Targeting tumor-associated endothelial cells: anti-VEGFR2-immunoliposomes mediate tumor-vessel disruption and inhibit tumor growth

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Translational relevance:

Targeting pharmaceutical compounds to the tumor or the tumor stroma is supposed to enhance efficacy and reduce side-effects of chemotherapy. Immunoliposomes can be used to stably encapsulate and selectively transport cytotoxic compounds in vivo. Our group has already performed a clinical trial with anti-EGFR-immunoliposomes (ASCO 2011 Abstract). In this article, we describe preclinical data supporting the development and clinical testing of anti-VEGFR2-immunoliposomes.
Abstract

Purpose: Angiogenesis is a key process in tumor progression. By binding vascular endothelial growth factor (VEGF), VEGF receptor-2 (VEGFR2) is a main signaling transducer in tumor-associated angiogenesis. Accordingly, therapeutic approaches against the VEGF/VEGFR2 signaling axis have been designed. However, an efficient and specific chemotherapeutic targeting of tumor-associated endothelial cells has not yet been achieved.

Experimental Design: We have employed anti-VEGFR2 antibodies covalently linked to pegylated liposomal doxorubicin (PLD) to specifically ablate tumor-associated endothelial cells in the Rip1Tag2 mouse model of insulinoma, in the MMTV-PyMT mouse model of breast cancer, and in the HT29 human colon cancer xenograft transplantation model.

Results: In each model, anti-VEGFR2-targeted immunoliposomes loaded with doxorubicin (anti-VEGFR2-ILs-dox) were superior in therapeutic efficacy to empty liposomes, empty anti-VEGFR2, antibodies alone, and PLD. Efficacy was similar to that of the oral VEGFR1, -2 and -3 inhibitor PTK787. Detailed histopathological and molecular analysis revealed a strong anti-angiogenic effect of anti-VEGFR2-ILs-dox, and the observed anti-angiogenic therapy was significantly more efficient in reducing tumor burden in well-vascularized transgenic mouse models as compared to the less-vascularized xenograft model.

Conclusions: Anti-VEGFR2 immunoliposomes provide a highly efficient approach to selectively deplete VEGFR2-expressing tumor vasculature. They offer a novel and promising anti-cancer strategy.
Introduction

The distribution of most current drugs in the body is determined by their hydrophilic or lipophilic nature. This constitutes a major obstacle for successful and safe therapies, since drug delivery is not limited to the diseased organ but encompasses the whole body. Nanoparticles, such as liposomes, have been generated for prolonged circulation and redirection of therapeutic drugs. They yield superior accumulation in tumors via a process referred to as the "enhanced permeability and retention" (EPR) effect (1).

Huge efforts were made to identify specific cell surface markers on tumor cells and to direct anti-cancer agents selectively to malignant cells. Immunoliposomes (ILs), in which monoclonal antibody (MAb) fragments are conjugated to the lipid membrane, represent a next generation of molecularly targeted drug delivery systems. By combining the targeting properties of MAbs with the pharmacokinetic and drug delivery advantages of liposomes, immunoliposomes offer the promise of selective drug delivery to tumor cells (2). Using this modular approach, we have developed immunoliposomes that successfully target epidermal growth factor receptor (EGFR) on cancer cells (3, 4).

Endothelial cells lining newly formed vessels in a growing tumor express a different set of genes in comparison to those lining physiological vessels, which translates into a different expression of surface markers (5). One of the antigens which are selectively upregulated on activated tumor-associated endothelial cells is VEGFR2 (reviewed by (6)). This upregulation probably is the consequence of a paracrine loop between tumor cells producing VEGF-A and endothelial cells (7). Not surprisingly, the activation of the VEGF-A/VEGFR2 axis and the differential expression of VEGFR2 on tumor-associated endothelial cells make it a prime target for anti-angiogenic treatments in malignant tumors (reviewed by (8)). The first anti-angiogenic drug introduced into the clinic was bevacizumab, a Mab that sequesters...
VEGF-A, the major VEGFR2-ligand. Clinical studies have proven the efficacy of bevacizumab in human colorectal, breast, kidney and central nervous system cancers (9, 10, 11, 12). Other experimental approaches designed to repress tumor-driven angiogenesis include small molecule inhibitors or monoclonal antibodies suppressing the activity of all or specific VEGF receptors, thus blocking various aspects of VEGF/VEGFR signaling.

In this study, we specifically targeted tumor-associated vasculature by anti-VEGFR2 immunoliposomes loaded with doxorubicine, a chemotherapeutic drug. The aim of this study was to assess the feasibility and efficacy of selective drug delivery to activated, VEGFR2 expressing endothelial cells in tumor-associated vessels. Here we describe the therapeutic potential of anti-VEGFR2 immunoliposomes in a series of transgenic and xenograft tumor models.

