Simultaneous targeting of VEGF-receptors 2 and 3 with immunoliposomes enhances therapeutic efficacy

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Abstract
Background: Tumor progression depends on angiogenesis. Vascular endothelial growth factor (VEGF) receptors (VEGFRs) are the main signal transducers that stimulate endothelial cell migration and vessel sprouting. At present, only VEGFR2 is targeted in the clinical practice.
Purpose: To develop new, anti-angiogenic nanoparticles (immunoliposomes, ILs), that redirect cytotoxic compounds to tumor-associated vascular cells.
Methods: Pegylated liposomal doxorubicin (PLD) was targeted against VEGFR2- and VEGFR3-expressing cells by inserting anti-VEGFR2 and/or anti-VEGFR3 antibody fragments into the lipid bilayer membrane of PLD. These constructs were tested in vitro, and in vivo in the Rip1Tag2 mouse model of human cancer.
Results: The combination treatment with anti-VEGFR2-ILs-dox and anti-VEGFR3-ILs-dox was superior to targeting only VEGFR2 cells and provides a highly efficient approach of depleting tumor-associated vasculature. This leads to tumor starvation and pronounced reduction of tumor burden.
Conclusion: Nanoparticles against VEGFR2 and VEGFR3 expressing tumor-associated endothelial cells represent a promising and novel anti-cancer strategy.

Keywords
Anti-angiogenic therapy, nanoparticle, targeted drug delivery, VEGFR2, VEGFR3

Introduction
Nanoparticles, including liposomes, circulate for a prolonged period in the blood stream, resulting in a superior accumulation of encapsulated drugs in tumor tissue. This process is called “enhanced permeability and retention (EPR)” effect and is thought to be due to the leaky and disorganized nature of tumor vasculature. However, nanoparticles per se do not provide targeted delivery of compounds into a specific cell type. Through conjugation of a monoclonal antibody (mAb) fragment to the surface of immunoliposomes (ILs), these nanoparticles achieve true targeted delivery on top of the EPR effect. The mAb fragment allows for highly specific binding and internalization of the liposomes into the target cell [1–3].

The initiation and maintenance of vascularization is a rate-limiting step in tumor progression. Angiogenesis (formation of new vessels from pre-existing ones) and vasculogenesis (de novo formation of vessels) are promoted by the tumor in order to meet its increasing demand for oxygen and nutrients.

Tumor-associated endothelial cells express a different set of genes than endothelial cells lining physiological vessels. Antigens that are selectively up-regulated on activated tumor-associated endothelial cells include vascular endothelial growth factor receptor 2 (VEGFR2) and vascular endothelial growth factor receptor 3 (VEGFR3) [4–6] making them potential targets for directed nanoparticulate anti-angiogenic cancer therapy. While VEGFR2 is expressed selectively on activated endothelial cells, VEGFR3 is also present on lymphatic endothelial cells. In blood vessels, VEGFR3 is selectively expressed on path-leading endothelial tip cells [4–8]. Activation of the VEGFR2 by VEGF-A, secreted predominantly by tumor cells and tumor-infiltrating inflammatory cells, is the prime event of the angiogenic switch in tumor vascularization. VEGF-C and VEGF-D are ligands for VEGFR3. They can also bind to VEGFR2 and induce heterodimerization of VEGFR2 and VEGFR3, which is a potent mechanism of inducing vessel sprouting.

Several anti-angiogenic drugs have been introduced into the clinic. The humanized anti-VEGF-A antibody bevacizumab was tested in clinical trials in combination with chemotherapy for metastatic cancers, such as breast, colorectal, renal, brain and non-small-cell-lung cancer [9–13]. Further, anti-angiogenic drugs in the clinic include the VEGFR tyrosine kinase inhibitors sunitinib, pazopanib, sorafenib and vandetanib.
However, none of those compounds directly deplete endothelial cells in vivo.

