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Cancer Therapy: Preclinical

Clinical Cancer Research

TNF-Related Apoptosis-Inducing Ligand (TRAIL)–Armed Exosomes Deliver Proapoptotic Signals to Tumor Site

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Abstract

Purpose: Exosomes deliver signals to target cells and could thus be exploited as an innovative therapeutic tool. We investigated the ability of membrane TRAIL-armed exosomes to deliver proapoptotic signals to cancer cells and mediate growth inhibition in different tumor models.

Experimental Methods and Results: K562 cells, transduced with lentiviral human membrane TRAIL, were used for the production of TRAIL$^+$ exosomes, which were studied by nanoparticle tracking analysis, cytofluorimetry, immunoelectronmicroscopy, Western blot, and ELISA. *In vitro*, TRAIL$^+$ exosomes induced more pronounced apoptosis (detected by Annexin V/propidium iodide and activated caspase-3) in TRAIL-death receptor (DR)5$^+$ cells (SUDHL4 lymphoma and INT12 melanoma), with respect to the DR5$^-$DR4$^+$KMS11 multiple myeloma. Intratumor injection of TRAIL$^+$ exosomes, but not mock exosomes, induced growth inhibition of SUDHL4 (68%) and INT12 (51%), and necrosis in KMS11 tumors. After rapid blood clearance, systemically administered TRAIL$^+$ exosomes accumulated in the liver, lungs, and spleen and homed to the tumor site, leading to a significant reduction of tumor growth (58%) in SUDHL4-bearing mice. The treatment of INT12-bearing animals promoted tumor necrosis and a not statistically significant tumor volume reduction. In KMS11-bearing mice, despite massive perivascular necrosis, no significant tumor growth inhibition was detected.

Conclusions: TRAIL-armed exosomes can induce apoptosis in cancer cells and control tumor progression *in vivo*. Therapeutic efficacy was particularly evident in intratumor setting, while depended on tumor model upon systemic administration. Thanks to their ability to deliver multiple signals, exosomes thus represent a promising therapeutic tool in cancer. *Clin Cancer Res; 22(14); 3499–512. ©2016 AACR.*

Introduction

Since the discovery of the selective sensitivity of cancer cells to TRAIL-mediated apoptosis, several agents targeting this pathway have been developed, including recombinant soluble TRAIL (sTRAIL) or TRAIL receptor agonists. Despite the encouraging results obtained at preclinical level, no convincing anticancer activity could be recorded in patients with cancer for any of the tested approaches (1–4). This could be attributed to the different sensitivity of tumors to TRAIL on one hand and to the limited activity of targeting molecules, due to the short half-life of recombinant TRAIL and the monospecificity of the agonistic antibodies (Abs), on the other (1, 5). To ameliorate TRAIL activity, several formulations of recombinant TRAIL, such as fusion to poly-histidine, Flag and leucin Zipper tags, or linked to Fc portion of IgG, have been developed and are currently tested at preclinical level (6, 7). The combination of recombinant TRAIL with chemotherapeutics, radiotherapy, small molecules, or natural compounds aimed at enhancing the sensitivity of cancer cells, have also found wide application in preclinical approaches (8, 9). In addition, liposomes, mesenchymal stem cells, leukocytes, or engineered CD34$^+$ cells have been recently tested in preclinical setting to deliver TRAIL, with the aim of optimizing bioavailability and stability of this molecule (10–15).

An innovate option to deliver TRAIL could be to embed it within vesicular structures directly generated by TRAIL-expressing cells. There is indeed convincing evidence that most cells release a large array of extracellular vesicles (EV) containing surface receptors, cytosolic and nuclear proteins, enzymes,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Driven by the extraordinary findings and technical developments, exosomes are lately attracting major interest as acellular and modifiable therapeutic devices. On the other hand, the selective sensitivity of cancer cells to TRAIL-mediated apoptosis confers this proapoptotic ligand a major role in clinical and preclinical approaches. Here, we intended to unite these two strategies and developed exosomes carrying functional membrane TRAIL as novel antitumor therapy. TRAIL exosomes induce potent target cell apoptosis *in vitro* and control cancer progression when directly injected into tumor lesion. Despite the massive accumulation in major organs when systemically administered, they reach the tumor site in sufficient quantities to mediate detectable apoptosis and reduce cancer growth. As exosomes can be easily produced and stored in large amounts, their use in clinical setting can be envisaged, particularly as intratumor therapy or in combination with drugs augmenting TRAIL sensitivity. TRAIL exosomes could also be loaded with genetic material to be delivered to cancer site through uptake process, offering the opportunity to integrate different treatments through this "natural delivery system."

RNAs, miRNAs, and DNAs of the originating cells (16). The family of EVs comprises vesicles of different size (30–1,000 nm or larger) and origin; it includes microvesicles, stemming from the cell membrane and characterized by the expression of surface markers of the cell of origin, and exosomes, deriving from the endosomal compartment and thus expressing proteins like the tetraspanins CD63, CD9, and CD81 and those related to their export machinery "endosomal sorting complexes required for transport" or ESCRT, such as TSG101 and Alix (17, 18). Exosomes originate in the lumen of multivesicular endosomes (MVE) from the invagination of the limiting membrane of endosomes, through pathways depending on the cell type and cargo (19). Although exosomes have been found to contain a multitude of apparently randomly assembled proteins and RNAs, their content is the result of a selective molecule-driven sorting, that only recently is starting to be elucidated (20). Once secreted into the extracellular milieu by fusion of the MVEs with the plasma membrane, exosomes can interact with recipient cells by receptor-ligand docking, fusion, or endocytosis (17). Cancer has been representing a crucial setting to study the shuttle properties of exosomes, as tumor cells have been shown to use EVs to deliver receptors and miRNAs to promote cell growth and motility, resistance to apoptosis, and even neoplastic transformation in tumor microenvironment and systemically (21, 22).

These features have paved the way to the hypothesis that exosomes could be exploited for the transfer of proteins or genetic material for therapeutic purposes, in cancer or in other pathologic conditions (23). Selective protein delivery or gene therapy approaches through exosomes are already ongoing in neurodegenerative and cardiovascular diseases, with quite exciting preclinical results (24, 25). One of the first clinical trials based on exosome administration was performed again in cancer several years ago, with exosomes produced by dendritic cells (Dexosomes), exploited to shuttle antigenic determinants of immune response, and to immunize patients in the context of cancer vaccines (26–28).

In the present work, we evaluated whether cells genetically modified for TRAIL expression can release homogenous exosomes cargoing active TRAIL (TRAIL exosomes), and if this strategy can be exploited for the delivery of proapoptotic signals to tumor site.

Materials and Methods

Antibodies, reagents, and cell lines

The following Abs were used: phycoerythrin (PE)–TRAIL, receptors (DR4, DR5, DcR1, and DcR2) and isotype controls (R&D Systems); PE-CD63, Fluorescein isothiocyanate (FITC)–caspase-3, Cytofix/Cytoperm Fixation/Permeabilization Kit and isotype controls (BioLegend; BD Biosciences); TRAIL neutralization: (Rik2; BD Pharmingen); Western blot: Rab 5B (Santa Cruz Biotechnology), GM130 (Transduction Laboratories), TRAIL (Peprotech), actin (Sigma-Aldrich). sTRAIL was purchased from AdipoGen. Cell lines included K562, SUDHL4, KMS11 described and authenticated by STR profiling (11). The INT12 melanoma cell line was generated in our laboratory from a human melanoma specimen. *Mycoplasma* contamination was tested periodically. Cell lines were maintained in RPMI-1640 medium supplemented with 10% FCS, 2 mmol/L L-glutamine, and 200 U/mL penicillin/streptomycin.

