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Evaluation of the human type-3 adenoviral dodecahedron as a vector to target acute myeloid leukemia

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33 **Abstract**

34 Intensive systemic chemotherapy is the gold standard of acute myeloid leukemia (AML)
35 treatment and is associated with considerable off-target toxicities. Safer and targeted delivery
36 systems are thus urgently needed. In this study, we evaluated a virus-like particle derived
37 from the human type-3 adenovirus, called the adenoviral dodecahedron (Dd) to target AML
38 cells. The vectorization of leukemic cells was proved very effective at nanomolar
39 concentrations in a time- and dose-dependent manner, without vector toxicity. The
40 internalization involved clathrin-mediated energy-dependent endocytosis and strongly
41 correlated with the expression of $\alpha\beta_3$ integrin. The treatment of healthy donor peripheral
42 blood mononuclear cells showed a preferential targeting of monocytes compared to
43 lymphocytes and granulocytes. Similarly, monocytes but also AML blasts were the best-
44 vectorized populations in patients while acute lymphoid leukemia blasts were less efficiently
45 targeted. Importantly, AML leukemic stem cells (LSCs) could be addressed. Finally, Dd
46 reached peripheral monocytes and bone marrow hematopoietic stem and progenitor cells
47 following intravenous injection in mice, without excessive spreading in other organs. These
48 findings reveal Dd as a promising myeloid vector especially for therapeutic purposes in AML
49 blasts, LSCs and progenitor cells.

50 **Keywords**

51 Adenoviral dodecahedron, virus-like particles, acute myeloid leukemia, leukemic stem cells,
52 delivery vector

53

54 **Introduction**

55 Acute myeloid leukemia (AML) has one of the lowest survival rates among childhood cancers
56 with a 5-year prognostic survival of 60 to 75%.^{1,2} In adults, AML is the most common
57 leukemia and patients under 60 years of age have a 5-year prognostic survival of 30% to
58 40% and this dramatically decreases below 15% above 60 years of age.³ For decades, AML
59 was classified according to cell morphology using the French-British-American (FAB)
60 classification (AML M0 to M7, *i.e.*: M3 acute promyelocytic leukemia). Since then, multiple
61 revisions from the World Health Organization (WHO) led to 4 new classes: AML with
62 recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related
63 AML and AML not otherwise specified.⁴

64 Although tremendous progress has been achieved over the past 40 years in the
65 understanding of AML physiopathology, treatment has not substantially changed. Indeed,
66 cytarabine and anthracyclines drugs, both impeding DNA replication, remain the gold
67 standard of intensive chemotherapy treatment used in combination with hematopoietic stem
68 cells (HSCs) transplantation but are associated with severe off-target toxicities and long-term
69 sequelae, especially in children and elderly patients.^{2,5-7} The specific targeting of AML cells is
70 thus a challenge of importance to enhance overall chemotherapy tolerability as well as for
71 the delivery of new therapies.⁸

72 So far, many synthetic or bioinspired drug carriers are currently in development and trials but
73 only few of them are in use with the most represented being liposomes and polymeric
74 nanoparticles.^{8,9} These carriers improve bioavailability, toxicity and may ameliorate
75 remission. For example, Vyxeos, a liposomal formulation of cytarabine and daunorubicin in a
76 5:1 molar ratio was approved by the FDA in 2017 and the EMA in 2018 for the treatment of
77 adults with newly diagnosed high-risk secondary AML.^{10,11} Among bio-inspired delivery
78 systems, virus-like particles (VLPs) are stable, self-assembled homogeneous nanoparticles,
79 composed of virus capsid or envelope proteins.¹² They are easily producible, carry no

80 genetic material and represent a versatile tool for biomedical applications including cargo
81 delivery for therapy.¹³

82 The capsid surface of the human serotype-3 adenovirus (Ad3) is composed of 3 structural
83 proteins: the hexon, the fiber and the penton base (Pb). The hexon is the major building
84 block of adenoviruses capsid but also the most immunogenic protein responsible for the bulk
85 of neutralizing antibody production¹⁴, especially against the hypervariable loops that differ
86 among adenovirus serotypes.¹⁵ The Ad3 fiber directs targeting properties through its specific
87 attachment to cellular receptors such as ubiquitous complement regulatory protein CD46¹⁶,
88 epithelial desmoglein-2¹⁷ and costimulatory molecules CD80/86¹⁸. The Ad3 Pb is meanwhile
89 responsible for the entry through the interaction of its RGD (Arg-Gly-Asp) motifs within $\alpha_v\beta_3$
90 and $\alpha_v\beta_5$ integrins present on cell membranes, which subsequently triggers endocytosis.¹⁹⁻²¹
91 In addition, an important feature of Ad3 (but not Ad2 or Ad5) is its ability to produce
92 dodecahedral VLPs during infection, consisting of fiber and Pb.²² Otherwise, the recombinant
93 expression of Ad3 Pb alone is sufficient to give rise to fibreless dodecahedron (Dd,
94 commonly referred as Base (Bs)-Dd).^{23,24} This simplified VLP is ~30 nm in diameter and is
95 exclusively composed of 12 copies of Ad3 Pb. Similarly to the Ad3 of origin, Dd shares the
96 entry mechanism connecting Pbs to α_v integrins.²⁵ Therefore, this VLP has been efficiently
97 used in the delivery of proteins^{26,27}, therapeutic compounds²⁸ and mRNA analogs.²⁹ Recently,
98 a study on human healthy blood demonstrated Dd natural affinity for leukocytes, ~45-fold
99 higher than for red blood cells (RBCs) or platelets.³⁰

