

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

Benjamin Caulier, Gaëlle Stofleth, Dalil Hannani, Mélanie Guidetti, Véronique Josserand, David Laurin, Jadwiga Chroboczek, Pascal Mossuz, Dominique Plantaz

▶ To cite this version:

Benjamin Caulier, Gaëlle Stofleth, Dalil Hannani, Mélanie Guidetti, Véronique Josserand, et al.. Evaluation of the human type-3 adenoviral dodecahedron as a vector to target acute myeloid leukemia. Molecular Therapy - Methods and Clinical Development, 2020, 10.1016/j.omtm.2020.11.009 . hal-03013800

HAL Id: hal-03013800 https://hal.science/hal-03013800

Submitted on 17 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

- 4
- Benjamin Caulier¹⁻³, Gaëlle Stofleth^{2,4}, Dalil Hannani¹, Mélanie Guidetti³, Véronique
 Josserand³, David Laurin^{3,5}, Jadwiga Chroboczek¹, Pascal Mossuz^{2,3} and Dominique
- 7 Plantaz⁴
- ¹Univ. Grenoble Alpes, CNRS, CHU Grenoble Alpes, Grenoble INP, TIMC-IMAG, 38000
 Grenoble, France;
- ²Institute of Biology and Pathology, Laboratory of Cellular Hematology, Univ. Grenoble Alpes
 Hospital, Grenoble, France;
- ³Institute for Advanced Biosciences, INSERM U1209, CNRS UMR 5309, Univ. Grenoble
 Alpes, Grenoble, France;
- ⁴Department of Pediatric Onco-Immuno-Hematology, Univ. Grenoble Alpes Hospital,
 Grenoble, France;
- ⁵Etablissement Français du Sang Auvergne Rhône-Alpes, Grenoble, France
- 17
- 18 Corresponding author:
- 19 Benjamin Caulier, PhD
- Institute of Biology and Pathology, Laboratory of Cellular Hematology, Univ. Grenoble Alpes
 Hospital, Grenoble, France
- 22 benjamin.caulier@gmail.com
- 23 Phone number: +33 4 76 76 94 93
- 24
- 25 <u>Place of work</u>: Grenoble, Isère, France
- 26 <u>Short title</u>: Adenoviral dodecahedron targets AML cells
- 27 Manuscript data:
- Text: 3618
- Abstract: 182
- Figure/table count: 4 figures + 2 tables (4 supplemental figures)
- Reference count: 49
- 32

33 Abstract

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

50 Keywords

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

52 delivery vector

54 Introduction

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

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

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

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

The capsid surface of the human serotype-3 adenovirus (Ad3) is composed of 3 structural 82 proteins: the hexon, the fiber and the penton base (Pb). The hexon is the major building 83 block of adenoviruses capsid but also the most immunogenic protein responsible for the bulk 84 of neutralizing antibody production¹⁴, especially against the hypervariable loops that differ 85 among adenovirus serotypes.¹⁵ The Ad3 fiber directs targeting properties through its specific 86 attachment to cellular receptors such as ubiquitous complement regulatory protein CD46¹⁶, 87 epithelial desmoglein-2¹⁷ and costimulatory molecules CD80/86¹⁸. The Ad3 Pb is meanwhile 88 responsible for the entry through the interaction of its RGD (Arg-Gly-Asp) motifs within $\alpha_V \beta_3$ 89 and $\alpha_{V}\beta_{5}$ integrins present on cell membranes, which subsequently triggers endocytosis.^{19–21} 90 In addition, an important feature of Ad3 (but not Ad2 or Ad5) is its ability to produce 91 dodecahedral VLPs during infection, consisting of fiber and Pb.²² Otherwise, the recombinant 92 expression of Ad3 Pb alone is sufficient to give rise to fibreless dodecahedron (Dd, 93 commonly referred as Base (Bs)-Dd).^{23,24} This simplified VLP is ~30 nm in diameter and is 94 exclusively composed of 12 copies of Ad3 Pb. Similarly to the Ad3 of origin, Dd shares the 95 entry mechanism connecting Pbs to α_v integrins.²⁵ Therefore, this VLP has been efficiently 96 used in the delivery of proteins^{26,27}, therapeutic compounds²⁸ and mRNA analogs.²⁹ Recently, 97 a study on human healthy blood demonstrated Dd natural affinity for leukocytes, ~45-fold 98 higher than for red blood cells (RBCs) or platelets.³⁰ 99

