Licence for Destruction: Tumor-specific Cytokine Targeting

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Abstract

Stroma is an integral part of solid tumors and plays a key role in growth promotion and immune suppression. Most current therapies focus on destroying tumors and/or abnormal vasculature. However, evidence is emerging that anti-cancer efficacy improves with vessel normalization rather than destruction. Specific targeting of cytokines into tumors provides proof-of-concept that tumor stroma is dynamic and can be remodeled to increase drug access and alleviate immune suppression. Changing the inflammatory milieu "opens" tumors for therapy and thus provides the license for destruction. This involves re-programming of paracrine signaling networks between multiple stromal components to break the vicious cycle of angiogenesis and immune suppression. With active immunotherapy rapidly moving into the clinic, local cytokine delivery emerges as an attractive adjuvant.

Glossary:

Cancer immunotherapy: cancer immunotherapy uses the immune system to reject tumors. Only recently, active immunotherapy has become available for cancer patients with the clinical approval of two agents: sipuleucel-T (Provenge, Dendreon) an autologous, dendritic cell vaccine for advanced prostate cancer, and Ipilimumab (Yervoy, Bristol-Myers Squibb) a monoclonal antibody to cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), for metastatic melanoma.

CD40: CD40 is a costimulatory molecule expressed on antigen presenting cells and in tumor stroma. CD40 agonists activate T cells and modulate tumor stroma. Anti-tumor effects of agonistic CD40 antibodies are currently assessed in clinical trials.

CpG-ODN: CpG-ODN is a synthetic reagent which consists of immunostimulatory oligodeoxynucleotides (ODN) with cytosine-guanine-rich (CpG) motifs and a phosphothioate-stabilized backbone. It mimics bacterial DNA and is a potent immune adjuvant. Through its interaction with Toll-like receptor-9 (TLR-9), CpG-ODN activates B cells and plasmacytoid dendritic cells (DC) in humans and a broader spectrum of DCs, B cells, and macrophages in mice.

Hypoxia: tumor hypoxia or low oxygen concentration is a consequence of vascular abnormalities and low oxygen supply in rapidly growing tumors which outgrow their blood supply. Anti-cancer drugs are often unable to penetrate into hypoxic areas.

Interstitial fluid pressure (IFP): IFP is regulated by stromal cells and extracellular matrix. Solid tumors have a raised IFP due to increased vessel permeability, lymphatic vessel abnormalities and interstitial fibrosis. Increased IFP in tumors reduces perfusion and drug penetration.

Pericytes: specialized mesenchymal cells which line and stabilize endothelial cells of small capillaries. Pericytes are part of the abnormal vascular bed in tumors and often loosely attached to tumor endothelial cells, reduced in numbers or less mature. Pericyte coverage is an important parameter for the assessment of tumor vessel remodeling/normalization.

Phage display libraries: phage display libraries are bacteriophage particles with randomly displayed peptides or antibody fragments of different binding specificities. Each peptide/antibody fragment recognizes different target molecules. Libraries can be injected intravenously to specifically screen for binding activities in a particular tissue or tumor. Phages that bind to target molecules in the tumor of interest are enriched after multiple rounds of biopanning. Subsequently, binding moieties are analysed and can be developed into targeting vehicles.

Regulator of G protein Signaling-5 (RGS5): RGS5 is a member of the regulator of G protein signaling family which is abundantly expressed in vascular smooth muscle cells and modulates vascular homeostasis by controlling G protein-coupled receptor signaling. RGS5 is specifically upregulated in tumor pericytes. Removing RGS5 from the tumor microenvironment in murine pancreatic tumors leads to normalization of the tumor vasculature and improved response to immunotherapy.

Tumor-associated macrophages (TAM): innate immune cells which are found in the majority of solid tumors. TAMs represent M2 activated macrophages which promote tumor growth by secreting factors that stimulate breakdown of extracellular matrix and vessel growth, and inhibit anti-cancer immunity. In contrast, M1 macrophages support anti-tumor immunity.