100 We postulate that Dd can represent a promising vector for AML cells. We thus characterized
101 its ability to target leukemic cells in vitro with attention for vector dose, subcellular
102 localization, intracellular vector maintenance, endocytosis pathway and toxicity. We
103 compared the internalization among peripheral blood mononuclear cells (PBMCs) in healthy
104 and patient settings with a focus on cell type targeting. Further, we assessed AML and acute
105 lymphoid leukemia (ALL) blasts targeting as well as the addressing of AML leukemic stem

106 cells (LSCs) ex vivo. Finally, we evaluated the in vivo biodistribution of Dd following IV
107 injection, especially in peripheral blood and bone marrow.

108

109 **Results**

110 **Dd efficiently vectorizes human leukemic cell lines**

111 Dodecahedral VLPs were produced upon recombinant Ad3 Pbs expression in baculovirus-
112 infected insect cells and purified by a two-step chromatography yielding soluble and stable
113 nanoparticles (**Figure S1**).

114 In this study, we first evaluated the vectorization of leukemic cell lines during 1 hour with a
115 labeled Dd (Dd-AF647). A significant increase of the percentage of Dd-AF647-positive cells
116 was observed between 0.1 nM and 1 nM with a mean of $22\pm 18\%$ and $94\pm 9\%$, respectively
117 (**Figure 1A**). At 5 nM Dd, that percentage was over 98% for all cell lines. In addition, the
118 relative amount of vector in cells (MFI) rose concomitantly with increasing concentration
119 above 0.1 nM. The MFI signal increased over incubation time (**Figure 1B**), until 24 hours (P
120 $<.05$) and showed only a slow decline over 72 hours indicating a good internal maintenance
121 of the AF647 cargo (**Figure 1C**).

122 The vector toxicity was assessed over a week with treatment every 48 hours (**Figure 1D**).
123 We used 1 μM of doxorubicin as a positive cell death control (10 nM of doxorubicin had no
124 effect, not shown) since it induced $85.7\pm 7.8\%$ of dead cells in 24 hours (not shown). A
125 metabolic assessment of the viability indicated that 1, 5 and 10 nM Dd had a minor toxic
126 effect with $93.6\pm 7.4\%$, $84.4\pm 14.3\%$ and $83.1\pm 13.9\%$ of alive cells at day 7, respectively.
127 Higher doses of 25 and 50 nM displayed much more toxicity, with $76.3\pm 11.5\%$ ($P <.001$) and
128 $55.7\pm 20.1\%$ ($P <.0001$) of alive cells at day 7.

129 Noteworthy, the AML cells vectorization featured high intracytoplasmic content of particles
130 often close to the nucleus, that was not assigned to membrane adsorption of Dd (**Figure 1E**).

131 Taken together, these results demonstrate Dd as a dose- and time-dependent vector,
132 efficiently targeting leukemic cell lines while displaying moderate toxicity.

133 **The Dd vectorization involves clathrin-mediated energy-dependent endocytosis**
134 **and strongly correlates with $\alpha_v\beta_3$ integrin expression in leukemic cells**

135 We investigated the effect of temperature and ATP depletion on the cellular uptake of
136 Dd-AF647 in leukemic cells. The vector accumulation was reduced upon treatment at 4°C,
137 with 87% of reduction in Dd-AF647 MFI compared to 37°C ($P <.01$; **Figure 2A**). Similarly,
138 when the cellular ATP pool was depleted by treatment with sodium azide and
139 2-deoxy-D-glucose, the vectorization was nearly abolished with 96% of reduction ($P <.01$).
140 These results indicated that the vectorization is temperature- and energy-dependent.

141 To clarify which endocytosis pathways is involved in leukemic cells vectorization, we
142 explored the effect of wortmannin, methyl- β -cyclodextrin (MBCD) and chlorpromazine that
143 specifically inhibit micropinocytosis/phagocytosis, caveolae/raft- and clathrin-mediated
144 endocytosis (CME).³¹⁻³³ When cells were treated with wortmannin or MBCD, Dd-AF647 MFI
145 slightly decreased while the treatment with chlorpromazine significantly reduced Dd uptake of
146 ~92% compared to control ($P <.05$; **Figure 2B**). Noteworthily, phagocytic Mono-mac-6 and
147 THP-1 cells as well as the chronic myeloid leukemia K-562 were less efficiently vectorized
148 compared to others. These inhibition results showed that the active transport of Dd is carried
149 out by CME.

