδ2 TCR on the SUP-T1 surface. Taken together, our results
211 showed that 107G3B5 has its own unique mechanism of action by directly and strongly
212 enhancingVγ9Vδ2 TCR binding to various target cells in a BTN2A-clustering dependent manner.
213 To better understand the molecular mechanism, we investigated the effect of 107G3B5 on
214 BTN3A1/BTN2A1 interaction. To this end, HEK293T cells were transfected with BTN3A1-GFP and
215 BTN2A1-mCherry constructs and incubated with agonist 107G3B5, antagonist 7.48 mAbs or control
216 isotype. 107G3B5 was able to significantly increase the colocalization of both molecules, as indicated
217 in yellow in merged images (Supplementary Fig 6a). Indeed, the fraction of total BTN2A1
218 fluorescence that colocalized with BTN3A1 fluorescence, as indicated by the Manders' Colocalization
219 Coefficient, was significantly increased on the HEK293T cell line after exposure to 107G3B5
220 (Supplementary Fig 6b). However, we observed no significant effect of the antagonist antibody 7.48
221 on BTN2A1/BTN3A1 clustering (Supplementary Fig 6b). To validate this idea, we used
222 BTN2A1KO/BTN3A1KO HEK293T cells transfected with CFP/YFP BTN2A1 and BTN3A1 fusion proteins
223 and incubated with increasing concentrations of 107G3B5 mAb or control isotype. We observed an
224 increasing FRET signal under anti BTN2A-treatment (Supplementary Fig 6c). The interaction of these
225 two molecules is part of the mandatory first steps of Vγ9Vδ2 T cell activation[11] and our results
226 demonstrate that 107G3B5 promotes the colocalization of both molecules.
227