Materials and Methods

Materials
Reagents for liposome preparation included: DilC<sub>18</sub>(3)-DS (Molecular Probes; Leiden, Netherlands); DSPC, cholesterol, and mPEG-DSPE (Avanti Polar Lipids; Alabaster, AL, USA); Mal-PEG(2000/3400)-DSPE (Nektar; Huntsville, AL, USA); organic solvents, and other chemicals of reagent purity (Sigma-Aldrich AG; Buchs, Switzerland). Doxorubicin (dox, Pfizer AG, Zürich, Switzerland) and pegylated liposomal doxorubicin (PLD, Caelyx®; Essex Chemie AG, Luzern, Switzerland) were obtained commercially. MAb DC101 was obtained from ImClone Systems Inc., New York, USA.

Liposome preparation
Liposomes were prepared by a lipid film hydration-extrusion method using repeated freeze-thawing to hydrate the lipid films (13, 14). Liposomes were composed of
DSPC and cholesterol (molar ratio 3:2) with mPEG-DSPE (5 mol% of phospholipid). The phospholipid concentration in the liposome solution was measured utilizing a standard phosphate assay (15).

For uptake studies, liposomes were labeled with 0.1-0.3 mol% DilC$_{18}$(3)-DS, a fluorescent lipid that can be stably incorporated into liposomal membranes (16, 17).

For biodistribution studies, Rip1Tag2 mice were treated with liposomes and anti-VEGFR2-ILs, which were labelled with [³H]-cholesteryl hexadecyl ether, injected via the tail vein at a dose of 31.25 µmol phospholipid/kg mouse (cohorts of 4-6 mice per time point and treatment). At 24 or 48 hours mice were euthanized, and tissues were collected after perfusion with PBS. Tissue samples were homogenized and [³H]-cholesteryl hexadecyl concentration were determined as described (4). The final distribution was expressed as % of injected dose (ID)/g tissue and as % ID for the concentration in the blood circulation. For encapsulation of doxorubicin, the remote-loading method with ammonium sulfate was used (18, 19).

**Preparation of MAb fragments and immunoliposomes**

DC101 is a rat MAb against the extracellular domain of mouse VEGFR2 (20). For preparation of DC101-Fab', intact DC101 MAb was cleaved and reduced as previously described (3).

For immunoliposomes, Fab' were covalently conjugated to maleimide groups at the termini of PEG-DSPE chains (Mal-PEG-DSPE) (21). 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 60 µg Fab'/µmol PL (22, 23). Unincorporated conjugates and free drug were separated from immunoliposomes by Sepharose CL-4B gel filtration. The incorporation efficiency of conjugated MAb fragments was estimated by SDS-PAGE (3).
Cell lines
MS-1 mouse endothelial, HT-29 human colon cancer and MDA-MB468 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD). MS-1 and MDA-MB-468 cells were maintained in "Improved MEM Zinc Option" medium (Invitrogen AG, Basel, Switzerland) and HT-29 in RPMI-1640 (Sigma-Aldrich AG, Buchs, Switzerland) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5 % CO₂ at 37 °C.

Specific binding of anti-VEGFR2-ILs in vitro
For flow cytometry studies, 150'000 MS-1 or MDA-MB468 cells were co-incubated in 12-well plates with saline (control), untargeted liposomes, Dil-Ls or VEGFR2-targeted immunoliposomes (anti-VEGFR2-Dil-ILs or anti-EGFR-Dil-ILs as a control), labeled with DilC₁₈(3)-DS for 2 h at 37 °C, washed extensively with PBS, followed by detaching and storing on ice until flow cytometry. DC101 anti-VEGFR2 or C225 anti-EGFR antibodies (ImClone Systems, Inc, Somerville, NJ) were inserted into the liposomes as described above.

Specific binding of anti-VEGFR2-ILs in vivo
For uptake studies in vivo, 12 weeks old Rip1Tag2 mice were injected i.v. with Dil-Ls or anti-VEGFR2-Dil-ILs. 36h after injection mice were sacrificed and a single cell suspension of pancreatic tumors was prepared by Dispase digestion. For subsequent FACS analysis cells were stained with PerCp-CD45 (Biolegend) and APC-CD31 (Biolegend) antibodies.