150 Among integrins, $\alpha_v\beta_3$ is characterized as the entry receptor for Dd.²⁵ Therefore, we
151 quantified its expression along Dd-AF647 internalization on leukemic cells. As expected, the
152 MFI displayed a very close linear correlation between $\alpha_v\beta_3$ expression and the cellular level
153 of Dd (**Figure 2C**). Conversely, although Dd was shown to interact with heparan sulfates³⁴, a
154 competitive inhibition of entry using heparin did not reduce Dd uptake in leukemic cells
155 (**Figure S2**).

156 Taken together, these results reveal the leukemic cells Dd-mediated vectorization as a
157 temperature- & energy-dependent phenomenon involving CME in $\alpha_v\beta_3$ integrin expressing
158 cells.

159 **Dd vectorizes human healthy donor peripheral blood mononuclear cells,**
160 **targeting preferentially monocytes**

161 PBMCs were purified from 25 healthy donor bloods and treated with Dd-AF647. Monocytes
162 appeared as the best-targeted cells followed by B lymphocytes with $96\pm 4\%$ and $47.7\pm 13.4\%$
163 of Dd-AF647-positive cells for 1 nM, respectively ($P < .0001$, **Figure 3A, left panel**).
164 Comparatively, granulocytes, T lymphocytes and NK lymphocytes exhibited very close Dd
165 penetration and were less efficiently vectorized. Importantly, T lymphocytes appeared to be
166 the least-vectorized population, with $53.5\pm 6.7\%$ of vector-positive cells at 10 nM ($P < .0001$,
167 not displayed). Similar results were obtained for MFI except for granulocytes that showed a
168 very low vector accumulation with a fold increase of 7 ± 2 at 10 nM (**Figure 3A, right panel**).
169 Thus, Dd vectorizes PBMCs ex vivo, targeting preferentially monocytes.

170 **Dd efficiently vectorizes AML blasts compared to ALL, targets monocytes and**
171 **all subtypes of AML blasts among PBMCs and is able to enter AML leukemic**
172 **stem cells**

173 We next analyzed the targeting of 33 leukemic patient PBMCs. Among these samples, 8
174 were obtained from ALL pediatric patients and 25 from AML patients, among which 84%
175 were adult and 16% were pediatric (**Table 1**). As expected, Dd preferentially vectorized AML
176 blasts compared to ALL, at 1 and 10 nM, both in terms of Dd-positive cells and MFI (**Figure**
177 **3B**). We thus focused on AML samples (**Table 2**) and showed that the vector targeted blasts
178 independently of the disease status (diagnosis versus relapse; **Figures S3 A-B**), the age
179 (pediatric versus adult patients; **Figures S3 C-D**) or whether the biological sample was
180 purified or not (**Figures S3 E-F**). These data suggest the Dd as a versatile vector for AML.

181 Lymphocytes, granulocytes and monocytes (LGM) were also monitored for Dd entry during
182 vectorization of AML blasts. There was 60.5±30.5% of Dd-AF647-positive cells for AML
183 blasts at 1 nM compared to 18±16% ($P <.0001$), 28±23% ($P <.0001$) and 81.7±19.9% (P
184 $<.01$) for LGM, respectively (**Figure 3C, left panel**). A ten-fold concentrated dose of vector
185 reduced these differences among populations. Quantitatively, data became statistically
186 significant with the use of 10 nM Dd, with 313±301-fold increase of MFI compared to 54±47
187 ($P <.0001$), 29±22 ($P <.0001$) and 608±427 ($P <.0001$) for LGM (**Figure 3C, right panel**). It
188 is thus noteworthy that Dd vectorizes massively AML blasts and monocytes compared to
189 lymphocytes and granulocytes.

190 To go further, we analyzed the vectorization of AML subtypes according to cell morphological
191 classification. All types of AML were vectorized with AML M4 displaying 802±322-fold
192 increase of MFI at 10 nM compared to 351±263 ($P <.05$), 151±202 ($P <.01$), 143±154 (P
193 $<.01$) and 130±192 ($P <.001$) for secondary AML, AML M0-1-2, M3 and M5, respectively
194 (**Figures 3D**). Furthermore, we analyzed Dd uptake according to the 2016-revised WHO
195 AML classification.⁴ There were no significant differences in Dd vectorization between these
196 new AML subtypes nor for 9 AML classified with recurrent genetic abnormalities regarding
197 *flt3*, *npm1* and *idh1/2* classical mutations (**Figure S4 A-B**). These results mean that the Dd
198 vectorization is efficient in AML regardless of the morphological and the WHO classification.

199 A key feature of AML physiopathology is the presence of renewable LSCs particularly those
200 found in granulocyte-macrophage progenitors (GMP-like LSCs).^{35,36} To explore the uptake of
201 this particular subpopulation, whole marrow samples were treated with Dd before being
202 enriched in CD34-positive cells. Overall, a similar number of Dd-AF647-positive cells and
203 MFI was observed for total stem cells, myeloid precursors and GMP-like LSCs (**Figure 3E**).