We have previously shown that anti-VEGFR2-ILs are highly efficient anti-neoplastic nanoparticles in several in vivo and in vitro models of human cancer [14]. In this study, we target tumor vasculature using anti-VEGFR3 with or without anti-VEGFR2-ILs in order to deliver doxorubicin, a cytotoxic anthracycline, directly to tumor-associated endothelial cells. We tested anti-VEGFR3 and anti-VEGFR2-ILs in the Rip1Tag2 transgenic mouse model of pancreatic β-cell tumorigenesis (insulinoma). All ILs-treated cohorts had a significant reduction in tumor burden and diminished blood vessel density compared to untargeted liposomal doxorubicin (PLD). The combination of anti-VEGFR3-ILs-dox with anti-VEGFR2-ILs-dox significantly reduced tumor burden compared to anti-VEGFR2-ILs-dox alone. Hence, a combined targeting of VEGFR2 and 3-positive endothelial cells with doxorubicin-loaded ILs is superior to isolated targeting of VEGFR2 and, thus, a promising approach for efficient anti-angiogenic therapy in the clinic.

Methods

Liposome preparation
Fluorescence-labeled and empty liposomes were prepared by a lipid film hydration–extrusion method using repeated freeze–thawing steps to hydrate the lipid films [15,16]. Liposomes were composed of DSPC, cholesterol and mPEG–DSPE in a molar ratio of 3:2:0.3 (for fluorescent-labeled liposomes) by co-incubation at 55°C for 30 min at a protein/liposome ratio of 60μg Fab'/μmol PL [21,22]. Unincorporated conjugates and free drug were separated from ILs by sepharose CL-4B gel filtration. The incorporation efficiency of conjugated Fab' fragments was determined by SDS–PAGE [19]. Reagents for liposome and IL preparation are listed in Supplementary File S1.

Preparation of MAb fragments and ILs
Anti-VEGFR2 and anti-VEGFR3 rat MAb against the extra-cellular domain of mouse VEGFR2 or VEGFR3 [18] were cleaved and reduced to Fab' as previously described [19]. For ILs, Fab' was covalently conjugated to maleimide groups at the termini of PEG–DSPE chains (Mal–PEG–DSPE) [20]. MAb fragment conjugates (Fab’–Mal–PEG–DSPE) were incorporated into liposomes by co-incubation at 55°C for 30 min at a protein/liposome ratio of 60μg Fab'/μmol PL [21,22]. Unincorporated conjugates and free drug were separated from ILs by sepharose CL-4B gel filtration. The incorporation efficiency of conjugated MAb fragments was determined by SDS–PAGE [19]. Reagents for liposome and IL preparation are listed in Supplementary File S1.

Specific binding of anti-VEGFR2-ILs and anti-VEGFR3-ILs in vitro
HeLa cells transfected to express mouse VEGFR3, MS-1 mouse blood endothelial and SV-LEC mouse lymphatic endothelial cells were plated in 12-well plates at a density of 150,000 cells/well. Cell lines were incubated for 2 h at 37°C with saline (control), untargeted, DiIC18(3)-DS labelled fluorescent liposomes (DiI-ILs) and VEGFR2 or VEGFR3-targeted fluorescent ILs (anti-VEGFR2-DiI-ILs or anti-VEGFR3-DiI-ILs). After incubation, cells were washed extensively with PBS, detached and stored on ice until analysis by flow cytometry via fluorescence activated cell sorting (FACS). More details concerning origin and handling of cell lines can be found in Supplementary File S1.

Specific binding of anti-VEGFR2-ILs and anti-VEGFR3-ILs in vivo
Rip1Tag2 mice were injected i.v. with anti-VEGFR2-ILs dox and/or anti-VEGFR3-ILs-dox for treatment. The specific binding of the ILs to the particular receptor was investigated on frozen sections by detection of the rat Fab' domain of the ILs with a specific monoclonal anti-rat Fab'-FITC labelled antibody (Sigma, Buchs, Switzerland) in combination with either anti-VEGFR3 (goat) or anti-VEGFR2 (goat)-directed antibody, respectively. The CD31 antigen staining was performed with a rat derived-anti-CD31 antibody in combination with anti-rat Fab'-FITC.