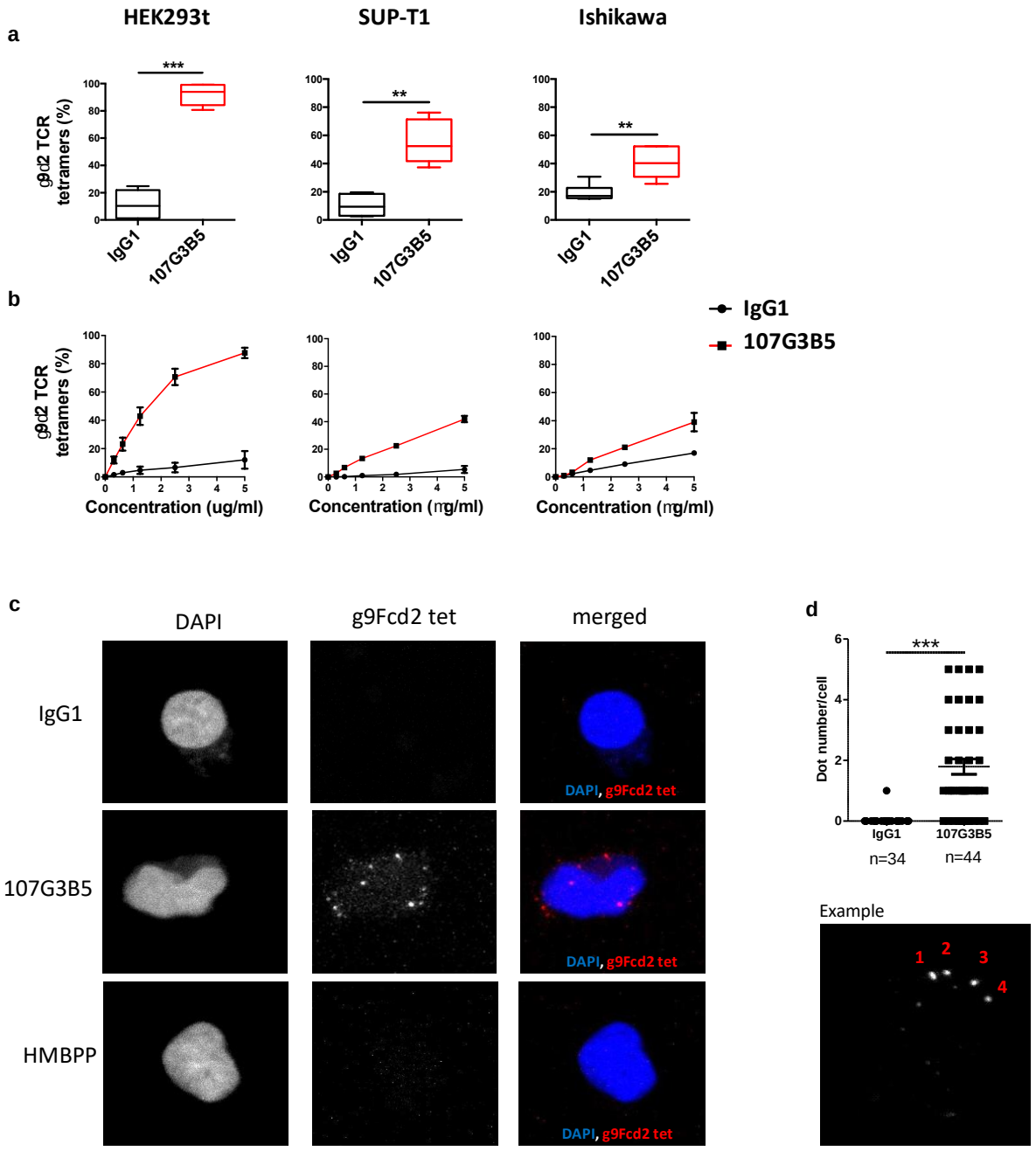


Fig. 5 107G3B5 induces a strong increase in Vγ9Vδ2 TCR binding which is associated with BTN2A1 clustering . (**a**,**b**) Binding of a recombinant, tetramerized Vγ9Vδ2 TCR to HEK293T IRR, SUP-T1 or Ishikawa, following saturation with 107G3B5 or an isotype control. Binding of Vγ9Vδ2 TCR (5µg/mL) (**a**) or dose dependent binding was monitored by flow cytometry (**b**). (**c**) Confocal microscopy of tetramerized Vγ9Vδ2 TCR binding to SUP-T1, with or without previous saturation with an isotype control ,107G3B5 or HMBPP. Evaluation of Vγ9Vδ2TCR binding by quantification of dots observed after treatment with IgG1 control isotype or 107G3B5. Statistical significance was established using Wilcoxon test (**a**) or Mann Whitney test (**d**). **p < 0.01; ***p < 0.001.

228 **Vγ9Vδ2 T cell activation by 107G3B5 triggers tumor cells pyroptosis**

229 Activation of caspase 3/7 can lead to apoptosis, a non-inflammatory form of programmed cell death
230 or pyroptosis, a form of pro-inflammatory cell death that has been discovered in recent years[40].
231 Interestingly, pyroptosis is preferentially triggered when CAR T cells have a high affinity for target
232 cells[28]. Because 107G3B5 increases interactions between target cells and Vγ9Vδ2 T cells we wanted
233 to determine whether pyroptosis may contributes to 107G3B5 activity. Pyroptosis is orchestrated by
234 the cleavage of Gasdermin (GSDM) family members such as GSDMD and GSDME. Indeed, GSDME, a
235 55kDa protein, can be cleaved by caspase-3/7 to produce a 35kDa active form that oligomerizes into
236 the plasma membrane causing pores formation and subsequent cellular pyroptosis[28]. When we co-
237 incubated Vγ9Vδ2 T cells with SUPT1, 697 or Ishikawa cell lines, we observed that 107G3B5 not only
238 increased caspase 3/7 activation, but also increased tumor cell permeabilization via Live/Dead+
239 marker (Fig. 6a and b). To better understand whether pyroptosis might be involved in this
240 permeabilization, we thought to visualize the GSDME forms by western blot. We observed that the
241 full-length form of GSDME was expressed in the 697 and ishikawa cell lines but was absent in Vγ9Vδ2
242 T cells. In target/effector co-cultures with control isotype, we observed the full-length form of
243 GSDME but also weakly the N-terminal active form, indicating that Vγ9Vδ2 T cells alone can induce
244 GSDME cleavage. Moreover, the relative expression of the full-length form of GSDME is reduced
245 under 107G3B5 treatment whereas the active form is significantly increased (Fig.6 c and d). Taken
246 together, these results suggest that 107G3B5 mediates the activation of Vγ9Vδ2 T cells, which can
247 mobilize GSDME to induce pyroptosis within the target cell.