Lentiviral vector construction and transduction of exosome-producing cells

Membrane-bound TRAIL-encoding lentiviral vector was constructed replacing the GFP sequence of pCCL sin.PPT.hPGK.GFP.pre (kind gift from L. Naldini, HSR, Italy) with human mTRAIL (NM_003810) coding sequence to produce the lenti-TRAIL vector or with ΔNGFR (kind gift from G. Ferrari, HSR, Italy), a truncated nerve growth factor receptor (NGFR) sequence, to produce the control lentiviral vector, lenti-NGFR. The viral stocks were prepared using standard methods (29). Infection was performed at different MOI, in presence of 8 μg/mL of polybrene. After 24 hours, the medium was replaced and efficiency/maintenance of infection tested by flow cytometry after 24 hours and 14 days.

Exosome isolation and nanoparticle tracking analysis

The supernatant of 10^9 K562 cells TRAIL$^+$ or NGFR$^+$, cultured 24 hours in serum-free RPMI-1640 medium, was sequentially centrifuged at 300 × *g* (10 minutes), 4,000 × *g* (20 minutes), 0.22-μm vacuum filtered (Millipore) to eliminate larger EVs and ultracentrifuged (Thermo Fisher Scientific) at 100,000 × *g* (4 hours) at 4°C. Exosomes were washed/concentrated in PBS at 100,000 × *g* (1 hour) at 4°C, suspended in PBS and after protein determination (Bradford Protein Assay; Bio-Rad), frozen at −80°C (200 and 400 μg aliquots). To minimize interpreparation variability, exosomes were routinely checked by nanoparticle tracking analysis (NTA), immunoelectronmicroscopy (TRAIL and exosomal markers), and ELISA. Viability, phenotype, and cytokine release of K562 TRAIL$^+$ cells cultured in the absence of FCS for exosome production was comparable with those cultured in complete medium (Supplementary Fig. S1A–S1C). The size and quantity of purified TRAIL exosomes (5 μg; diluted 1:10,000) was determined using a LM10-HS NanoSight instrument and NTA

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software (NanoSight). Preparations were analyzed five times for 30 seconds.

Electron and confocal microscopy

For routine staining of exosomes, preparations, fixed with 4% paraformaldehyde and deposited on Formvar-carbon-coated Nickel grids, were incubated with anti-TRAIL Ab (1:5) or anti-CD63, LAMP-2 (BD Pharmingen), and Rab 5B (1:10) Mix Abs, followed by gold-conjugated goat anti-mouse IgG (H+L) 6 nm and goat anti-rabbit IgG (H+L) 5 nm Abs (Jackson ImmunoResearch) and examined by transmission electron microscope CM 10 Philips (FEI). For confocal microscopy, frozen tissue sections tissues (6 μm) were evaluated by a Radiance 2100 microscope (Bio-Rad Laboratories).

Western blot and ELISA

Standard Western blot analysis was performed using 4% to 12% bis-Tris precast gels (Invitrogen), nitrocellulose membranes (Amersham), and enhanced chemiluminescence (SuperSignal). Exosomal TRAIL was quantified by ELISA (Human TRAIL, Quantikine; R&D Systems), according to the manufacturer's instructions, without applying the lysing procedure.

Flow cytometry

Flow cytometry of cells was performed according to standard procedures. Flow cytometry of exosomes was performed using sulfate/aldehyde latex beads (5 μm; Life Technologies; ref. 30). Apoptosis was detected by Annexin V/propidium iodide (PI) staining, performed according to the manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit; Bender MedSystems GmbH). Activated caspase-3 was detected in permeabilized cells. Samples were evaluated using a FACSCalibur flow cytometer (BD Biosciences) and the FlowJo software (TreeStar Inc.).

In vivo studies

Mice were maintained at the Fondazione IRCCS Istituto Nazionale dei Tumori under standard conditions according to institutional guidelines. All procedures were approved by the Institute Ethical Committee for animal use and by the Italian Ministry of Health. A total of 20×10^6 SUDHL4 cells were subcutaneously xenotransplanted into 8-week-old female SCID mice. Treatments were started at 200 to 300 mm^3 for intratumor and at 100 mm^3 for intravenous injections. INT12 melanoma cells were injected subcutaneously at the dose of 2×10^6 cells and treatments started at 100 mm^3 tumor volume (TV). Cells were injected with Matrigel (BD Biosciences) in a mixture 1:1 in 200 μL. Tumors were imaged by VisualSonics Vevo 770 to distinguish between Matrigel and growing tumors and later measured by Vernier caliper. For the KMS11 model, cells were injected subcutaneously to 8-week-old female NOD-SCID for intravenous treatments and SCID mice for intratumor treatments at the dose of 5×10^6 cells or 20×10^6 cells, respectively. Treatments were started when tumors reached 100 mm^3. If not otherwise specified, the treatment schedule consisted in 4 injections, every 48 hours, of 200 μg/injection of TRAIL or NGFR exosomes, sTRAIL (intra-tumor: 200 ng or 300 μg/injection; i.v.: 30 mg/kg/injection) or saline. Mice were sacrificed 24 hours after the last treatment or during follow-up at the first signs of distress and lesions and organs collected. TV was calculated by the formula: TV (mm^3) = $d_2 \times D/2$, where d and D are the shortest and the longest diameter, respectively. The antitumor activity was assessed as TV inhibition percentage (TVI%) in treated versus control mice, calculated as follows: TVI% = 100-(mean TV treated/mean TV control × 100). For *in vivo* treatments, that is, 200 μg exosomes per injection, corresponding to 200 ng TRAIL, were chosen on the basis of literature data showing that this dose is generally well tolerated whereas higher dosages are associated with death due to pulmonary embolism (31–33). The schedule of 4 intravenous injections for systemic therapy was designed on the basis of previous experience with TRAIL-expressing $CD34^+$ cells, introducing a precautionary 48 hours interval, instead of the 24 hours there used, to minimize embolic risk (34). If not otherwise specified, each experiment was performed at least two times, using 5 to 7 animals per group.

IHC

Formalin-fixed and paraffin-embedded tissue sections (3–4 μm) were incubated with the following Abs: Ki67 (Immunological Sciences), caspase-3 (Cell Signaling Technology), and anti-human TRAIL-DR5 (Novus Biologicals), cross-reacting with mouse TRAIL-DR5. UltraVision Quanto Detection System HRP (Thermo Fisher Scientific Inc.) and DAB (Liquid DAB+ Substrate Chromogen System; Dako) were used to develop the reaction. TUNEL staining (ApopTag Peroxidase In Situ Apoptosis Detection Kit; Millipore) was performed according to the manufacturer's instructions. Images were acquired by Aperio ScanScope XT systems (Aperio Technologies, Leica Microsystems Srl) or Eclipse E600 microscope (Nikon).

Statistical analysis

Data were analyzed using GraphPad Prism 5.0 Software (GraphPad Software Inc.). Results are shown as mean ± SD or SEM and analyzed using two-way ANOVA and unpaired Student t test, as specified.

Results

Generation of TRAIL-expressing exosomes

K562 cells were transduced with a lentiviral vector containing human membrane TRAIL (lenti-huPGK-TRAIL) or encoding for a human truncated non-functional NGF receptor (lenti-huPGK-NGFR) to obtain $TRAIL^+$ K562 and $NGFR^+$ K562 control cells (Fig. 1A and B). $TRAIL^+$ K562 cells expressed no TRAIL death receptors and the stability of TRAIL expression (40 MOI) during large-scale expansion, was confirmed (Supplementary Fig. S1D–S1F). Exosomes isolated by sequential ultracentrifugation were at first evaluated by NTA technology, showing a vesicle population of 140 nm as mean size (Fig. 1C). TRAIL and NGFR expression was detected at significant level on purified vesicles by flow cytometry, confirming the export of these molecules via exosomes, together with CD63 exosomal marker (Fig. 1D and E). Electron and immunoelectronmicroscopy analysis revealed a population of vesicles of slightly smaller dimensions than measured by NTA technology (60–100 nm), ascribable to the preparation method of the samples (Fig. 1F), and displaying a high positivity for TRAIL (Fig. 1G) and the exosomal markers Rab 5B, CD63 and Lamp-2 (Fig. 1H). The expression of membrane TRAIL (32 kDa) was confirmed by Western blot analysis in exosome fractions, as compared with their cells of origin together with

