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Human acute myeloid leukemia blast-derived exosomes in patient-derived xenograft mice mediate immune suppression

Chang-Sook Hong[a], Gwenn Danet-Desnoyers[b], Xiaochuan Shan[b], Priyanka Sharma[a], Theresa L. Whiteside[a,1], and Michael Boyiadzis[a,1]

[a]*University of Pittsburgh School of Medicine, UPMC Hillman Cancer Center, Pittsburgh, Pennsylvania;* [b]*Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*

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Exosomes are virus-size membrane-bound vesicles of endocytic origin present in all body fluids. Plasma of AML patients is significantly enriched in exosomes, which carry a cargo of immunosuppressive molecules and deliver them to recipient immune cells, suppressing their functions. However, whether these exosomes originate from leukemic blasts or from various normal cells in the bone marrow or other tissues is unknown. In the current study, we developed an AML PDX model in mice and studied the molecular cargo and immune cell effects of the AML PDX exosomes in parallel with the exosomes from plasma of the corresponding AML patients. Fully engrafted AML PDX mice produced exosomes with characteristics similar to those of exosomes isolated from plasma of the AML patients who had donated the cells for engraftment. The engrafted leukemic cells produced exosomes that carried human proteins and leukemia-associated antigens, confirming the human origin of these exosomes. Furthermore, the AML-derived exosomes carried immunosuppressive proteins responsible for immune cell dysfunctions. Our studies of exosomes in AML PDX mice serve as a proof of concept that AML blasts are the source of immunosuppressive exosomes with a molecular profile that mimics the content and functions of the parental cells.

Immune cell dysfunctions are a common feature of acute myeloid leukemia (AML). We and others have reported that AML patients have low natural killer (NK) cell activity, elevated levels of circulating regulatory T (Treg) cells, high circulating levels of transforming growth factor β (TGF-β) and alterations in a profile of other endogenous cytokines [1–4]. Whether the immune aberrations seen in AML are a result of the profound cytopenia these patients experience or are manifestations of dysfunctions in remaining immune cells remains unclear. Studies in mouse cancer models suggest that the immune cells differentiating in the tumor microenvironment acquire an inefficient effector phenotype, allowing for immune escape of the tumor [5,6]. Recently, it was discovered that tumor-driven reprogramming of immune cells is mediated by small extracellular vesicles (EVs) [7]. EVs are present in all body fluids and are a heterogeneous collection of differently sized vesicles with distinct cellular origins. Exosomes are one of the smallest (30–150 nm) subsets of EVs [8]. They differ from larger microvesicles and apoptotic bodies not only because of the smaller size but also because of their unique origin in the endocytic compartment of parent cells. Consequently, exosomes carry endocytic markers, and their molecular cargo mimics that of their parent cells.

The exosome biogenesis begins with early and then late endosomes that fuse, forming multivesicular bodies (MVBs) [9]. Inward budding of the MVB membrane leads to the formation of intraluminal vesicles which,

Offprint requests to: Michael Boyiadzis, Division of Hematology and Oncology, University of Pittsburgh School of Medicine, UPMC Hillman Cancer Center, 5150 Center Avenue, Suite 564, Pittsburgh, PA 15232; E-mail: boyiadzism@upmc.edu

[1]TLW and MB contributed equally to this work.

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in the process of invagination, enclose cytoplasmic components. Fusion of MVBs with the parent cell membrane leads to the release of exosomes into extracellular space. Exosomes circulate freely in all body fluids, delivering messages to circulating and/or tissue-bound recipient cells and reprogramming their activities. Exosome plasma levels are increased relative to those of healthy donors in plasma of patients with AML [10,11]. These plasma-derived exosomes co-express leukemia-associated antigens (LAAs), such as CD123, CLL-1, and CD96; myeloid markers; and immunosuppressive proteins, including TGF-β and ligands such as PD-L1 or FasL [10,11]. Ex vivo functional studies with normal human immune cells co-incubated with tumor-derived exosomes have confirmed the capability of exosomes to mediate immune suppression. Exosomes produced by tumor cells exert direct or indirect effects on immune cells. They interfere with normal differentiation of immune cells, induce apoptosis of activated effector T cells, polarize immune cells to tumor-promoting phenotypes, and regulate mobilization of immune cells to the tumor [12–14]. Indirectly, they expand proliferation of Treg and myeloid-derived suppressor cells and upregulate suppressor activity of these cells, thus contributing to tumor-induced immune suppression and the tumor immune escape [12–14]. Their effects are mediated by interaction of ligand-carrying exosomes with surface receptors on immune cells and internalization of exosomes by target cells and release of their content. In NK cells, downregulation in expression of the activating receptors, especially NKG2D, is induced by exosomes carrying MICA and MICB ligands. NK-cell activation and cytotoxicity is inhibited by TGF-β [11]. Tumor-derived exosomes, which are able to make adenosine from ATP by virtue of carrying CD39 and CD73, are implicated in inducing suppressive activity in activated B cells. Co-incubation of T cells with tumor-derived exosomes results in downregulation of the TcR ζ chain [15]. Additionally, exosomes reduce phosphorylation in activated T cells and JAK expression, which is essential for interleukin (IL-2), IL-7, and IL-15 function [12–14].