We postulate that Dd can represent a promising vector for AML cells. We thus characterized its ability to target leukemic cells in vitro with attention for vector dose, subcellular localization, intracellular vector maintenance, endocytosis pathway and toxicity. We compared the internalization among peripheral blood mononuclear cells (PBMCs) in healthy and patient settings with a focus on cell type targeting. Further, we assessed AML and acute 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

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

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

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

129 Noteworthily, 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**).

Taken together, these results demonstrate Dd as a dose- and time-dependent vector,
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

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

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

Among integrins, $\alpha_{V}\beta_{3}$ is characterized as the entry receptor for Dd.²⁵ Therefore, we quantified its expression along Dd-AF647 internalization on leukemic cells. As expected, the MFI displayed a very close linear correlation between $\alpha_{V}\beta_{3}$ expression and the cellular level of Dd (**Figure 2C**). Conversely, although Dd was shown to interact with heparan sulfates³⁴, a competitive inhibition of entry using heparin did not reduce Dd uptake in leukemic cells (**Figure S2**).

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

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

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

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

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

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

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

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

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

206 Dd reaches peripheral monocytes and bone marrow precursors in vivo

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

Additionally, peripheral blood and bone marrow were analyzed. Although we used a small injected amount of Dd, a qualitative analysis of targeted cells could be performed. Leukocytes were the major vectorized population with regard to platelets and RBCs at 1 hour and the signal decreased rapidly over 12 hours (**Figure 4B**). The study of leukocytes subsets 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 time points-mean of 3.42±1.51% of vector-positive cells, compared to 0.85±0.63%, 219 0.62±0.41%, 0.97±0.72% and 0.55±0.36% for the common-lymphoid progenitors (CLPs), the 220 common-myeloid progenitors (CMPs), the GMPs and the megakaryocyte-erythrocyte 221 222 progenitors (MEPs), respectively (P < .001). HSCs displayed a mean of $1.43 \pm 1.13\%$ of Dd-AF647-positive cells that was lower than for MPPs (P <.01). Comparisons between CLPs, 223 CMPs, GMPs and MEPs were difficult as these populations showed similar but lower 224 225 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

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

231 **Discussion**

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

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

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

expression and especially $\alpha_{V}\beta_{3}$ such as for highly expressing leukemic cell lines as well as 259 monocytes^{40,41}, AML cells⁴²⁻⁴⁴ and bone marrow progenitors⁴⁵⁻⁴⁷ that were efficiently 260 261 targeted. However, dedicated mechanistic studies could deeper characterized this $\alpha_{V}\beta_{3}$ correlated, CME-mediated endocytosis in AML cells, since partial entrapment in lysosomes 262 has been described and that could mitigate vectorized molecules effectiveness.²⁷ Besides, it 263 was shown that Dd preferentially penetrates leukocytes than platelets or RBCs in healthy 264 donor blood.³⁰ That distinct vectorization efficiency was confirmed and we further revealed 265 monocytes as well as AML blasts to be better vectorized than ALL blasts, lymphocytes (T, B 266 and NK) and granulocytes. Additionally, Dd slightly spread outside the bloodstream with most 267 of filtration occurring in 12 hours, which support a rapid clearance after IV injection. It is 268 269 noteworthy that Dd entered the heart only weakly, which is promising for AML to circumvent the cardiotoxicity induced by anthracyclines.⁶ The limited vector spreading is of great 270 importance to allow optimal leukocytes targeting while reducing systemic toxicity. Altogether, 271 these features strengthen the use of Dd to target leukemic cells compared to others carriers -272 with no selective cell delivery properties - such as liposomes, already evaluated in AML.^{10,11} 273

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

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

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

In conclusion, these results highlight promising features of Dd vector for myeloid and monocytes cell delivery applications as well as for AML therapy-aiming at targeting preferentially blasts and HSPCs. Further studies should now consider a therapeutic form of Dd such as Dd bearing doxorubicin and assess its full potential in AML engrafted mice 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