RT-PCR
A single cell suspension of pancreatic tumors of 12 week old Rip1Tag2 mice was prepared by Dispase digestion. For isolation of GLP-1R+ β-tumor cells and tumor-
derived CD31+ blood endothelial cells by FACS, cells were stained with FITC labeled
exendin-4 (Phoenix Pharmaceuticals, Inc.) (24), and with APC-CD31 (Biolegend).
Total RNA was extracted from isolated cells, cDNA prepared and the expression of
the mRNA evaluated by PCR.
Primers semiquantitative PCR:
mActin: ACACTGTGCCCATCTACGAGG and CATGCATGCCACAGGATTCC
mCD31: GGAGTCAGAACCCATCAGGA and TACTGGGCTTCGAGAGCATT
mGLP-1R: TCAGAGACGGTGACGAAATG and CAAGCCGGAGAAAGAAAGTG
mVEGFR1: CGGCAGACCAATAACAATCCT and CCGCTGCTCTATAGATGCTC
mVEGFR2: GGGCTGTTGGATGACATCTTand GTCACTGACAGAGCGATGA
mVEGFR3: GCTGTTGGTTGGAGAGAAGC and GAGCCACTAGACACTCTGATGA
Primers qRT-PCR:
mCD31: CGGTGTTTCAAGCAGATCC and CGACGAGATGGAAATCACAA
mVEGF-R2: CGAGCCGCCTCTGTGGGTTTG and
CAAAGCCAGTCCAGGTCCCGC
mRPL19: GCCAGTACCCTTCTCTCTCC and AGCCTGTGACTGTCCATTCC

Mice
Phenotypic and genotypic analyses of Rip1Tag2 and MMTV-PyMT transgenic mice
in a C57Bl/6J and FVB/N background have been described previously (25, 26). Wild-
type C57Bl/6J mice were used for organ toxicity studies. Athymic nude mice were
hosts for the HT-29 xenograft experiments. Animals were maintained and treated in
compliance with the guidelines of the Swiss Federal and the Cantonal Veterinary
Office Basel-Stadt (permissions No. 1878, 1907, 1908, and 2030).

For therapeutic studies, mice were randomized according to their gender.
Rip1Tag2 mice were 10 and PyMT mice 8 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
PLD (Caelyx®) or the same quantity of empty liposomes or empty
immunoliposomes, respectively, i.v. at day 1, 4, 8, and 11. Additional cohorts of Rip1Tag2 mice were injected with free DC101 alone (50 μg/mouse) or with a combination of PLD and free DC101 at the same concentration as present in the anti-VEGFR2-ILs-dox cohort (membrane-bound DC101) or with 100 mg/kg bodyweight PTK787 dissolved in polyethylene glycol 300 (PEG300; Sigma) by daily oral administration for 2 weeks. Tumor size was assessed after sacrifice (day 15) using a caliper or a grid (for small tumors). The mass was calculated assuming an ellipsoid shape of the tumor.

For xenograft experiments, 1x10^6 HT-29 colon cancer cells in 100μl cell culture medium were injected subcutaneously in the flanks of athymic mice. Once tumor size reached a volume of 150-250 mm^3, treatment was started following the protocol described for the transgenic mouse models. Tumor size was assessed twice weekly for 6 weeks.

**Histologic and toxicity analysis**

Tumor tissue analysis was performed as described before (24, 27). Blood and lymphatic vessels were visualized using anti-CD31 or anti-Lyve1 primary antibodies, respectively (Sigma, Buchs, Switzerland). For the analysis of metastasis in Rip1Tag2 mice lung, liver, kidney and spleen were isolated and fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. Histologic analysis was performed on H&E-stained paraffin sections. The entire organs were cut into 5 μm sections and every 5th section was stained with H&E for microscopic examination. Frozen sections were incubated with rat anti-CD31, anti-NG2 or anti-smo (Sigma, Buchs, Switzerland) as primary antibody. For VEGFR2 staining, goat anti-VEGFR2 Mab (R&D Systems Europe Ltd., London) was used. Fab’ fragments of DC101 were visualized with monoclonal anti-rat Fab’-FITC (Sigma, Buchs, Switzerland).

Anti-Caspase-3, cleaved (Ab-2) (Calbiochem Merck, Darmstadt, Germany), a rabbit polyclonal antibody, was utilized for apoptotic cell labelling.
Proliferation and apoptosis assay

For bromodeoxyuridine (BrdU) staining (proliferation assay), BrdU was injected at 100 µg BrdU per gram body weight i.p. 2h before sacrifice (24). For terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL; apoptosis) assays, slides were incubated for 1 h in TUNEL reagent (Roche, Basel, Switzerland) and apoptotic cells were visualized with the AEC-kit (Vector Laboratories, Servion, Switzerland).