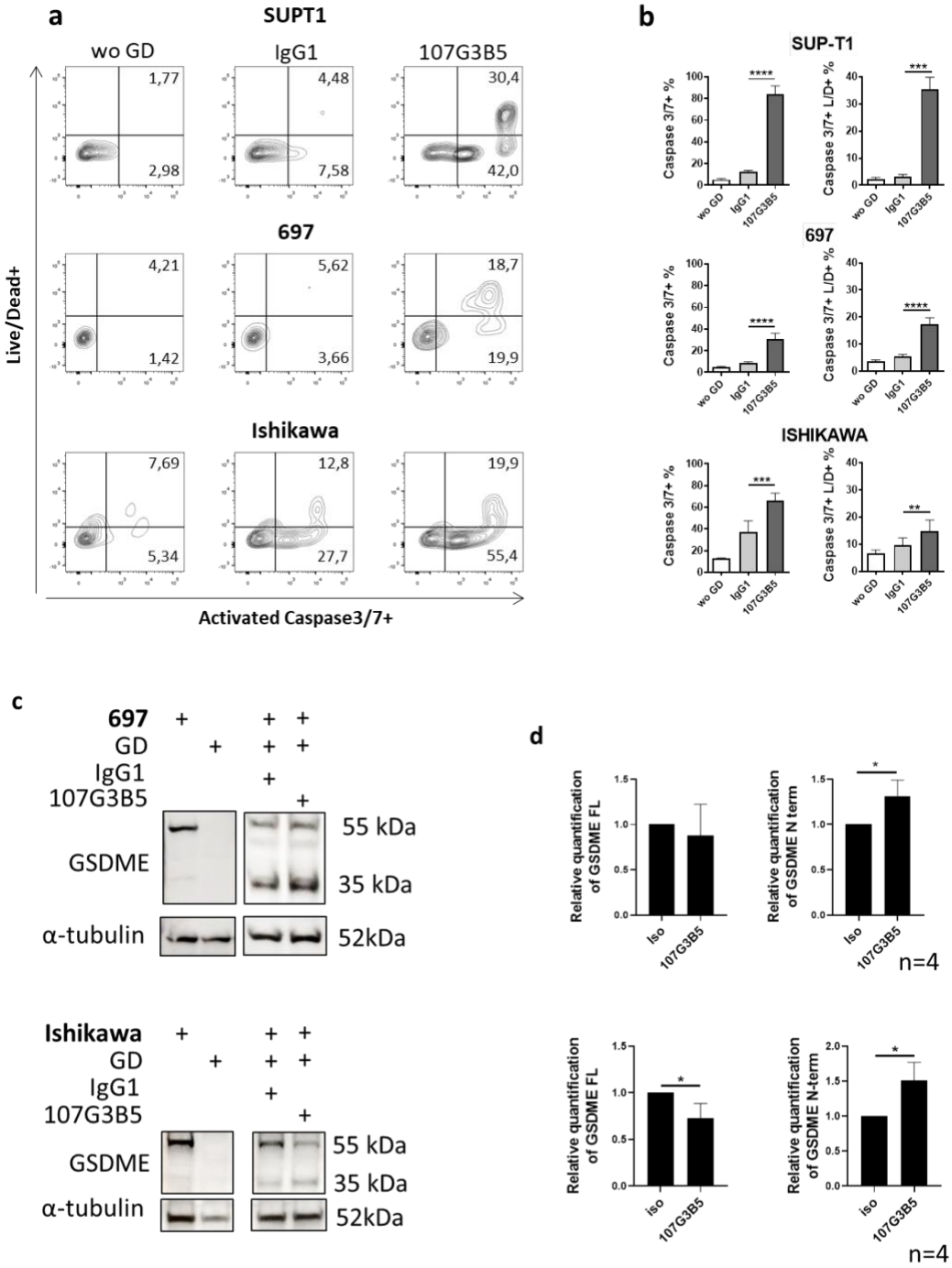


Figure 6. Activation of Vγ9Vδ2 T cells by 107G3B5 induces cancer cell pyroptosis. Vγ9Vδ2 T cells were cocultured with SUPT1, 697 or Ishikawa cells at an effector/target ratio (E/T), 2/1, 2/1 and 3/1, respectively. Cells were treated with 10ug/ml of mIgG1 control isotype or anti-BTN2A1 107G3B5 for 5 hours. The percentage of activated caspase 3/7+ and or permeabilized Live/Dead + tumor cells was determined by flow cytometry. (**a**) Representative staining. (**b**) Data are means ±SD of 3 independent experiments (n>5). The statistical significance was established using a paired T-test. (**c,d**) GSDME and tubuline were analyzed by Western blot. (**c**) Visualization of representative Western blot. (**d**) Data are means of relative quantification ±SD of 4 independent experiments. Statistical significance was established using a paired T-test. *p< 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

248 **Discussion**

249 VγV9δ2 T cells provide a natural bridge between innate and adaptive immunity, responding
250 rapidly and strongly to cancer cells or pathogenic infections. Expression of BTN2A1 is required to
251 trigger activation of Vγ9Vδ2 T cells by pAgs. However, current data on BTN2A1 expression are very
252 sparse. Indeed, BTN2A1 expression has been shown on circulating B, T, NK cells, monocytes and
253 VγV9δ2 T cells from healthy volunteers[13]. Expression on tumor was known for only a few cell lines [10].
254 To develop new therapeutic tools targeting BTN2A1, it is necessary to study BTN2A1 in a patient
255 cohort. Therefore, we carefully investigated the expression of BTN2A in normal and tumor tissues.
256 Our results demonstrate that BTN2A1 is ubiquitously expressed at the transcriptomic and protein
257 level. Moreover, BTN2A1 is found on the cell surface of tumor from different origins including
258 primary ALL tumor cells. These data confirm that BTN2A1 can be targeted in a wide range of tumors.

259 Indeed our results show that in hematological cell lines the activity profile of 107G3B5 was
260 promising in both lymphoid and myeloid malignancies. Next, we confirmed that 107G3B5 enhanced
261 effector functions of Vγ9Vδ2 T cells against primary ALL cells at diagnosis. These findings suggest that
262 BTN2A1 should be considered as a novel immune-checkpoint to target in cancer immunotherapy.

