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Targeting TRAIL Receptors with Genetically-Engineered CD34+ Hematopoietic Stem Cells

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1. Introduction

Dysregulated apoptosis plays a key role in the pathogenesis and progression of neoplastic disorders, allowing tumor cells to survive beyond their normal life-span, and to eventually acquire chemo-radioresistance (Laconi et al., 2000; Pommier et al., 2004). Thus, targeting either the intrinsic or the extrinsic pathways of apoptosis represent attractive therapeutic strategies for restoring apoptosis sensitivity of malignant cells, or activating agonists of apoptosis (Waxman & Schwartz, 2003). Due to the ability of death receptor ligands to induce cell death, there has been considerable interest in the physiological roles and therapeutic potential of these cytokines as anti-cancer agents. Death receptor ligands of the tumor necrosis factor α (TNF α) superfamily are type II transmembrane proteins that signal to target cells upon cell-cell contact, or after protease-mediated release to the extracellular space (Ashkenazi, 2002). Members of this family, including Fas ligand (FasL), TNF α , and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), stand out because of their ability to induce cell death (Wajant, 2003; Wiley et al., 1995).

2. Soluble TRAIL

Unlike other apoptosis-inducing TNF family members, soluble TRAIL appears to be inactive against normal healthy tissue (Ashkenazi et al., 1999; Lawrence et al., 2001). A variety of preclinical data clearly show that soluble TRAIL is a cancer cell-specific molecule exerting a remarkable antitumor activity both in vitro (Ashkenazi et al., 1999; Gazitt, 1999; Jin et al., 2004; Mitsiades et al., 2001; Pollack et al., 2001; Rieger et al., 1998) as well as in vivo in athymic nude mice or in non-obese diabetic/severe combined immunodeficient (NOD-SCID) mice (Ashkenazi et al., 1999; Daniel et al., 2007; Kelley et al., 2001).

The physiological functions of TRAIL are not yet fully understood, but mouse gene knockout studies indicate that this agent has an important role in antitumor surveillance by immune cells, mediates thymocyte apoptosis, and is important in the induction of autoimmune diseases (Cretney et al., 2002; Lamhamedi-Cherradi et al., 2003; Smyth et al., 2003).

TRAIL signals by interacting with its receptors. So far, five receptors have been identified, including the two agonistic receptors TRAIL-R1 (Pan et al., 1997b) and TRAIL-R2 (Walczak et al., 1997), and the three antagonistic receptors (Sheridan et al., 1997) TRAIL-R3 (Pan et al.,

1997a), TRAIL-R4 (Degli-Esposti et al., 1997), and osteoprotegerin (OPG) (Emery et al., 1998). Both TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins containing a cytoplasmic death domain (DD) motif that engage apoptotic machinery upon ligand binding (Almasan & Ashkenazi, 2003), whereas the other three receptors either act as decoys or transduce antiapoptotic signals (Wang & El-Deiry, 2003). TRAIL-R3 and TRAIL-R4 have close homology to the extracellular domains of agonistic receptors. TRAIL-R4 has a truncated, nonfunctional cytoplasmic DD, while TRAIL-R3 exists on the plasma membrane as a glycosphospholipid-anchored protein lacking the cytosolic tail. The physiological relevance of OPG as a soluble receptor for TRAIL is unclear, but a recent study suggests that cancer-derived OPG may be an important survival factor in hormone-resistant prostate cancer cells (Holen et al., 2002).

3. TRAIL-induced apoptosis signaling

Soluble TRAIL forms homotrimers that bind three receptor molecules, each at the interface between two of its subunits. A Zn atom bound to cysteine residues in the trimeric ligand is essential for trimer stability and optimal biologic activity. Binding of TRAIL to the extracellular domain of agonistic receptors results in trimerization of the receptors and clustering of the intracellular DDs, which leads to the recruitment of the adaptor molecule Fas-associated protein with death domain (FADD). Subsequently, FADD recruits initiator caspase-8 and -10, leading to the formation of the death-inducing signaling complex (DISC), where initiator caspases are autoactivated by proteolysis. Once they become enzymatically active, caspase-8 and/or -10 are released from the DISC and signal through two different proteolytic pathways that converge on caspase-3 and lead to cellular disassembly (Kaufmann & Steensma, 2005). In type I cells, activation of initiator caspases upon death receptors ligation is sufficient to directly activate downstream effector caspases, such as caspase-3 and/or -7 (Scaffidi et al., 1998). This extrinsic pathway is independent of the mitochondria and is not blocked by overexpression of Bcl-2. In type II cells, the commitment from death receptor ligation to apoptosis is less direct (Scaffidi et al., 1998). The amount of initially cleaved caspase-8 and/or -10 is not enough to directly trigger effector caspases activation. Consequently, apoptotic signaling requires an amplification loop by mitochondrial pathway engagement through caspase 8-mediated cleavage of Bid (BH3 interacting death domain agonist), which, in turn, induces the cytosolic Bcl-2 family member Bax (Bcl-2-associated X protein) and/or the loosely bound mitochondrial homolog Bak (Bcl-2 antagonist/killer) to insert into the mitochondrial membrane, where they contribute to the mitochondrial release of cytochrome c (Lucken-Ardjomande & Martinou, 2005). In the cytosol, cytochrome c binds the adaptor protein Apaf-1 (apoptotic protease activating factor 1) to form an apoptosome with recruitment and activation of the apoptosis-initiating caspase-9, which proteolytically activates additional caspase-3. These events are further amplified by apoptogenic factors released from the mitochondrial space, including Smac/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein with low pI) (Verhagen & Vaux, 2002).

4. Enhancing the antitumor efficacy of soluble TRAIL

Despite very promising preclinical in vitro and in vivo antitumor evidences, phase I/II clinical trials have demonstrated limited antitumor activity of soluble TRAIL likely due to

its short half-life and the consequent short exposure of tumor cells to the molecule (Ashkenazi et al., 2008). Because of soluble TRAIL's short half-life (Ashkenazi et al., 1999; Kelley et al., 2001; Walczak et al., 1999), it seems unlikely that the recommended soluble TRAIL dose of 8 mg/kg body weight will allow adequate exposure of tumor cells at high drug concentrations (Ashkenazi et al., 2008). Strategies to enhance the therapeutic activity of soluble TRAIL include combining it with conventional chemotherapy (Ballestrero et al., 2004) or with new agents such as histone deacetylase inhibitors that upregulate TRAIL-R1 and/or TRAIL-R2 (Inoue et al., 2004).