RT-PCR
Total RNA was isolated from the above-mentioned cell lines, cDNA prepared and the expression of the mRNA evaluated by qRT-PCR.
Primers qRT-PCR:
• mCD31: CGGTTGTTAGCGGATCC and CGACGAGA TGGAAAATCACA
• mVEGFR3: CGACCGCCCTCTGTTGGTTTG and CAA AGCCAGTCCAGGTCCGC
• mVEGFR3: GCTTTGTTGGTTGGAGAAGC and GAGC CACTCGACACTGATGA
• mGAPDH: TTGATGCAACAATCTCCAC and GTCC CTGAACAAAAATGTT
• hGAPDH: AATGAAGGGGTCAATGTGAG and AAGGT GAAGGTCGGGTCA

Mice
Phenotypic and genotypic analyses of Rip1Tag2 in a C57Bl/6J background have been described previously [23,24]. For therapeutic studies, mice were randomized according to their age and gender. Rip1Tag2 mice were in the median 10 weeks old at the onset of therapy. Mice were injected into the tail vein with 5 mg dox/kg anti-VEGFR2-ILs-dox, 5 mg dox/kg anti-VEGFR3-ILs-dox or 5 mg dox/kg anti-VEGFR2-ILs-dox and anti-VEGFR3-ILs-dox in a 1:1 ratio. The control groups were treated with 5 mg dox/kg PLD (Caelyx®) or the same quantity of empty liposomes or empty ILs, respectively, i.v. at day 1, 4, 8, and 11. Tumor size was assessed after sacrifice (day 15 after initial injection) using a caliper or a grid (for small tumors). The mass was calculated assuming an ellipsoid shape of the tumor.

Histologic analysis and microscopy
Tumor tissue analysis was performed as described [14,25,26]. Blood and lymphatic vessels were labelled on frozen sections with rat anti-CD31, rabbit anti-LYVE-1 or anti-NG2 as primary antibody. For VEGFR2 and/or VEGFR3, primary antibody AF644 or AF743 (R&D Systems Europe Ltd., London, UK) was used. Apoptosis was detected with an antibody against cleaved-Caspase-3 [27], and proliferation
was determined with an anti-phospho-H3 antibody. Conventional stainings were performed with frozen tissue sections of 7 μm thickness, for confocal microscopy analysis sections were of 80 μm thickness. Immunohistochemical stainings were analyzed on an AxioVert microscope. Immunofluorescence stainings were analyzed on a LSM510 META confocal microscope.

Statistical analysis
Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Tumor volume and mass were compared using non-parametric statistical analysis (Kruskal–Wallis test) with Dunn’s post-test. Proliferation, apoptosis, and blood and lymphatic vessel density were analyzed by parametric testing (one-way ANOVA).

Results

In vitro binding of anti-VEGFR2 and anti-VEGFR3 ILs

VEGFR2 and 3-expressing endothelial cells were targeted with ILs containing a rat-derived Fab’ of specific monoclonal anti-VEGFR2 or VEGFR3 antibodies, binding to the extra-cellular domain of their respective receptor. MS-1, a pancreatic islet mouse endothelial cell line expressing endogenously VEGFR2 and VEGFR3; SV-LEC, a mouse lymphatic endothelial cell line expressing only low levels of VEGFR2 and HeLa-VR3, a human epithelial cervical adenocarcinoma cell line stably transfected to express murine VEGFR3 were tested for binding and uptake of fluorescently tagged ILs containing DiIC$_{18}(3)$-DS and DiIC$_{18}(3)$-DS liposomes without antibody fragments using flow cytometry.

Anti-VEGFR2-ILs showed a one order-of-magnitude higher accumulation in MS-1 cells than did control liposomes (Figure 1A). There was no uptake in VEGFR2-negative cells like SV-LEC (Figure 1B) and HeLa-VR3 (Figure 1C). Binding of DiI-labeled anti-VEGFR3-ILs correlated with the expression level of VEGFR3 in the various cell lines. In MS-1 cells, a one order-of-magnitude higher accumulation was detected (Figure 1A), in SV-LEC only a twofold accumulation was observed when compared to control liposomes (Figure 1B), while HeLa-VR3 showed a 3-log higher uptake (Figure 1C). Expression of VEGFR2 and -3 on MS-1, SV-LEC and HeLa-VR3 was assessed by RT-PCR (Figure 1D).