263 The tumor models used in this study showed different profiles of susceptibility to Vγ9Vδ2 T
264 cell killing, either in basal condition or after stimulation with 107G3B5. In solid tumors, we found that
265 PC-3, a prostate cancer cell line was particularly sensitive to the cytolytic activity of Vγ9Vδ2 T cells
266 after the use of 107G3B5. Therefore, targeting BTN2A1 may be a good alternative to overcome the
267 limited cytotoxic effects of Vγ9Vδ2 T cells on PC-3 cells under zoledronate treatment [41].

268 BTN2A1 is a surface protein, that has been described as a key molecule that binds directly to
269 the γ9 chain of the TCR. However, the molecular mechanisms involved in the regulation of Vγ9Vδ2 T
270 cell activation are still unclear. We have recently shown that BTN2A1 mAbs can inhibit the ability of
271 Vγ9Vδ2 T cells to kill cancer cells[10]. Indeed, BTN2A1 mAbs are able to completely prevent the binding
272 of BTN2A1 to the Vγ9Vδ2 TCR. Surprisingly, we report here for the first time that a unique anti-
273 BTN2A 107G3B5 mAb can induce Vγ9Vδ2 T cell activation. Our work demonstrates that the binding

274 of 107G3B5 to BTN2A1 expressing cells increases the interaction of recombinant Vγ9Vδ2 TCR. To
275 better understand the underlying molecular mechanisms of action, we analyzed the impact of
276 107G3B5 on BTN2A1 clustering and on its association with BTN3A1. We observed that under
277 107G3B5 treatment, BTN2A1 appears to form clusters at the surface of cancer cells. Given that TCR
278 avidity depends on both the affinity of a single TCR molecule for its ligand and the number of TCR-
279 ligand engagements, we hypothesis that BTN2A1 clusters increase the avidity for Vγ9Vδ2 TCR . We
280 also observed that 107G3B5 treatment increased the interaction between BTN2A1 and BTN3A1. We
281 do not exclude that other associated molecules may be directly required. Our findings support a
282 model in which BTN2A1 is more than a passive interacting molecule, BTN2A1 appears to be an on/off
283 switch molecule for Vγ9Vδ2 T cells. Therefore, BTN2A1 represents a fundamentally different class of
284 immune recognition compared to the well-known antigen presenting molecules such as MHC and
285 MHC-like molecules.

286 The pAgs bind to the B30.2 intracellular domain of BTN3A1 and this has recently been
287 defined as a key step in initiating an intracellular heterodimeric association between BTN3A1 and
288 BTN2A1[11]. We demonstrate here that 107G3B5 is also able to enhance BTN2A1 and BTN3A1
289 association. However, we did not ruled out the possibility that their intracellular domains is involved
290 in such phenomena. The involvement of intracellular or extracellular domains in the observed
291 colocalization induced by the 107G3B5 should be further investigated.

292 Standards in onco-immunology for the visualization and characterization of lymphocyte's
293 effector functions and cytotoxic effects on cancer cells are based on the immunolabeling of specific
294 membrane or intracellular proteins with fluorescent antibodies or on the use of chemical dyes. This
295 has two disadvantages: first, the labels alter cellular functions[42,43]; second, the excitation light used to
296 stimulate fluorescence produces phototoxicity and photobleaching[43]. Holotomographic microscopy
297 (HTM) used in this study does not have these drawbacks as it is label-free and uses extremely low
298 power light[37]. HTM reports the refractive index distribution of the observed sample and enables
299 previously impossible long-term time lapse imaging of lymphocytes and tumor cells. Combined with

300 advanced computer vision, HTM provides unique quantitative information, particularly in this study,
301 on the dynamic interactions between effector and target cells. Specifically, we are able to show that
302 107G3B5 promoted a long-lasting anti-tumor response of Vγ9Vδ2T cells and illustrate the effect of
303 107G3B5 on the establishment and stabilization of the effector-target cell interaction. One of the
304 promising applications of HTM is to monitor, quantify and compare the effects of multiple
305 immunotherapeutic treatments on malignant cell death over time.

306 Despite the growing importance of immunotherapy in cancer, its applications is severely
307 limited by the fact that a high percentage of patients do not respond to these treatments or respond
308 only transiently. Many cancer tumors are not inflamed or "cold," meaning that the immune system
309 does not recognize them. Induction of apoptosis has long been considered as one of the most
310 important strategy for cancer treatment, as the regulation of apoptosis allows tumors elimination. In
311 the last decade, many new forms of non-apoptotic cell death including pyroptosis has been
312 discovered. This offers new strategies for cancer treatment. Interestingly, the immunogenicity of
313 tumor cells can be maximized by inducing non-apoptotic cell death. Indeed, recent studies have
314 found that GSDME-mediated pyroptosis enhances the phagocytic function of tumor associated
315 macrophages on tumor cells and increases the number and function of tumor-infiltrating T CD8+ /NK
316 cells as well[27]. In melanoma patients, gene signature associated to the increased pyroptosis was
317 correlated with a better prognosis and was predictive of response to anti-PD-1 immunotherapy.
318 Consequently, the understanding of pyroptosis could greatly enhance the efficacy of
319 immunotherapy.