Furthermore, studies using AML mouse models have reported that exosomes released from leukemia blasts carry leukemia-related miRNAs that suppress hematopoietic progenitor cell functions directly via the delivery of these miRNAs to hematopoietic progenitor cells or indirectly through reprogramming of stromal cells to produce niche-retention factors preventing progenitor cell evolution [16–18].

The origin of exosomes found in the plasma of patients with AML has not been confirmed. Whether these exosomes originate from leukemic blasts or from various normal cells in the bone marrow or other tissues is unknown. The major barrier has been the lack of strategies for the isolation from AML plasma of blast-derived vesicles versus exosomes derived from other, normal cells. So far, no specific markers have been identified on blast-derived exosomes that would differentiate them from exosomes produced by nontumor cells. Without the capability to separate blast-derived exosomes from other vesicles in patients' plasma, the real source of circulating vesicles remains unclear [19].

To address this confounding situation, we developed an AML PDX model in mice and studied the molecular cargo and immune cell effects of the AML PDX exosomes in parallel with the exosomes isolated from plasma of the corresponding AML patients.

Methods

Patients and cells

Leukapheresis was performed in five patients with AML (2 newly diagnosed AML, 1 refractory AML, 2 relapsed AML). The patients' characteristics are summarized in Supplementary Table E1 (online only, available at www.exphem.org). The collected cells following leukapheresis were diluted to 100 million/mL, red blood cells were lysed using ammonium chloride, and cells were frozen in 7% dimethyl sulfoxide (DMSO), 2% bovine serum albumin (BSA), and 2% Hetastarch. AML sample collection, banking, and distribution were conducted according to institutional guidelines (IRB Protocol No. 703185). All patients signed an informed consent form allowing for the use of their cells for research.

Generation of AML PDX mice

To generate primary AML PDX mice, each NOD-SCID-IL-2/Rγcnull (NSG) mouse was conditioned with 30 mg/kg Busulfan administered as intraperitoneal injections 24 hours before intravenous delivery of 5 million T cell-depleted mononuclear cells obtained from an AML patient, as previously described [20]. Femoral bone marrow aspirates were collected 6 weeks post-AML injection and every 4 weeks thereafter and analyzed by flow cytometry to follow engraftment by determining the percentage of human leukemic blasts ($huCD45^+CD3^-$). Mice engrafted with human leukemic blasts in the bone marrow were exsanguinated, and the molecular content and functions of exosomes isolated from plasma were determined.

Exosome isolation from plasma by mini size-exclusion chromatography

Exosomes were isolated from thawed, precleared plasma samples using size-exclusion chromatography (SEC) on Sepharose 2B columns as previously described by us [21]. The exosomes eluting in fraction 4 were harvested, and their protein content; nanoparticle numbers, size, and morphology; and molecular profiles were determined. Isolation of exosomes from samples of human and murine plasma were handled in the same way.

Protein determination

Protein content and concentration of the isolated exosome fractions were determined using the Pierce BCA protein

assay kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions. The protein concentrations were calculated as micrograms of protein per 1 mL of precleared plasma that was loaded onto each mini-SEC column.

Exosome size and concentration assessment by tunable resistive pulse sensing

Size ranges and concentrations of isolated exosome fractions were measured using tunable resistive pulse sensing (TRPS) as recommended by the system manufacturer Izon (Cambridge, MA). The measurement conditions and details were described by us earlier [21,22].