Taken together, anti-VEGFR2 and -3 ILs bind selectively to their target receptors, thus providing a source basis for targeted delivery of cytotoxic compounds to tumor-associated endothelial cells.

Localization of VEGFR2 and VEGFR3 in tumor-associated endothelial cells in RipTag2 mice

Tumor-associated angiogenesis is characterized by up-regulated expression of VEGFR2 and VEGFR3. To investigate tumor vessel morphology and VEGFR expression in tumors of Rip1Tag2 transgenic mice, confocal microscopy analysis of Rip1Tag2 pancreatic sections was performed using antibodies directed against VEGFR2 and/or VEGFR3 in combination with the endothelial cell marker CD31 and the pericyte marker NG2. Co-localization of VEGFR2 with CD31 was close to 100%. This confirmed the expression of VEGFR2 in a broader variety of endothelial cells, such as leading tip cells and trailing stalk cells (Figure 2A(ii)). Only a subset of CD31-positive endothelial cells expressed VEGFR3, but all intratumoral VEGFR3-expressing cells were CD31-positive. This result confirmed the notion that these cells were blood vessel endothelial cells and not of lymphatic origin (Figure 2A(ii)). No exocrine CD31 positive vessels were positive for VEGFR3 expression. VEGFR3-positive structures in the exocrine pancreas were identified as lymphatic vessels by their co-staining with Lyve1, a marker for lymphatic vessel endothelial cells (Supplementary Figure S1).

Simultaneous stainings for VEGFR2 and VEGFR3 showed a high percentage of intratumoral co-localization. All VEGFR3-positive cells were also positive for VEGFR2 expression (Figure 2A(iii)). Co-expression of the two VEGFR most likely identified tumor-associated angiogenic tip cells in angiogenic sprouts [6]. While the staining of VEGFR2 and NG2 showed an adjacent, partly overlapping localization (Figure 2A(iv)), no co-localization was found with VEGFR3 and NG2 (Figure 2A(v)).

The co-localization of VEGFR2 with VEGFR3 in sprouting angiogenic tumor-associated vessels was further validated in vivo in Rip1Tag2 mice treated with anti-VEGFR2-ILs, anti-VEGFR3-ILs or PLD. Pancreatic sections of Rip1Tag2 mice treated with anti-VEGFR2-ILs or anti-VEGFR3-ILs were co-stained with anti-VEGFR3 or anti-VEGFR2 antibodies (both goat derived) in combination with a specific anti-rat-Fab’ antibody detecting directly Fab’ fragments of the ILs. In both stainings, co-expression of the two VEGF receptors was confirmed (Figure 2B, upper and middle row). As a positive control, pancreatic sections of Rip1Tag2 mice treated with PLD were primarily stained for CD31 (a rat MAb) and thereafter with the anti-rat-Fab’ antibody (Figure 2B, bottom row) confirming specificity of the anti-rat-Fab’ antibody. The negative control did not show any background (data not shown).

Additional supporting evidence for the co-expression of VEGFR2 and VEGFR3 in tumor-associated sprouting cells was obtained by the analysis of intratumoral VEGFR3 expression in Rip1Tag2 mice treated with anti-VEGFR2-ILs-dox (n = 8 mice, n = 27 tumors), anti-VEGFR3-ILs-dox (n = 8 mice, n = 28 tumors), PLD (n = 8 mice, n = 48 tumors) or anti-VEGFR3-ILs-empty (n = 8 mice, n = 35 tumors). Rip1Tag2 mice were sacrificed at day 15 and pancreatic sections were stained with a rat anti-VEGFR3 antibody. Immunohistochemical stainings and computerized analysis of the microphotographs revealed a significant decrease in the VEGFR3-positive area within tumor tissue of anti-VEGFR2-ILs-dox-treated mice (median 1.7; percentile range 0.8–2.9) and anti-VEGFR3-ILs-dox-treated mice (median 1.1; 0.6–3.4) compared to PLD-treated mice (median 5.2; 3.7–7.1). PLD was able to reduce the VEGFR3-positive area compared to empty anti-VEGFR3-ILs, probably due to its EPR-enhanced, unspecific anti-tumor activity. However, anti-VEGFR2-ILs and anti-VEGFR3-ILs were significantly more efficient than PLD in depleting VEGFR3 expressing cells (Figure 2C). Importantly, both anti-VEGFR2-ILs and anti-VEGFR3-ILs led to the reduction of intratumoral VEGFR3 expression without showing a significant difference between VEGFR2-
ILs-dox and VEGFR3-ILs-dox-treated mice. The reduction of VEGFR3-positive area in anti-VEGFR2-ILs-dox-treated mice strengthened the hypothesis of VEGFR2/VEGFR3 co-expression in the cells of sprouting blood vessels.