320 Cytotoxic cells such as Natural Killer (NK) cells, CD8+ T cells and CAR T cells induce tumor cells
321 pyroptosis through pyroptosis[27,28]. Importantly here we show here for the first time that Vγ9Vδ2 T
322 cells are able to convert non-inflammatory apoptosis into pyroptosis in GSDME-expressing cancer cell
323 lines. Interestingly, pyroptosis induced by the GSDME cleavage is strongly enhanced by anti-BTN2A1
324 treatment.

325 Tumor cell death by pyroptosis is often dampened by epigenetic silencing of GSDME due to
326 hypermethylation of the GSDME promoter which is found in approximately 52-65% of primary
327 cancers [44]. However, treatment with the demethylating agent 5-aza-2'-deoxycytidine or decitabine
328 can induce the expression of GSDME [45,46] . γδ T cells are attractive effector cells for cancer
329 immunotherapy because they exert potent cytotoxicity against a wide range of cancer cells. Although
330 these approaches have been well tolerated, the clinical responses have generally been infrequent
331 and of short duration. In this context, enhancing immunogenic cell death such as pyroptosis appears
332 to be a promising strategy to improve the efficacy of γδ T cell-based immunotherapy.

333 In conclusion, our results demonstrate that targeting BTN2A is a promising option as it (i)
334 allows the sensitization of ALL in both allogeneic and autologous settings as well as solid tumor
335 models to Vγ9Vδ2 T cells, (ii) by quantitatively and qualitatively improving the interaction between
336 target and effector cells, associated with (iii) BTN2A1 clustering and BTN3A1 colocalization, triggering
337 (iv) the conversion of cancer cell apoptosis into inflammatory pyroptosis. Future work is needed to
338 determine the potential role of other immune cytotoxic cell subsets such as CD8 T cells, B cells and
339 NK cells that express the target. Finally, our findings are the first to demonstrate the great potential
340 of targeting BTN2A for the treatment of cancer. Additional preclinical data will guide the future
341 development of anti BTN2A treatments. It is tempting to speculate that this discovery may help to
342 improve clinical outcomes for patients with cancers, including non-inflammatory tumors that have so
343 far failed to respond to the most promising immunotherapy treatments.

344

345 Material and methods

346 **Patients**

347 PBMCs from 28 adult ALL patients at diagnosis (10 B-ALL, 9 T-ALL and 9 Ph+ ALL) were analyzed for
348 their BTN expression level, and some were also tested in functional assays (7 B-ALL, 7 T-ALL and 3
349 Ph+ ALL). Heparinized blood from ALL patients was obtained from the Hematology Department of

350 the Institut Paoli Calmettes. Informed consent was obtained from all donors in accordance with the
351 Declaration of Helsinki, and the study was approved by our Institutional Review Board (BTN-LAL-
352 IPC2021-049 - Immunomodulation dans les leucémies aigues - June 22, 2021). Baseline
353 characteristics of ALL patients are provided in supplementary Table 1.
354
355 **Cell lines**
356 Cell lines derived from acute lymphoblastic leukemia (697, RS4;11, NALM-6, HPB-ALL, SUP-T1; CCRF-
357 CEM), acute myeloid leukemia (GF-D8, HL-60, Kasumi-3, MOLM-13, SKM-1), anaplastic large-cell
358 lymphoma (COST, KARPAS-299, SUDHL-1), non-Hodgkin lymphoma (DAUDI), CML (K562), breast
359 cancer (MCF-7), colorectal cancer (HT-29), prostate cancer (PC-3), pancreatic cancer (PANC-1),
360 cervical cancer (DoTc2, CaSki), and endometrial cancer (Ishikawa) were obtained from the American
361 Type Culture Collection or from DSMZ. In addition, we used HEK293T for microscopy experiments
362 and binding assays.
363 697, GF-D8, Kasumi-3, SKM-1 were cultured in RPMI 1640 with 20% Fetal Bovine Serum (FBS). PC-3,
364 PANC-1, DoTc2, CaSki and Ishikawa were cultured in DMEM 20% FBS. RS4;11, NALM-6, HPB-ALL, SUP-
365 T1; CCRF-CEM, HL-60, MOLM-13, COST, KARPAS-299, SUDHL-1, DAUDI, K562, MCF-7, HT-29 were
366 cultured in RPMI 1640 with 10% FBS.
367 Generation of BTN3AKO/BTN2AKO HEK293T cells using CRISPR-Cas9-mediated inactivation of BTN2A
368 and/or BTN3A as previously described[10]. The absence of BTN2A and/or BTN3A was confirmed by flow
369 cytometry.
370 All cell lines were tested negative for mycoplasma.
371
372 **Tumor spheroids generation**
373 MCF-7, HT-29, DoTc2, PC-3, CaSki and Ishikawa cell lines were cultured to form spheroids. Depending
374 on the type of functional assay, $1x10^4$ to $2 x10^5$ cells were plated in 96 well low-attachment U-bottom