Gene therapy approaches have also been proposed to enhance TRAIL-mediated tumor cell targeting. Recently, a TRAIL-expressing adenoviral vector (Ad-TRAIL) has been shown to cause direct tumor cell killing, as well as a potent bystander effect through presentation of TRAIL by transduced normal cells (Lee et al., 2002). Thus, using Ad-TRAIL might be an alternative to systemic delivery of soluble TRAIL possibly resulting in better tumor cell targeting and increased tumoricidal activity (Armeanu et al., 2003; Griffith et al., 2000; Griffith & Broghammer, 2001; Kagawa et al., 2001; Lee et al., 2002). However, systemic Ad-TRAIL-based gene therapy requires efficient infection of target tumor cells as well as avoidance of immune clearance, and is limited by several safety and toxicity issues related to intravenous adenovector administration (Harrington et al., 2002). Intratumoral injection of TRAIL-encoding adenovectors has been successfully explored in a number of experimental models; however, this approach results in local antitumor activity and has little, if any, value in the treatment of disseminated tumors.

Alternatively, cell-based vehiculation of the full-length, membrane-bound (m)TRAIL (Griffith et al., 2009) has been proposed to achieve an optimal systemic delivery. Indeed, genetically modified stem/progenitor cells represent an innovative approach for delivery of anticancer molecules (Harrington et al., 2002; Introna et al., 2004). Due to their homing properties, systemically injected stem/progenitor cells could infiltrate both primary and metastatic tumor sites, thus allowing tumor-specific targeting (Burger & Kipps, 2006; Jin et al., 2006; Kaplan et al., 2007; Kucia et al., 2005; Loebinger et al., 2009; Najbauer et al., 2007; Rafii et al., 2002), and potentially overcoming limitations inherent to the pharmacokinetic profile of soluble drugs (Aboody et al., 2008; Griffith et al., 2009; Sasportas et al., 2009). Neural or mesenchymal stem cell-mediated mTRAIL delivery has been investigated in solid tumors (Grisendi et al., 2010; Kim et al., 2008; Loebinger et al., 2009; Menon et al., 2009; Mohr et al., 2008; Uzzaman et al., 2009).

In order to optimize the use of TRAIL-encoding adenovectors for the treatment of systemic tumors, we have recently investigated a cell-based approach using mobilized CD34+ hematopoietic cells transduced with a replication-deficient Ad-TRAIL (CD34-TRAIL+) encoding a full-length mTRAIL under the control of the CMV promoter (Carlo-Stella et al., 2006; Griffith et al., 2000). Several lines of evidence support the use of gene-modified CD34+ cells as optimal vehicles of antitumor molecules. In fact, CD34+ cells are already widely used in the clinical setting. Additionally, they can migrate from the bloodstream into tumor tissues due to the expression of adhesion receptors that specifically interact with counter-receptors on endothelial cells in the tumor microenvironment (Burger & Kipps, 2006; Kaplan et al., 2005; Verfaillie, 1998). Moreover, up-regulation of inflammatory chemo-attractants in the tumor microenvironment provides with a permissive milieu that potentially allows for homing of systemically delivered CD34-TRAIL+ cells and efficient tumor targeting (Jin et al., 2006). Using a multiplicity of infection (MOI) of 500, the transduction protocol optimized for the transduction of CD34+ cells consistently results in a transduction efficiency higher

than 80% (range 70% - 96%), a high level expression of mTRAIL, and a cell viability $\geq 85\%$. Flow cytometry analysis of CD34-TRAIL+ cells shows significant levels of transgene expression for at least 96 hours after transduction, and Western blot analysis reveals the presence of 32- and 55-kDa proteins, which are the expected products for full-length monomer and dimer TRAIL, respectively (Carlo-Stella et al., 2006).

5. Antitumor activity of mTRAIL-expressing cells

The antitumor activity of CD34-TRAIL+ cells has been investigated in a variety of localized and disseminated tumor models in NOD/SCID mice. Using a localized, subcutaneous multiple myeloma model (KMS-11 cell line), intravenously-injected mTRAIL-expressing cells significantly reduced tumor growth over controls as well as soluble TRAIL.¹ In fact, compared with untreated controls, both CD34-TRAIL+ cells and soluble TRAIL significantly inhibited tumor growth by day 28 after tumor injection, when tumor volumes were reduced by 38% ($P < .05$) and 31% ($P < .05$), respectively. However, on day 35, CD34-TRAIL+ cells induced a 40% reduction in tumor growth over controls (4.2 ± 1.2 vs 7.0 ± 2.0 g, $P < .001$), whereas a 29% reduction of tumor growth was detected in mice receiving soluble TRAIL (5.0 ± 1.7 g vs 7.0 ± 2.0 g, $P < .001$) (Lavazza et al., 2010). Even more importantly, an efficient antitumor activity of intravenously injected mTRAIL-expressing CD34+ cells was also detected in NOD/SCID mice bearing disseminated, systemic multiple myeloma and non-Hodgkin lymphoma xenografts (Carlo-Stella et al., 2006; Carlo-Stella et al., 2007; Carlo-Stella et al., 2008). Using KMS-11 as model system, treatment of advanced-stage disease with CD34-TRAIL+ cells resulted in a significant increase of median survival over controls (83 vs 55 days, $P \leq 0.0001$), with 28% of NOD/SCID mice alive and disease-free at the end of the 150-day observation period (Carlo-Stella et al., 2006).²

6. In vivo homing of CD34-TRAIL+ cells

Homing properties of transduced cells in healthy tissues as well as tumor nodules were extensively investigated in tumor-bearing NOD/SCID mice who received a single

¹ Six- to eight-week-old female NOD/SCID mice with body weight of 20 to 25 g were purchased from Charles River (Milano, Italy, EU). Mice were housed under standard laboratory conditions according to our institutional guidelines. Animal experiments were performed according to the Italian laws (D.L. 116/92 and following additions), and were approved by the institutional Ethical Committee for Animal Experimentation. KMS-11 cells (5×10^6 cells/mouse) were inoculated subcutaneously in the left flank of each mouse. When tumor reached approximately 7 - 10 mm in diameter (usually 10-12 days after tumor inoculation), mice were randomly assigned to planned treatments consisting of daily injections of either CD34-TRAIL+ cells or mock-transduced CD34+ cells (1×10^6 cells/mouse/injection/day, intravenous, days 12-15), or a 4-day course of recombinant soluble TRAIL (30 mg/kg/day, intraperitoneal, days 12-15). Mice were checked twice weekly for tumor appearance, tumor dimensions, body weight, and toxicity. Tumor volumes were measured with calipers and their weights calculated using the formula: $(a \times b^2)/2$, where a and b represented the longest and shortest diameters, respectively. Mice were followed up for 3 weeks after the end of the treatments. The endpoint of the subcutaneous model was tumor weight. Each experiment was performed on at least two separate occasions, using five mice per treatment group.