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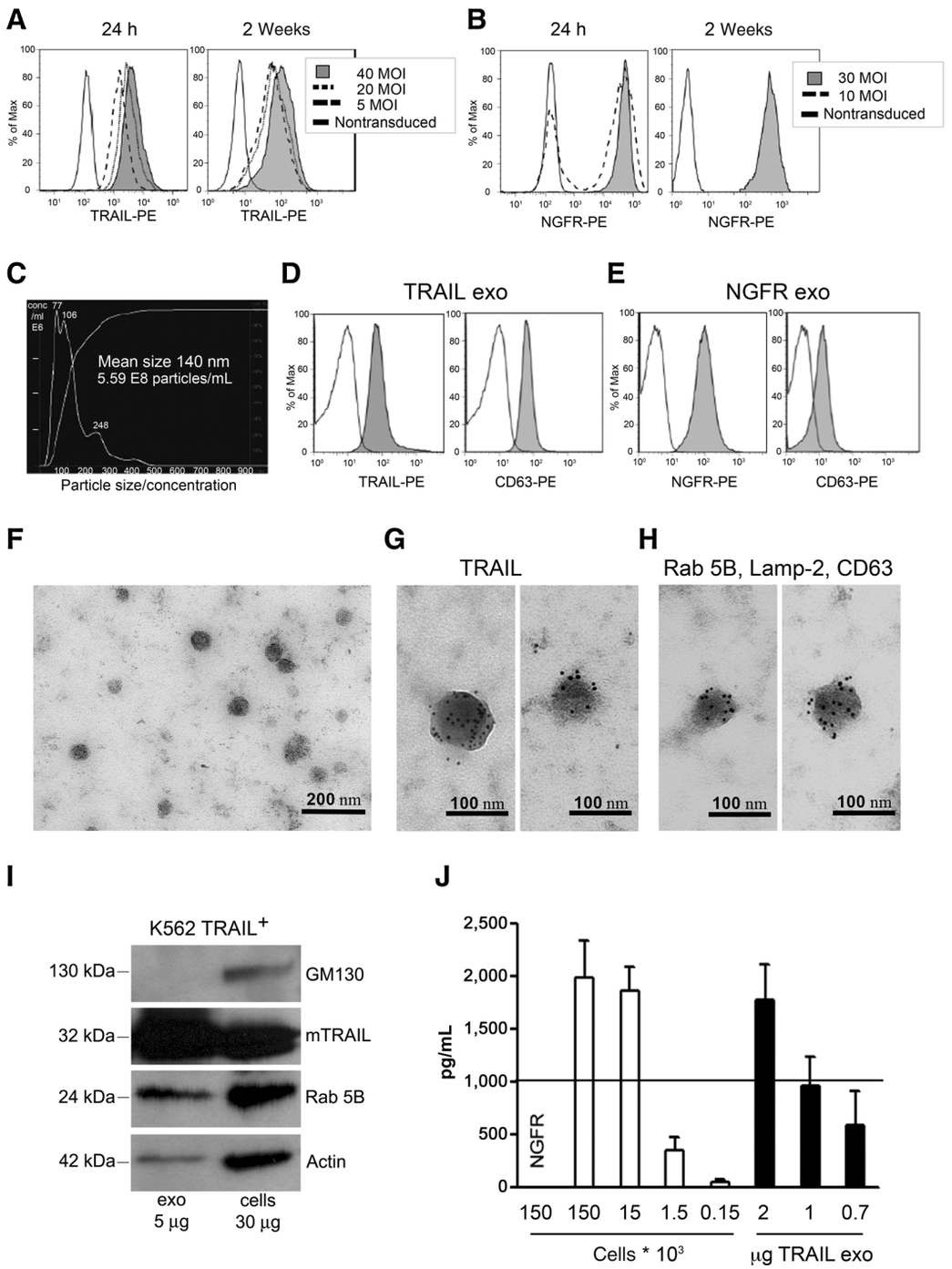

Figure 1.
Generation of TRAIL-expressing exosomes. A, transduction of K562 cells with human membrane TRAIL. K562 cells were infected with different MOI (5, 20, and 40) of lenti huPGK-TRAIL. Flow cytometry analysis of TRAIL expression 24 hours posttransduction and after 2 weeks of *in vitro* culture. K562 lenti-TRAIL 40 MOI cells were chosen for subsequent studies. B, transduction of K562 cells with human NGFR. K562 cells were infected with different MOI (10 and 30) of lenti-NGFR. Flow cytometry analysis of NGFR expression 24 hours posttransduction and after 2 weeks of culture. K562 lenti-NGFR 30 MOI cells were chosen for subsequent studies. C, characterization of TRAIL exosomes. Assessment of size, number, and distribution of TRAIL exosomes by NTA technology. D and E, flow cytometry of exosome-bead complexes for the expression of TRAIL and CD63 on purified TRAIL exosomes and NGFR and CD63 on purified NGFR exosomes. Filled histograms represent the positivity for molecule tested, lines represent IgG controls. F, electronmicroscopy of exosomes purified from conditioned media of TRAIL-transduced K562 cells. G and H, immunoelectronmicroscopy of TRAIL exosomes labeled for TRAIL and CD63, Rab 5B, and Lamp-2 exosomal markers. I, Western blot analysis of TRAIL exosomes and originating K562 TRAIL$^+$ cells for the expression of membrane (m) TRAIL, Rab 5B exosomal marker, actin and GM130 Golgi marker protein. J, quantification of exosomal TRAIL by ELISA in $n = 10$ TRAIL exosome fractions (in μg) and in lysates of originating TRAIL$^+$ K562 cells or NGFR$^+$ K562 cells (negative control).

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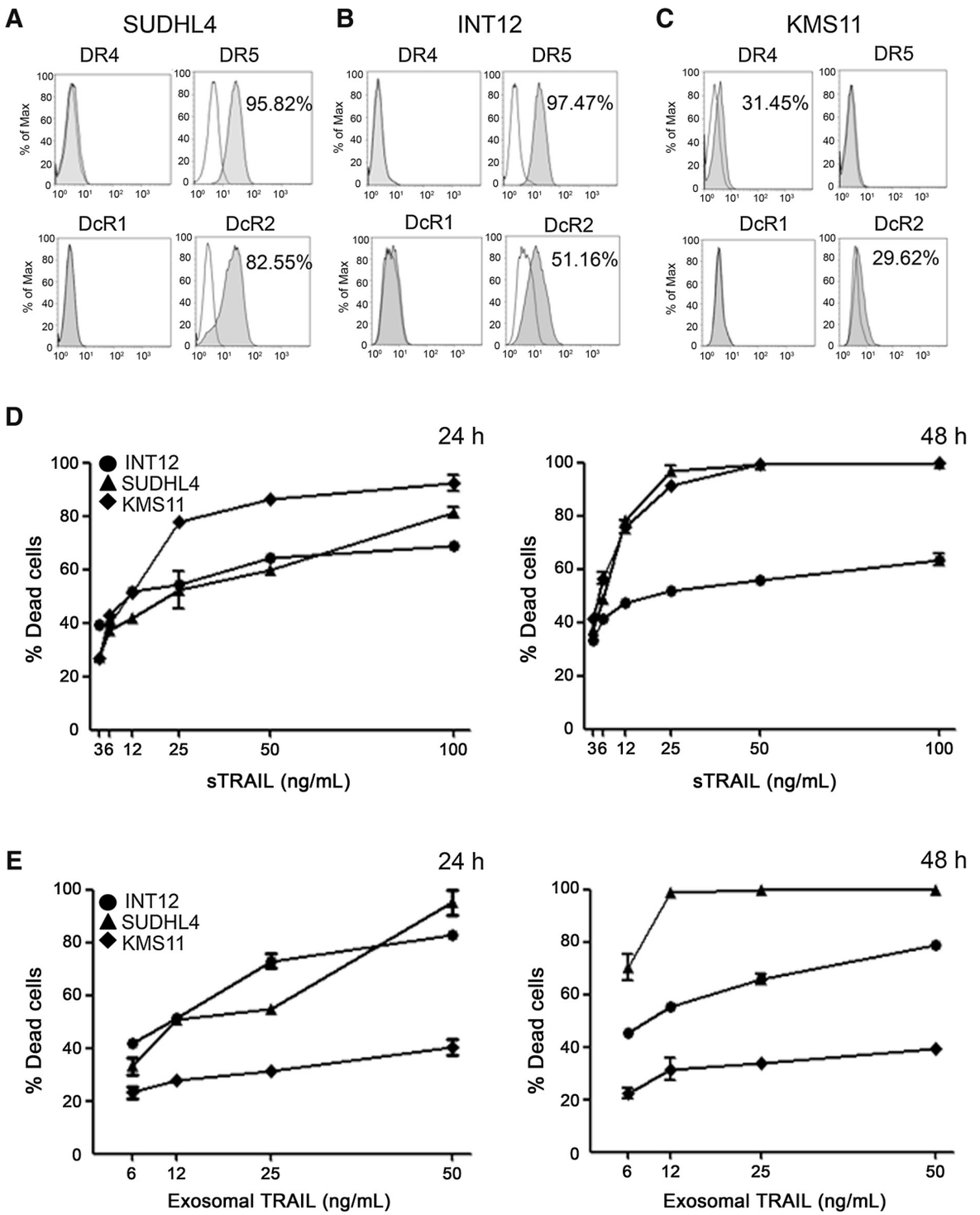