Transmission electron microscopy

Transmission electron microscopy was performed as previously described at the Center for Biologic Imaging at the University of Pittsburgh. Exosomes were visualized with a JEOL JEM-1011 transmission electron microscope [21,22].

Western blots

In preparation for Western blots, exosome fraction 4 was concentrated by centrifugation on a 100K Amicon Ultra 0.5-mL centrifugal filter (EMD Millipore, Billerica, MA) at 5,000*g*. Western blots were performed as previously described [10]. Polyvinyl difluoride (PVDF) membranes were incubated overnight at 4°C with antibodies (Abs) purchased from various vendors. Exosomes were tested for the presence of exosome markers, including TSG101, leukemia blasts markers, and LAAs as previously described [10,23]. All Abs used for the detection of antigens carried by exosomes isolated from AML patient plasma or from plasma of AML PDX mice were specific for human proteins. The following Abs were used: anti-CD123 (1:1,000, AF841), anti-CLL-1 (1:2,000, AF2946), anti-PD-1 (1:125, MAB1086), anti-TGFb1 (1:200, AF-246), from R&D Systems; anti- CD33 (1:500, WM53), and anti-TSG101 (1:500, PA5-31260) from ThermoFisher; anti-FasL (1:1,000, 4273) from Cell Signaling; anti-CD34 (1:2,000, 81289), anti-Fas (1:1,000, 133619), anti- Ku80 (1:600, 87860) from Abcam.

NKG2D expression in NK cells by AML exosomes

NK cells were isolated from normal human PBMCs using AutoMACS (Miltenyi Biotec, Auburn, CA), as previously described, with >95% cell purity [21,22]. $CD56^+/CD3^-$ NK cells were activated with IL-2 (200 IU/mL) for 24 hours. Exosomes isolated from plasma of the AML patients whose cells were used for injections into mice or exosomes isolated from plasma of the PDX mice (10 μg) were added to wells of a 96-well plate, each well containing 200×10^3 NK cells. The cells were co-incubated with the exosomes for 24 hours at 37°C. Cultures without exosomes served as controls. The expression of NKG2D was assessed by staining cells with phycoerythrin (PE)-conjugated anti-NKG2D antibody or isotype controls (Beckman Coulter, Atlanta, GA) and flow cytometry analysis. The data were presented as mean fluorescence intensity values (MFI). Due to the small amount of plasma obtained from the AML PDX mice and the total exosomes required for the experiments, the NK cell assays were performed only once with exosomes of each patient.

Apoptosis induction in $CD8^+$ T cells by AML exosomes

$CD8^+$ T cells were isolated from normal human PBMCs using AutoMACS (Miltenyi Biotec), as previously described, with >95% cell purity [21]. Following T-cell isolation from PBMCs, $CD8^+$ T cells were activated with IL-2 (150 IU/mL) and CD3/CD28 T-cell activator (25 μL/mL, Stemcell, Vancouver, BC, Canada) in RPMI for 6 hours. Briefly, 200,000 cells per well were plated in a 96-well plate in an exosome-depleted RPMI medium. After stimulation, exosomes isolated from plasma of the AML patients whose cells were used for injections into mice or exosomes isolated from plasma of the AML PDX mice (10 μg) were added to the cells and co-incubated for 12 hours. Co-cultures containing no exosomes served as controls. Apoptosis of $CD8^+$ T cells was measured by flow cytometry (Beckman Coulter) using an Annexin V assay as previously described [21,22]. Because of the small amount of plasma obtained from the AML/PDX mice and the total exosomes required for the experiments, the T-cell assays were performed only once with exosomes of each patient.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 6, San Diego, CA). Flow analyses were performed with Kaluza (Version 1.5, Beckman Coulter).

Results

Engraftment of human AML cells in immunodeficient mice

T-cell depleted mononuclear cells obtained from patients with AML ($n = 5$) were injected IV into NOD-SCID-IL-2/Rγc null (NSG) recipient mice. All 5 mice developed AML within 9–14 weeks of engraftment and exhibited splenomegaly. The range of AML engraftment levels observed in the experiments was consistent with the diversity of engraftment levels we previously reported [20]. Supplementary Table E2 (online only, available at www.exphem.org) provides a summary of the tumor burden in mouse bone marrow at the time of sacrifice, and Supplementary Figure E1 (online only, available at www.exphem.org) provides the flow cytometry gating strategy illustrating engraftment.