Microenvironmental therapy

Tumor cells are embedded in stroma which is composed of blood vessels, immune cells, and connective tissue including fibroblasts and extracellular matrix. Stroma is crucially involved in tumor growth, invasion and metastasis [1-2]. The tumor microenvironment also impedes drug delivery and thus reduces the efficacy of conventional anti-tumor therapies such as chemo- and radiation therapy [3-4]; similar mechanisms contribute to a general lack of cytotoxic T cell function and anti-tumor immunity [5-6].

Tumors create their own microenvironments which are diverse and tumor type- and stage-dependent; however, tumors also share common stromal features and signaling themes. In particular, intricate relationships between inflammatory factors, macrophages and blood vessels exist in most solid tumors which modulate growth, therapeutic response and ultimately relapse [7-9]. Disruption of these relationships and remodeling of stroma opens tumors for cytotoxic drugs or immune destruction and thus creates new and exciting opportunities for anti-cancer therapy. As new stromal markers and functional relationships are discovered, potential therapeutic strategies include local delivery of drugs/toxins into the tumor microenvironment via peptides or antibodies. In vivo screening of phage-display libraries of peptides or antibodies have identified unique targets for stroma-specific molecules in situ, resulting in successful development of targeted delivery of diagnostic or bioactive fusion compounds [10-12]. These compounds may have efficacy on their own, but in the right context, can also enhance other anti-cancer drugs and, importantly, anti-tumor immunity. Here, we discuss the latest aspects of "microenvironmental therapy" which exploits the conventional concept of peptide or antibody-mediated targeting in the context of tumor stroma modulation and vascular normalization for improved combination therapies.

Targeting tumor vasculature

The majority of peptides or antibodies with high stromal affinity identified by in vivo perfusion methods bind to the angiogenic vasculature in solid tumors [10 , 11] (Box 1). This is not surprising since tumor blood vessels are morphologically and functionally different from normal blood vessels [13]. Moreover, intravenous injection and blood circulation facilitate binding of ligands on the luminal side of endothelial cells. Such peptides which generically home to angiogenic vessels have the RGD (Arg-Gly-Asp) or NGR (Asn-Gly-Arg) motifs which bind to $\alpha_v\beta_3/\alpha_v\beta_5$ integrins and the metalloprotease aminopeptidase N (CD13),

respectively [14-15]. Most prominent vessel-targeting antibody fragments (single-chain variable fragments, scFv) are directed against specific splice variants of fibronectin (L19, F8) and tenascin C (G11, F16), both part of the extracellular matrix (ECM) surrounding tumor neovasculature [16-19]. Thus, these targeting reagents commonly recognize molecules actively involved in cell-cell/cell-matrix interactions and angiogenic vessel remodeling, and are frequently overexpressed in the tumor vasculature. Importantly, the value of these peptides/antibodies is in their potential to deliver therapeutic payloads into precise tumor stromal compartments.

Therapeutic tumor targeting

Peptide or antibody fusion compounds when used as carrier molecules provide a unique opportunity to improve anti-cancer therapy whilst reducing harmful side effects. An impressive spectrum of fusion compounds for delivery of toxic agents, radionuclides, pro-coagulation factors and cytokines have been tested in preclinical tumor models and some clinical trials are underway (Table 1). For instance, application of the NGR motif fused with pro-apoptotic factors such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [20] or the D-amino acid peptide $_{D}$[KLAKLAK]$_2$ [21] induces tumor endothelial cell apoptosis. Vessel-targeted truncated tissue factor (tTF) causes thrombosis and vessel collapse in animal models and some reduction in tumor perfusion in a clinical case [22]. Chemotherapeutic drugs have also been directly conjugated to targeting moieties or used in various combination therapies [23-24]. Indeed, most targeting efforts to date have been directed to improve cytotoxic drug delivery into tumors and enhance drug penetration into parenchyma to amplify anti-tumor cytotoxicity (Box 2).