Tumor burden is significantly reduced by IL treatment

To examine the anti-tumor activity of the specific targeted transport of doxorubicin to the tumor-associated vasculature, we treated Rip1Tag2 transgenic mice with anti-VEGFR2-ILs-dox (cohort \( n = 12 \)), anti-VEGFR3-ILs-dox (cohort \( n = 16 \)) or anti-VEGFR2/3-ILs-dox (cohort \( n = 9 \)) and compared them to Rip1Tag2 mice treated with PLD (cohort \( n = 15 \)) (5 mg doxorubicin/kg mice for all ILs-dox and PLD) or anti-VEGFR3-ILs-empty (cohort \( n = 7 \)). Rip1Tag2 transgenic mice express SV40 large T antigen under the control of the rat insulin promoter and develop pancreatic \( \beta \)-cell tumors [24]. Repeated treatments were necessary since the in vivo (blood) half-life of ILs was 21 h (data not shown).

Thus, injections were performed twice weekly for two weeks. Mice were sacrificed at day 15 and tumor volumes were determined. For the combination therapy of anti-VEGFR2-ILs-dox with anti-VEGFR3-ILs-dox the total concentration of doxorubicin was maintained at 5 mg doxorubicin/kg by mixing half the amounts of anti-VEGFR2-ILs-dox and anti-VEGFR3-ILs-dox as compared to the individual treatments.

The tumor volume was significantly reduced in all experimental cohorts compared to the control groups (Figure 3). The median tumor volumes in anti-VEGFR2-ILs-dox-treated animals were 1.5 mm\(^3\) (0.425–3.0, values = median and interquartile range), 0.5 mm\(^3\) (0.175–1.75) in anti-VEGFR3-ILs-dox, and 0.0 mm\(^3\) (0.0–0.5) in anti-VEGFR2/3-ILs-dox-treated mice as compared to the individual treatments.

The tumor volume was significantly reduced in all experimental cohorts compared to the control groups (Figure 3). The median tumor volumes in anti-VEGFR2-ILs-dox-treated animals were 1.5 mm\(^3\) (0.425–3.0, values = median and interquartile range), 0.5 mm\(^3\) (0.175–1.75) in anti-VEGFR3-ILs-dox, and 0.0 mm\(^3\) (0.0–0.5) in anti-VEGFR2/3-ILs-dox-treated mice as compared to the individual treatments.

Figure 1. Anti-VEGFR2 and anti-VEGFR3 immunoliposomes specifically bind their targets in vitro. The uptake of anti-VEGFR2-ILs and anti-VEGFR3-ILs was evaluated in several cell lines by flow cytometry. Cells were incubated with DiIC\(_{18}(3)\)-DS-labelled immunoliposomes tagged either with the anti-VEGFR2-Fab\(^0\) (orange open histogram) or the anti-VEGFR3-Fab\(^0\) (blue filled histogram) or with control liposomes prepared identically as ILs except for omission of the MAb fragment (green open histogram). The red-filled histograms show the analyzed cell lines without any treatment. (A) MS1 endothelial cells express high endogenous levels of VEGFR2 and -3. A one-log shift is observed with both anti-VEGFR2 and -3 ILs. (B) SV-Lec expresses low levels of VEGFR3. A shift is only seen with anti-VEGFR3-ILs. (C) HeLa VR3 has been stably transfected to express murine VEGFR3. A three-log shift is observed with anti-VEGFR3-ILs. (D) Quantitative RT-PCR has been performed on the cell lines described above to assess the relative levels of CD31, VEGFR2 and VEGFR3 mRNAs. (Please refer to the online edition of this article for the color code.)
When comparing the three interventional ILs cohorts, a statistically relevant difference was detected between anti-VEGFR2-ILs-dox and the combination treatment. There was a numerical, but no statistically significant difference between single anti-VEGFR3-ILs-dox versus combination treatment.