Microscopy

Immunohistochemical stainings were analyzed on an AxioVert microscope (Zeiss, Göttingen, Germany). Immunofluorescence stainings were analyzed on a LSM510 META confocal microscope (Zeiss). Histologic grading of islet and mammary tumors follows the WHO International Classification of Rodent Tumors, Part II, The Mouse.

Statistical analysis

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

Results

Target expression, cellular uptake and biodistribution of VEGFR2-targeted immunoliposomes

Immunoliposomes against activated endothelial cells were coated with Fab’ derived from DC101, a monoclonal rat antibody binding the extracellular domain of
mouse VEGFR2 (28). Binding and uptake of VEGFR2-targeted immunoliposomes was evaluated by flow cytometry in MS-1 cells, an endothelial cell line expressing high levels of VEGFR2 (29), and in MDA-MB-231 human breast cancer cells, which lack VEGFR2 expression but express high levels of EGFR instead. In this assay, liposomes were fluorescently labeled with DiIC18(3)-DS with and without conjugated MAb fragments, and incubated for 2 h with MS-1 or MDA-MB468 cells. Immunoliposomes containing DC101-Fab’ showed a one order-of-magnitude higher accumulation in MS-1 cells than did control liposomes (Figure 1A). In contrast, MDA-MB468 cells had a two-log higher uptake of anti-EGFR-ILs compared to anti-VEGFR2-ILs (Supplementary Figure S1A).

We next asked whether this specific uptake in VEGFR2-expressing cells could also be observed in vivo. Staining of tissue sections of Rip1Tag2 mice treated with anti-VEGFR2-ILs using a FITC-labeled anti-rat-Fab’ antibody showed specific binding of the targeted immunoliposomes to VEGFR2-positive cells (Supplementary Figure S1B). Further confirmation of in vivo binding and uptake resulted from FACS sorting of explanted tumor-associated endothelial cells from Rip1Tag2 mice (Supplementary Figure S1C). CD31-expressing vascular cells treated with anti-VEGFR2-ILs loaded with Dil had a threefold increase in Dil positivity compared to treatment with untargeted Dil-positive liposomes.

We further evaluated the specificity of VEGFR2 expression on tumor-associated endothelial cells in the Rip1Tag2 mouse model. There, tumor cells can be sorted based on their selective expression of the glucagon-like 1 receptor (GLP-1R, (24)). Quantitative PCR of GLP-1R or CD31 sorted primary cells from tumors of untreated Rip1Tag2 mice showed no expression of VEGFR2 in tumor cells, whereas VEGFR2 was detectable in endothelial cells (Fig. 1B and Supplementary Figure S2A). Co-staining of sections of the pancreas of Rip1Tag2 mice confirmed the presence of VEGFR2 in intra-tumoral endothelial cells (Supplementary Figure S2B).
Biodistribution studies of non-targeted liposomes versus anti-VEGFR2 immunoliposomes were conducted in the Rip1Tag2 mouse model. Tissues were assayed as described before (4) at 24 or 48 hours. Comparable to previously published preclinical models (2, 4), analysis of tissue samples showed a non-significant trend to a higher sequestration of ILs in parenchymal organs (lung, liver and spleen) but not in the tumors. Therefore, a difference in tumor targeting efficiency is rather due to an enhanced and specific uptake of immunoliposomes than to distribution (Supplementary Figure S3).

Effect of anti-VEGFR2 immunoliposomes on tumor burden

To investigate the anti-tumor activity of anti-VEGFR2 immunoliposomes, we treated three different mouse models of cancer: (i) Rip1Tag2 mice express SV40 large T antigen under the control of the insulin promoter and develop pancreatic β-cell tumors; (ii) MMTV-PyMT mice express polyoma middle T antigen under the control of the MMTV promoter and develop breast cancer similar to human ductal carcinoma; and (iii) athymic nude mice subcutaneously xenografted with HT 29 human colon cancer cells. Cohorts of Rip1Tag2 (n=6 each), MMTV-PyMT (n=9 each) and nude mice xenografted with HT29 colon cancer cells (n=8 each, 1x10⁶ cells per flank) were injected with 5 mg doxorubicin/kg anti-VEGFR2-ILs i.v. at day 1, 4, 8, and 11. Control groups were treated by the same time schedule as the interventional group with non-targeted empty liposomes, targeted empty immunoliposomes and non-targeted, doxorubicine-loaded liposomes (PLD, 5 mg doxorubicin/kg). In the experiment with Rip1Tag2 mice, we also analyzed one group treated with DC101 antibody alone, one treated with a combination of PLD and the free DC101 antibody (using the same concentration of the antibody as in the immunoliposomal group) and one treated with the oral VEGFR1, 2 and 3 inhibitor PTK787. Rip1Tag2 and MMTV-PyMT mice were sacrificed at day 15 and the tumor volume was determined. The
size of the xenografted tumors in nude mice was determined twice weekly for 6 weeks.