² KMS-11 (0.5×10^6 cells/mouse) cell line was inoculated intravenously. CD34-mock or CD34-TRAIL+ cells (1×10^6 cells/mouse/injection) were inoculated intravenously weekly for 4 weeks starting either on day 7 (early-stage tumor model), or 14 (advanced-stage tumor model) after tumor cell injection.

intravenous injection of CD34-TRAIL+ cells (3×10^6 cells/mouse) (Lavazza et al., 2010). Tumor and healthy tissue sections were immunostained with an anti-human CD45 antibody and digitally recorded to count transduced cells on entire tissue sections.³ Early following injection, transduced cells were detected at high frequencies in the lung, liver and spleen (Figure 1). CD34-TRAIL+ cells progressively decreased and were no longer detectable in these tissues 24 hours after injection. Bone marrow CD34-TRAIL+ cells peaked 5 hours after injection and were detectable up to 24 hours. Low frequencies of transduced cells were detected within tumors as early as 30 minutes following injection. They progressively increased and peaked 48 hours post-injection when on average 188 ± 25 CD45+ cells per 10^5 tumor cells (i.e., $0.2 \pm 0.03\%$) were recorded (Figure 1). Overall, kinetics data suggest that transduced cells transiently circulate through healthy tissues, whereas they are preferentially recruited within tumor nodules, allowing to hypothesize that homing signals by tumor endothelial cells actively promote intratumor homing of transduced cells.

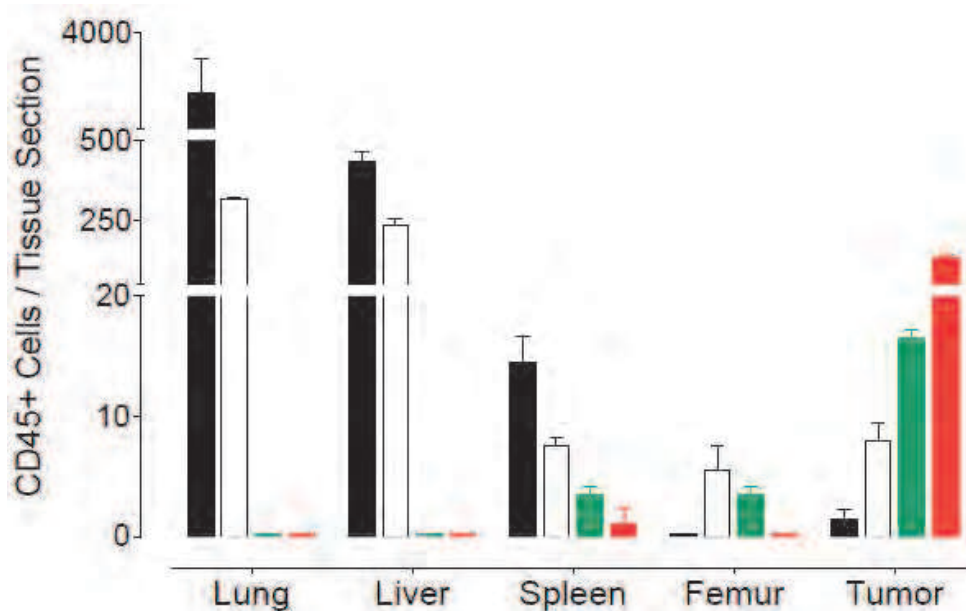


Fig. 1. Tissue kinetics of CD34-TRAIL+ cells. Lung, liver, spleen, femur, and tumor nodules were harvested from tumor-bearing NOD/SCID mice 0.5 (■), 5 (□), 24 (■), and 48 (■) hours after a single intravenous injection of CD34-TRAIL+ cells (3×10^6 cells/mouse). Shown is the quantification of CD34-TRAIL+ cells on digitally acquired tissue sections stained with anti-CD45. Frequency of CD34-TRAIL+ cells is expressed as the mean (\pm SD) number of CD45+ cells per tissue section.

³ Images of tissue sections were acquired at 20 \times magnification with an automatic high-resolution scanner (dotSlide System, Olympus, Tokyo, Japan) and subdivided into a collection of non-overlapping red, green, and blue (RGB) images in TIFF format (final resolution 3.125 pixels/ μ m). Image analysis was carried out using the open-source ImageJ software (<http://rsb.info.nih.gov/ij/>). Routines for image analysis were coded in ImageJ macro language and executed on RGB images without further treatment. Per each experimental condition, at least three sections from different tumor nodules or healthy tissues were analyzed. Intratumor frequency of CD34-TRAIL+ cells was expressed as the number of CD45+ cells per total cells per tissue section. Total cells were counted by the ImageJ internal function for particle analysis, whereas CD45+ cells were manually counted in all images from whole scanning of histochemically stained tissue sections.

7. Vascular signals involved in tumor homing

This issue was investigated by evaluating the expression of homing receptors on tumor vasculature. Confocal microscopy⁴ revealed that 30% of tumor vessels expressed high levels of VCAM-1 on the luminal surface (**Figure 2_{b-c}**) (Jin et al., 2006), whereas SDF-1 was ubiquitously expressed on tumor vessels and tumor cells (**Figure 2_{e-f}**). Thus, $\alpha 4\beta 1$ integrins and the CXCR4 chemokine (De Raeye et al., 2004; Peled et al., 1999) seem to play a critical role in regulating intratumor homing of mTRAIL-expressing cells. To further investigate the functional relevance of SDF-1/CXCR4 and VCAM-1/VLA-4 pathways in mediating tumor homing of transduced cells, inhibitory experiments with an anti-VCAM-1 antibody and the CXCR4 antagonist AMD3100 were performed.⁵ As compared to controls, tumor homing of CD34-TRAIL⁺ cells was significantly reduced in mice administered with anti-VCAM-1 antibody [$0.2 \pm 0.03\%$ vs $0.09 \pm 0.01\%$ ($P = .001$)] or the CXCR4 antagonist AMD3100 (Fricker et al., 2006) [$0.2 \pm 0.03\%$ vs $0.05 \pm 0.006\%$ ($P = .0003$)]. Tumor vasculature was also analyzed for the expression of TRAIL-R2 receptor. Indeed, confocal microscopy revealed that approximately 8 - 12% of tumor endothelial cells expressed TRAIL-R2 receptor on their luminal surface (**Figure 2_{h-i}**), suggesting that mechanisms other than SDF-1/CXCR4 and VCAM-1/VLA-4, such as the mTRAIL/TRAIL-R2 interactions, might be involved in regulating intratumor homing as well as functional activity of CD34-TRAIL⁺ cells (Lavazza et al., 2010).