Inflammatory factors such as cytokines represent attractive compounds for specific, high dose delivery into tumors. Anti-tumor effects of cytokines have been well documented over the last decades. In particular high-dose TNFα disrupts angiogenic vessels and is currently used in isolated limb perfusion to treat locally advanced melanoma and soft tissue sarcoma [25-26]. However, the clinical application of cytokines has been restricted to local treatment due to high toxicity. Precise targeting of tumor vessels is therefore a promising strategy and has been employed for various peptide/antibody-cytokine chimeric compounds which include interleukin (IL) 2, IL12, interferon γ (IFNγ) and TNFα (Table 1). Most cytokines are not cytotoxic for cancer cells, but exert direct anti-vascular effects at high doses and also modulate the host's immune system. Thus, tumor rejection in preclinical models requires immune-competency, frequently leads to tumor infiltration by adaptive and innate immune

cells, and may also induce immunological memory [27-28]. Interestingly, efficacy of targeted cytokine therapy can be enhanced when combined with conventional chemotherapy possibly by promoting intratumoral accumulation of cytotoxic drugs and/or tumor antigen presentation [29-31]. Selected compounds are currently being evaluated in clinical trials with encouraging results especially in combination with chemotherapy (Table 1). Given the clinical application of TNFα in isolated limb perfusion, TNFα is one of the best-studied cytokines which has also been conjugated to ligands such as NGR, RGD and L19, and successfully used as a ligand-directed vascular targeting agent (Table 1). Anti-cancer effects of vessel-targeted TNFα were attributed to increased tumor vessel leakage and enhanced drug uptake [29-30, 32]. Whilst these mechanisms are difficult to demonstrate in the clinic [33], low dose NGR-TNFα is currently the only peptide compound with vessel-targeting capability which shows promising effects in early clinical trials when combined with chemotherapy [34-35].

Notably, most selective vascular targeting approaches induce vessel death. Destroying tumor vasculature to restrict blood supply has been validated clinically leading to the approval of several drugs which block vascular endothelial growth factor (VEGF) signaling pathways. When used in combination with chemotherapy, disease stabilization and overall survival benefits are observed for some tumor types. However, whilst destruction of tumor vessels is a logical anti-cancer approach, the optimism of three decades has been tempered by merely transient anti-tumor effects and disappointing long term responses [36]. Conceivably the major value of anti-vascular therapies will only occur by further improving vascular targeting strategies and in combination with synergistic treatment modalities such as chemo- and immunotherapy. Interestingly, it may well be that restoration of tumor vessel function, so called normalization, rather than destruction may be an alternative and more sustained approach to enhance anti-tumor effects of vessel targeting strategies; this requires further investigation to explore its full therapeutic potential.

Tumor targeting of immune modulatory cytokines re-visited

Earlier studies using NGR-TNFα in conjunction with cytotoxic drugs for anti-cancer therapy were based on the assumption that cytokines such as TNFα damage the tumor vasculature, increase vessel leakiness and thus drug and immune cell penetration [32, 37-38]. However, this is difficult to reconcile with the exceedingly low therapeutically effective doses of NGR-TNFα (nanograms (ng) or picograms in mice [38] and 0.8 microgram (μg)/m^2 in humans [39-40]). Also, increased vessel leakiness for plasma molecules does not necessarily enhance

drug perfusion deep into solid tumors. Instead, leaky tumor vessels create a hypoxic environment with increased interstitial fluid pressure (IFP) [3]. High IFP in turn acts as a barrier for effective drug delivery and may also prevent infiltration of immune effector cells. This raises the question what other vascular changes may render tumors permissive for drug or immune cell uptake. Cytokines including TNFα activate vessels which play a crucial role for leukocyte trafficking into sites of inflammation and conceivably into tumor vascular beds. Interestingly, TNFα has also been shown to reduce IFP, and low dose systemic injection facilitates uptake of circulating liposomes indicative of improved blood flow, tumor perfusion and vessel function [41-42]. Indeed, recent studies have re-visited intratumoral cytokine effects and provide new insights how tumor-targeted cytokines may be exploited to activate and/or remodel the tumor vasculature for improved therapy [43-45].