Figure 2.

Functionality of TRAIL exosomes. A to C, expression of TRAIL receptors. SUDHL4, INT12, and KMS11 cells were labeled with PE-conjugated mAbs against DR4, DR5, DcR1, and DcR2 or with an appropriate IgG-PE as control. Filled histograms represent the positivity for each receptor tested, lines represent IgG controls. Results show representative histograms for each cell line. D, sensitivity to sTRAIL. SUDHL4, INT12 and KMS11 cells were incubated with increasing concentrations of sTRAIL and evaluated by Annexin V/PI staining after 24 (left) and 48 hours (right) by flow cytometry. Results are shown as mean ± SEM of three independent experiments. E, sensitivity to exosomal TRAIL. SUDHL4, INT12, and KMS11 cells were incubated with increasing concentrations of exosomal TRAIL and evaluated by Annexin V/PI staining after 24 (left) and 48 hours (right) by flow cytometry. Exosomal TRAIL was determined on TRAIL exosomes by TRAIL ELISA. Results are shown as mean ± SEM of three independent experiments.

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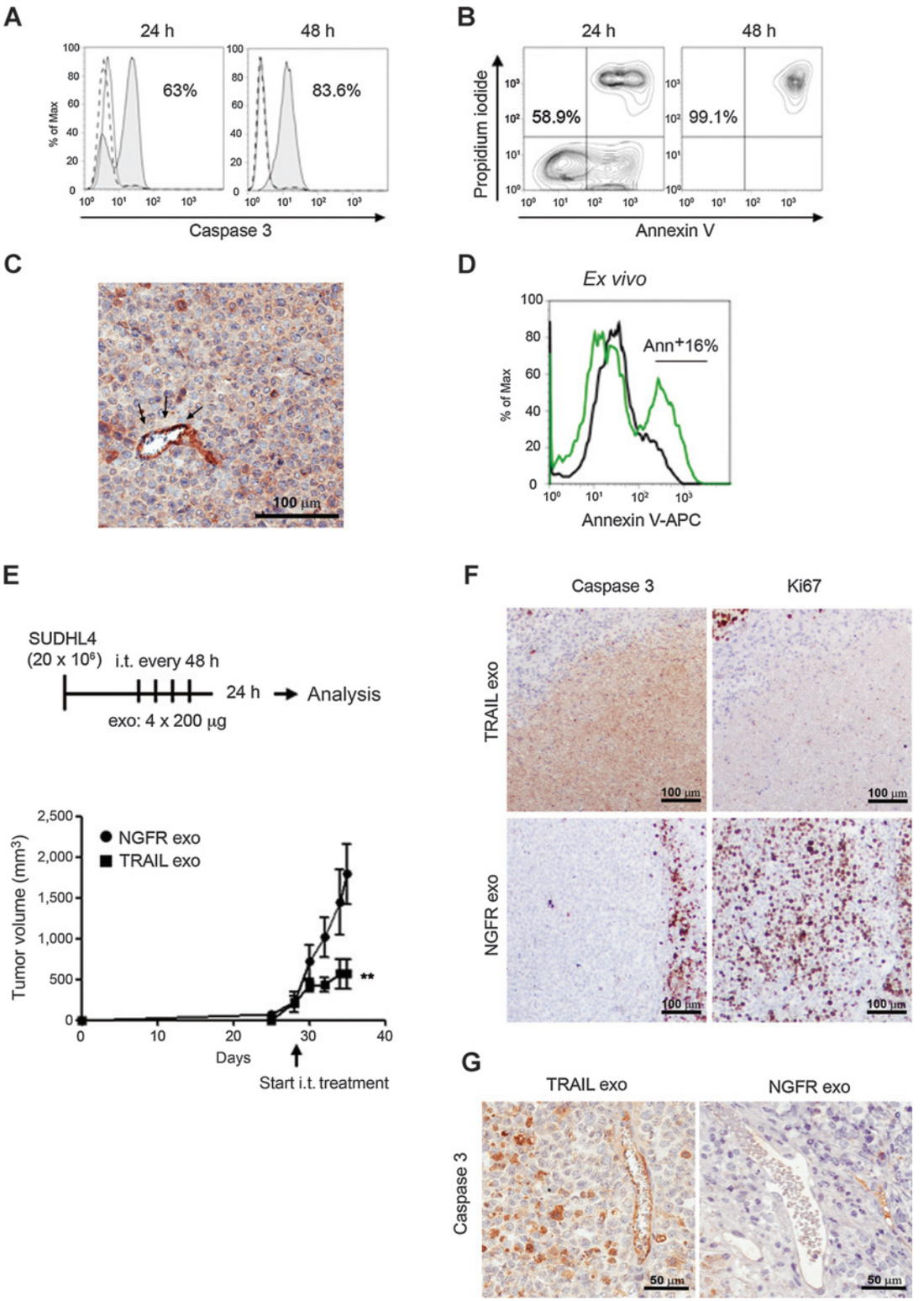

Figure 3.
Antitumor activity of TRAIL exosomes on SUDHL4 B cell lymphoma. A, caspase-3 activation. SUDHL4 cells were incubated with TRAIL exosomes (20 ng/mL exosomal TRAIL) for 24 and 48 hours and evaluated for caspase-3 activation by flow cytometry after intracellular staining. Filled histograms represent the positivity for caspase-3, dashed lines represent caspase-3 positivity in cells treated with TRAIL exosomes pre-incubated with anti-TRAIL Ab (Rik2) and lines represent caspase-3 in untreated controls. B, detection of cell death. (*Continued on the following page.*)