375 plates (Costar) in cell culture medium containing 10% FBS. Cells were incubated for 5 days at 37°C
376 and 5% $C0_2$ before use in functional assays.
377
378 **Expansion of Vγ9Vδ2 T cells**
379 PBMCs from healthy volunteers (HV) were provided by the local Blood Bank (French Blood
380 Establishment). PBMCs from ALL patients and HV were isolated by density gradient (Lymphoprep,
381 REF). For ALL patients, cells were frozen until use. For establishment of allogenic Vγ9Vδ2 T cells, fresh
382 PBMCs from HV were stimulated with zoledronate 1µM (Sigma-Aldricht), and rhIl-2 (Miltenyi) at day
383 0. From day 5, rhIL-2 was renewed every two days and cells were kept at 1.5x106 /ml for 14 days.
384 Only cell cultures that reached more than 80% of Vγ9Vδ2 T cells purity at day 14 were selected and
385 frozen until use. Allogeneic Vγ9Vδ2T-cells were thawed and cultured overnight in rhIL-2 (200 UI/mL)
386 for use in functional assays.
387 To generate autologous Vγ9Vδ2 T cells, frozen PBMCs from ALL patients were stimulated by adding
388 rhIL-15 (10ng/mL) using the same protocol, as previously described to enhance proliferative
389 capacities of Vγ9Vδ2 T cells[47,48], including in PBMCs from renal cell carcinoma patients[17] or from AML
390 patients [49]. Fresh autologous expanded cells were then used for functional assays, depending on the
391 quantity of Vγ9Vδ2 T cells. The fold increase of viable Vɣ9Vδ2 T cells was calculated according to the
392 formula: (%Vɣ9Vδ2 at day 14 × total cell number at day 14)/ (% Vγ9Vδ2 at day 0 × total cell number
393 at day 0).
394
395 **Transcriptomic expression of BTN2A and BTN3A1**
396 RNA-seq of human normal or tumor tissue samples were respectively selected from the Genotype-
397 Tissue Expression (GTEx) Project portal, and from the PanCancer Genome Atlas, respectively.
398 For scRNA-Seq analysis multiple datasets of normal and tumor origin were analyzed (bone marrow
399 from healthy volunteers and AML patients: GSE1166256-6; colorectal cancer patients: GSE144735;
400 breast normal and tumor tissues: GSE164898; non squamous cells Lung carcinoma Investigation

401 description E-MTAB-8107; hepatocellular carcinoma GSE 140228; basocellular carcinoma GSE11625;
402 renal Cell Carcinoma phs002065.v1.p1).
403 For bulk RNA-seq datasets, data were log2 normalized, quantile normalized, and collapsed by using
404 median values, using Phantasus **v1.11.0** software.
405 For sc-RNA seq, data were log2, batch effect was corrected using mutual nearest neighbors
406 (MNN)method, only transcripts found in more than 5 cells and expressed in more than 200 cells were
407 analyzed. The fraction of transcripts from mitochondrial genes was set up to 5% for normal tissues
408 study and 10% for the tumor tissue study. Identification of subpopulations was realized using UMAP
409 algorithm and was analyzed using Bioturing software.
410
411 **Generation of anti-human BTN2A and BTN3A mAbs**
412 To generate anti-BTN3A 20.1 and anti-BTN3A 108.5 mAbs, BALB/c mice were immunized with a
413 soluble BT3.1-Ig fusion protein, as previously described[50].
414 Anti-BTN2A mAbs were produced by mouse immunization using a recombinant human BTN2A1-Fc
415 fusion protein (R&D Biotech), as described in patent WO2019057933A1 (clone 7.48) and patent
416 WO2020188086A1.
417
418 **ELISA**
419 Vγ9Vδ2 T cells and target cells were cocultured with at an Effector:Target (E:T) ratio of 10:1.
420 Supernatants were collected and frozen at -80°C for further cytokine measurement. Supernatants
421 were further analyzed for TNFα and/or IFNγ production using respectively BD OptEIA Human TNFα
422 and IFNγ ELISA kits (BD Biosciences). Flat-bottom 96-well plates (COSTAR) were coated with capture
423 Ab overnight andthen blocked with PBS 10% FCS for 1h. After 2h of incubation with standards or
424 supernatants, plates were incubated with detection Ab and streptavidin HRP and then developed
425 using 3,3',5,5'-tetramethylbenzidine (TMB) Liquid Substrate System (BD biosciences). The reaction

426 was stopped withHCL 2N, and the absorbance was quantified within 30 minutes using a CLARIOstar
427 microplate reader at a wavelength of 450 nm.