In the Rip1Tag2 model, tumor burden in the experimental group was significantly reduced compared to all control groups except the treatment with PTK787 (Dunn’s test, p<0.05, Figure 2A). The median tumor volumes in the experimental group were 3.6 (0.9-4.6) mm$^3$, as compared to 21.8 (14.4-37.5) mm$^3$ in the PLD group, 36.8 (23.5-50.4) mm$^3$ in the non-targeted empty liposome group, 39.8 (31.9-84.6) mm$^3$ in the targeted empty immunoliposome group, 28.0 (21.7-45.4) mm$^3$ in the group with the antibody alone, 11.3 (11.3-37.5) in the PLD plus DC101 group and 6.3 (6.2-7.8) mm$^3$ in the group treated with PTK787.

In the MMTV-PyMT mouse, the median tumor volumes in the experimental group were 162 (99-233.5) mm$^3$, as compared to 588 (479-769) mm$^3$ in the PLD group, 3624 (2852-4435) mm$^3$ in the empty liposome group, and 3945 (3539-4677) mm$^3$ in the targeted empty immunoliposome group. Tumor volumes in the anti-VEGFR2-ILs-dox group were significantly smaller than in all included control groups (p<0.05, Dunn’s test, Figure 2B) (values = median and interquartile range).

In nude mice bearing xenografted HT29 colon carcinoma cells, tumor growth was effectively inhibited in both the groups injected with PLD and with anti-VEGFR2-ILs-dox (Figure 2C). However, the onset of the growth inhibition occurred earlier in the mice treated with targeted doxorubicin than in mice injected with PLD. In a multivariate rank analysis, the liposomal and anti-VEGFR2 immunoliposomal groups were not significantly different from one another (p > 0.05; Figure 2C). The two groups with empty (immuno-) liposomes had a significantly higher tumor load than the groups with doxorubicin-containing (immuno-) liposomes (p<0.001).

Analysis of the tumors by H&E staining of histological sections revealed smaller tumors and occasional hemorrhagic necrosis in tumors treated with anti-VEGFR2-immunoliposomes (Supplementary Figure 4). Quantification of nuclear atypia in tumors indicated that atypia (anisokaryosis, hyperchromasia or nuclear enlargement)
was significantly more frequent in the group treated with PLD (median: 3% of the tumor cells) than in the anti-VEGFR2-immunoliposomes (median 1.5%, p<0.05 ANOVA testing) or in the empty (immuno-) liposomes groups (median 0.5% of the cells, p<0.05, ANOVA testing, data not shown). Whole organ examination of lung, liver, kidney and spleen in Rip1Tag2, PyMT and xenografted athymic nude mice of all cohorts did not reveal metastases.

**Tumor cell proliferation, apoptosis and blood microvessel density**

In each experimental system, we analyzed tumor cell proliferation and apoptosis by BrdU and TUNEL assay, respectively (Figure 3).

In both the Rip1Tag2 and the PyMT model, tumor cell proliferation was significantly reduced if compared to targeted or untargeted empty ILs, but there was no significant reduction in the anti-VEGFR2-ILs group if compared to the PLD group (Figure 3, p>0.05, Newman-Keuls test). In contrast, apoptosis was significantly increased in mice treated with anti-VEGFR2-immunoliposomes as compared to all control groups (p<0.05, Newman-Keuls test). In the HT-29 xenograft model, at the time of analysis (day 42, 4 weeks after termination of the therapy), there was no difference in proliferation or apoptosis when comparing the anti-VEGFR2-ILs-dox group with the PLD group (Figure 3).

We next determined blood microvessel density for all groups in all models by staining histological sections with antibodies against CD31 (Figure 3). In Rip1Tag2 mice, blood microvessel density was reduced by 25-31% in the anti-VEGFR2-ILs-dox group compared to the other groups (p < 0.05, Newman-Keuls post test). In MMTV-PyMT breast cancer mice, blood vessel density in the anti-VEGFR2-ILs-dox group was reduced by 31-33% and in the HT-29 xenograft model by 24-28% (p < 0.05, Newman-Keuls test).

Immunohistochemical stainings with antibodies against the lymphatic marker LYVE-1 did not reveal significant differences in lymphatic vessel density between the
treatment protocols in all three cancer models (data not shown). However, these models exhibit a low frequency of lymphangiogenesis and, hence, may not be suitable to assess the effect of immunoliposomes on tumor lymphangiogenesis.