Vascular activation and anti-tumor immunity

Despite the presence of inflammatory cytokines in solid tumors, transmigration through endothelial barriers and tumor homing of effector T cells seems to be a rate limiting step for tumor cell lysis [46]. However, a common underlying theme of successful immune therapy is the activation of tumor blood vessels [47]. Indeed, experimental induction of vascular adhesion molecules such as E-/P-selectins, vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) in tumor vascular beds enhances T cell transmigration and tumor rejection [48-53]. For instance, in ovarian cancer, expression of endothelin B receptor (ETBR) on tumor vessels is inversely correlated with tumor infiltrating lymphocytes and patient survival. Inhibition of ETBR increases ICAM-1 expression on endothelia and concomitantly T cell influx [49]. Similarly, inert tumor vessels in several tumor models are rendered susceptible for T cell penetration and immune-mediated tumor killing by IL-6-dependent induction of inflammatory adhesion molecules such as E/P-selectin and ICAM-1 [53]. Thus, anergic tumor blood vessels can be activated to permit immune cell invasion and tumor cell killing. Intriguingly, low dose TNFα when targeted to tumor vessels in a mouse model of pancreatic endocrine tumors activates the vasculature resulting in high expression of adhesion molecules. This enables effector cell trafficking into tumor parenchyma, but more importantly, shows strong therapeutic effects when combined with anti-tumor vaccination or adoptive T cell transfers [45]. Thus, vascular activation induced by changing the intratumoral cytokine profile can act as a strong adjuvant to immunotherapy (Table 2). Moreover, synergistic efficacy of a triple therapy combining low dose NGR-TNFα

with anti-tumor vaccination and chemotherapy is observed in a mouse model of subcutaneously growing melanoma [54]. These studies demonstrate that a combination of cytokine targeting with immunotherapy is a highly effective approach; in combination with chemotherapy, induction of immunogenic cancer cell death may further enhance activation of anti-tumor immunity [55]. This warrants further investigation in particular in light of increasing clinical applications of anti-cancer immune therapy [56].

Vascular normalization and anti-tumor immunity

Recently, a new concept has emerged where vessel stabilization and reduced vascular permeability, a phenomenon described as vessel normalization [57], improves anti-cancer therapies; remarkably, this includes chemo- and radiation therapies, delivery of nanoparticles and immunotherapy [50, 58-63]. Proof-of-principle that vessel remodeling per se, even in the absence of inflamed vascular endothelium, dramatically improves the outcome of immunotherapy comes from genetic deletion of the regulator of G protein signaling (RGS) 5. This gene is highly upregulated in angiogenic vessels; loss of RGS5 causes pericyte maturation, vascular normalization and importantly, activated T effector cell influx in quantities sufficient for tumor rejection in a mouse model of pancreatic endocrine cancer [50]. Subsequently, it has been demonstrated that blockade of VEGF/VEGFR pathways for instance in B16 melanoma and orthotopic breast cancer models temporarily normalizes vessels and improves active immunotherapy, in particular with low dose angiostatic treatment [60, 63]. It is conceivable that improved tumor blood flow concomitant with reduced hypoxia and IFP enhances delivery of therapeutics including tumor-specific T cells.

In preclinical models, evidence for enhanced therapeutic efficacy with vessel modulation and normalization rather than destruction is compelling. Ongoing clinical studies show promising synergy between angiostatic treatment and chemotherapy. Whether this is the result of vessel destruction and increased leakiness or vessel remodeling and improved perfusion is still unclear. So far, only a limited number of clinical studies have been conducted which assess tumor perfusion/vessel permeability in the context of classical anti-angiogenesis therapy [64]. Currently, NGR-TNFα is the only targeting compound where imaging of vascular changes was incorporated in a clinical trial. In a patient cohort undergoing a phase I dose escalation study, dynamic contrast enhanced (DCE)-MRI was used to analyse early effects (2 h) of NGR-TNFα on vessel permeability [33]. Overall, DCE-MRI failed to predict the optimal treatment dose. However, interesting observations include heterogeneity in vascular response

depending on tumor size, e.g., small nodules are more responsive. Also observed are reduced leakiness of vessels in liver metastases after treatment, which may be indicative of vascular normalization, and off-target effects on normal liver tissue despite a tumor-specific vascular targeting approach. Since animal studies document the dynamic nature of drug-induced vessel remodeling (Figure 1) imaging of acute, disease stabilizing and relapse phases for different human tumors is warranted in particular in anticipation of immune combination therapies.