8. CD34-TRAIL⁺ cells induce tumor cell apoptosis and hemorrhagic necrosis

Tumor-homing of CD34-TRAIL⁺ cells is associated with significant levels of tumor cell apoptosis (Carlo-Stella et al., 2006). To obtain an objective quantification of apoptosis, a computer-aided image analysis using ImageJ software was performed.⁶ As compared to controls, TUNEL⁺ cells were increased by 8- ($2.4 \pm 1.4\%$ vs $0.3 \pm 0.3\%$, $P < .0001$) and 4-fold ($1.2 \pm 0.7\%$ vs $0.3 \pm 0.3\%$, $P < .0001$) following treatment with CD34-TRAIL⁺ cells and soluble TRAIL, respectively (**Figure 3A**). Interestingly, apoptotic effects of CD34-TRAIL⁺ cells resulted significantly more potent than those exerted by soluble TRAIL ($P < .0001$). Additionally, TUNEL staining of tumor sections from untreated, mock- and soluble TRAIL-

⁴ Cryosections were fixed with cold acetone, rinsed with PBS, and then blocked with 2% BSA. Sections were first incubated with the appropriate primary antibody, including mouse anti-human stromal cell-derived factor-1 (SDF-1) (R&D Systems), rat anti-mouse VCAM-1 (Southern Biotech), or hamster anti-mouse TRAIL-R2 (BD Pharmingen). After washing, sections were incubated with the appropriate Alexa Fluor 568-conjugated secondary antibody (Invitrogen). Biotinylated tumor vessels were revealed with Alexa Fluor 488-conjugated streptavidin (Invitrogen). Sections were examined under an epifluorescent microscope equipped with a laser confocal system (MRC-1024, Bio-Rad Laboratories). Image processing was carried out using LaserSharp computer software (Bio-Rad Laboratories).

⁵ To inhibit intratumor homing of CD34-TRAIL⁺ cells, mice received either one single intraperitoneal dose of anti-VCAM-1 (vascular cell adhesion molecule-1) antibody (clone M/K-2; Southern Biotech, Birmingham, AL, USA) at 0.5 mg/mouse, 3 hours before cell administration, or two doses of AMD3100 (5 mg/kg, subcutaneous, 1 hour prior to and 3 hours after cell administration).

⁶ The number of total and TUNEL⁺ cells per section was counted as follows. Briefly, the dynamic range of images was expanded to full by contrast enhancement, and cells were identified by appropriate filtering in the red, green, and blue (RGB) channels. Resulting black and white images were combined to represent only pixels selected in every color channel. For each image, both total and TUNEL⁺ cells were counted by the ImageJ internal function for particle analysis.

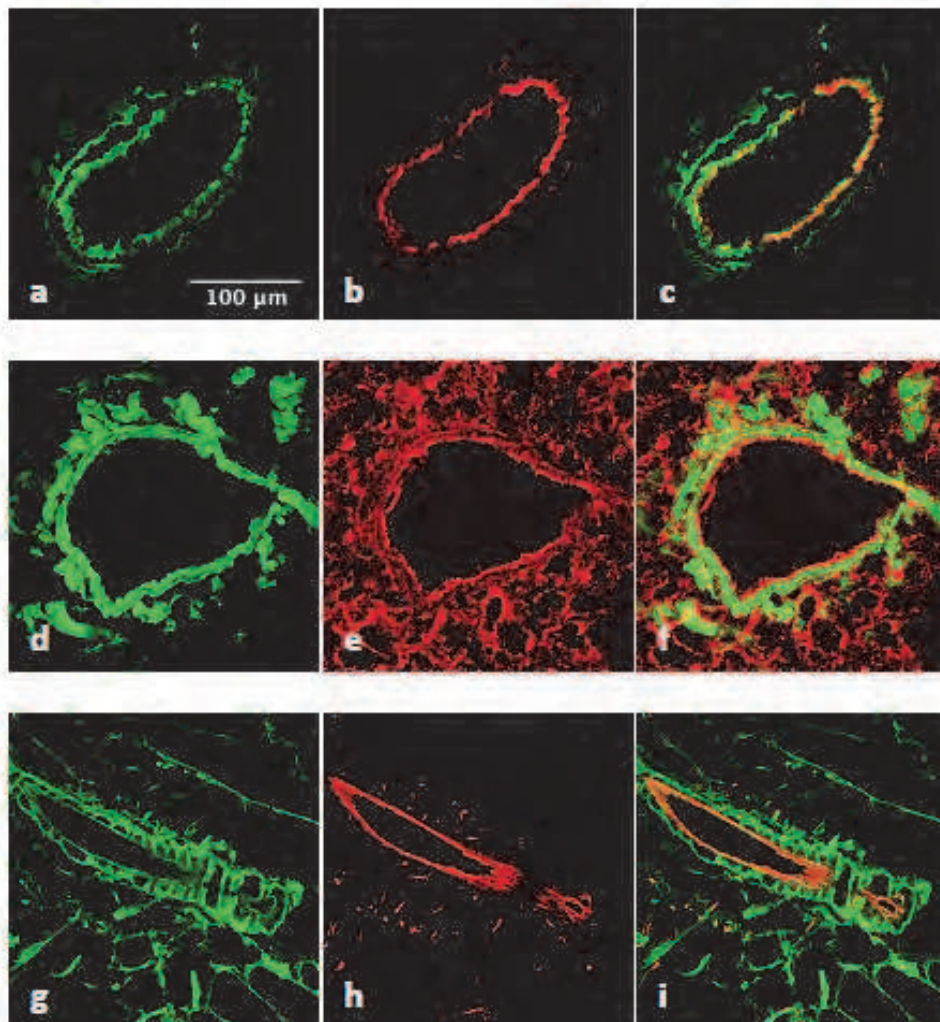


Fig. 2. Vascular molecules involved in intratumor homing of CD34-TRAIL+ cells. Confocal microscopy analysis of intratumor recruiting signals was carried out on 4- μ m cryosections from in vivo biotinylated tumors. Cryosections were stained with Alexa Fluor 488-conjugated streptavidin (*green*) to detect tumor vasculature (*a, d, g*). Cryosections were also stained with anti-VCAM-1 (*b*), anti-SDF-1 (*e*), or anti-TRAIL-R2 (*h*) followed by the appropriate Alexa Fluor 568-conjugated secondary antibody for indirect detection of the corresponding antigen (*red*). Merged images demonstrate VCAM-1 (*c*), SDF-1 (*f*), or TRAIL-R2 (*i*) expression by endothelial cells. Objective lens, 40 \times .