**Tumor-associated endothelial apoptosis**

Next we asked whether anti-VEGFR2-ILs-dox could induce apoptosis specifically in tumor-associated endothelial cells (Figure 4). Pancreatic sections of Rip1Tag2 mice were co-stained with anti-CD31 and anti-cleaved caspase 3 (Figure 4A). In anti-VEGFR2-ILs-dox treated mice, the analysis of endothelial cells within the tumor perimeter indicated a median of 20 apoptotic endothelial cells per visual field. In the exocrine pancreas, anti-VEGFR2-ILs-dox did not induce endothelial cell death. In untreated controls and PLD treated mice, no significant number of apoptotic endothelial cells was observed, be it in the tumor or in the exocrine pancreas (Figure 4B, Dunn’s test, p < 0.001). The results indicate that anti-VEGFR2-ILs-dox exhibit a selective toxicity on activated endothelial cells, while quiescent endothelial cells of the nearby exocrine pancreas are not targeted by the compound.

**Disruption of small blood vessels**

The injection of anti-VEGFR2-ILs-dox induced a decline of tumor-associated microvessels. However, when staining for CD31-positive vessels, remnants of the tumor vasculature were still present after treatment with anti-VEGFR2-ILs-dox. To investigate the mechanisms of tumor vessel disruption, we analyzed vessel morphology by immunofluorescence stainings using antibodies directed against endothelial cells (anti-CD31), pericytes (anti-NG2, not shown) or smooth muscle cells (anti-smooth muscle actin; Figure 5).

We found that major vessels and small capillaries in the exocrine pancreas were unaffected by the treatment with anti-VEGFR2-ILs-dox (Figure 5A). While morphologically normal capillaries were present in tumors of mice treated with PLD
(Figure 5B), the number of intact capillaries was reduced by one third in the tumors treated with anti-VEGFR2-ILs-dox (Figure 5D). Notably, DAPI staining showed pyknotic nuclei in the center of interspersed cells that still expressed CD31 (Figure 5D). However, in the tumors of mice injected with anti-VEGFR2-ILs-dox we could still detect small vessels lined by a layer of smooth muscle cells (Figure 5C), suggesting that mature microvessels, such as arterioles, are not targeted by anti-VEGFR2-ILs-dox. However, no increased pericyte coverage of microvessels was detected in tumors of mice treated with anti-VEGFR2-ILs-dox, suggesting that vessel normalization did not occur upon this anti-angiogenic treatment regimen. This is in contrast with results found with other anti-angiogenic modalities (30).

Toxicity of anti-VEGFR2-immunoliposomes

To evaluate long-term toxicity of doxorubicin-loaded anti-VEGFR2-ILs, we injected cohorts of C57Bl/6 mice (n=3) either with anti-VEGFR2-ILs-dox, PLD, or empty immunoliposomes. The injection dose and schedule was the same as for the efficacy experiments.

H&E staining of all internal organs, CAB staining of the liver and PAS staining of the kidneys 3 days after termination of the therapy did not reveal signs of acute toxicity. Analysis of peripheral blood in Rip1Tag2 mice of all cohorts showed a normal blood count throughout and after treatment (data not shown). H&E staining of histological sections of hearts one month after therapy revealed no signs of fibrosis (data not shown).

All animals survived for 18 months without apparent signs of distress. The body weight corresponded to that of age-matched untreated controls. Histological analysis of internal organs, including pancreas, liver and kidneys after this prolonged time period showed no morphological differences between treated mice and healthy controls (Figure 6).
Discussion

Immunoliposomal strategies combine the advantages of an encapsulated drug with the targeting modalities of a monoclonal antibody on its surface. In this study, we have evaluated the feasibility and the efficacy of targeting the tumor-associated endothelium by anti-VEGFR2-ILs loaded with doxorubicin.

The treatment with anti-VEGFR2-ILs loaded with doxorubicin induced a reduction of blood vessel density within 2 weeks. The response of the targeted blood vessels was dependent on both localization and morphology of the vessel. First, vessels localized in the tumor were most vulnerable, whereas vessels in healthy tissue were not affected. This probably reflects the differential expression of VEGFR2 between quiescent and tumor-associated vasculature. Secondly, capillaries consisting of a single layer of endothelial cells strongly responded to anti-VEGFR2-ILs-dox whereas endothelial cells within vessels covered by smooth muscle cells were protected from the effect of anti-VEGFR2-ILs-dox.