Leukemic cell lines, PBMCs, bone marrow mononuclear cells and whole blood/bone marrow were incubated with 0.01 to 10 nM Dd-AF647 for up to 7 days at 37°C. Untreated cells or whole blood/bone marrow were used as controls. After incubation, purified cells were treated with 0.5% Trypsin-EDTA (Gibco) during 5 min at 37°C to remove surface-bound Dd. For LSCs vectorization, CD34 enrichment was performed using CD34 MicroBeads kit
 (autoMACS Pro, Miltenyi Biotech).

314 **Cytotoxicity assays**

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

319 **Confocal microscopy**

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

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

untreated cells. For measurement of $\alpha_{V}\beta_{3}$ integrin expression, cells were labeled with anti- $\alpha_{V}\beta_{3}$ -PE monoclonal antibody. Data were acquired on FACS Canto II & FACS LSR II (Beckton Dickinson) and analyzed with FlowJo V10 software.

340 In-vivo biodistribution in blood and bone marrow

The project was authorized by the French ministry of research with the permit number: APAFIS#18922-2019013112124432v4. Twelve 5-weeks old C57Black6 female mice were purchased (Janvier Laboratory) and housed for 1 week before the day of experiment. Mice 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 PBS alone (n = 3). Three mice were anesthetized per time point at 1, 5 and 12h postinjection, 500 μ L of blood were collected and mice were sacrificed for organs and bone marrow sampling.⁴⁹

348 Statistics

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

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,

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.

362 Acknowledgment

363 This work was funded by Société Française de lutte contre les Cancers et les leucémies de l'Enfant et de l'adolescent, La Ligue contre le Cancer 38, Association Grenobloise pour le 364 Développement d'Etudes et de Recherche en Médecine Infantile, Association Espoir, 365 GEFLUC Grenoble Dauphiné-Savoie and IDEX Univ. Grenoble Alpes. The authors thanks 366 Sylvie Berthier of the Institute of Biology and Pathology (Grenoble) for access and technical 367 assistance with flow cytometers, Alexei Grichine and Mylène Pezet of the Institute for 368 369 Advanced Biosciences (Grenoble) for technical assistance with confocal microscopes and 370 flow cytometers, Gautier Szymancki, Claire Vettier, Christine Lefebvre and the technicians of the Laboratory of Cellular Hematology (Grenoble), for providing patient samples, Pascal 371 Fender of the Institut de Biologie Structurale (IBS, Grenoble) for helpful advice and 372 discussions on the adenoviral Dd, Marta Jedynak and Ewa Szolajska of the Institute of 373 374 Biochemistry and Biophysics (Warsaw, Poland) for materials and discussions on the adenoviral Dd, Karine Laulagnier from Remy Sadoul group of the Grenoble Institute of 375 Neuroscience (Grenoble), for access to Nanosight and advice on particle analysis, Daphna 376 Fenel and Guy Schoehn of the electron microscopy (EM) IBS platform (Grenoble). This work 377 used the EM facilities at the Grenoble Instruct-ERIC Center (ISBG; UMS 3518 CNRS CEA-378 UJF-EMBL) with support from the French Infrastructure for Integrated Structural Biology 379 (FRISBI; ANR-10-INSB-05-02) and the Grenoble Alliance for Integrated Structural Cell 380 Biology (GRAL; ANR-10-LABX-49-01) within the Grenoble Partnership for Structural Biology. 381 The IBS EM platform is supported by the Rhône-Alpes Region, the Fonds Feder, the 382 Fondation pour la Recherche Médicale and GIS-IBiSA. 383