Vessel modulation is context-dependent

Changes in tumor vessels, be it death, activation or normalization, are not mutually exclusive. Instead, modulation of the tumor vasculature is a dynamic process which can induce several phenotypes simultaneously or consecutively (Figure 1). Moreover, the actual vascular effects of peptide targeted inflammatory mediators vary significantly depending on compound, dose, and treatment duration. This is exemplified in a series of studies which used a prototypic mouse model of pancreatic endocrine cancer and the RGR peptide to correlate vascular effects with anti-tumor immunity (Figure 1). In this model, even fully activated anti-tumor effector cells fail to penetrate highly angiogenic tumors as is often observed in human cancers. Interestingly, vascular targeting of µg quantities of IFNγ destroys the tumor vasculature resulting in some therapeutic efficacy reminiscent of anti-angiogenic therapy; intratumoral IFNγ fails, however, to improve T cell extravasation and anti-tumor immune responses [45]. Even though IFNγ has potentially strong immune-enhancing properties, it predominantly affects angiogenesis in the tumor microenvironment. This is consistent with other models where IFNγ specifically destroys stroma during immune-mediated tumor rejection [65]. Thus, cytokines with strong anti-vascular effects do not necessarily enhance leukocyte influx and tumor immunity. Similarly, no synergy is observed between vascular disruption agents which cause hemorrhagic tumor necrosis and active anti-cancer immune therapy [66]. In contrast, agonistic CD40 antibodies when conjugated with RGR peptide specifically activate CD40-positive tumor vessels to express VCAM, ICAM and E-selectin. Activation of endothelia alone has no anti-tumor efficacy. However, when combined with adoptive transfers of anti-tumor T cells, these effector cells efficiently enter otherwise inaccessible tumors. Survival was further enhanced by using an IL2-RGR fusion protein which increases effector cell activity once they reach the tumor site [43]. Effective vessel activation is also achieved by vascular targeting of RGR-coated liposomes containing toll-

like receptor (TLR) 9 ligands (e.g., oligodeoxynucleotides with CpG motifs, CpG-ODNs). Remarkably, CpG-ODN not only inflames vessel walls but also primes anti-tumor cytotoxicity, most likely due to uptake by intratumoral macrophages and efficient antigen presentation. Again, spontaneous anti-tumor immunity is further enhanced with adoptively transferred anti-tumor T cells [44]. As discussed above, low dose RGR-TNFα therapy (ng and µg quantities) induces strong expression of inflammatory adhesion molecules on tumor vessels. Simultaneously, vessels are stabilized with reduced permeability and increased perfusion. This stage also permits influx of adoptively transferred, pre-activated effector cells. However, continuous, low dose RGR-TNFα kills vessels and stroma and ultimately limits effector cell penetration [45]. Stromal destruction as a consequence of prolonged treatment is observed in most preclinical models and demonstrates the unsustained nature of current treatment modalities [43-44, 57]. Thus, the challenge ahead is to develop targeting strategies which modulate rather than destroy stroma for improved anti-tumor immunity.

Immune-amplification in the tumor environment

Vessel targeting strategies naturally focus on direct changes in the tumor vascular bed. However, recent evidence demonstrates an intricate network of paracrine signals between vessels and other stromal cells, most notably macrophages, which indirectly shapes the vascular phenotype, and the tumor immune milieu. Tumor-associated macrophages (TAMs) display a high degree of plasticity and have been implicated in vascular remodeling. TAMs normally exhibit an M2 phenotype and, without intervention, foster angiogenesis, tumor growth and immune evasion [9]. However, TAMs can be re-educated to reverse the tumor promoting program [45, 67-69]. For instance, genetic deletion of VEGF specifically in myeloid cells results in vascular normalization in a transgenic mouse model of breast cancer which in turn increases susceptibility to chemotherapy [67]. Experimental tumors with forced expression of histidine-rich glycoprotein harbor M1-type macrophages which produce less placental growth factor (PlGF) and display a normalized vasculature; re-programming of TAMs improves chemotherapy response and also anti-tumor immunity [69]. Moreover, IFNα^{high} myeloid cells which specifically home to tumor vessels in an orthotopic glioma model stimulate TAMs to secrete proinflammatory cytokines such as IL-1 and TNFα concomitant with vascular normalization [68].