Exosomes from plasma of the AML patients who donated cells for PDX generation

We first evaluated the exosomes isolated from plasma of the AML patients whose PBMCs were used to generate the AML PDX mice. Abs specific for human proteins were used to establish the exosome profiles by Western blots. As illustrated in Figure 1A, these profiles were qualitatively or quantitatively distinct for each AML patient. All exosomes carried the LAAs and several immunoinhibitory molecules previously reported by us to be present on AML exosomes [10,11,23] All exosomes carried TSG101 protein, confirming their endocytic origin.

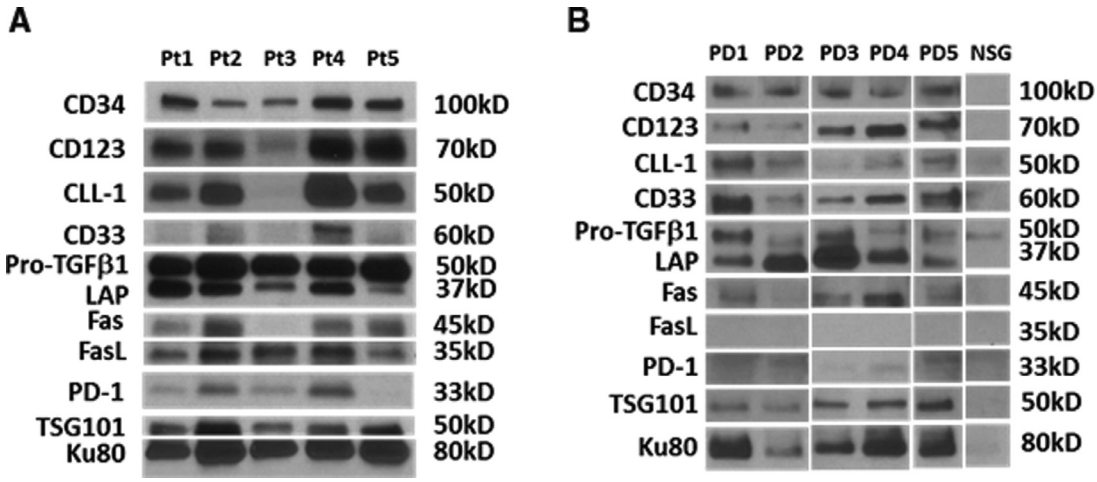

Figure 1. **(A)** Western blots of exosomes isolated from the plasma of five AML patients whose mononuclear cells were individually injected into five immunodeficient mice. **(B)** Western blots of exosomes isolated from plasma of fully engrafted immunodeficient mice. All antibodies used for antigen detection were specific for human proteins. In the right lane of (B), exosomes isolated from the control, non-injected NSG mouse were blotted with antibodies specific for human proteins.

Exosomes isolated from plasma of AML PDX mice after engraftment

Exosomes were isolated from plasma of the AML PDX mice after engraftment using miniSEC and studied in parallel with the exosomes derived from plasma of the corresponding AML patient. Transmission electron microscopy of exosomes isolated from plasma of the AML PDX mice revealed the presence of vesicles sized at 30–150 nm (Figure 2) that were similar to vesicles present in plasma of AML patients, as previously reported [16].

Antibodies used for Western blots were specific for *human* proteins. Exosomes isolated from plasma of the AML PDX mice contained LAAs and inhibitory molecules similar to the exosomes isolated from plasma of the corresponding AML patients (Figure 1B). All exosomes were Ku80+, confirming their *human* origin. Ku80 is a helicase used to detect engraftment, migration, and differentiation of human cells after transplantation into mice and rats [24]. As leukemic blasts are the only human cells in the PDX mice, these data provide evidence that exosomes circulating in AML PDX mice are derived from human blasts.

Exosomes from AML PDX mice reduce NKG2D expression on human activated NK cells

We have previously reported downregulation of the NK cell-activating receptor, NKG2D, upon co-incubation of activated normal human NK cells with AML exosomes