204 Together, these results confirm the potential of Dd to vectorize AML blasts. Importantly, the
205 vector is able to penetrate LSCs.

206 **Dd reaches peripheral monocytes and bone marrow precursors in vivo**

207 Finally, an in vivo biodistribution study was conducted to confirm Dd vectorization properties.
208 Mice were IV injected with Dd-AF647 and sacrificed at 1-, 5- and 12-hours post-injection. Ex
209 vivo fluorescence imaging was performed on isolated organs (**Figure 4A**). An important
210 uptake was observed in the kidney at 1 and 5 hours and in the liver at the 3 time points while
211 only a weak signal arose from other organs including heart, brain, skin, and spleen.

212 Additionally, peripheral blood and bone marrow were analyzed. Although we used a small
213 injected amount of Dd, a qualitative analysis of targeted cells could be performed.
214 Leukocytes were the major vectorized population with regard to platelets and RBCs at 1 hour
215 and the signal decreased rapidly over 12 hours (**Figure 4B**). The study of leukocytes subsets
216 revealed that monocytes were also preferentially targeted in vivo (**Figure 4C**).

217 Bone marrow hematopoietic stem and progenitor cells (HSPCs) showed interesting results
218 (**Figure 4D**). The best-targeted population was the multipotent progenitors (MPPs) with a 3
219 time points-mean of $3.42 \pm 1.51\%$ of vector-positive cells, compared to $0.85 \pm 0.63\%$,
220 $0.62 \pm 0.41\%$, $0.97 \pm 0.72\%$ and $0.55 \pm 0.36\%$ for the common-lymphoid progenitors (CLPs), the
221 common-myeloid progenitors (CMPs), the GMPs and the megakaryocyte-erythrocyte
222 progenitors (MEPs), respectively ($P < .001$). HSCs displayed a mean of $1.43 \pm 1.13\%$ of
223 Dd-AF647-positive cells that was lower than for MPPs ($P < .01$). Comparisons between CLPs,
224 CMPs, GMPs and MEPs were difficult as these populations showed similar but lower
225 percentage of Dd penetration. Thus, all bone marrow-resident HSPCs were reached by Dd
226 upon IV injection with MPPs accumulating more Dd than other cells

227 Taken together, these results recapitulate ex vivo observations and further demonstrate Dd
228 ability to vectorize peripheral leukocytes in vivo, especially monocytes. Importantly, Dd
229 reaches the bone marrow where it vectorizes progenitors, which is an important property for
230 AML treatment delivery and targeting of LSCs.

231 **Discussion**

232 The targeted delivery of AML cells is crucial for the improvement of chemotherapy tolerability
233 by reducing unspecific off-target effects and subsequent acute and chronic toxicities
234 mediated by intensive treatment.^{5,6,8} Furthermore, myeloid delivery systems are scarce and
235 urgently needed for the avenue of new therapies. In this study, we fully evaluated the use of
236 a non-infectious VLP as a vector to target AML cells since it presents a natural affinity for
237 leukocytes.³⁰ Dd highlights several advantageous properties to represent an interesting
238 delivery system in AML:

239 The vector is only composed of Ad3 Pbs, and 12 copies are sufficient to trigger the assembly
240 of a dodecahedral VLP that is massively produced upon recombinant expression.^{23,24} In
241 addition, the vector is easily purifiable using size-exclusion followed by anion-exchange
242 chromatography. Hence, it could be efficiently manufactured. Comparatively to the virus of
243 origin, Dd is devoid of genome, hexon and fiber proteins thus simpler and non-infectious.
244 Although pre-existing or de novo generation of neutralizing anti-Ad3 antibodies could mitigate
245 efficient drug vectorization, it has been shown that the hexon was the main target of pre-
246 existing antibodies as well as adenovirus specific CD4+ and CD8+ T cells.^{14,37,38} Moreover,
247 Zochowska et al.²⁹ showed only a slow and moderate build-up of anti-Dd antibodies following
248 5 IV injections in rats. This suggest a limited vector neutralization risk at therapy initiation and
249 overtime.

250 The Ad3 infects epithelial and hematopoietic cells using macropinocytosis as a major entry
251 pathway.³⁹ Comparatively, the endocytic targeting of Dd mainly involved energy-dependent
252 CME in tested leukemic cells. This behavior might be thus related to the simpler vector
253 composition of only Pbs. The use of a fibreless Dd also restrains undesired cell tropism by
254 reducing the number of attachment receptors.¹⁶⁻¹⁸ In this sense, it has been shown that the
255 Ad3 enters platelets and granulocytes using fiber-mediated binding of desmoglein-2¹⁷ while,
256 in this study, fibreless Dd showed only a slight internalization in these cells. Importantly, Dd
257 keeps the ability to trigger specific endocytosis through the interaction of Pb RGD motifs with
258 $\alpha_v\beta_3$ integrins.^{20,25} This feature confers, at least, a relative selectivity toward α_v integrins