Anti-VEGFR2-ILs-dox tumor therapy was more potent than treatment with PLD or PLD combined with free DC101. In our study we used free DC101 at a concentration that matches the amount of DC101 found in the anti-VEGFR2-ILs-dox group. This corresponds to about 50 μg of DC101 per animal and is about 20 times less than the concentration used in trials of DC101 monotherapy (31). However, our results clearly show that anti-VEGFR2-ILs-dox are superior to PLD and anti-VEGFR2 antibody at the same dose level. The efficacy of anti-VEGFR2-ILs-dox is similar to that of a strong oral angiogenesis inhibitor, such as PTK787. Of the many treatment regimens tested in Rip1Tag2 mice, including genetic ablation or sequestration of tumor- and angiogenesis promoting factors (VEGF, insulin-like growth factor II, fibroblast growth factors), and classical chemotherapy, the treatment with anti-VEGFR2-ILs-dox is one of the most efficient, with up to 91% smaller tumor volume in
the interventional group, compared to 67-89% in previous studies (24). This also compares favorably with the treatment of Rip1Tag2 mice with another anti-angiogenic compound, the multi-kinase inhibitor sunitinib, which resulted in a tumor burden of 1/5 compared to control mice (31).

One limitation of the transgenic mouse models employed is the continuous transgenic expression of oncogenes, such as SV40 large T-antigen in Rip1Tag2 and the polyoma middle T antigen in MMTV-PyMT mice. Tumors re-grow after termination of therapy, thus precluding a long-term observation of mice. Experimental results from our and other laboratories (32, 33) indicate that tumor-associated vessels can become independent of VEGFR2-signaling through adopting FGFR-signaling, through vascular co-option or through loss of PTEN expression in vascular endothelial cells. These mechanisms may account for failure of anti-angiogenic therapy in preclinical models and in patients, yet it should be investigated whether this also applies to anti-VEGFR2 immunoliposomal strategies.

Recent reports have raised a fundamental concern about the safety of anti-angiogenic treatment (31). Therefore, we carefully evaluated side effects of the therapy in our preclinical tumor models. Regarding tumor bearing mice models, the analysis of nuclear atypia after therapy indicates that treatment with PLD but not anti-VEGFR2-ILs-dox elicits pronounced changes in morphology. No increase in tumor progression and in metastasis formation was apparent after treatment with anti-VEGFR2-ILs-dox and no notable long-term toxicity occurred.

A particular concern with anti-angiogenic therapy is the possibility of promoting tissue hypoxia and upregulated HIF-1α/2α signaling. This might counteract the beneficial effect of anti-angiogenic therapy (34). First experimental approaches against HIF-1α/2α signaling have been developed, and it will be interesting to test these compounds in conjunction with anti-angiogenic therapy.
In conclusion, we have demonstrated that the selective targeting of tumor-associated vascular cells with immunoliposomes is feasible and highly effective in repressing tumor growth. These results set the stage for the design of strategies that specifically target other components of tumor-associated vasculature, such as vascular tip cells, pericytes and smooth muscle cells. In addition, it is conceivable to direct immunoliposomes against other cells of the tumor microenvironment, such as lymphatic endothelial cells or tumor-associated fibroblasts, which play important roles in tumor genesis and propagation. Thus, immunoliposomes offer a versatile and efficient technological platform to test therapeutic strategies against different cellular compartments of malignant tumors.

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**Figure legends**

**Figure 1:**

**(A) Uptake of VEGFR2-targeted immunoliposomes in MS-1 cells.** The uptake of anti-VEGFR2-ILs was evaluated in VEGFR2-expressing MS-1 endothelial cells by flow cytometry. Cells were incubated with DiIC$_{18}$(3)-DS-labeled immunoliposomes containing DC101-Fab’ or with control liposomes prepared identically as ILs except for omission of the MAb fragment. A shift of one log was detected for anti-VEGFR2 ILs in target cells compared to non-targeted liposomes.

Open histogram: MS-1 cells only without treatment; filled histogram: control untargeted DiIC$_{18}$(3)-DS-labeled liposomes; dashed histogram: anti-VEGFR2-ILs-Dil.

**(B) PCR performed on a single cell suspension of pancreatic tumors of 12 week old Rip1Tag2 mice.** GLP-1R$^+$ β-tumor cells and tumor-derived CD31$^+$ blood endothelial cells were sorted by FACS and analysed for the expression of VEGFR1, VEGFR2 and VEGFR3. VEGFR2 is expressed by endothelial, but not by tumor cells.