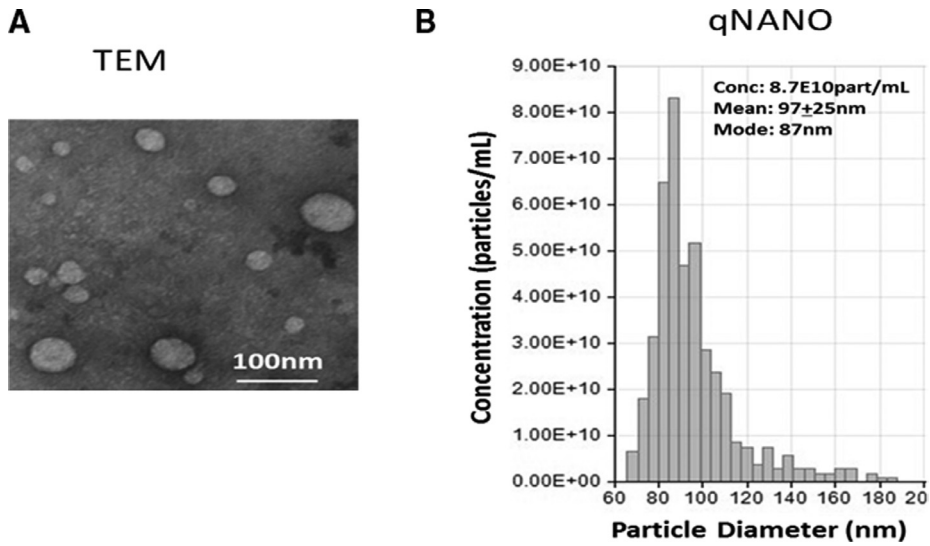

Figure 2. Characteristics of exosomes isolated from plasma of AML-PDX mice. **(A)** Transmission electron microscopy (TEM) of the isolated exosomes. **(B)** Size and concentration of the exosomes as determined by tunable resistive sensing using q-Nano.

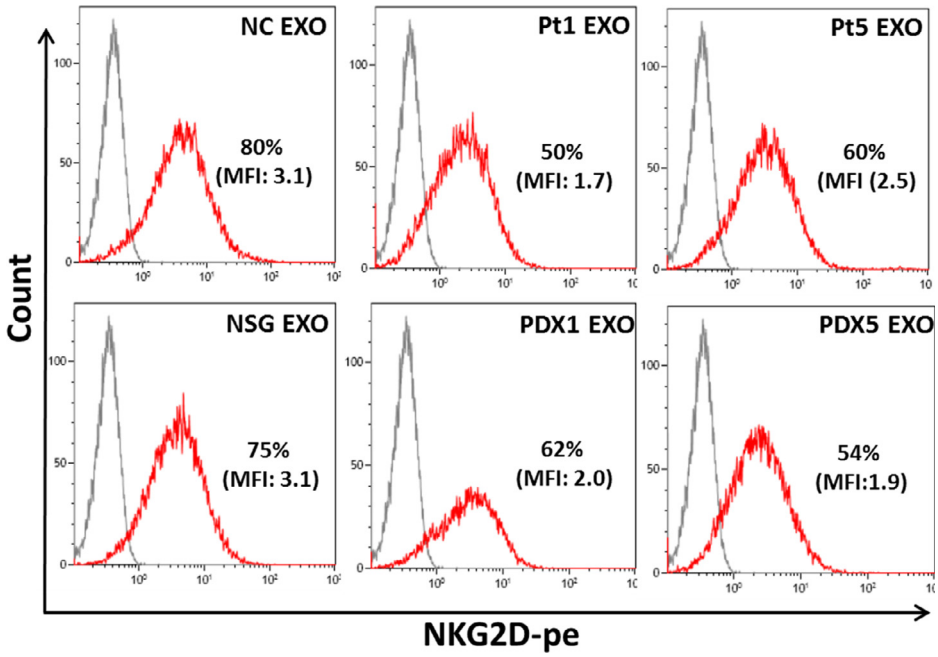

Figure 3. Downregulation of the NKG2D receptor expression level in normal human NK cells co-incubated with AML exosomes. Flow cytometry illustrates downregulation of NKG2D on NK cells co-incubated with plasma exosomes isolated from AML-PDX (PDX1, PDX5 EXO) mice or exosomes isolated from the plasma of the corresponding AML patients (Pt1, Pt5 EXO). Exosomes isolated from plasma of normal control (NC EXO) or non-injected NSG mouse (NSG EXO) were used as controls.

[10,11]. As illustrated in Figure 3, exosomes isolated from plasma of AML PDX mice also induced significant downregulation of NKG2D expression levels in normal human NK cells.