treated mice revealed a homogeneous mass of viable cells with necrotic areas accounting only for $1.4 \pm 1.0\%$, $1.8 \pm 1\%$, and $2.9 \pm 1\%$ of total tissue, respectively (**Figure 3B**). In contrast, tumors from CD34-TRAIL-treated mice displayed a significant increase of necrotic areas as compared to controls, with percentages of necrotic areas per tissue section ranging from 6% to 18%, and a mean 8-fold increase over controls ($11 \pm 3.8\%$ vs $1.4 \pm 1.0\%$, $P < .0001$), and 4-fold increase over soluble TRAIL-treated mice ($11 \pm 3.8\%$ vs $2.9 \pm 1\%$, $P = .0001$) (**Figure 3B**). Pharmacological inhibition of intratumor recruitment of CD34-TRAIL+ cells using AMD3100, or anti-VCAM-1 antibody significantly reduced necrotic areas by 37% ($P = .02$) and 56% ($P = .002$), respectively (**Figure 3C**), suggesting that intratumor recruitment of CD34-TRAIL+ cells specifically triggered tumor necrosis.

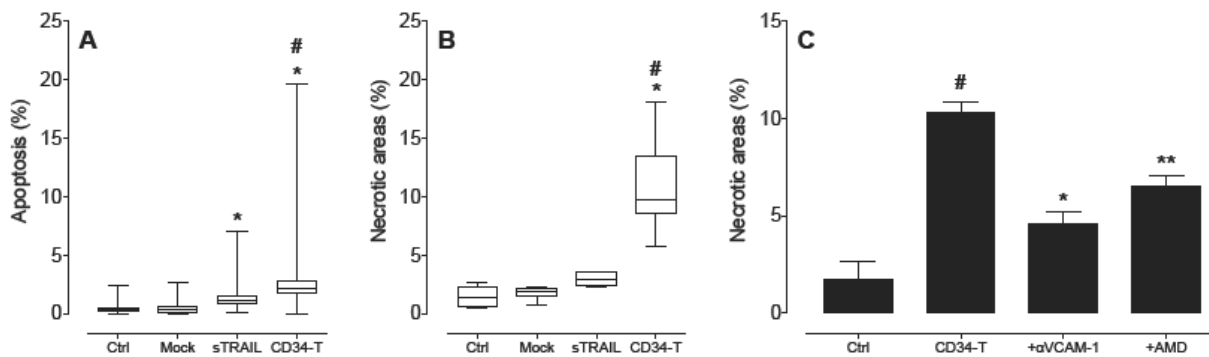


Fig. 3. Pro-apoptotic and necrotic effects of CD34-TRAIL+ cells. NOD/SCID mice bearing subcutaneous tumor nodules 10 mm in diameter were randomly assigned to receive CD34-TRAIL+ cells, mock-transduced CD34+ cells (3×10^6 cells/mouse, intravenous), recombinant soluble TRAIL (500 μ g/mouse, IP), or control vehicle. (A) Percentages of apoptotic cells in tumors from untreated or treated animals were computationally calculated on digitally acquired images (objective lens, 20 \times) using ImageJ. At least three sections from different animals were analyzed. The boxes extend from the 25th to the 75th percentiles, the lines indicate the median values, and the whiskers indicate the range of values. * $P < .0001$, compared to controls. # $P < .0001$, compared to soluble TRAIL. (B) Quantification of necrotic areas by ImageJ analysis on tissue sections stained with TUNEL. At least six sections from different animals were analyzed per treatment group. * $P < .0001$, compared to controls. # $P = .0001$, compared to soluble TRAIL. (C) Anti-VCAM-1 and AMD3100 reduced tumor necrosis in mice treated with CD34-TRAIL+ cells. # $P < .0001$, compared to control. * $P = .002$, compared with CD34-TRAIL+ cells. ** $P = .02$, compared with CD34-TRAIL+ cells.

A distinctive and prominent feature of tumors treated with CD34-TRAIL+ cells was represented by hemorrhagic phenomena within necrotic areas close to damaged vessels which were detected by immunohistochemical staining with glycophorin A (Figure 4A). Hemorrhagic phenomena exactly matched TUNEL+ necrotic areas and closely associated with apoptotic endothelial cells (Figure 4B_L). In striking contrast, apoptotic vessels and hemorrhagic phenomena could not be detected neither in tumors from mice treated with soluble TRAIL (Figure 4B_K and 4A), nor in healthy tissues (Figure 5), suggesting a tumor-restricted antivascular activity by CD34-TRAIL+ cells.

9. Antivascular effects of CD34-TRAIL+ cells

To better understand the relationship between the antitumor effects of CD34-TRAIL+ cells and apoptosis of endothelial cells, an extensive vascular analysis⁷ was performed on

⁷ Tumor vasculature was analyzed on cryosections using the open-source ImageJ software (<http://rsb.info.nih.gov/ij/>) from in vivo biotinylated mice stained with HRP-conjugated streptavidin. To calculate endothelial area, i.e., the percentage of tissue section occupied by endothelium, endothelial cells were identified by contrast enhancement and appropriate filtering. Background signal was removed considering only structures larger than an arbitrary minimal value. To analyze vessel wall thickness, we manually selected rectangular regions of RGB input images containing at least a hollow vessel. An automatic routine computed vessel thickness according to the formula: Thickness = $2 \times (\text{vessel area}) / [(\text{vessel perimeter}) + (\text{lumen perimeter})]$. At first, endothelial tissue was identified applying a threshold on the blue channel and obtaining a binary image representative of its