428 **Flow cytometry**

429 Relative surface expression of BTN2A (107G3B5 and 7.48 mAbs) and BTN3A (20.1 and 108.5 mAbs)
430 on cancer cell lines and ALL blasts was assessed by flow cytometry using calibrator beads allowing
431 quantification of antigen density (Quantum™ Simply Cellular® (QSC) kits, Bang Laboratories). To avoid
432 bias related to modulation of BTN surface expression in correlation analysis, relative quantification of
433 BTN was performed concurrently withwith functional assays.
434 For analysis of CD107 expression, Vγ9Vδ2 T cells and target cells were cocultured in a Effector:Target
435 (E:T) ratio of 1:1 with anti-CD107a, anti-CD107b and Golgi stop, with 107G3B5 or isotype control (1
436 µg/mL). After 4 hours, cells were harvestedand analyzed by flow cytometry. To study IFNγ and TNFα
437 production, cells were permeabilized with Permwash (BD Biosciences) to allow intracellular staining.
438 Cells were acquired on a FACS CANTO II and analysed using Flowjo software.
439

440 **Apoptosis assays**

441 Cell lines and primary ALL blasts

442 Target cells were first labelled with the Cell Proliferation Dye eFluor™ 670 (LifeTechnologies) and
443 then cocultured for4, 6 or 10 hours with effector cells at an E:T ratio of 5:1 (cell lines), 10:1 or 15:1
444 (primary ALL blasts), in the presence of an isotype control, 107G3B5 or anti-BTN3A 20.1 agonist mAb
445 (1 or 10 µg/mL). In caspase assays with cancer cell lines, target cells preincubated overnight (O/N)
446 with zoledronate (45 µM) were used as a positive control. At the end of coculture, target cells were
447 labeled with CellEvent™ caspase-3/7 green detection reagent for 30 min and analyzed by flow
448 cytometry (FACSCanto II). The percentage of specific apoptosis was calculated using the formula
449 [(experimental – spontaneous caspase 3/7 activation)/ (100 – spontaneous caspase 3/7 activation) x
450 100].

451 Tumor spheroids

452 Ishikawa, HT-29, PC-3 and CaSki were cocultured with Vγ9Vδ2 T cells from 6 HV for 10 hours at an E:T
453 ratio of 5:1, in the presence of an isotype control or 107G3B5 (10µg/mL). Caspase-Glo 3/7 reagent
454 was added at the end of the coculture and was incubated for 1 hour. Luminescence was quantified
455 within 30 minutes using a CLARIOstar microplate reader.

456

457 **3-dimensional (3D) tumor spheroids live-cell imaging**

458 After 5 days of spheroid generation, PC-3 were incubated for 4 hours in serum-free medium
459 containing calcein (Sigma) at a final concentration of 2 µM. PC-3 spheroids were then washed
460 extensively and cocultured with Vγ9Vδ2 T cells from 6 HV for 48 hours at an E:T ratio of 5:1, in
461 presence of an isotype control or 107G3B5 (10µg/mL).

462 Real-time calcein intensity was monitored using fluorescence live-cell imaging combined with a
463 phase-contrast setup using the 4×objective (Sartorius AG, Gottingen, Germany). Images were
464 acquired hourly and analyzed using Incucyte software and ImageJ.

465

466 **Live-cell imaging of effector and target cell interactions**

467 The target cells (T); HEK293T, SUP-T1 and Ishikawa; were cultured O/N or 1h for SUP-T1 on dishes
468 (Ibidi, Cat.No: 80136) precoated with poly-L-lysine. Non-adherent cells were removed by washing
469 with PBS. Vγ9Vδ2 T cells expanded from healthy volunteers were added to the target cells (E:T ratio =
470 2:1 for HEK293T and SUPT-1, 3:1 for Ishikawa) in 1 ml of RMPI 10% SVF. Control isotype or 107G3B5
471 was added to the final concentration of 10 µg/mL. Dishes were incubated at 37°C with 5% CO_2 within
472 Tokaihit stage top incubator throughout the acquisitions, which were performed with a CX-A
473 automated microscope. The CX-A (Nanolive) consists of an holotomographic microscope coupled to
474 an automated stage that allows grid scanning and multi-wel acquisition. The microscope uses a 60X
475 air objective (NA = 0.8) at a wavelength of λ = 520 nm (Class 1 low power laser, sample exposure 0.2
476 mW/mm^2) and USB 3.0 CMOS Sony IMX174 sensor, single field of view 90 × 90 × 30 µm, axial