In summary, in all experimental cohorts of Rip1Tag2 mice a significant reduction of the tumor volume was achieved through anti-angiogenic treatment with doxorubicin-loaded ILs directed against VEGFR2 and/or 3-positive endothelial cells. Combined anti-VEGFR2 and anti-VEGFR3-ILs therapy was superior to single treatment with anti-VEGFR2-ILs or anti-VEGFR3-ILs in suppressing tumor growth.

**Anti-VEGFR2-ILs and anti-VEGFR3-ILs reduce tumor vessel density**

Next we analyzed the microvessel density in the tumor perimeter and in the exocrine pancreas to evaluate potential angiotoxicity of the dual therapy against VEGFR3- and VEGFR2-expressing vascular cells. Staining of pancreatic sections of Rip1Tag2 mice with anti-CD31 antibody treated with anti-VEGFR2-ILs-dox, anti-VEGFR3-ILs-dox or the combination of the two demonstrated a significant reduction in tumor-associated vessel density in all the interventional groups (Figure 4). Microvessel density decreased approximately 30% in anti-VEGFR2-ILs-dox, 40% in anti-VEGFR3-ILs-dox and 50% in anti-VEGFR2/3-ILs-dox-treated cohorts compared to PLD (**p < 0.01, ***p < 0.001; ANOVA, Kruskal–Wallis test with Dunn's Multiple Comparison Test). None of the interventional groups showed a decrease of vessel density in the exocrine pancreas, underlining the exquisite specificity of anti-VEGFR2 and three targeted ILs.

**ILs decrease proliferation of tumor cells and induce endothelial and tumor cell apoptosis**

Tumor cell proliferation was measured on pancreatic sections of Rip1Tag2 mice treated with anti-VEGFR2 and/or anti-
VEGFR3-ILs by immunohistochemical staining for phosphorylated histone 3 (pH3) [28,29]. Immunostaining of pancreatic sections of Rip1Tag2 mice treated with PLD revealed a median of 5.6% (1.6–14.9, values = median and interquartile range) of the intratumoral area positive for pH3 (Figure 5A and B), while significant reduction of the pH3 positive areas was detected in mice treated with anti-VEGFR2 ILs-dox (2.0%, 0–5.6%), anti-VEGFR3 ILs-dox (2.5%, 0–5.7%) and anti-VEGFR2/3 ILs-dox (2.0%, 0.4–4.2%) (**p < 0.001, ANOVA, Kruskal–Wallis test with Dunn’s Multiple Comparison Test).

Next, we examined whether apoptosis was specifically induced in tumor-associated endothelial cells. Pancreatic sections of Rip1Tag2 mice treated with ILs or PLD were stained with an antibody specifically recognizing cleaved caspase-3 (Supplementary Figure S2). Co-staining of Rip1Tag2 pancreatic sections for CD31 and cleaved Caspase-3 showed highly significant co-localization of both proteins in ILs-treated mice, which underlines the specificity and efficacy of this anti-angiogenic approach.

As shown previously, the median of CD31/cleaved Caspase-3-positive endothelial cells (per visual field) in anti-VEGFR2-ILs-dox-treated mice was 20 cells within the tumor perimeter [14]. In the current cohort, this number amounts to 1.5 cells in the anti-VEGFR3-ILs-dox group and five cells in the anti-VEGFR2/3-ILs-dox group (**p < 0.01, ***p < 0.001; ANOVA, Kruskal–Wallis test with Dunn’s Multiple Comparison Test; Figure 6B). The high frequency of apoptotic endothelial cells in the anti-VEGFR2-ILs group can be explained by the fact that nearly all CD31-positive tumor-associated vessels express VEGFR2 and, hence, treatment with VEGFR2-ILs leads to a higher number of apoptotic vessels. Since VEGFR3 is less frequently expressed in CD31-positive tumor-associated vessels, less apoptotic tumor-associated endothelial cells are detected in anti-VEGFR3-ILs-dox-treated tumors. The dual anti-angiogenic treatment resulted in an intermediate value, since half the dose of each individual treatment was applied.