259 expression and especially $\alpha_v\beta_3$ such as for highly expressing leukemic cell lines as well as
260 monocytes^{40,41}, AML cells⁴²⁻⁴⁴ and bone marrow progenitors⁴⁵⁻⁴⁷ that were efficiently
261 targeted. However, dedicated mechanistic studies could deeper characterized this $\alpha_v\beta_3$ -
262 correlated, CME-mediated endocytosis in AML cells, since partial entrapment in lysosomes
263 has been described and that could mitigate vectorized molecules effectiveness.²⁷ Besides, it
264 was shown that Dd preferentially penetrates leukocytes than platelets or RBCs in healthy
265 donor blood.³⁰ That distinct vectorization efficiency was confirmed and we further revealed
266 monocytes as well as AML blasts to be better vectorized than ALL blasts, lymphocytes (T, B
267 and NK) and granulocytes. Additionally, Dd slightly spread outside the bloodstream with most
268 of filtration occurring in 12 hours, which support a rapid clearance after IV injection. It is
269 noteworthy that Dd entered the heart only weakly, which is promising for AML to circumvent
270 the cardiotoxicity induced by anthracyclines.⁶ The limited vector spreading is of great
271 importance to allow optimal leukocytes targeting while reducing systemic toxicity. Altogether,
272 these features strengthen the use of Dd to target leukemic cells compared to others carriers -
273 with no selective cell delivery properties - such as liposomes, already evaluated in AML.^{10,11}

274 The relevance of LSCs has been thoroughly described in AML³⁶ but novel directed systems
275 do not always evaluate the vector ability to reach these cells. The vector presented here is
276 able to target human GMP-like LSCs ex vivo which, along with lymphoid-primed multipotent
277 progenitors LSCs, coexist in 80% of patients.³⁵ Furthermore, upon IV injection in mice, the
278 vector reached the bone marrow and HSPCs, among which MPPs were the best-vectorized.
279 Since 10 to 15% of AML cases are characterized by a dominant population resembling
280 MPPs³⁵, that population would be potentially targeted by Dd. These features emphasize the
281 use of Dd to target HSPCs and by extension the place where LSCs reside.

282 The vectorization of model leukemic cells revealed an efficient targeting at low nanomolar
283 concentration as well as a good intracytoplasmic maintenance of the fluorescent cargo.
284 These points are of importance since intensive chemotherapy treatment induces efflux-
285 mediated multidrug resistance (MDR) mechanisms that greatly contribute to AML relapses.⁴⁸

286 Of note, the vectorization of AML blasts obtained from relapsed patients was as effective as
287 the vectorization of patient blasts at diagnosis. Thus, the enhanced retaining time of cargo
288 provided with low concentration of Dd could circumvent those hurdles, similarly to
289 doxorubicin in the context of human MDR sarcoma cells.³⁰ Since our data also indicate a
290 moderate toxicity of Dd, its utilization as a vector might be safe in vivo. Hence, Dd and
291 chemotherapy could be associated to enhance the effect of free therapeutic form, thereby
292 reducing off-targets and side effects. Using this strategy, the effective cytotoxic concentration
293 of bleomycin delivered with Dd to human cervical cancer cells was 100 times lower than that
294 of free drug.²⁸ Thus, the vector is effective from very low molecular concentrations,
295 supporting its use as an efficient enhancer for therapeutics in AML cells also via
296 improvement of cell retention while cytotoxicity of Dd remains acceptable.

297 In conclusion, these results highlight promising features of Dd vector for myeloid and
298 monocytes cell delivery applications as well as for AML therapy-aiming at targeting
299 preferentially blasts and HSPCs. Further studies should now consider a therapeutic form of
300 Dd such as Dd bearing doxorubicin and assess its full potential in AML engrafted mice
301 models in comparison to free-drug doxorubicin or another reference therapy.

302 **Materials & Methods**

303 **Dd labelling**

304 Dd was conjugated to AlexaFluor 647 (Dd-AF647) using AF647 Protein Labeling Kit
305 (Molecular Probes) and the structure was verified by nanoparticle tracking analysis on a
306 NanoSight NS300 (Malvern Panalytical).

307 **Dd cell vectorization**

308 Leukemic cell lines, PBMCs, bone marrow mononuclear cells and whole blood/bone marrow
309 were incubated with 0.01 to 10 nM Dd-AF647 for up to 7 days at 37°C. Untreated cells or
310 whole blood/bone marrow were used as controls. After incubation, purified cells were treated
311 with 0.5% Trypsin-EDTA (Gibco) during 5 min at 37°C to remove surface-bound Dd. For

312 LSCs vectorization, CD34 enrichment was performed using CD34 MicroBeads kit
313 (autoMACS Pro, Miltenyi Biotech).

314 **Cytotoxicity assays**

315 The Dd toxicity was assessed on cell lines using a metabolic assessment of cell viability by a
316 chromogenic assay. Cells were quantified using CellTiter 96 Aqueous One Solution Cell
317 Proliferation Assay (Promega) on a Varioskan Flash (Thermo Scientific). Doxorubicin
318 (Accord) was used as a positive control for cell death.