Exosomes from AML PDX mice induce apoptosis in human activated T cells

Co-incubation of the exosomes isolated from plasma of AML PDX mice with activated human $CD8^+$ T cells resulted in apoptosis of these targeted cells, similar to results previously reported for exosomes derived from AML plasma [22]. Apoptosis levels were similar to those induced by co-incubation of T cells with exosomes from plasma of the corresponding patients who donated cells for PDX generation (Figure 4). No apoptosis was observed in human $CD8^+$ T cells co-incubated with exosomes isolated from control NSG mice.

Discussion

In AML, the presence of several different immunosuppressive mechanisms results in immune cell deregulation and escape of leukemia from immune control. These mechanisms include downregulation of major histocompatibility complex (MHC) class I and class II expression, extensive consumption of amino acids essential for T-cell growth through activities of arginase-2 (ARG2) or indoleamine 2,3-dioxygenase-1 (IDO1), expansion of Treg cells with increased suppressor functions, and upregulation in expression of negative checkpoint molecules [25–27]. We have previously reported downregulation of the NK cell-activating receptor, NKG2D, and a concomitant decrease in NK-cell cytotoxicity upon co-incubation of activated normal human NK cells with AML exosomes [11]. Exosomes isolated from plasma of AML PDX mice also induced significant downregulation of NKG2D expression levels in normal human NK cells. Co-incubation of the exosomes isolated from plasma of AML PDX mice with activated human $CD8^+$ T cells resulted in apoptosis of these cells, similar to results previously reported for exosomes derived from AML plasma [22]. Apoptosis levels were similar to those induced by co-incubation of T cells with exosomes from plasma of the corresponding patients who donated cells for PDX generation. As human AML cells were the only source of human exosomes in these mice, we concluded that AML-derived exosomes carried an immunosuppressive cargo of ligands able to activate complementary receptors on human NK cells or $CD8^+$ T cells, leading to their dysfunction. These data confirm the ability of AML blast-derived exosomes to mediate immune cell suppression.

Several investigators have recently developed in vivo AML mouse models using human primary AML blasts to study the effects of exosome secretion on hematopoiesis and leukemia development [17,28,29]. These studies have found that exosomes released from leukemia blasts carried leukemia-related miRNAs that suppressed hematopoietic progenitor cell functions directly via the

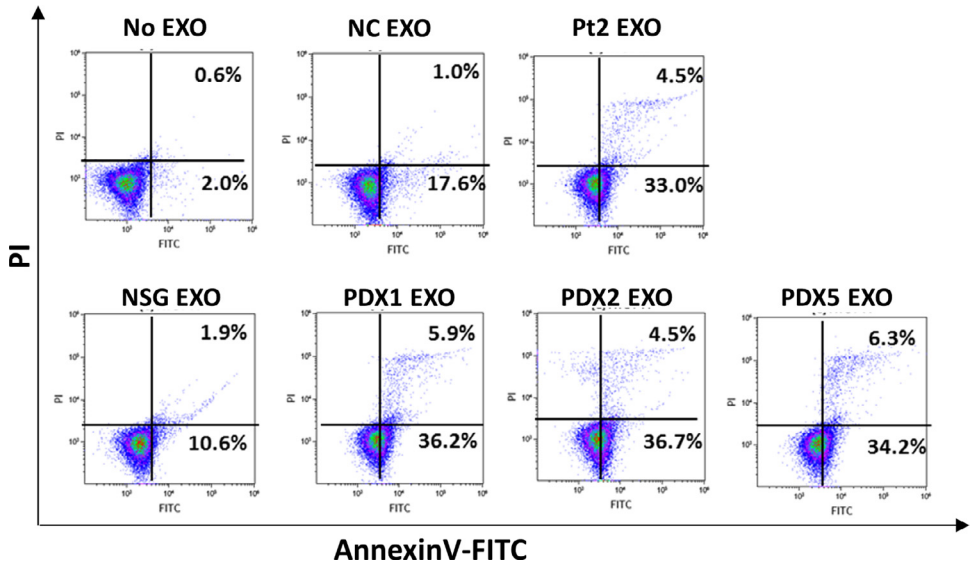

Figure 4. Exosomes isolated from plasma of AML-PDX mice induce apoptosis in human activated $CD8^+$ T cells. Flow cytometry reveals apoptosis of $CD8^+$ T cells upon co-incubation with exosomes isolated from the plasma of AML/PDX mice (PDX1, PDX2, PDX5 EXO) or with exosomes isolated from the plasma of an AML patient (Pt2 EXO). No significant apoptosis was observed with exosomes of normal control (NC EXO) or with NSG mouse exosomes (NSG EXO).

delivery of these miRNAs to hematopoietic progenitor cells or indirectly through reprogramming of stromal cells to produce niche-retention factors preventing progenitor cell evolution. Thus, exosomes secreted by AML blasts were implicated in remodeling of the bone marrow niche into a leukemia growth-permissive microenvironment. However, whether these effects are due solely to blast-derived exosomes or to additional involvement of exosomes produced by reprogrammed tissue cells has not been determined.