385 **References**

- Rasche, M., Zimmermann, M., Borschel, L., Bourquin, J.-P., Dworzak, M., Klingebiel, T.,
 Lehrnbecher, T., Creutzig, U., Klusmann, J.-H., and Reinhardt, D. (2018). Successes and
 challenges in the treatment of pediatric acute myeloid leukemia: a retrospective analysis of the
 AML-BFM trials from 1987 to 2012. Leukemia *32*, 2167–2177.
- Creutzig, U., van den Heuvel-Eibrink, M.M., Gibson, B., Dworzak, M.N., Adachi, S., de Bont, E.,
 Harbott, J., Hasle, H., Johnston, D., Kinoshita, A., et al. (2012). Diagnosis and management of
 acute myeloid leukemia in children and adolescents: recommendations from an international
 expert panel. Blood *120*, 3187–3205.
- Döhner, H., Estey, E.H., Amadori, S., Appelbaum, F.R., Buchner, T., Burnett, A.K., Dombret, H.,
 Fenaux, P., Grimwade, D., Larson, R.A., et al. (2010). Diagnosis and management of acute
 myeloid leukemia in adults: recommendations from an international expert panel, on behalf of
 the European LeukemiaNet. Blood *115*, 453–474.
- Arber, D.A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M.J., Le Beau, M.M., Bloomfield, C.D.,
 Cazzola, M., and Vardiman, J.W. (2016). The 2016 revision to the World Health Organization
 classification of myeloid neoplasms and acute leukemia. Blood *127*, 2391–2405.
- 5. Döhner, H., Weisdorf, D.J., and Bloomfield, C.D. (2015). Acute Myeloid Leukemia. New England
 Journal of Medicine *373*, 1136–1152.
- 403 6. McGowan, J.V., Chung, R., Maulik, A., Piotrowska, I., Walker, J.M., and Yellon, D.M. (2017).
 404 Anthracycline Chemotherapy and Cardiotoxicity. Cardiovasc Drugs Ther *31*, 63–75.
- Leung, W., Hudson, M.M., Strickland, D.K., Phipps, S., Srivastava, D.K., Ribeiro, R.C., Rubnitz,
 J.E., Sandlund, J.T., Kun, L.E., Bowman, L.C., et al. (2000). Late Effects of Treatment in Survivors
 of Childhood Acute Myeloid Leukemia. Journal of Clinical Oncology *18*, 3273–3279.
- 408 8. Briot, T., Roger, E., Thépot, S., and Lagarce, F. (2018). Advances in treatment formulations for 409 acute myeloid leukemia. Drug Discovery Today *23*, 1936–1949.
- 410 9. Torchilin, V.P. (2014). Multifunctional, stimuli-sensitive nanoparticulate systems for drug
 411 delivery. Nature Reviews Drug Discovery *13*, 813–827.
- Krauss, A.C., Gao, X., Li, L., Manning, M.L., Patel, P., Fu, W., Janoria, K.G., Gieser, G., Bateman,
 D.A., Przepiorka, D., et al. (2019). FDA Approval Summary: (Daunorubicin and Cytarabine)
 Liposome for Injection for the Treatment of Adults with High-Risk Acute Myeloid Leukemia. Clin
 Cancer Res 25, 2685–2690.
- 416 11. European public assessment reports (2018). Vyxeos liposomal (previously known as Vyxeos).
 417 European Medicines Agency. https://www.ema.europa.eu/en/medicines/human/EPAR/vyxeos418 liposomal.
- Yoo, J.-W., Irvine, D.J., Discher, D.E., and Mitragotri, S. (2011). Bio-inspired, bioengineered and
 biomimetic drug delivery carriers. Nature Reviews Drug Discovery *10*, 521–535.
- 421 13. Koudelka, K.J., Pitek, A.S., Manchester, M., and Steinmetz, N.F. (2015). Virus-Based
 422 Nanoparticles as Versatile Nanomachines. Annu. Rev. Virol. 2, 379–401.
- Sumida, S.M., Truitt, D.M., Lemckert, A.A.C., Vogels, R., Custers, J.H.H.V., Addo, M.M., Lockman,
 S., Peter, T., Peyerl, F.W., Kishko, M.G., et al. (2005). Neutralizing antibodies to adenovirus
 serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. J.
 Immunol. *174*, 7179–7185.