319 **Confocal microscopy**

320 Cells were treated with PBS or 10 nM Dd-AF647 for 2h at 37°C. After incubation, cells were
321 fixed with 1x FACS lysing solution (BD Biosciences). Nuclei were stained with DAPI (1:3000;
322 Sigma) and membranes with AlexaFluor 555-conjugated wheat germ agglutinin (WGA;
323 1:200; Invitrogen) during 10 min at room temperature. Images were acquired after sequential
324 laser excitation using an LSM 710 NLO laser-scanning microscope (Zeiss) onto optical
325 sectioning of 1 µm and analyzed with ImageJ software.

326 **Drug treatment**

327 Cells were pre-incubated during 1h prior to Dd addition in PBS containing the following
328 drugs: 100 nM wortmannin, 30 µM chlorpromazine hydrochloride, 500 µM MBCD, 10 mM
329 2-deoxy-D-glucose and 10 mM sodium azide (Sigma).

330 **Flow cytometry**

331 Human and mouse antibodies were purchased from BD Biosciences. In all experiments, cells
332 were selected by a viability labelling using Zombie Aqua Fixable Viability Kit (BioLegend).
333 The percentage of Dd-AF647 positive cells and AF647 mean fluorescence intensity (MFI)
334 were measured for each population. The threshold percent gate was set-up by cell type for
335 background fluorescence of untreated cells. The MFI of Dd-AF647-treated cells was
336 compared to control by dividing (fold increase) or subtracting the MFI of the corresponding

337 untreated cells. For measurement of $\alpha_V\beta_3$ integrin expression, cells were labeled with anti-
338 $\alpha_V\beta_3$ -PE monoclonal antibody. Data were acquired on FACS Canto II & FACS LSR II
339 (Beckton Dickinson) and analyzed with FlowJo V10 software.

340 **In-vivo biodistribution in blood and bone marrow**

341 The project was authorized by the French ministry of research with the permit number:
342 APAFIS#18922-2019013112124432v4. Twelve 5-weeks old C57Black6 female mice were
343 purchased (Janvier Laboratory) and housed for 1 week before the day of experiment. Mice
344 were injected in the tail vein with 100 μ g Dd-AF647 in 200 μ L of PBS (n = 9) or with 200 μ L of
345 PBS alone (n = 3). Three mice were anesthetized per time point at 1, 5 and 12h post-
346 injection, 500 μ L of blood were collected and mice were sacrificed for organs and bone
347 marrow sampling.⁴⁹

348 **Statistics**

349 Data are reported as the mean values \pm standard deviation (SD). Student *t*-test was used in
350 univariate analysis and Pearson *r* coefficient was calculated for correlation. One-way or two-
351 way ANOVA tests were performed for multivariate analysis and *P*-value were conservatively
352 adjusted for multiple comparisons using a Bonferroni correction. *P*-value $<.05$ was
353 considered significant and the abbreviations were: n.s., not significant; *, $P \leq .05$; **, $P \leq .01$;
354 ***, $P \leq .001$; ****, $P < .0001$. Statistics were calculated using GraphPad Prism V7 software.

355 **Author Contributions**

356 Conceptualization, B.C, D.H, D.L and J.C; Methodology, B.C, D.H and J.C; Investigation, B.C
357 G.S, D.H and M.G; Validation, Formal Analysis, Visualization and Writing – Original Draft,
358 B.C; Writing – Review & Editing, B.C, D.H, V.J, D.L, J.C, P.M and D.P; Funding Acquisition,
359 D.L, J.C and D.P; Resources, J.C, P.M and D.P; Supervision, B.C, P.M and D.P.

360 **Conflict of interest**

361 The authors declare no competing interests.

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384

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522 **Figures Legends**

523 **Figure 1. The adenoviral Dd efficiently vectorizes human leukemic cells,**
524 **displaying robust intracellular maintenance and low toxicity.**