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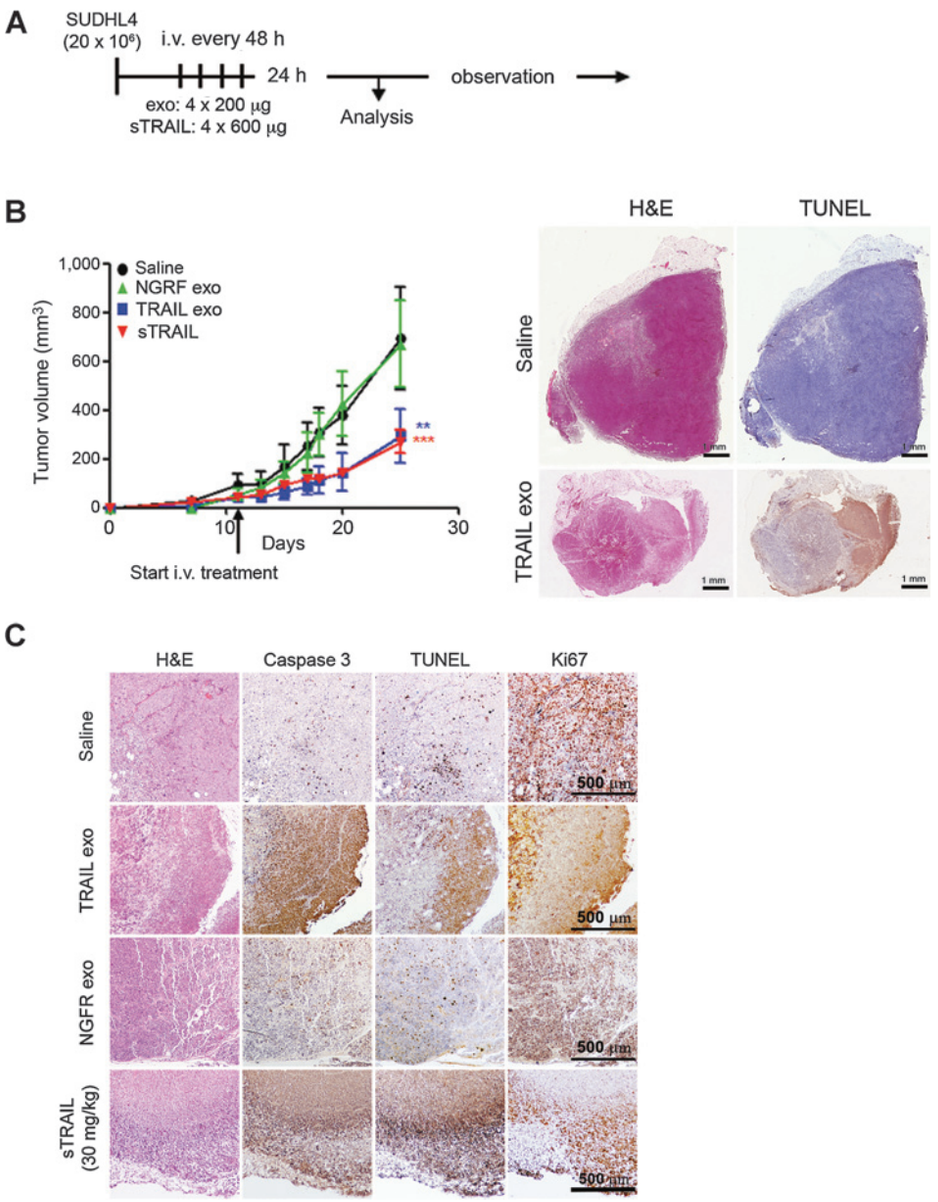

Figure 4.
Systemic antitumor activity of TRAIL exosomes. A, treatment schedule. SCID mice received 200 μg/injection (corresponding to 200 ng/injection of exosomal TRAIL) of exosomes (NGFR or TRAIL), saline or sTRAIL at 30 mg/kg/injection. B, effect on tumor growth. Left, growth curves of SUDHL4 subcutaneous nodules in SCID mice. Statistical significance was achieved by TRAIL exosome and sTRAIL treatment versus control groups (saline and NGFR exosomes) using two-way ANOVA; **, $P < 0.01$; ***, $P < 0.001$. Right, H&E and TUNEL staining of nodules collected from TRAIL exosomes and saline-treated mice. Images are adapted to the same scale. C, representative IHC analysis of tumors removed 24 hours after the last intravenous injection of saline, TRAIL exosomes, NGFR exosomes, or sTRAIL. Sections were stained for H&E, caspase-3, TUNEL, and Ki67. Images show the same area of serial sections stained for the above-mentioned markers.

Rab 5B (Fig. 1I). The absence of Golgi protein (GM130) ascertained the purity of vesicle populations (Fig. 1I). The quantification of TRAIL in whole exosome preparations by commercial ELISA allowed to measure 1 ± 0.25 ng TRAIL in 1 μg of TRAIL exosomes ($n = 10$; Fig. 1J).

Functionality of TRAIL exosomes

The proapoptotic potential of membrane TRAIL expressed by exosomes was next tested *in vitro* on SUDHL4 B-cell lymphoma and INT12 melanoma cells, expressing DR5 at almost 100% and DcR2 at 80% and 50%, respectively (Fig. 2A and B). The third

(*Continued.*) Annexin V/PI staining of SUDHL4 cells after 24 and 48 hours co-culture in the presence of TRAIL exosomes (20 ng/mL exosomal TRAIL). C, DR5 expression in tumor tissues. IHC staining of DR5 on SUDHL4 tumor sections, subcutaneously grown in SCID mice ($n = 2$). D, *in vivo* interaction of TRAIL exosomes with SUDHL4 tumor cells. Annexin V-APC staining of tumor cell suspensions collected from SCID mice ($n = 2$/treatment) 24 hours after intratumor injection of TRAIL exosomes. The green line represents the staining of cells obtained from tumors of animals that received TRAIL exosomes. The percentage of Annexin V^+ cells was obtained by subtracting the background (suspensions from saline-treated mice; black line). E, intratumor treatment. Treatment schedule of tumor-bearing mice (top), tumor growth curve of SUDHL4-bearing animals treated with TRAIL or NGFR exosomes according to the depicted schedule. Treatment was started when nodules reached 200 to 300 mm^3. Statistical significance was achieved by TRAIL exosomes versus NGFR exosomes using two-way ANOVA; **, $P < 0.01$. F, representative IHC staining of lesions collected 24 hours after the last injection of exosomes. Images show the same area of serial sections stained for caspase-3 and Ki67 proliferation index marker. G, IHC detection of activated caspase-3–positive endothelial cells forming tumor vessels on lesions of TRAIL or NGFR exosome–treated mice.

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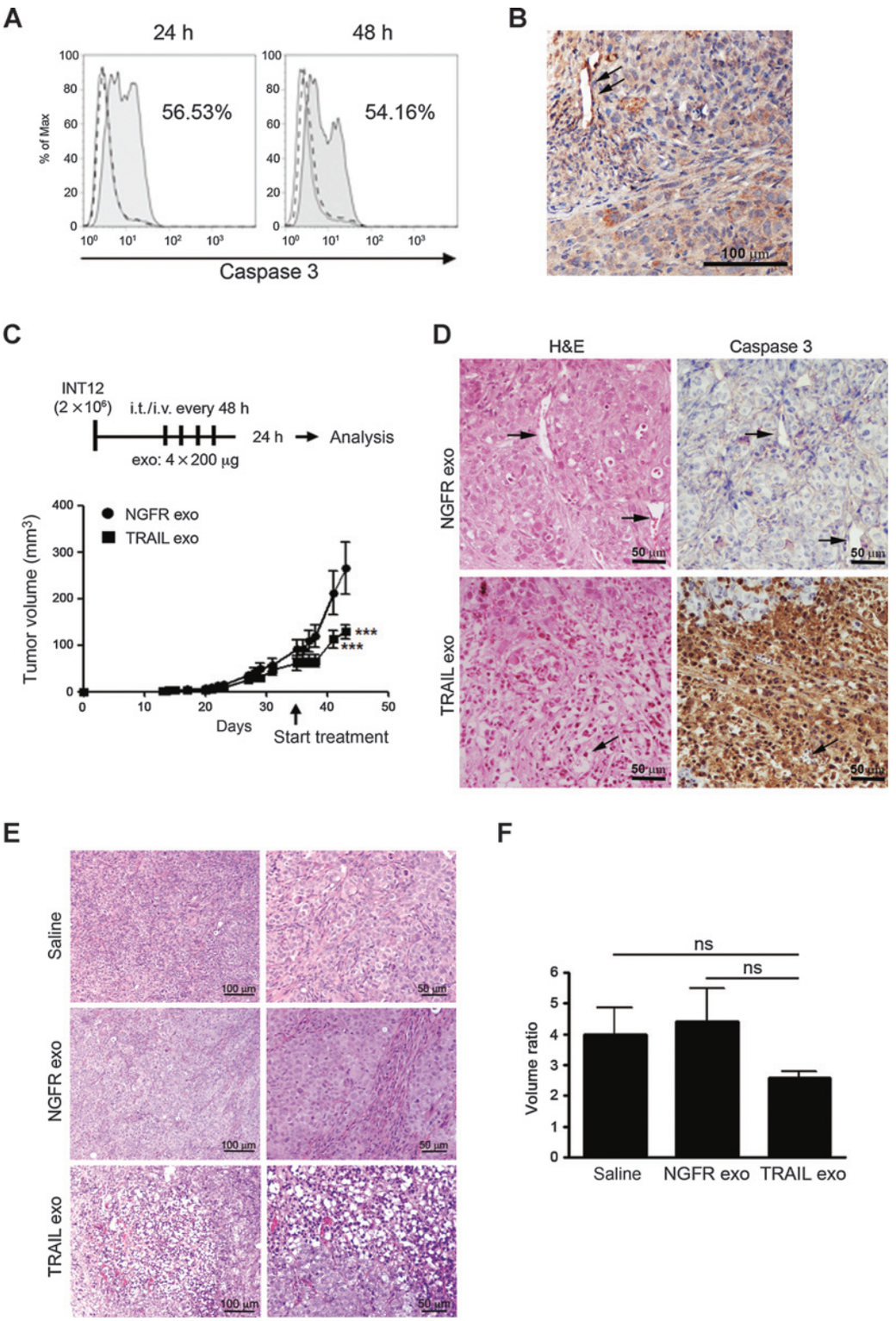