histological sections from tumors treated with transduced cells and subsequently in vivo biotinylated to detect tumor vasculature (Lavazza et al., 2010; Rybak et al., 2005). In untreated mice, tumor vasculature was abundant, tortuous, and evenly distributed throughout the tumor (**Figure 6A**). In striking contrast, in NOD/SCID mice treated with CD34-TRAIL+ cells, viable tumor cells surrounding necrotic areas appeared deficient in capillaries and small-caliber blood vessels, which were less tortuous and had fewer branches and sprouts (**Figure 6A**). Globally, mean percentages of endothelial areas from control and mock-treated tumors were $8.8 \pm 5.6\%$ and $8.2 \pm 3.3\%$, respectively (**Figure 6B**). Administration of soluble TRAIL did not affect endothelial area compared to controls ($8.1 \pm 2.9\%$ vs $8.8 \pm 5.6\%$, $P = \text{ns}$). In contrast, a single intravenous injection of 3×10^6 CD34-TRAIL+ cells caused a 37% decrease of endothelial area compared to control ($5.6 \pm 3.2\%$ vs $8.8 \pm 5.6\%$, $P < .0001$) (**Figure 6B**). Additionally, blood vessels from tumors treated with CD34-TRAIL+ cells were thicker than those observed in untreated or soluble TRAIL-treated animals (**Figure 6A**). Based on these findings, we isolated images of transversally oriented vessels in streptavidin-HPR stained sections and calculated vessel wall thickness by processing images with ImageJ and specifically written macros. As shown in **Figure 6C**, wall thickness was 1.7-fold increased compared to control (5.5 ± 1.4 vs $3.2 \pm 0.8 \mu\text{m}$, $P < .0001$), whereas no increases emerged after soluble TRAIL administration (3.3 ± 0.7 vs $3.2 \pm 0.8 \mu\text{m}$).

10. Conclusions

Experimental data obtained in a variety of preclinical models of both localized and disseminated disease strongly suggest that TRAIL-expressing CD34+ cells can efficiently vehiculate mTRAIL within the tumors where they exert potent antivasculature and antitumor activities resulting in a significant reduction of tumor growth. Analysis of tumor nodules obtained 48 hours after a single administration of transduced cells showed that TRAIL-expressing cells were 2-fold more effective than soluble TRAIL in inducing apoptosis of tumor cells. Broad necrotic events, involving up to 18% of tumor tissue, were detected only after administration of CD34-TRAIL+ cells and were associated with a hemorrhagic component which was not detectable after soluble TRAIL administration. Hemorrhagic

distribution. Then, the lumen of each vessel in the image was identified as a non-endothelial area, ringed by endothelial tissue and greater than an arbitrary threshold. This procedure rejected smaller artifacts and allowed for recognition of hollow vessels even when erythrocytes or other cells occupied the lumen. Subsequently, we identified the endothelium surrounding a given lumen by an iterative procedure. At first, we subdivided the binary representation of stained tissue into areas by means of a watershed algorithm. Then we selected only those regions adjacent to the lumen, obtaining a minimal image of the vessel wall. This minimal image was used to compute a working thickness according to the previously stated formula. Then, to avoid arbitrary removal of bona fide portions of the walls, we calculated a theoretical vessel contour expanding the lumen outline by a number of pixels equivalent to the working thickness. We next turned back to the watersheded image of endothelium distribution, selecting only those areas connected with the new, theoretical contour. Inclusion of the new regions in the minimal image produced the final vessel image. Both images were saved to allow for manual appreciation of proper vessel identification. At last, the final vessel thickness was calculated after assessment of the final vessel area and external perimeter. Per each parameter, the accuracy and appropriate cut-off levels were determined by comparing processed images to the RGB originals. In all instances, automatic routines were validated by comparing results with those obtained by visual counting of up to 10% of the total images by two independent pathologists.