- Fausther-Bovendo, H., and Kobinger, G.P. (2014). Pre-existing immunity against Ad vectors:
 humoral, cellular, and innate response, what's important? Hum Vaccin Immunother *10*, 2875–
 2884.
- 430 16. Gaggar, A., Shayakhmetov, D.M., and Lieber, A. (2003). CD46 is a cellular receptor for group B
 431 adenoviruses. Nat Med *9*, 1408–1412.
- Wang, H., Li, Z.-Y., Liu, Y., Persson, J., Beyer, I., Möller, T., Koyuncu, D., Drescher, M.R., Strauss,
 R., Zhang, X.-B., et al. (2011). Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11 and
 14. Nat. Med. *17*, 96–104.
- 435 18. Short, J.J., Pereboev, A.V., Kawakami, Y., Vasu, C., Holterman, M.J., and Curiel, D.T. (2004).
 436 Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors.
 437 Virology *322*, 349–359.
- 438 19. Wickham, T.J., Mathias, P., Cheresh, D.A., and Nemerow, G.R. (1993). Integrins alpha v beta 3
 439 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell *73*, 309–
 440 319.
- Cuzange, A., Chroboczek, J., and Jacrot, B. (1994). The penton base of human adenovirus type 3
 has the RGD motif. Gene *146*, 257–259.
- Xiong, J.-P. (2002). Crystal Structure of the Extracellular Segment of Integrin alpha Vbeta 3 in
 Complex with an Arg-Gly-Asp Ligand. Science *296*, 151–155.
- Fender, P., Boussaid, A., Mezin, P., and Chroboczek, J. (2005). Synthesis, cellular localization, and quantification of penton-dodecahedron in serotype 3 adenovirus-infected cells. Virology 340, 167–173.
- 448 23. Fender, P., Ruigrok, R.W.H., Gout, E., Buffet, S., and Chroboczek, J. (1997). Adenovirus 449 dodecahedron, a new vector for human gene transfer. Nature Biotechnology *15*, 52–56.
- 450 24. Szolajska, E., Burmeister, W.P., Zochowska, M., Nerlo, B., Andreev, I., Schoehn, G., Andrieu, J.451 P., Fender, P., Naskalska, A., Zubieta, C., et al. (2012). The Structural Basis for the Integrity of
 452 Adenovirus Ad3 Dodecahedron. PLoS ONE *7*, e46075.
- 453 25. Fender, P., Schoehn, G., Perron-Sierra, F., Tucker, G.C., and Lortat-Jacob, H. (2008). Adenovirus
 454 dodecahedron cell attachment and entry are mediated by heparan sulfate and integrins and
 455 vary along the cell cycle. Virology *371*, 155–164.
- Garcel, A., Gout, E., Timmins, J., Chroboczek, J., and Fender, P. (2006). Protein transduction into
 human cells by adenovirus dodecahedron using WW domains as universal adaptors. The
 Journal of Gene Medicine *8*, 524–531.
- 459 27. Sumarheni, Gallet, B., and Fender, P. (2016). The Use of Adenovirus Dodecahedron in the
 460 Delivery of an Enzymatic Activity in the Cell. Biotechnology Research International 2016, 1–11.
- Zochowska, M., Paca, A., Schoehn, G., Andrieu, J.-P., Chroboczek, J., Dublet, B., and Szolajska, E.
 (2009). Adenovirus Dodecahedron, as a Drug Delivery Vector. PLoS ONE *4*, e5569.
- 29. Zochowska, M., Piguet, A.-C., Jemielity, J., Kowalska, J., Szolajska, E., Dufour, J.-F., and
 Chroboczek, J. (2015). Virus-like particle-mediated intracellular delivery of mRNA cap analog
 with in vivo activity against hepatocellular carcinoma. Nanomedicine: Nanotechnology, Biology
 and Medicine *11*, 67–76.
- Jedynak, M., Laurin, D., Dolega, P., Podsiadla-Bialoskorska, M., Szurgot, I., Chroboczek, J., and
 Szolajska, E. (2018). Leukocytes and drug-resistant cancer cells are targets for intracellular
 delivery by adenoviral dodecahedron. Nanomedicine: Nanotechnology, Biology and Medicine
 14, 1853–1865.