**Figure 2:**

**Effect of doxorubicin-loaded anti-VEGFR2-immunoliposomes on tumor growth.** Treatment of Rip1Tag2 and MMTV-PyMT transgenic mice and HT29 xenotransplanted mice with anti-VEGFR2-ILs-dox effectively inhibits tumor growth.
(A) Tumor volumes in the Rip1Tag2 tumor mouse model, (B) the MMTV-PyMT breast cancer mouse model, (C) the HT-29 xenograft model. The tumor volumes in panels A-C were assessed after bi-weekly injections for a period of two weeks. The p value was calculated using the Kruskal-Wallis test.

(D) Kinetic of tumor growth in the HT-29 xenograft model. Tumor growth arrest is observed first in the anti-VEGFR2-ILs-dox group and later in the PLD group.

Figure 3:

Changes in tumor cell proliferation and apoptosis and in blood microvessel density in the Rip1Tag2 (top row), the MMTV-PyMT (middle row) and the HT-29 cancer model (bottom row). There is a trend for reduced proliferation in the anti-VEGFR2-ILs-dox group compared to the PLD group. Apoptosis is significantly increased in the anti-VEGFR2-ILs-dox group compared to all other treatment protocols. Blood microvessel density is significantly and specifically reduced in the anti-VEGFR2-ILs-dox cohorts in all three models.

Bars = mean ± standard deviation, p values were calculated using one-way anova testing with Newman-Keuls post test.

Figure 4:

Anti-VEGFR2 ILs-dox treatment leads to endothelial apoptosis in the tumor.

(A) Immunflourescence analysis of Rip1Tag2 mice of tumor-associated and physiological vessels in the endocrine and exocrine pancreas. Untreated control (top row), PLD (middle row) or anti-VEGFR2-ILs-dox (bottom row). Apoptosis was visualized with an anti-cleaved caspase3 antibody (red) and vessels were stained with an anti-CD31 antibody (green), blue=Dapi. Specific endothelial cell apoptosis was analysed (insert, bottom row). Scale bar = 50 µm. (B) Apoptosis significantly increases in CD31 positive tumor-associated endothelial cells of Rip1Tag2 mice treated with anti-VEGFR2-ILs-dox compared to PLD or untreated control mice, but
had no effect in the vasculature of the exocrine pancreas. Apoptotic cells were assessed per 400x high power field (hpf).
Bars = mean ± standard deviation, *** p<0.001 (Kruskal-Wallis post-test).

**Figure 5:**

**Immunofluorescence analysis of tumor-associated and physiological vessels.**

**(A)** Vessel in the exocrine pancreas of a Rip1Tag2 mouse treated with anti-VEGFR2-ILs-dox. This is an intact microvessel formed of a single layer of endothelial cells. Green = staining for CD31, blue = DAPI.

**(B)** Capillaries in the tumors of a Rip1Tag2 mouse treated with PLD. The endothelial cells form again a single layer tube. Green = staining for CD31, blue = DAPI.

**(C)** Vessel at the rim of a tumor in a Rip1Tag2 mouse treated with anti-VEGFR2-ILs-dox. This is a larger vessel composed of an endothelial and a smooth muscle cell layer that stayed intact despite the treatment with anti-VEGFR2-ILs-dox. Green = CD31, red = smooth muscle, blue = DAPI.

**(D)** Apoptotic endothelial cells in a tumor of a Rip1Tag2 mouse treated with anti-VEGFR2-ILs-dox. The endothelial cells still express CD31 but they have become pyknotic and do not form capillaries any more. Green = CD 31, blue = DAPI.

Scale bar **A-D** = 10 µm.

**Figure 6:**

**Treatment with anti-VEGFR2-ILs-dox does not cause organ toxicity.**

Microphotographs of H&E stained sections of pancreas, liver and kidneys from C56Bl/6 mice injected with anti-VEGFR2-ILs-dox or saline are shown. The mice were sacrificed 18 months after treatment and the internal organs were analysed morphologically for signs of toxicity. There was no morphological difference between organs in the saline cohort and in the anti-VEGFR2-ILs-dox cohort.

Scale bar = 100 µm.
Figure 3

**proliferation**

% BrdU positive cells

Ls-empty, Ls-dox (PLD), anti-VEGFR2-ILs-empty, anti-VEGFR2-ILs-dox, DC101 MAb

**apoptosis**

% TUNEL positive cells

Ls-empty, Ls-dox (PLD), anti-VEGFR2-ILs-empty, anti-VEGFR2-ILs-dox, DC101 MAb

**blood microvessel density**

vessels per visual field

Ls-empty, Ls-dox (PLD), anti-VEGFR2-ILs-empty, anti-VEGFR2-ILs-dox, DC101 MAb

ns, p < 0.05
Targeting tumor-associated endothelial cells: anti-VEGFR2-immunoliposomes mediate tumor-vessel disruption and inhibit tumor growth

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