The dearth of markers typifying AML blasts has been the major barrier in identifying AML-derived exosomes [19]. Most of the AML-associated antigens are overexpressed on leukemia blasts and are also expressed, albeit weakly, in a variety of normal cells [30]. This means that the presence of LAAs on exosomes does not verify their origin from leukemic blasts. Bearing this in mind, we sought to confirm the blast origin of AML exosomes in patients' plasma. AML blasts were implanted in immunodeficient mice and engrafted and became the only source of AML blast-derived exosomes in PDX mice. In fact, fully engrafted AML PDX mice produced exosomes that had characteristics similar to those of exosomes isolated from plasma of the AML patients who had donated the cells for engraftment. The engrafted leukemic cells produced exosomes that carried human proteins detectable with anti-human Abs, confirming the human origin of these exosomes. Although it is possible that exosomes in mouse plasma are of mouse origin and carry recycled human proteins, Western blots of these exosomes in Figure 1B reveal unique expression levels for every protein in each mouse. This does not seem to be consistent with nonspecific protein adsorption of human proteins, including LAAs, to the exosome surface. Also, the median exosome level in the NSG mice was 32 μg protein/mL, whereas in the AML PDX mice, it was 73 μg protein/mL, suggesting that leukemia blasts present in the AML PDX mice contribute to the elevated exosome levels, similar to the increased exosome levels of newly diagnosed AML patients. Hence, exosomes of human origin could account for the observed increase. The exosomes were isolated from the plasma of the AML PDX mice using size-exclusion chromatography on Sepharose 2B columns. The isolated total exosomes are a mix of blast-derived and normal mice cell-derived exosomes present in plasma of the AML PDX mice. In the experiments performed, exosomes isolated from plasma of NSG mice did not affect immune cell functions, whereas the exosomes isolated from plasma of AML PDX mice induced changes in immune cell functions. This suggests that blast-derived exosomes and not normal cell-derived (i.e., mouse) exosomes are responsible for the observed immune cell effects. However, the possibility that human blast-derived exosomes could reprogram the mouse bone marrow microenvironment and modify immune functions of the mouse exosomes will also need to be investigated.

Our data also suggest that AML PDX mice can be used as a source of human leukemia-derived exosomes, which appear to faithfully reproduce the phenotypic and functional characteristics of the original human leukemic blasts. It will be possible in the future to use these leukemic exosomes in AML PDX mice for in vivo studies to explore silencing of immunosuppressive exosomes with drugs or their removal to enhance responses of mice to experimental immune therapies.

Our studies demonstrated that fully engrafted AML PDX mice produced exosomes with characteristics that mimic those of exosomes in the plasma of AML patients who had donated cells for engraftment. The engrafted leukemic cells produced exosomes that carried human proteins and LAAs, confirming the exosome origin in human leukemic blasts. Furthermore, AML-derived exosomes carried immunosuppressive proteins responsible for immune cell dysfunction. Our studies of exosomes in AML PDX mice serve as a proof of concept that AML blasts are the source of immunosuppressive exosomes with a molecular profile that mimics the content and functions of the parental cells.

Acknowledgments

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Conflict of interest disclosure

The authors declare that they have no competing interests.