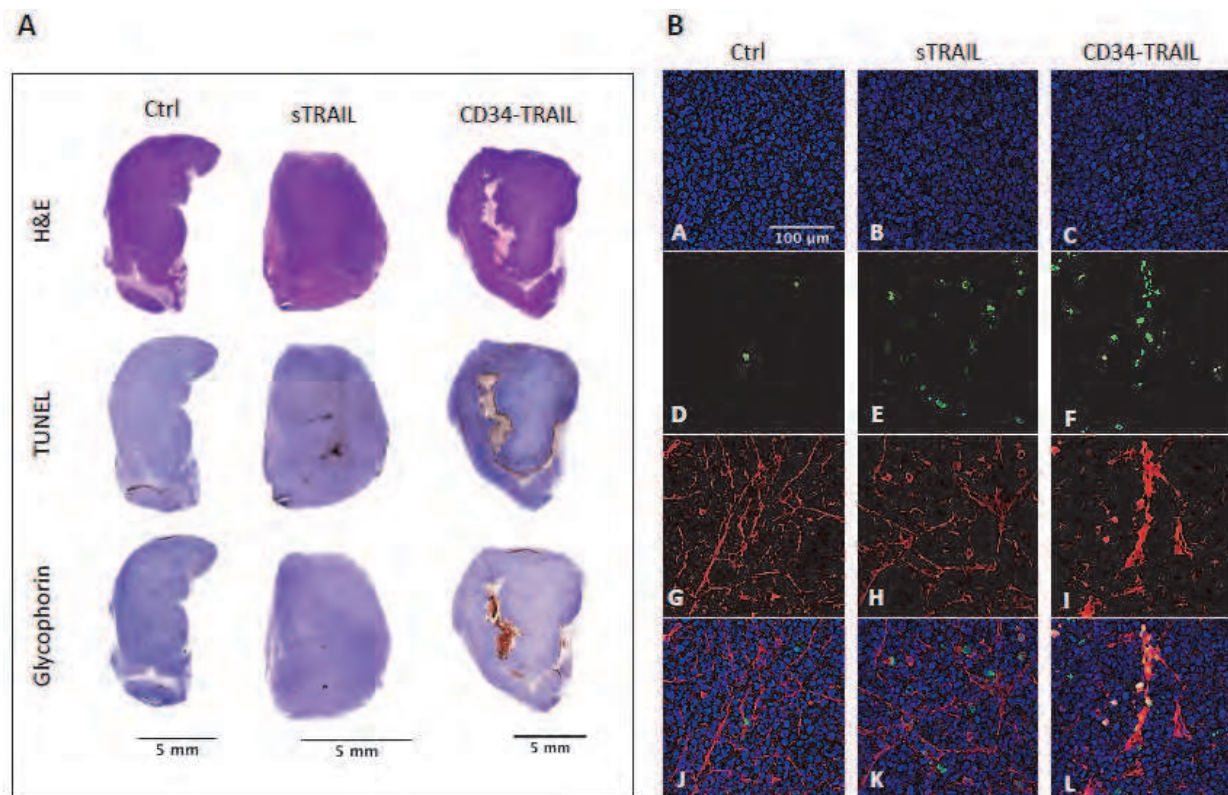


Fig. 4. Tumor hemorrhagic necrosis and endothelial cell apoptosis induced by CD34-TRAIL+ cells. NOD/SCID mice bearing subcutaneous tumor nodules 10 mm in diameter were randomly assigned to receive CD34-TRAIL+ cells, recombinant TRAIL (500 μg/mouse, IP), or control vehicle. Tumors were harvested forty-eight hours after treatment. (A) Hematoxylin and eosin (H&E), TUNEL and glycophorin A staining were performed. Objective lens, 2×. (B) Just before sacrifice NOD/SCID mice were intravenously injected with sulfo-NHS-LC-biotin to biotinylate tumor vasculature. Representative confocal images of tumors from untreated and treated animals processed by triple immunofluorescence staining. (A–C) Cell nuclei were detected in blue by TO-PRO-3; (D–F) apoptotic cells were detected in green by TUNEL staining; (G–I) tumor endothelial cells were detected in red by Alexa 568-conjugated streptavidin. (J–L) After merging of single-color images, apoptotic nuclei (green) were detectable throughout tumor parenchyma after treatment with either soluble TRAIL or CD34-TRAIL+ cells, whereas endothelial cells with apoptotic nuclei (yellow) could be detected only in CD34-TRAIL+ cell-treated animals. Objective lens, 40×.

necrosis was localized near TUNEL+ blood vessels, suggesting that apoptosis of tumor endothelial cells represents an early event triggered by CD34-TRAIL+ cells. Overall, these findings support the hypothesis that CD34-TRAIL+ cells exert their cytotoxic activity not only by targeting parenchymal tumor cells but also by targeting tumor vasculature (Carlo-Stella et al., 2006; Lavazza et al., 2010). Indeed, the vascular-disrupting activity of mTRAIL might represent a major concern in view of clinical applications. Notwithstanding the intratumor vascular-disrupting activity of mTRAIL, extensive analysis of healthy tissues failed to detect any evidence of hemorrhagic necrosis, suggesting that vascular damage was tumor-restricted.

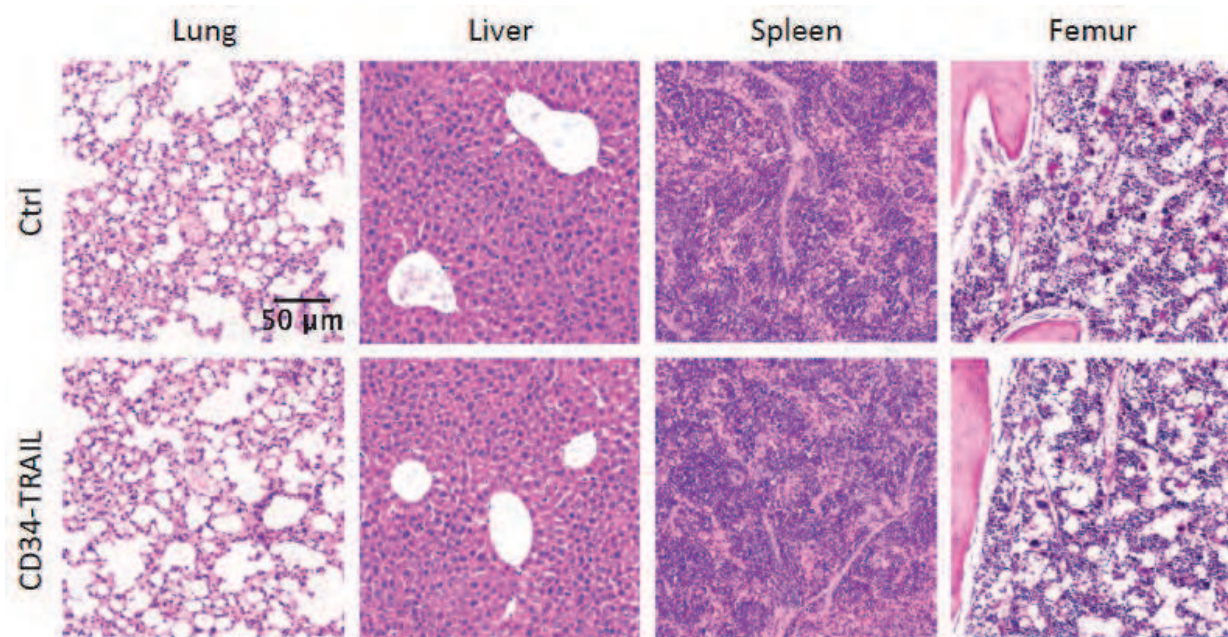


Fig. 5. Tissue and vascular toxicity in healthy tissue after CD34-TRAIL+ cells administration. NOD/SCID mice bearing subcutaneous tumor nodules received a single intravenous injection of CD34-TRAIL+ cells (3×10^6 cells/mouse), or control vehicle. Forty-eight hours after treatment, lung, liver, spleen, and femur were harvested and analyzed. Hematoxylin and eosin staining demonstrated the absence of tissue or vascular damage. Representative histological images are shown. Objective lens, 10x.

Increasing evidences suggest that recruitment of CD34+ cells in the tumor microenvironment is due to homing signals similar to those found in the bone marrow hematopoietic niches (Jin et al., 2006; Kaplan et al., 2007; Rafii et al., 2002; Wels et al., 2008). Both SDF-1/CXCR4 and VCAM-1/VLA-4 pathways play a key role in regulating bone marrow homing of transplanted hematopoietic stem cells (Aiuti et al., 1997; Peled et al., 1999) as well as intratumor recruitment of CXCR4-expressing cells and neovascularization during acute ischemia and tumor growth (Burger & Kipps, 2006; Jin et al., 2006; Petit et al., 2007). Kinetics data obtained in our models clearly show that intravenously injected transduced cells circulate in normal tissues up to 24 hours, but they progressively and preferentially home at tumor sites where they can be detected up to 48 hours after injection. Lack of intratumor detection of CD34-TRAIL+ cells beyond 48 hours after injection (data not shown) may be due to destruction of mTRAIL-expressing cells in the context of antitumor activities (i.e., disruption of tumor vasculature, hemorrhagic necrosis, tumor necrosis, etc.). Pharmacological manipulation of adhesion receptor expression using either AMD3100 or anti-VCAM-1 antibodies significantly reduced both the frequency and the antitumor efficacy of CD34-TRAIL+ cells strongly suggesting that SDF-1 and VCAM-1 expressed by tumor vasculature efficiently recruit transduced CD34+ cells within tumors by challenging their trafficking and homing properties. The role of additional binding systems, such as mTRAIL/TRAIL-R2, in mediating tumor tropism of CD34-TRAIL+ cells may be hypothesized on the basis of our data. Binding of CD34-TRAIL+ cells to TRAIL-R2 expressed by tumor vasculature could significantly contribute to initiation of a cascade of events that induce early endothelial damage, leading to extensive tumor cell death (Arafat et al., 2000).