Intriguingly, TAMs also play a crucial role in therapeutic efficacy of low dose TNFα targeting. Besides vessel remodeling, intratumoral RGR-TNFα elicits profound stromal

activation which also includes TAMs. TAMs change their expression profile after TNFα treatment and are polarized to secrete M1-like inflammatory factors, e.g., monocyte chemotattractant protein (MCP)-1, inducible nitric oxide synthase (iNOS), and angiopoietin (Ang)2. This in turn has dual effects on the tumor environment: first, M1-like TAMs act as a strong adjuvant to $CD8^+$ T cell effector function, and second, Ang2 in conjunction with TNFα upregulates the expression of endothelial adhesion molecules which in turn facilitated leukocyte transmigration [45] (Figure 2). This immune amplification cascade which triggers profound changes in the tumor micromilieu explains some of the remarkable effects of low dose intratumoral TNFα. It also provides proof-of-concept that although vascular targeting has obvious limitations, such as non-specific uptake, heterogeneous binding to tumor vessels and insufficient payload delivery, cytokine targeting is a viable and promising approach for further exploration.

Concluding remarks and future perspective

Over the past decade tumor targeting has been explored and refined concomitant with increasing knowledge about tumors and their microenvironments. Whilst the idea of fighting tumors from the inside is compelling, it is transpiring that therapeutic use of tumor targeting will depend on strategies to maximize intratumoral effects. This includes optimized bioengineering for improved ligand-receptor interactions, use of targeting moieties which allow deeper and more homogeneous access into the tumor parenchyma, and simultaneous or sequential targeting of multiple stromal components. Discovery of new ligands which bind to non-vascular stromal targets such as cancer-associated macrophages and fibroblasts [70-71] will facilitate this process. Multi-targeting strategies could then be explored to eliminate or remodel stromal cells in primary tumors and also metastatic lesions [72].

Ultimately, however, stromal targeting requires further refinement to enhance other anti-cancer modalities. Vascular destruction without complete eradication of cancer cells will at best delay tumor growth but may also induce resistance, relapse and increased invasiveness as witnessed with anti-angiogenesis drugs. New approaches which exploit the dynamic nature of tumor stroma to re-program rather than destroy vessels may provide longer lasting anti-tumor effects, in particular, if stromal remodeling is combined with strategies to eliminate cancer cells such as cytotoxic, radiation, molecular targeted and immune therapies.

Exciting new developments demonstrate that immune signaling cascades can be activated to amplify anti-tumor activity by re-education of tumor-resident cells and recruitment of

immune cells into tumors which promote rejection [44]. In this context it is remarkable that minute amounts of selected inflammatory factors when delivered into tumors can have profound effects on both tumor perfusion and anti-tumor immunity [45]. This is consistent with an active immune-suppressive role of stroma and the observation that soluble factors in untreated tumors simultaneously promote angiogenesis and immunosuppression. Overall, this encourages further development of combination therapies which first create an angiostatic and immunostimulatory environment followed by cell lysis for complete tumor destruction.

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Box 1. Tumor peptide-targeting up to date

Binding activities of peptide-ligands can be based on overexpression and also on cancer-specific cellular localization of their receptors which differs from normal cells. For example, the F3 peptide, a fragment of the human high mobility group protein 2, binds to nucleolin which can be aberrantly exposed on the surface of endothelial and tumor cells during carcinogenesis [73]. Tumor endothelial cells are surrounded by pericytes and a basement membrane, both of which are commonly altered in solid tumors. Collagen IV for instance is modified by matrix metalloproteinases (MMPs) during angiogenic vessel remodeling and a peptide sequence (TLTYTWS) has been identified that specifically binds to collagen IV modified by MMP-2 [74]. Peptide ligands such as CPRECES (receptor: aminopeptidase A (CD249)), CRGRRST (RGR peptide, putative receptor: platelet-derived growth factor receptor (PDGFR)β), CSRNLIDC (pBP peptide, receptor: PDGFRβ) or indeed NGR (receptor CD13) with vascular targeting properties may also bind to tumor pericytes [54, 75-78]. As a consequence of leaky tumor blood vessels, blood clotting complexes are located in tumor vessels and surrounding stroma which provide specific docking signals for the pentapeptide CREKA [79]. Besides blood vessels, lymphatic vessels are also an integral part of solid tumors and intimately involved in metastatic spread. Peptides such as LyP-1 (CGNKRTRGC) which have been identified as ligands for the molecule p32, when expressed on the cell surface, bind to tumor-associated lymphatics, some p32-positive tumor cells and tumor-associated macrophages [70].