Figure 5.
Antitumor activity of TRAIL exosomes on INT12 melanoma. A, caspase-3 activation. INT12 cells were incubated with TRAIL exosomes (15 ng/mL exosomal TRAIL) for 24 and 48 hours and evaluated for caspase-3 activation by flow cytometry after intracellular staining. Filled histograms represent the positivity for caspase-3, dashed lines represent caspase-3 positivity in cells treated with TRAIL exosomes pre-incubated with anti-TRAIL Ab (Rik2), and lines represent caspase-3 expression in untreated controls. B, DR5 expression in tumor tissues. IHC staining for DR5 on INT12 tumor sections subcutaneously grown in SCID mice ($n = 2$). Arrows show the positive staining on tumor vessels. (*Continued on the following page.*)

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target included in the analysis, that is, the multiple myeloma KMS11, expressed instead DR4 and DcR2, both detectable in about 30% cells (Fig. 2C). Exposure to increasing amounts of sTRAIL showed that KMS11 was highly sensitive to sTRAIL, with an ED_{50} of 15.12 and 10.77 ng/mL at 24 and 48 hours, respectively (Fig. 2D). SUDHL4 reached 50% of cell death with 22 ng/mL sTRAIL at 24 hours and with 9.33 ng/mL at 48 hours (Fig. 2D). Thus, both the cell lines displayed a substantial increase of cell death if the culture was prolonged to 48 hours. In contrast, INT12 melanoma cells, among the most sensitive within a panel of 10 melanoma cell lines tested (data not shown), displayed no increased apoptosis with prolonged incubation time and never reached 100% even at the highest sTRAIL concentration tested of 100 ng/mL (Fig. 2D).

The activity of TRAIL expressed by exosomes was tested under the same conditions using increasing concentrations of exosomal TRAIL, according to ELISA quantification. Figure 2E shows that exosomal TRAIL was more efficient in inducing apoptosis of SUDHL4 cells than sTRAIL, with an ED_{50} of 5.9 ng/mL compared with 9.3 ng/mL for sTRAIL at 48 hours. Similarly, in INT12 melanoma cells we observed a tendency to an enhanced sensitivity to exosomal TRAIL with respect to sTRAIL after 48 hours incubation, with an ED_{50} of 8.9 versus 19 ng/mL, respectively. In contrast, KMS11 cells appeared to be much less sensitive to TRAIL exosomes as compared with the soluble molecule, both at 24 and 48 hours (Fig. 2E). This evidence suggests a preferential interaction of exosomal TRAIL with DR5, as KMS11 was the only cell line tested that expressed DR4 and stained negative for DR5.

Antitumor activity of TRAIL exosomes on SUDHL4 B-cell lymphoma

In SUDHL4 cells, apoptosis was associated with a rapid and efficient activation of caspase-3, here shown at 24 and 48 hours (63% and 83.6%, respectively), in the presence of 20 ng/mL exosomal TRAIL (Fig. 3A and B). Preincubation of exosomes with TRAIL-neutralizing Ab (Rik2) completely abrogated this effect, demonstrating the specific involvement of TRAIL (Fig. 3A). In contrast, no apoptosis was detected in the presence of control NGFR exosomes (data not shown).

For *in vivo* testing, SCID mice were subcutaneously injected with 20×10^6 SUDHL4 cells and, when nodules reached 200–300 mm^3, they were evaluated for DR5 *in vivo* expression using an anti-human Ab that cross-reacts with murine TRAIL receptor. IHC analysis depicted a diffuse positivity in both cancer cells and vessels (Fig. 3C), confirming the expression of TRAIL receptor in the tumor microenvironment as a potential target of proapoptotic receptor agonists (35). The dose for *in vivo* treatment, that is, 200 μg exosomes, corresponding to 200 ng TRAIL, was chosen on the basis of literature data as maximal tolerated dose (as detailed in Materials and Methods). Of note, one single intratumor injection of TRAIL exosomes led to measurable apoptosis (16% ± 10%) in tumor cell suspensions prepared from nodules extracted 24 hours post-administration (Fig. 3D).

To test the therapeutic efficacy of TRAIL exosomes upon local delivery, mice bearing SUDHL4 tumors of 200 to 300 mm^3 volume were assigned to receive multiple intratumor injections of NGFR or TRAIL exosomes. TRAIL exosome administration led to a rapid and persisting inhibition of tumor growth (maximal TVI 68%; Fig. 3E), with respect to the injection of NGFR exosomes. IHC of lesions removed 24 hours after last treatment showed that large areas of necrosis and few Ki67-positive cells could be detected in TRAIL exosome–receiving mice (Fig. 3F), whereas tumor lesions from NGFR exosome-treated animals displayed high-proliferation index (Ki67) and limited apoptosis, as depicted by activated caspase-3 expression. Caspase-3 expression was also evident at tumor vessel level upon TRAIL exosome administration, suggesting a possible direct or indirect effect on endothelial cells, as previously demonstrated with $CD34^+$ $TRAIL^+$ cells in a comparable xenograft setting (Fig. 3G; ref. 34).

The antitumor activity of TRAIL exosomes was then analyzed upon systemic administration. To verify their actual homing to tumor site, PKH26-labeled TRAIL exosomes were injected intravenously in SUDHL4-bearing mice twice (Supplementary Fig. S2). Confocal microscopy showed that red fluorescently labeled cancer cells could be detected in tumor lesions and analysis of *ex vivo* tumor cell suspensions revealed the presence of Annexin V–positive cells (23.3% ± 3.4%; Supplementary Fig. S2).

Systemic treatment of SUDHL4-bearing mice (Fig. 4A) induced a rapid and progressive inhibition of tumor growth in mice receiving TRAIL exosomes or sTRAIL, reaching 58% reduction in tumor size at the end of treatment (Fig. 4B, left). sTRAIL, here administered at the effective dose of 30 mg/kg/injection, provided comparable results (Fig. 4B, left), although it should be pointed out that this dose (600 μg/injection) was remarkably higher than the TRAIL content of exosomes (200 ng/injection). Conversely, NGFR exosomes did not influence tumor growth with respect to saline treated animals. TUNEL staining of tumor nodules removed 24 hours after the fourth treatment depicted large areas of necrosis covering almost 50% of the lesion in animals receiving TRAIL exosomes, but not in controls (Fig. 4B, right). Corroborative results were obtained by IHC staining of tumor lesions for caspase-3 and Ki67 (Fig. 4C).