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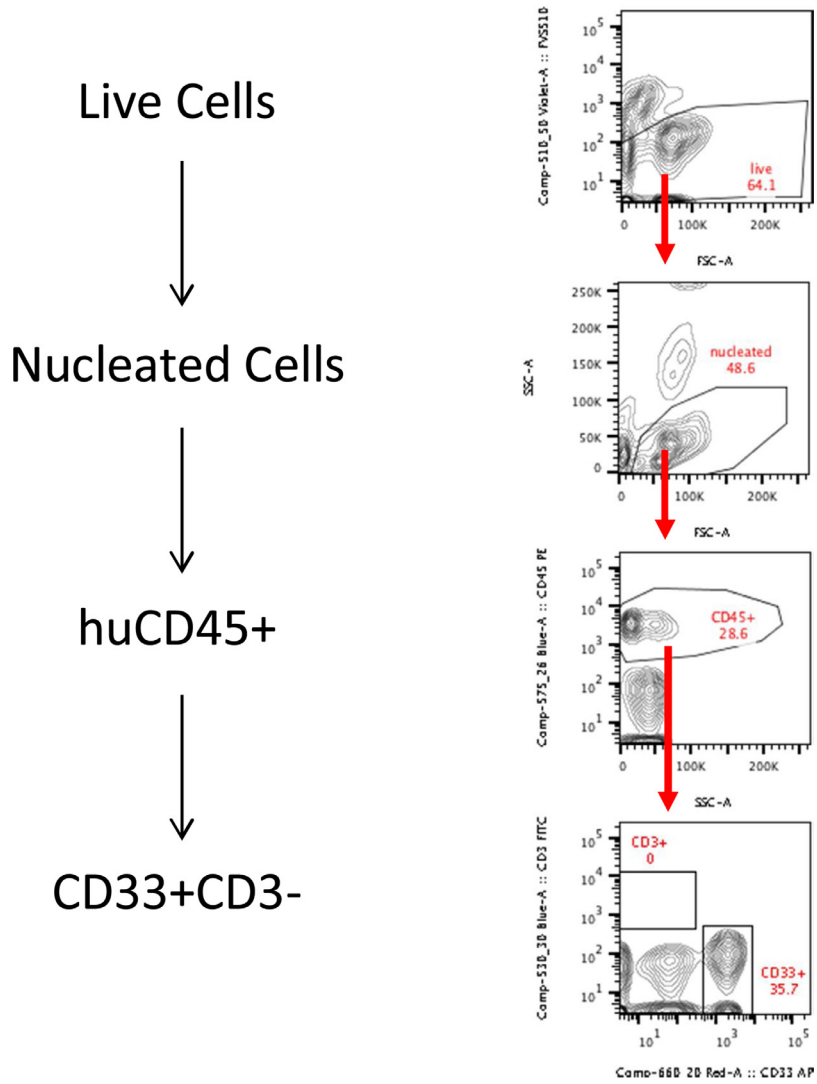

Supplementary Figure E1.

Supplementary Table E1. Patients' characteristics.

Pt	Age (y)	Sex	AML	Disease status	WBC	Blasts %	Cytogenetics	FLT3 status
1	66	M	AML-Multilineage dysplasia with prior MDS	Refractory	161	89	Normal	Wildtype
2	64	M	AML-Multilineage dysplasia, no prior MDS	Refractory	130	90	47,XY,+8[2]/46,XY, t(3;17)(q21;q25)[2]/46, XY[16]	ITD
3	64	M	Myelomonocytic	De Novo	241	74	Normal	Wildtype
4	59	F	Myelomonocytic	Relapsed	46	98	NA	NA
5	55	M	Myelomonocytic	De Novo	180	89	Normal	ITD

F, female; M, male; FLT3, FMS-like tyrosine kinase-3; ITD, internal tandem duplication
Pt, patient; WBC, white blood cell; NA, not available; MDS, Myelodysplastic Syndrome

Supplementary Table E2.

Primary AML Engraftment in Mouse BM									
AML3965/Pt1 (12 wk)		AML3252/Pt2 (9 wk)		AML3221/Pt3 (12 wk)		AML3370/Pt4 (11 wk)		AML3954/Pt5 (14 wk)	
Mouse #	$CD45^+CD3^-$ (%)	Mouse #	$CD45^+CD3^-$ (%)	Mouse #	$CD45^+CD3^-$ (%)	Mouse #	$CD45^+CD3^-$ (%)	Mouse #	$CD45^+CD3^-$ (%)
0645	2.31	0642	43.16	0641	7.20	0643	24.50	0649	21.30
0650	1.52	0647	72.09	0642	9.15	0648	9.67	0684	27.00
0685	0.82	0682	70.19	0681	10.60	0683	9.04	0664	27.70
0665	2.15	0652	53.59	0651	4.28	0653	0.23	0674	3.93
0675	3.72	0672	11.29	0671	6.29	0673	0.24		
Avg	**2.10**		**50.06**		**7.50**		**2.10**		**19.98**