Box 2. Vascular homing peptides: optimizing cytotoxic anti-tumor effects

One strategy to improve vascular targeting and anti-tumor effects is to use nanoparticles decorated with multiple vascular homing peptides and loaded with cytotoxic drugs for release at the tumor site [11]. Multivalent targeting of cytotoxic nano-carriers can overcome some pharmacological limitations of directly conjugated peptide ligands. However, access into tumors beyond the vasculature remains challenging. Recently, Ruoslahti and colleagues described a series of peptides which specifically bind to tumor stroma and also penetrate into tumor tissue [94-96]. Most remarkable is the capacity of prototypic iRGD peptide (CRGDK/RGPD/EC) to deliver payloads such as doxorubicin into tumors simply by co-administration, thus abolishing the need to produce fusion compounds. This approach results in a 14-fold increase in doxorubicin containing liposomes in tumors as compared to injection of doxorubicin liposomes without iRGD and significantly enhances anti-tumor effects [94].

Another recent development elegantly harnesses biological effector cascades to combine traditional chemotherapy with vascular destruction. For instance, rod-shaped gold nanoparticles (nanorods) which passively home into tumors are used to induce coagulation under near-infrared light irradiation. This in turn significantly enhances accumulation of doxorubicin-loaded liposomes conjugated with the peptide substrate for the coagulation factor FXIII in human breast cancer xenotransplants [97]. Specific targeting of coagulated tumor vessels for delivery of cytotoxic drugs may overcome some limitations arising with anti-angiogenic therapy such as decreased tumor perfusion and limited drug access.

Figure Legend

Figure 1: Dynamic vessel remodeling is context dependent. Solid tumors create a proinflammatory, pro-angiogenic environment which promotes angiogenesis, tumor growth and immune suppression. However, the tumor microenvironment can be modulated by targeted delivery of inflammatory factors with different therapeutic implications. In a mouse model of pancreatic endocrine cancer, targeting of IFNγ specifically to the angiogenic vasculature using the RGR peptide results in vessel death and reduced tumor growth without immune involvement [44]. Tumor-targeted agonistic antibodies against CD40 (RGR-αCD40) or inflammatory agents such as RGR-CpG-ODN predominantly activate tumor vessels which in turn promotes effector cell infiltration and immune-mediated tumor regression [42-43]. RGR-TNFα activates and normalizes tumor vessels, and also supports anti-tumor immunity [44]. Vessel activation and normalization are not mutually exclusive. However, most long term treatment with vessel-activating agents leads to stromal destruction which ultimately limits therapeutic efficacy [42-44]. Abbreviations: CD31, vascular marker (also known as platelet endothelial cell adhesion molecule (PECAM-1); CpG-ODN, oligodeoxynucleotides with CpG motifs; IFNγ, interferon γ; RGR, peptide sequence (CRGRRST) which confers specific binding to angiogenic tumor vessels; TNFα, tumor necrosis factor α; VCAM, vascular cell adhesion molecule.

Figure 2: Intratumoral immune amplification. Local, low dose TNFα acts on multiple stromal cells, including blood vessels and macrophages, to improve tumor perfusion, leukocyte extravasation and anti-tumor immunity. TNFα-stimulated macrophages, which cluster around the vasculature and play an important role in amplifying vessel activation by secreting Ang2. Ang2, in conjunction with TNFα, upregulates the expression of endothelial adhesion molecules such as VCAM. TNFα also skews tumor-resident macrophages to produce M1-like factors which support T cell activity (MCP1, IL6, iNOS). Thus, immune amplification triggered by tumor- targeted TNFα enhances spontaneous anti-tumor immunity and active immunotherapy [45]. Abbreviations: Ang2, angiopoietin 2; IL6, interleukin 6; iNOS, inducible nitric oxide synthase; MCP1, monocyte chemoattractant protein 1, TNFα, tumor necrosis factor α, VCAM, vascular cell adhesion molecule.