- 471 31. Araki, N. (1996). A role for phosphoinositide 3-kinase in the completion of macropinocytosis
 472 and phagocytosis by macrophages. The Journal of Cell Biology *135*, 1249–1260.
- 473 32. Nabi, I.R., and Le, P.U. (2003). Caveolae/raft-dependent endocytosis. The Journal of Cell Biology
 474 161, 673–677.
- Wang, L.H. (1993). Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch
 for coated pit formation. The Journal of Cell Biology *123*, 1107–1117.
- 477 34. Vivès, R.R., Lortat-Jacob, H., Chroboczek, J., and Fender, P. (2004). Heparan sulfate
 478 proteoglycan mediates the selective attachment and internalization of serotype 3 human
 479 adenovirus dodecahedron. Virology *321*, 332–340.
- 480 35. Goardon, N., Marchi, E., Atzberger, A., Quek, L., Schuh, A., Soneji, S., Woll, P., Mead, A., Alford,
 481 K.A., Rout, R., et al. (2011). Coexistence of LMPP-like and GMP-like Leukemia Stem Cells in
 482 Acute Myeloid Leukemia. Cancer Cell *19*, 138–152.
- 483 36. Thomas, D., and Majeti, R. (2017). Biology and relevance of human acute myeloid leukemia 484 stem cells. Blood *129*, 1577–1585.
- Serangeli, C., Bicanic, O., Scheible, M.H., Wernet, D., Lang, P., Rammensee, H.-G., Stevanovic, S.,
 Handgretinger, R., and Feuchtinger, T. (2010). Ex vivo detection of adenovirus specific CD4+ Tcell responses to HLA-DR-epitopes of the Hexon protein show a contracted specificity of
 T(HELPER) cells following stem cell transplantation. Virology *397*, 277–284.
- 489 38. Leen, A.M., Christin, A., Khalil, M., Weiss, H., Gee, A.P., Brenner, M.K., Heslop, H.E., Rooney,
 490 C.M., and Bollard, C.M. (2008). Identification of hexon-specific CD4 and CD8 T-cell epitopes for
 491 vaccine and immunotherapy. J. Virol. *82*, 546–554.
- Amstutz, B., Gastaldelli, M., Kälin, S., Imelli, N., Boucke, K., Wandeler, E., Mercer, J., Hemmi, S.,
 and Greber, U.F. (2008). Subversion of CtBP1-controlled macropinocytosis by human
 adenovirus serotype 3. EMBO J 27, 956–969.
- 495 40. Weerasinghe, D., McHugh, K.P., Ross, F.P., Brown, E.J., Gisler, R.H., and Imhof, B.A. (1998). A 496 role for the alphavbeta3 integrin in the transmigration of monocytes. J. Cell Biol. *142*, 595–607.
- 41. Brilha, S., Wysoczanski, R., Whittington, A.M., Friedland, J.S., and Porter, J.C. (2017). Monocyte
 Adhesion, Migration, and Extracellular Matrix Breakdown Are Regulated by Integrin αVβ3 in
 Mycobacterium tuberculosis Infection. J.I. *199*, 982–991.
- 42. Al-Asadi, M.G., Brindle, G., Castellanos, M., May, S.T., Mills, K.I., Russell, N.H., Seedhouse, C.H.,
 and Pallis, M. (2017). A molecular signature of dormancy in CD34+CD38- acute myeloid
 leukaemia cells. Oncotarget *8*, 111405–111418.
- 43. Miller, P.G., Al-Shahrour, F., Hartwell, K.A., Chu, L.P., Järås, M., Puram, R.V., Puissant, A.,
 Callahan, K.P., Ashton, J., McConkey, M.E., et al. (2013). In Vivo RNAi Screening Identifies a
 Leukemia-Specific Dependence on Integrin Beta 3 Signaling. Cancer Cell 24, 45–58.
- Johansen, S., Brenner, A., Bartaula-Brevik, S., Reikvam, H., and Bruserud, Ø. (2018). The
 Possible Importance of β3 Integrins for Leukemogenesis and Chemoresistance in Acute Myeloid
 Leukemia. IJMS *19*, 251.
- 50945.Umemoto, T., Matsuzaki, Y., Shiratsuchi, Y., Hashimoto, M., Yoshimoto, T., Nakamura-Ishizu, A.,510Petrich, B., Yamato, M., and Suda, T. (2017). Integrin $\alpha\nu\beta$ 3 enhances the suppressive effect of511interferon- γ on hematopoietic stem cells. EMBO J. *36*, 2390–2403.
- 46. Kräter, M., Jacobi, A., Otto, O., Tietze, S., Müller, K., Poitz, D.M., Palm, S., Zinna, V.M., Biehain,
 U., Wobus, M., et al. (2017). Bone marrow niche-mimetics modulate HSPC function via integrin
 signaling. Sci Rep 7, 2549.