477 resolution 400 nm, and maximum temporal resolution of 0.5 3D RI volume per second. The
478 theoretical sensitivity of the RI measurement was 2.71×10^{-4}. The time lapse acquisition regime was
479 one image every 9 minutes for 20 hours and was the same for all imaging experiments. The dynamics
480 of the lethal fraction was realized as previously described [51], using ML-based dead cell identification.
481
482 **Quantification of effector/target cells interactions**
483 Quantifications were performed using proprietary computer vision developed at Nanolive. This
484 analysis consists of identifying each cell object and its type (effector cells on one side, target cells on
485 the other) and to quantifying the dynamic interactions between the two populations. The dynamic
486 evolution of four parameters was exploited; the ratio of γδ T cells in contact with target cells, the
487 average minimum distance between γδ T cells and target cell centroids, the percentage of target cell
488 area covered by γδ T cells and the global movement speed of γδ T cells in um/sec. The lethal fraction
489 of target cells, i.e. the cumulative number of dead cells over time, was also quantified. To do this, we
490 used machine learning to train the computer to distinguish the typical shape and texture signatures
491 of living and of dead target cells observed by HTM.
492
493 **Recombinant Vγ9Vδ2 TCR binding assays**
494 A recombinant Vγ9Vδ2 TCR (clone G115)[52] with a C-terminal Fc-tag or His-tag on the Vγ9 chain was
495 produced in CHO cells, then purified using protein A column chromatography followed by buffer-
496 exchange into PBS. Vγ9Vδ2 TCR were biotinylated using protein labeling kit according to the
497 manufacturer's instructions (Thermofisher). Biotinylated proteins were then tetramerized with
498 streptavidin-APC (Bio- legend) at 4:1 molar ratio by adding the streptavidin over 10 times intervals to
499 maximize tetramer formation. HEK293T, SUP-T1 or Ishikawa cells were saturated with 107G3B5 or
500 isotype control (10 µg/mL) for 4 hours at 37°C. The cells were then stained with recombinant
501 tetrameric Vγ9Vδ2 TCR- Fc at 10 µg/ml for 30min at 37°C. After two washes, AF647 mean
502 fluorescence intensity (MFI) was assessed by flow cytometry (Fortessa).

503 **Confocal microscopy**

504 To study the colocalization of BTN2A1 and BTN3A1, HEK293T cells were transiently transfected with
505 either with BTN3A1 GFP or BTN2A1 mcherry using PEI max reagent (Fisher Scientific), according to
506 the manufacturer's instructions. For both constructs, a linker was inserted between the
507 BTN2A1/BTN3A1 C-terminal end and the -eGFP or -mCherry molecules in order to provide flexibility
508 and not interfere with molecular function. The next day, the cells were plated on precoated
509 coverslips with poly-L-lysine. After overnight incubation, cells were preincubated with either an
510 isotype control or anti-BTN2A 107G3B5/7.48 or anti-BTN3A 20.1 mAb (10µg/mL) during 1 hour. After
511 two washes, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS pH 7.4),
512 saturated with 2% BSA in phosphate-buffered saline (PBS pH 7.4) for 2 hours and labeled with DAPI
513 for 2min. After 2 washes with phosphate-buffered saline (PBS pH 7.4) and 1 wash with deionized
514 water, coverslips were mounted with prolong Gold (Invitrogen). Cells were imaged using an inverted
515 ZEISS LSM 880-Spectral-Airyscan with the 63X objective. BTN2A1 and BTN3A1 distribution and
516 colocalization were analyzed using ImageJ software and the JACoP plugin.

517 In order to visualize and quantify the colocalization of BTN2A and BTN3A and the binding of the
518 Vγ9Vδ2 TCR on a more relevant model, SUP-T1 cells were preincubated with either an isotype
519 control or 107G3B5 (10µg/mL) or HMBPP (10µM) for 4 hours at 37 °C. After two washes, cells were
520 incubated with either an anti BTN2A 7.48 AF647 mAb or anti-BTN3A 103.2 AF488 mAb (10µg/mL) for
521 1 hour at 4°C or with Vγ9Vδ2 TCR- Fc at 10 µg/ml for 30min at 37°C. After two washes, cells were
522 fixed, then mounted with prolong Gold and covered with a coverslip overnight. Cells were imaged
523 using an inverted ZEISS LSM 880-Spectral-Airyscan with the 63X objective.

524

525 **Western blotting**

526 The cells were lysed in M2 lysis buffer. The protein concentrations were determined using Bradford
527 reagent. Then, the proteins were run on an SDS-PAGE gel and transferred to PVDF membrane which
528 were blocked in 5% milk and probed with antibodies overnight with antibodies : anti α-tubulin and

529 anti GSDME (cell signaling). Secondary antibody conjugated with biotin and third antibody
530 conjugated with horseradish peroxidase were followed by enhanced chemiluminescence (Cytivia).
531 Results were confirmed by at least three independent experiments and quantified using Fiji.

532

533 **Statistics**

534 All analyses were performed using the GraphPad Prism 5.0 software package.
535 The normality distribution of the data was determined by using the D'Agostino test. Appropriate
536 statistical tests were used as indicated in the figure legends. All replicates' data are biological except
537 for ELISA experiments, where technical replicates are indicated.

538

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658

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665

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667 A.C.L.F, C.I., C.E.C., M.F. and D.O. conceived and designed the study. A.C.L.F, C.I., N.B., L.G., S.F., F.O.,
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669 C.E.C., M.F. and D.O. interpreted the data. N.V. treated patients and coordinated the study. A.C.L.F,

670 C.I., C.E.C, G.G, M.F. and D.O. coordinated the research and wrote the manuscript. All authors
671 critically reviewed the manuscript.
672
673 **Competing interests**
674 D.O. is cofounder and shareholder of Imcheck Therapeutics. C.E.C is employee and shareholder of
675 Imcheck Therapeutics. The remaining authors declare no competing interests.