Antitumor activity of TRAIL exosomes on INT12 melanoma

TRAIL exosomes induced TRAIL-dependent caspase-3 activation in 56% and 54% of INT12 cells after 24 or 48 hours, respectively (Fig. 5A). DR5 expression detected by flow cytometry was confirmed by IHC of tumor nodules from xenotransplanted SCID mice (2×10^6 INT12 cells injected subcutaneously), showing a consistent positivity of tumor cells and tumor vessels (Fig. 5B). Intratumor injection of

(*Continued.*) C, intratumor treatment. Treatment schedule of tumor-bearing mice (top), tumor growth curve of INT12-bearing animals treated with TRAIL or NGFR exosomes (bottom). Treatment was started when nodules reached 100 mm^3. Statistical significance was achieved by TRAIL exosomes versus NGFR exosomes using two-way ANOVA; ***, $P < 0.001$. D, representative H&E and caspase-3 stainings on sections of tumors removed after the end of treatments. Arrows indicate tumor vessels. Images show the same area of serial sections. E, systemic treatment. H&E-stained representative sections of tumors collected after the end of treatment with intravenously injected TRAIL or NGFR exosomes or saline. F, effect on tumor growth. Ratios were calculated as volume at the end/volume at the beginning of treatment. Statistical analysis was performed by the Student *t* test (NS not significant).

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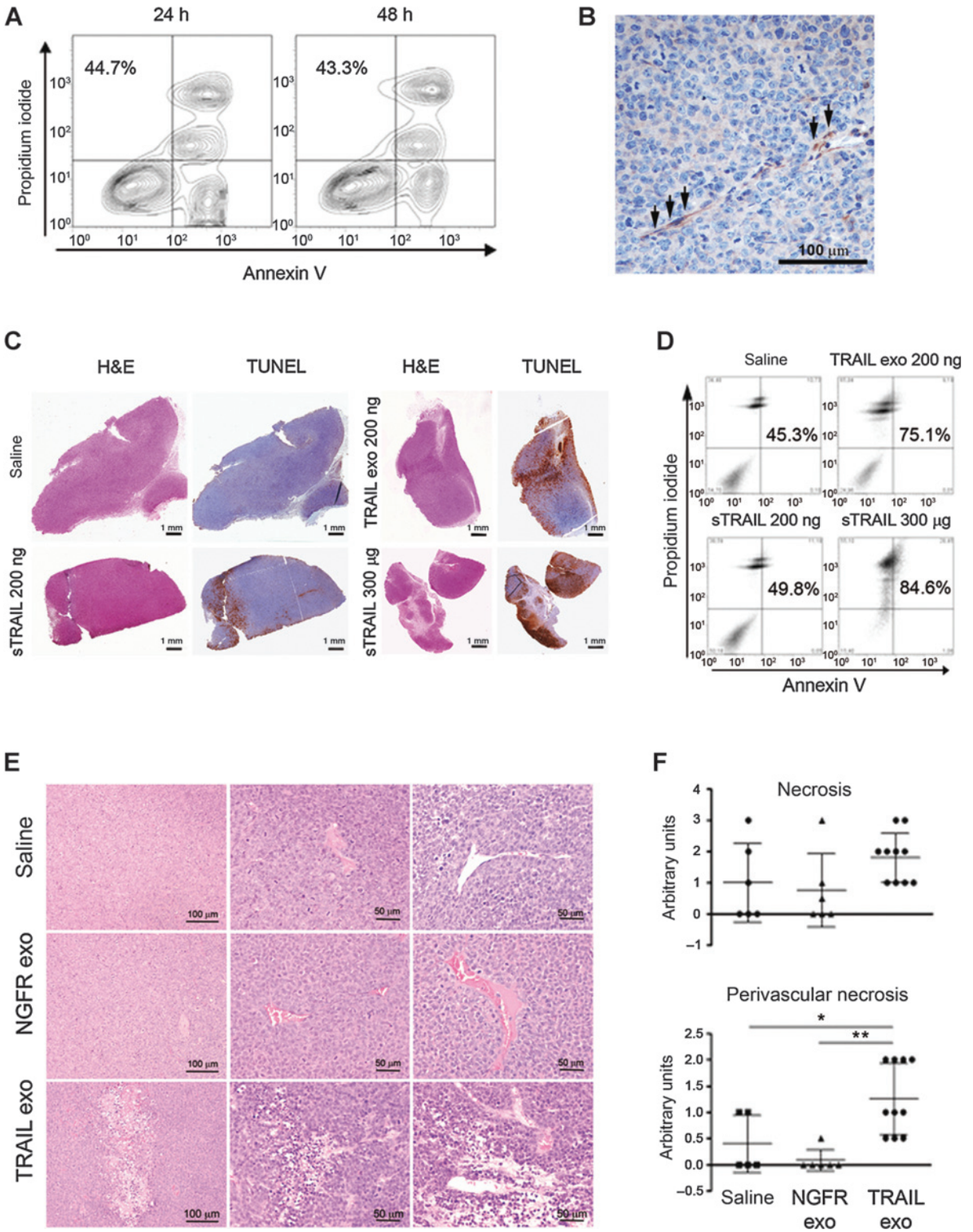

Figure 6.

Antitumor activity of TRAIL exosomes on KMS11 multiple myeloma. A, detection of cell death. Annexin V/PI staining of KMS11 cells after 24 and 48 hours coculture in the presence of TRAIL exosomes (200 ng/mL exosomal TRAIL). B, DR5 expression on tumor vessels. IHC staining of KMS11 tumor sections for DR5 ($n = 2$). The positivity was confined to endothelial vessel forming cells (arrows). C, intratumor treatment. KMS11-bearing mice received 4 treatments, every 48 hours, of TRAIL exosomes (exosomal TRAIL 200 ng/injection), sTRAIL (200 ng/injection), sTRAIL (300 μg/injection), or saline. (*Continued on the following page.*)

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TRAIL exosomes (4 × 200 μg/injection every 48 hours) into INT12 melanoma nodules (100 mm^3) mediated a significant reduction of TV (maximal TVI 51%; Fig. 5C), with respect to NGFR exosome–treated mice. This tumor kinetics was confirmed by IHC, depicting necrotic and caspase-3–positive areas extended also to tumor vessels, in TRAIL exosome, but not NGFR exosome–treated lesions (Fig. 5D).

Systemic treatment gave similar histologic results as intratumor treatment, with large necrotic areas for mice receiving TRAIL exosomes in contrast with intact tissue in control mice (NGFR exosomes and saline, Fig. 5E). However, this scenario was not associated with a statistically significant inhibition of tumor growth, although a reduction of TV could be observed upon administration of TRAIL exosomes, as shown by the volume ratios at end versus start of treatment, with respect to the controls (NGFR exosomes and saline; Fig. 5F).

Antitumor activity of TRAIL exosomes on KMS11 multiple myeloma

KMS11 cells showed poor sensitivity to TRAIL exosomes *in vitro* (Fig. 2), even if we were able to measure about 40% of cell death by increasing the dose of exosomal TRAIL to up to 200 ng/mL (Fig. 6A).

In vivo, IHC staining of KMS11 tumor nodules confirmed the negativity for DR5 expression detected *in vitro* (Fig. 2), although the receptor was instead detectable on tumor vessels (Fig. 6B). Intratumor treatment of SCID mice bearing KMS11 nodules was performed according to the described schedule of 4 injections every 48 hours and included exosomal TRAIL at 200 μg/injection (corresponding to 200 ng TRAIL), sTRAIL at 200 ng/injection for comparison and sTRAIL at the effective dose of 300 μg/injection. Monitoring of tumor size revealed that a growth arrest could be detected only in animals receiving sTRAIL at the higher dose of 300 μg (data not shown). Nodules removed from these mice showed reduced size, high levels of necrosis hematoxylin and eosin (H&E and TUNEL; Fig. 6C) and cell death (80% ± 10% PI^+; Fig. 6D). In lesions from mice receiving TRAIL exosomes, we could also observe increased areas of necrosis (H&E and TUNEL; Fig. 6C) together with augmented levels of dead cells (30% ± 10% PI^+) (Fig. 6D). In contrast, no major change with respect to controls was detected in lesions from mice receiving sTRAIL at the exosome-equivalent dose of 200 ng (Fig. 6C and D), suggesting a higher efficacy of TRAIL exosomes in the induction of tumor apoptosis when injected locally.