- 47. Wang, J., Liu, Z., Zhang, S., Wang, X., Bai, H., Xie, M., Dong, F., and Ema, H. (2019). Lineage marker expression on mouse hematopoietic stem cells. Experimental Hematology *76*, 13-23.e2.
- 517 48. Zhang, J., Gu, Y., and Chen, B. (2019). Mechanisms of drug resistance in acute myeloid 518 leukemia. OTT *Volume 12*, 1937–1945.
- 49. Liu, X., and Quan, N. (2015). Immune Cell Isolation from Mouse Femur Bone Marrow. BioProtoc 5.

522 Figures Legends

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

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

Figure 2. The vectorization is an energy-dependent phenomenon, involving clathrin-coated pit-mediated endocytosis and strongly correlating with $\alpha_{\nu}\beta_{3}$ integrin highly expressing cells.

Leukemic cell lines were either pre-incubated at 4°C or with inhibitor drugs at 37°C in 547 complete medium during 1 h before incubation with 1 nM Dd-AF647 during 1 h. Control cells 548 549 were directly exposed to Dd without inhibitors at 37°C and untreated cells were used as negative control. Measures were done in triplicates for each cell lines and the mean ± SD is 550 indicated. P-values were determined by one-way ANOVA. ns, not significant; *, P ≤.05; **, P 551 $\leq .01$; ***, $P \leq .001$; ****, P < .0001. (A) Effect of low temperature (4°C) and cellular ATP 552 553 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 Dd-AF647 (MFI). (C) Scatterplot showing linear correlation between Dd-AF647 MFI and $\alpha_{V}\beta_{3}$ 555 integrin MFI. The 7 cell lines were treated with 1 nM Dd-AF647 during 1 h at 37°C (without 556 inhibitors) and then labeled with anti- $\alpha_{V}\beta_{3}$ -PE monoclonal antibody. Pearson r coefficient of 557 correlation and P-value (two-tailed) are indicated. 558

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 from AML patients (E) were incubated with Dd-AF647 during 2h. Cells positive (%) for Dd-563 AF647 (A, C left panel; B, E in blue). Fold increase in Dd-AF647 MFI (A, C right panel; B in 564 red; D) or Dd-AF647 MFI (E in red). Results represent the mean ± SD and the corresponding 565 566 number of samples is indicated. P-values were determined by Student t-tests (B) or two-way ANOVA (A, C, D). ns, not significant; *, *P* ≤.05; **, *P* ≤.01; ***, *P* ≤.001; ****, *P* <.0001. (**A**) Dd 567 penetration within subpopulations of healthy donor PBMCs. Min/Max values (external lines) 568 are also indicated. Cells were identified as follow: CD45+ CD66b+ for remaining 569 570 granulocytes after PBMCs purification (blue); CD45+ CD3+ for T lymphocytes (red); CD45+ CD3- CD19- CD56+ for NK lymphocytes (purple); CD45+ CD19+ for B lymphocytes (green) 571 and CD45+ CD33+ CD14+ for monocytes (orange). (B) Vectorization of SSC^{low} CD45^{low} 572 blasts from acute lymphoblastic leukemia (ALL, n = 8) versus acute myeloblastic leukemia 573

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

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

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

612 **Tables**

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)

	PEDIATRIC (n, %)	ADULT (n, %)
AML at		I
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 CLASSFICIATION		
AML with recurrent genetic abnormalities	2 (50)	7 (33)
flt3	1 (25)	3 (14)
npm1	0	2 (10)
idh1/2	0	2 (10)
Others	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)

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