**Toxicity of ILs**

To assess toxicity of anti-VEGFR2-ILs and/or anti-VEGFR3-ILs, Rip1Tag2 transgenic mice were analyzed at the end of their treatment, while C57BL/6 non-transgenic mice were treated with the same regimen and analyzed after 6 months. Importantly, H&E stainings of histological sections of lung and liver did not provide evidence of metastatic infiltration. In this set of experiments, anti-angiogenic therapy obviously does not have any pro-metastatic effects. In addition, pancreatic sections of Rip1Tag2 mice treated with ILs or PLD were also stained with an antibody against LYVE-1, a marker for lymphatic endothelial cells and tumor-associated macrophages (TAM; data not shown). Statistical analysis did not reveal a significant change in lymphatic vessel density or macrophage infiltration in tumors of mice treated with anti-VEGFR3-ILs as compared to PLD-treated mice (data not shown). Taken together, a very low toxicity profile was observed, confirming earlier observations with anti-VEGFR2-ILs [14].

**Discussion**

Previously, we have shown that loading liposomal nanoparticles with chemotherapeutic drugs and coupling these liposomes with anti-VEGFR2 antibodies are a feasible and highly efficient approach to inhibit tumor angiogenesis and tumor growth in mouse models of human cancer [14]. However, it has remained unclear which endothelial cell surface antigens are most efficacious for targeted delivery. In this study, we have compared ILs targeted against VEGFR2 and VEGFR3, two main tyrosine kinase receptors specifically expressed on blood endothelial cells. VEGFR2 is expressed on all activated endothelial stalk cells, while VEGFR3 is selectively expressed on path-leading endothelial tip cells [4–8]. Considering the different biological role of VEGFR2 and −3 in tumor angiogenesis, we hypothesize that it is favorable to deplete both tumor-associated endothelial cell populations in order to hamper tumor-induced angiogenesis and maintain tumor starvation.

Employing cultured cells in vitro, we demonstrate that anti-VEGFR2-ILs and anti-VEGFR3-ILs target their receptors with high specificity and with a dependency on the expression levels of the respective receptors. In vivo analysis on pancreatic tumor sections of Rip1Tag2 mice confirms a high co-expression of VEGFR2 and VEGFR3.
Figure 4. Reduction of intratumoral microvessel density in ILs-treated Rip1Tag2 mice. Quantification of CD31-positive microvessels in pancreatic sections of Rip1Tag2 mice treated with either anti-VEGFR2-ILs-dox, anti-VEGFR3-ILs-dox or the combination thereof demonstrates a significant reduction in tumor-associated vessel density in all the interventional groups. Analyses of sections stained with anti-CD31 antibody were performed within the single tumor perimeter and additionally in the surrounding, non-tumoral tissue (exocrine pancreas). **p < 0.01, ***p < 0.001; ANOVA, Kruskal–Wallis test with Dunn’s Multiple Comparison Test. ns = not significant.

Figure 5. Reduced proliferation in tumors of the ILs-treated Rip1Tag2 mice. (A) Histological pancreatic sections stained with anti-CD31 antibody (green), and anti-phospho-H3 (pH3). Nuclei are visualized with DAPI. Mice were treated with PLD (upper row), anti-VEGFR2-ILs-dox (second row), anti-VEGFR3-ILs-dox (third row), anti-VEGFR2/3-ILs-dox (bottom row). Bars, 50 μm. (B) Quantification of cells staining with anti-phospho H3 antibody within tumors of Rip1Tag2 mice treated with PLD (n = 49), anti-VEGFR2-ILs-dox (n = 32), anti-VEGFR3-ILs-dox (n = 46), anti-VEGFR2/3-ILs-dox (n = 17). ***p < 0.001; ANOVA, Kruskal–Wallis test with Dunn’s Multiple Comparison Test. (Please refer to the online edition of this article for the color code.)
Exocrine pancreas is confined to comparatively rare endothelial tip cells. All CD31-positive endothelial cells, while VEGFR3 expression pattern of VEGFR2 and anti-VEGFR2-ILs. This probably reflects the expression of apoptotic endothelial cells is highest in the cohort treated with combination treatment groups. Interestingly, the percentage of lymphial cells is high in the anti-VEGFR2, the anti-VEGFR3 and the CD31/cleaved Caspase-3 double-positive apoptotic endothelial cells and augmented apoptosis in tumors. In particular, the level of CD31/cleaved Caspase-3 double-positive apoptotic endothelial cells is high in the anti-VEGFR2, the anti-VEGFR3 and the combination treatment groups. Interestingly, the percentage of apoptotic endothelial cells is highest in the cohort treated with anti-VEGFR2-ILs. This probably reflects the expression pattern of VEGFR2 and −3. VEGFR2 is expressed by nearly all CD31-positive endothelial cells, while VEGFR3 expression is confined to comparatively rare endothelial tip cells.