525 KG-1a, THP-1, Mono-Mac-6, SupB15, KG-1, OCI-AML-3, HEL, K-562, Jurkat, U-937 and
526 HL-60 cells were incubated with Dd-AF647. The corresponding untreated cells were used as
527 negative control. All measures were done in triplicates for each cell lines and the mean \pm SD
528 is indicated. *P*-values were determined by one-way ANOVA (C) or two-way ANOVA (D). ns,
529 not significant; *, *P* \leq .05; **, *P* \leq .01; ***, *P* \leq .001; ****, *P* $<$.0001. (A) Effect of Dd
530 concentration on the 11 cell lines during 1 h. In blue, cells positive (%) for Dd-AF647. In red,
531 fold increase in Dd-AF647 MFI compared to untreated. (B) Effect of incubation time. KG-1a,
532 THP-1, Mono-Mac-6, SupB15, KG-1, OCI-AML-3 and Jurkat were treated with 1 nM Dd-
533 AF647 up to 180 minutes. (C) Persistence of Dd-AF647 inside treated cells. Cells were
534 treated with 1 nM Dd-AF647 and analyzed at 2, 24, 48 and 72 h for AF647 MFI. (D) Effect of
535 Dd on cell viability. The 11 cell lines were treated with repetitive doses of unlabeled-Dd every
536 24 h and assessed for viability by measurement of cell metabolism at days 0, 3, 5 and 7.
537 Doxorubicin (DOX; 1 μ M) was used as a cell death control. Left panel: DOX, 1, 5 and 10 nM
538 Dd. Right panel: 25 and 50 nM Dd. (E) Cellular localization of Dd. HEL & OCI-AML-3 cells
539 were incubated with 10 nM Dd-AF647 (red signal) during 2 h. Membranes were labeled with
540 AF555-conjugated wheat germ agglutinin (green signal) and nuclei counterstained with DAPI
541 (blue signal). Visualization was performed on a Zeiss LSM710 NLO microscope with a
542 63x/1.4 oil-immersion objective. Signals were collected after sequential laser excitation onto
543 optical sectioning of 1 μ m. Scale bars represent 10 μ m.

544 **Figure 2. The vectorization is an energy-dependent phenomenon, involving**
545 **clathrin-coated pit-mediated endocytosis and strongly correlating with $\alpha_v\beta_3$**
546 **integrin highly expressing cells.**

547 Leukemic cell lines were either pre-incubated at 4°C or with inhibitor drugs at 37°C in
548 complete medium during 1 h before incubation with 1 nM Dd-AF647 during 1 h. Control cells
549 were directly exposed to Dd without inhibitors at 37°C and untreated cells were used as
550 negative control. Measures were done in triplicates for each cell lines and the mean \pm SD is
551 indicated. *P*-values were determined by one-way ANOVA. ns, not significant; *, *P* \leq 0.05; **, *P*
552 \leq 0.01; ***, *P* \leq 0.001; ****, *P* $<$ 0.0001. **(A)** Effect of low temperature (4°C) and cellular ATP
553 depletion (w/o ATP) on the internalization of Dd-AF647 (MFI). **(B)** Effect of endocytic
554 inhibitors (chlorpromazine, wortmannin and methyl- β -cyclodextrin) on the internalization of
555 Dd-AF647 (MFI). **(C)** Scatterplot showing linear correlation between Dd-AF647 MFI and $\alpha_v\beta_3$
556 integrin MFI. The 7 cell lines were treated with 1 nM Dd-AF647 during 1 h at 37°C (without
557 inhibitors) and then labeled with anti- $\alpha_v\beta_3$ -PE monoclonal antibody. Pearson *r* coefficient of
558 correlation and *P*-value (two-tailed) are indicated.

559 **Figure 3. Dd targets preferentially monocytes among PBMCs and blasts from**
560 **AML compared to ALL, addressing all AML subtypes with a higher enrichment**
561 **in acute myelomonocytic leukemia and is able to enter AML LSCs.**

562 Healthy donor (A) and leukemic patient (B, C, D) PBMCs or whole bone marrow obtained
563 from AML patients (E) were incubated with Dd-AF647 during 2h. Cells positive (%) for Dd-
564 AF647 (A, C left panel; B, E in blue). Fold increase in Dd-AF647 MFI (A, C right panel; B in
565 red; D) or Dd-AF647 MFI (E in red). Results represent the mean \pm SD and the corresponding
566 number of samples is indicated. *P*-values were determined by Student *t*-tests (B) or two-way
567 ANOVA (A, C, D). ns, not significant; *, *P* \leq 0.05; **, *P* \leq 0.01; ***, *P* \leq 0.001; ****, *P* $<$ 0.0001. **(A)** Dd
568 penetration within subpopulations of healthy donor PBMCs. Min/Max values (external lines)
569 are also indicated. Cells were identified as follow: CD45+ CD66b+ for remaining
570 granulocytes after PBMCs purification (blue); CD45+ CD3+ for T lymphocytes (red); CD45+
571 CD3- CD19- CD56+ for NK lymphocytes (purple); CD45+ CD19+ for B lymphocytes (green)
572 and CD45+ CD33+ CD14+ for monocytes (orange). **(B)** Vectorization of SSC^{low} CD45^{low}
573 blasts from acute lymphoblastic leukemia (ALL, n = 8) versus acute myeloblastic leukemia