Systemic administration of TRAIL exosomes did not induce any significant impact on tumor growth (data not shown), with only marginal increase of overall tumor necrosis detected by histologic analysis in mice treated with TRAIL exosomes, with respect to NGFR exosomes or saline (Fig. 6E and F, top). Interestingly, in lesions from mice receiving TRAIL exosomes we could observe significant perivascular necrosis (Fig. 6E and F, bottom).

Discussion

Herein, we depict the strategy for the delivery of functional TRAIL to sensitive cancers via genetically engineered exosomes. Our report shows that cells, modified to express TRAIL, can produce exosomes that incorporate the proapoptotic ligand in their membranes in an active form. TRAIL exosomes displayed a significant killing activity *in vitro* and *in vivo*, in local and systemic treatment approaches, although therapeutic efficacy varied in the different tumor models analyzed.

Thanks to the ability to shuttle their cargo and cross biologic barriers, EVs are recently being exploited as drug delivery vehicles in several diseases, including cancer. We sought to generate exosomes expressing high levels of functional TRAIL, to combine the advantage of a transmembrane conformation with nanovesicular structures for systemic delivery (11). K562 cells transduced with a human lentiviral vector were chosen as exosome producers, for their resistance to TRAIL-mediated apoptosis, the ability to grow at large scale level *in vitro* and the approved use for human application (36–38). Nevertheless, other donor cells, such as CD34 from healthy volunteers and different transfection tools (i.e., AdenoTRAIL vectors; refs. 34, 39), produced in our hands comparable exosomes (Supplementary Fig. S3), proving a broad applicability of the exosomal TRAIL approach.

Exosomes released by lenti-TRAIL K562 cells displayed a rather homogeneous structure and size (140 nm), and remarkable levels of TRAIL protein on their surface, as clearly depicted by immunoelectronmicroscopy. Once incubated with TRAIL-susceptible cells, they triggered rapid caspase-3–mediated cell death, indicating the ability of exosome-embedded TRAIL to efficiently crosslink its cognate receptor and initiate the apoptotic cascade. Usually the interaction of exosomes and cells can be of different nature, depending not only on the exosome surface composition but also on the type of target cell. In fact, exosomes interact through receptor-ligand docking, direct fusion, or endocytosis and are thereby taken up by the recipient cells. Our results, showing that apoptosis induced by TRAIL exosomes was completely abrogated by neutralization with TRAIL Ab, suggest that the proapoptotic activity of TRAIL exosomes relies principally on a surface-to-surface interaction of TRAIL with its cognate receptor. Interestingly, the activity of exosomal TRAIL, but not sTRAIL tested for comparison, appeared to be superior in target cells expressing DR5 (SUDHL4 and INT12), with respect to those expressing DR4 (KMS11 cells). This evidence suggests that DR4 might harbor a conformational structure less suitable to interact with TRAIL embedded in nanosized-membrane particles.

(*Continued.*) Animals were sacrificed 24 hours after the last treatment and sections analyzed by IHC (representative images of H&E and TUNEL staining are shown). Images are adapted to the same scale. D, Annexin V/PI staining of tumor cell suspensions ($n = 2$) prepared *ex vivo* from nodules collected after the end of intratumor injections. E, systemic treatment of KMS11-bearing animals. KMS11-bearing mice received 4 intravenous treatments, every 48 hours, of TRAIL exosomes (exosomal TRAIL 200 ng/injection), NGFR exosomes (200 μg/injection) or saline ($n = 5$–6 saline and mice receiving NGFR exosomes; $n = 10$ animals receiving TRAIL exosomes). H&E staining of representative sections of tumors extracted after the end of treatment. F, quantification of necrotic areas. Graphs show tumor necrosis (top) and perivascular necrosis (bottom) quantified in tumor sections of animals for each group (saline, TRAIL exosomes, and NGFR exosomes). Statistical significance was achieved by TRAIL exosomes versus NGFR exosomes and saline using the Student *t* test; *, $P < 0.05$; **, $P < 0.01$; NS, not significant.

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In vivo administration of TRAIL exosomes led to clear signs of antitumor activity in the three tumor models here analyzed. Local multiple treatments were associated with a significant inhibition of tumor growth, paralleled by remarkable caspase-3 activation and necrosis. These data indicate a potential suitability of our approach for intratumor therapy, particularly in melanoma where the strategy of achieving disease control through local injection of proapoptotic agents (e.g., oncolytic viruses) has been recently approved by the FDA (40).

The systemic administration of TRAIL exosomes, leading to detectable but undoubtedly inferior homing to tumor site, also determined evident antitumor effects. However, only the highly sensitive SUDHL4 tumor was remarkably affected by the treatment, whereas no significant impact on the growth of INT12 melanoma and KMS11 myeloma could be observed, in spite of the signs of necrosis and vessel damage detected by histologic analysis. These latter results could be explained by the evidence that exosomes homing to tumor site represent only minor fractions of those administered, which are instead largely sequestered by the major organs (33). Indeed, a pharmacokinetic study performed with Near-Infrared (NIR)–labeled or unlabeled TRAIL exosomes revealed that injected vesicles were almost immediately cleared from the blood and principally localized in the liver, lungs, and spleen, being detectable subsequently also in kidneys and bone marrow (Supplementary Fig. S4).

At this regard, we must underline that an impact of the xenogeneic nature of TRAIL exosomes on the observed treatment efficacy cannot be ruled out in our experimental setting. Hence, studies in syngeneic models are in progress to confirm a potential clinical translatability of our approach. In addition, the homing properties of TRAIL exosomes could be improved by inserting tumor-specific receptors or ligands, or applying strategies to avoid their clearance by macrophages (41, 42). TRAIL-mediated tumor apoptosis in immunocompetent mice would also allow engaging systemic immunity, ideally promoting an amplified antitumor effect (43, 44). Importantly, these experiments would shed light on the potential toxicity generated by systemic administration of TRAIL exosomes.

Exosomes are acknowledged to bear advantages above synthetic nanovesicles for *in vivo* drug delivery, mostly related to the high stability in body fluids and their properties of "natural delivery system" (45–47). Furthermore, their elevated plasticity in terms of molecular manipulation makes exosomes more appealing than for instance liposomes, also tested for TRAIL delivery (12–14). Notably, covalently bound TRAIL carried by liposomes increased its therapeutic potential with respect to the recombinant soluble counterpart, sustaining our system of exosomes released by their donor cells with "natural" membrane TRAIL (48). Indeed, in addition to molecules for homing improvement, proteins or genetic material like miRNAs could also be cargoed into TRAIL exosomes to concomitantly overcome TRAIL resistance directly at tumor site. Another interesting strategy is represented by combining TRAIL exosomes with emerging anticancer natural compounds like piperlongumine, to increase DR5 expression and thereby sensitivity to TRAIL-mediated apoptosis (49). Obviously, it must be mentioned that exosomes, as likely independent entities, mediate a broad array of functions specific of the originating cells and in cancer they often promote disease progression (50). At this regard, we would like to underline that no major protumor effect was instead observed in the mouse models used in this study (Fig. 4B).

In summary, the delivery of TRAIL to sensitive cancers by exosomes appears as an attractive and efficient therapeutic approach, particularly for local treatment. TRAIL exosomes can be easily produced in large amounts and stored before administration. They could be combined with chemotherapeutics, small molecules, or natural compounds, aimed at augmenting TRAIL sensitivity by inducing death receptor expression, or loaded with drugs and genetic material to be delivered to cancer cells through uptake process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Study supervision: A.M. Gianni, V. Huber
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TNF-Related Apoptosis-Inducing Ligand (TRAIL)–Armed Exosomes Deliver Proapoptotic Signals to Tumor Site

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