Therefore, the absolute number of apoptotic cells in the anti-VEGFR3-ILs-dox treated group is low. Although, VEGFR2 positive tumor-associated endothelial cells are larger in number, single therapy with anti-VEGFR3-ILs-dox seems to be more effective, at least numerically. This emphasizes once more the different biological functions of these cell populations, including their role as targets for targeted delivery. However, strengthening our hypothesis, it is the combined treatment with anti-VEGFR2 and anti-VEGFR3-ILs that is most efficient in reducing blood vessel density and tumor burden. Thus, it appears that the dual targeting of VEGFR2 and −3 expressing cells represents an efficacious anti-angiogenic therapy. Intriguingly, we did not observe a significant decrease of VEGFR3 positive lymphatic vessels in mice treated with anti-VEGFR3-ILs. It remains unclear whether this is due to a lower expression of VEGFR3 on lymphatic endothelial cells, a lower proliferation rate of the lymphatic endothelial cells in comparison to the tumor-associated blood endothelial cells or to another reason.

To address general toxicity of ILs therapy we have analyzed CD31-positive (quiescent) vessels in the exocrine pancreas of ILs-treated Rip1Tag2 mice. No morphological changes and no endothelial-specific apoptosis were detected in the vessels of the exocrine pancreas. This result underlines the high specificity of anti-VEGFR2 and −3-targeted therapy to tumor-associated, activated blood vessel endothelial cells.

Another issue is the question whether anti-angiogenic therapies have a rebound effect and can paradoxically promote tumor metastasis [30,31]. In our experimental approach,
H&E stainings on lung and liver did not provide any evidence for increased metastatic infiltration induced by anti-angiogenic treatment, indicating that anti-VEGFR2 and −3 ILs do not increase the risk for metastasis formation in this model. Rather, this serious side-effect may depend on the type of anti-angiogenic therapy, its efficacy and the type of tumor concerned.

Conclusions

We have assessed the selective elimination of tumor-associated vascular cells with ILs directed to the two VEGF receptors 2 and 3. The combination of the ILs targeted for the VEGF receptors demonstrates an additive effect in reducing tumor burden. These results demonstrate a remarkably high efficacy in selectively targeting different components of tumor-associated vasculature, such as vascular tip cells (VEGFR3-positive) and stalk cells (VEGFR2-positive). In addition, it may be worthwhile to direct ILs against other cells of the tumor microenvironment, such as tumor-associated fibroblasts or tumor-infiltrating inflammatory cells. Since ILs are also in clinical development, they may offer a versatile and efficient technological platform to test and develop therapeutic strategies against different cellular compartments of malignant tumors not only in animal models but also in patients [32].

Declaration of interest

This work was supported by the Gebert-Rüf Stiftung (GRS-038/07) (to AW and CM), the Desiree and Niels Yde Foundation (to AW), the Schoenmakers-Müller Foundation (to AW and CM), the Swiss Cancer League (CCRP OCS-01812-12-2005), the Nora van Meeuwen-Haefliger Foundation and the EU-FP7 TuMIC HEALTH-F2-2008-201662 (to GC).

The authors have no conflicts of interest to declare.

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Supplementary material available online
Supplementary File S1 and Supplementary Figures S1 and S2.