574 (AML, n = 25). **(C)** Dd penetration within subpopulations of 25 AML PBMCs. Min/Max values
575 (external lines) are also indicated. Subpopulations were identified as follow: SSC^{low} CD45^{high}
576 CD3+ and CD19+ and CD56+ for lymphocytes (red); SSC^{high} CD45+ CD66b+ for remaining
577 granulocytes after PBMCs purification (blue); SSC^{int} CD45+ CD33+ CD14+ for monocytes
578 (orange) and SSC^{low} CD45^{low} distinct population for AML blasts (grey). **(D)** Vectorization of
579 SSC^{low} CD45^{low} AML blasts according to the morphological French-American-British (FAB)
580 classification (n = 25). Single patient values are displayed and the mean is indicated by a
581 black line. Secondary AML represent AML contracted after a myelodysplastic syndrome,
582 myeloproliferative syndrome, chronic myelomonocytic leukemia and chemotherapy. FAB M0-
583 1-2 define undifferentiated AML or with minimal differentiation/maturation; FAB M3 = acute
584 promyelocytic leukemia; FAB M4 = acute myelomonocytic leukemia; FAB M5 = acute
585 monocytic leukemia and FAB M7 = acute megakaryoblastic leukemia. **(E)** Vectorization of
586 stem cells with 1 nM Dd-AF647. Cells were enriched for CD34 expression after Dd
587 incubation using magnetic sorting. Total stem cells = CD34+, Myeloid precursors = CD34+
588 CD33+ and GMP-like LSCs = Lin- CD34+ CD38+ CD123+ CD45RA+.

589 **Figure 4. Dd demonstrates limited vector spreading outside the bloodstream**
590 **and reach peripheral monocytes and bone marrow precursors in vivo.**

591 Mice were injected IV with 100 µg Dd-AF647 in 200 µL of PBS (n = 9) or with 200 µL of PBS
592 alone (n = 3). Three mice were sacrificed at 1-, 5- and 12-hours post-injection (p.i). Organs
593 and cells from PBS-injected mice were used as vehicle control. Results represent the mean
594 (n = 3) ± SD. *P*-values were determined by Student *t*-tests (B) or two-way ANOVA (C-D). ns,
595 not significant; *, *P* ≤ .05; **, *P* ≤ .01; ***, *P* ≤ .001; ****, *P* < .0001. **(A)** 2D fluorescence imaging
596 performed ex vivo on isolated organs, displayed in [photon/sec]/cm². Insert: a sample image
597 of a mouse sacrificed 1 h p.i. **(B)** Principal blood constituents positive (%) for Dd-AF647.
598 Platelets, red blood cells and leukocytes were gated by their specific expression of CD41,
599 TER-119 (Ly-76) and CD45, respectively. **(C)** Lymphoid and myeloid lineages positive (%)
600 for Dd-AF647. Subpopulations were identified with the following markers: CD45^{high} and CD3+

601 for T lymphocytes or CD335+ (NKp46) for NK lymphocytes or CD19+ for B lymphocytes;
602 CD45+ CD11c- MHCII- GR1+ (Ly-6G) for granulocytes and CD45+ CD11c^{low/neg} MHCII+
603 F4/80+ (Ly-71) for monocytes. **(D)** Stem cells and early precursors positive (%) for Dd-
604 AF647. Subpopulations were identified as follow: Lin- Sca1+ c-Kit+ CD135- CD34+ for
605 hematopoietic stem cells (HSCs); Lin- Sca1+ c-Kit+ CD135+ CD34+ for multipotent
606 progenitors (MPPs); Lin- CD127+ Sca1+ c-Kit+ for common lymphoid progenitors (CLPs);
607 Lin- CD127- Sca1- c-Kit+ CD16/32- CD34+ for common myeloid progenitors (CMPs); Lin-
608 CD127- Sca1- c-Kit+ CD16/32+ CD34+ for granulocyte-monocyte progenitors (GMPs) and
609 Lin- CD127- Sca1- c-Kit+ CD16/32- CD34- for megakaryocyte-erythrocyte progenitors
610 (MEPs).

611

612 **Tables**

613 **Table 1. Description of Acute Lymphoid Leukemia (ALL) and Acute Myeloid**
614 **Leukemia (AML) patient population.**

	ALL (n, %)	AML (n, %)
NUMBER OF SAMPLES	8 (16)	25 (84)
SEX (male %)	62,5	68
AGE AT DIAGNOSIS		
<2	0	2 (8)
2-10	5 (62,5)	1 (4)
10-18	3 (37,5)	1 (4)
18-60	N/A	9 (36)
>60	N/A	12 (48)

615

616 **Table 2. Classification of pediatric and adult AML patient population.**

	PEDIATRIC (n, %)	ADULT (n, %)
AML at		
Diagnosis	4 (100)	21 (71)
Relapsing	0	6 (29)
FAB CLASSIFICATION		
M0	1 (25)	5 (24)
M1	0	2 (10)
M2	1 (25)	7 (33)
M3	0	1 (5)
M4	0	3 (14)
M5	1 (25)	3 (14)
M6	0	0
M7	1 (25)	0
WHO CLASSIFICATION		
AML with recurrent genetic abnormalities	2 (50)	7 (33)
<i>flt3</i>	1 (25)	3 (14)
<i>npm1</i>	0	2 (10)
<i>idh1/2</i>	0	2 (10)
<i>Others</i>	1 (25)	0
AML with myelodysplasia related changes	0	8 (38)
therapy-related AML	1 (25)	0
AML not otherwise specified	1 (25)	6 (25)

617