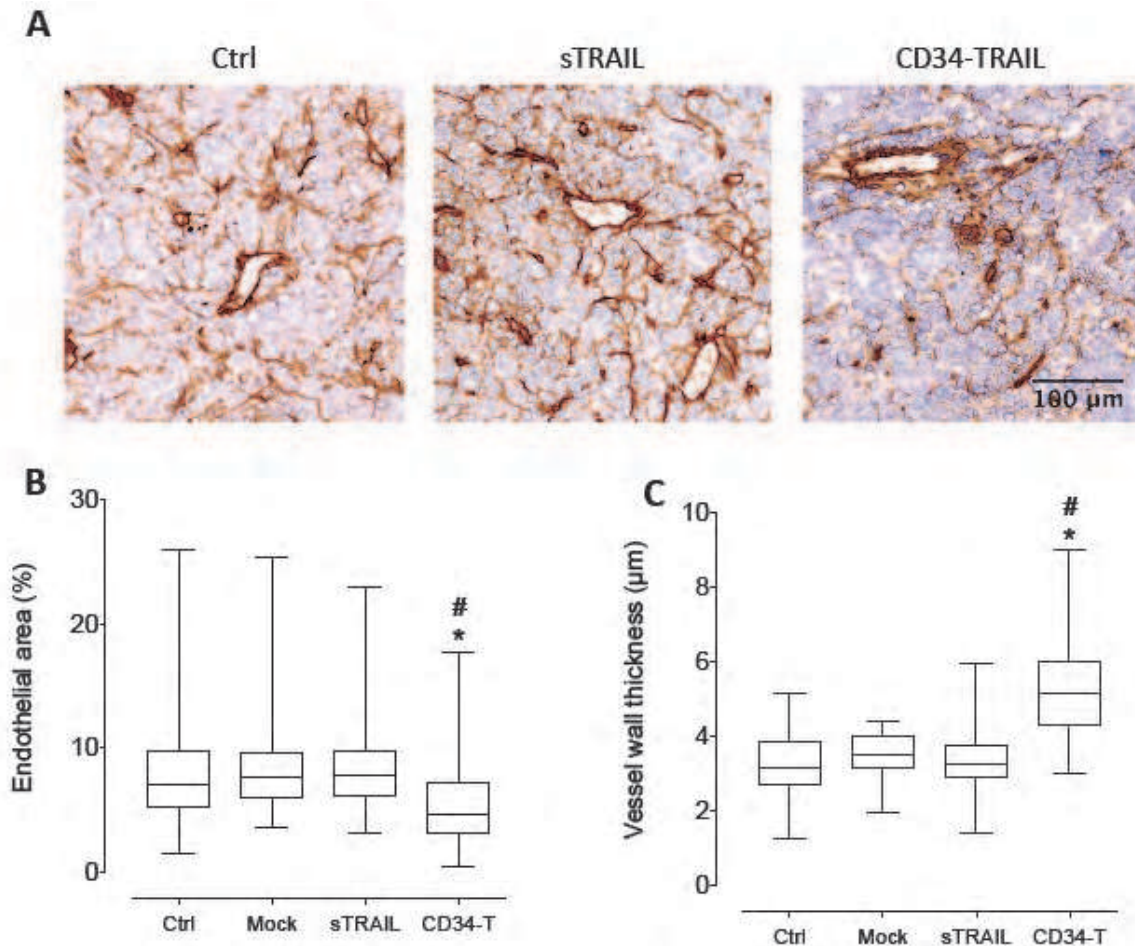


Fig. 6. Antivascular effects of CD34-TRAIL⁺ cells. NOD/SCID mice bearing subcutaneous tumor nodules 10 mm in diameter were randomly assigned to receive CD34-TRAIL⁺ cells, mock-transduced CD34⁺ cells (3×10^6 cells/mouse, intravenous), recombinant soluble TRAIL (500 μg /mouse, IP), and control vehicle. (A) Forty-eight hours after treatment, NOD/SCID mice were intravenously injected with 0.2 mL of sulfo-NHS-LC-biotin (5 mg/mL) to biotinylate tumor vasculature. Tumors were then excised, and biotinylated endothelium was revealed by HRP-streptavidin and 3,3'-diaminobenzidine for light microscopy analysis. Representative histological images of *in vivo* biotinylated mice receiving the different treatments are shown. (B) Sections were analyzed using ImageJ for quantification of vascular parameters. Endothelial area was calculated on whole tissue sections as (streptavidin-HRP stained area)/(total tissue area) \times 100. * $P < .0001$, compared to controls. # $P < .0001$, compared to soluble TRAIL. (C) Vessel wall thickness was calculated on transversally oriented vessels. * $P < .0001$, compared to controls. # $P < .0001$, compared to soluble TRAIL.

In conclusion, under our experimental conditions the use of transduced CD34⁺ cells as a vehicle of mTRAIL resulted in an antitumor effect greater than that exerted by soluble TRAIL, likely because of an antivascular action. Our findings appear to be of outstanding interest in the context of the increasing need for therapeutic strategies targeting not only tumor cells but also the tumor microenvironment (De Raeve et al., 2004; Joyce, 2005; Rafii et al., 2002). Finally, the clinical feasibility of such a systemic CD34⁺ cell-based gene therapy

approach could be exploited to develop effective autologous or allogeneic anticancer treatments.

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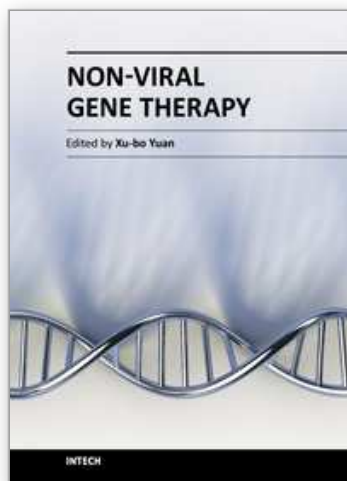
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