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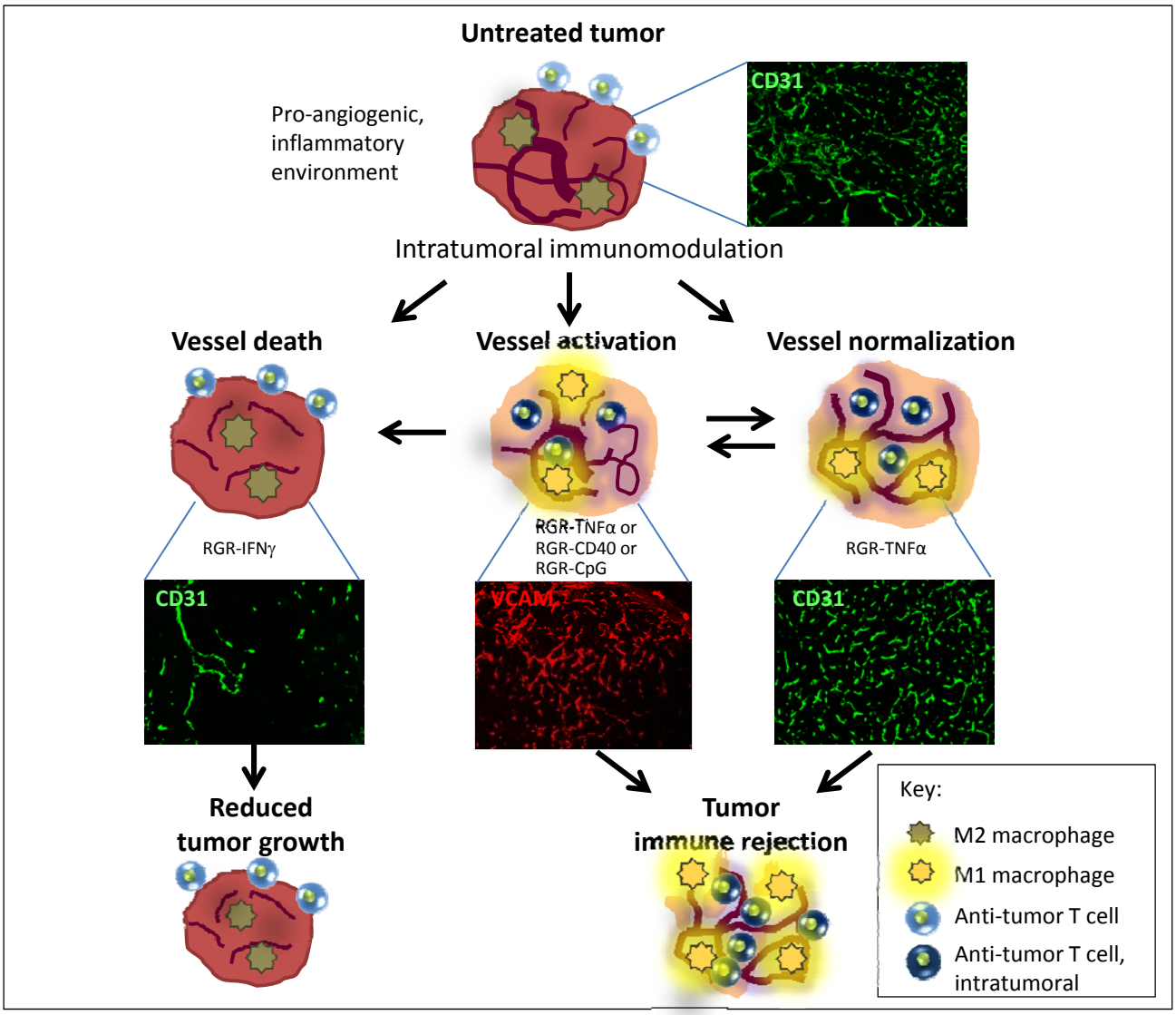
Untreated tumor
Pro-angiogenic, inflammatory environment
CD31
Intratumoral immunomodulation
Vessel death
Vessel activation
Vessel normalization
RGR-IFNγ
CD31
RGR-TNFα or RGR-CD40 or RGR-CpG
VCAM
RGR-TNFα
CD31
Reduced tumor growth
Tumor immune rejection
Key:
M2 macrophage
M1 macrophage
Anti-tumor T cell
Anti-tumor T cell, intratumoral

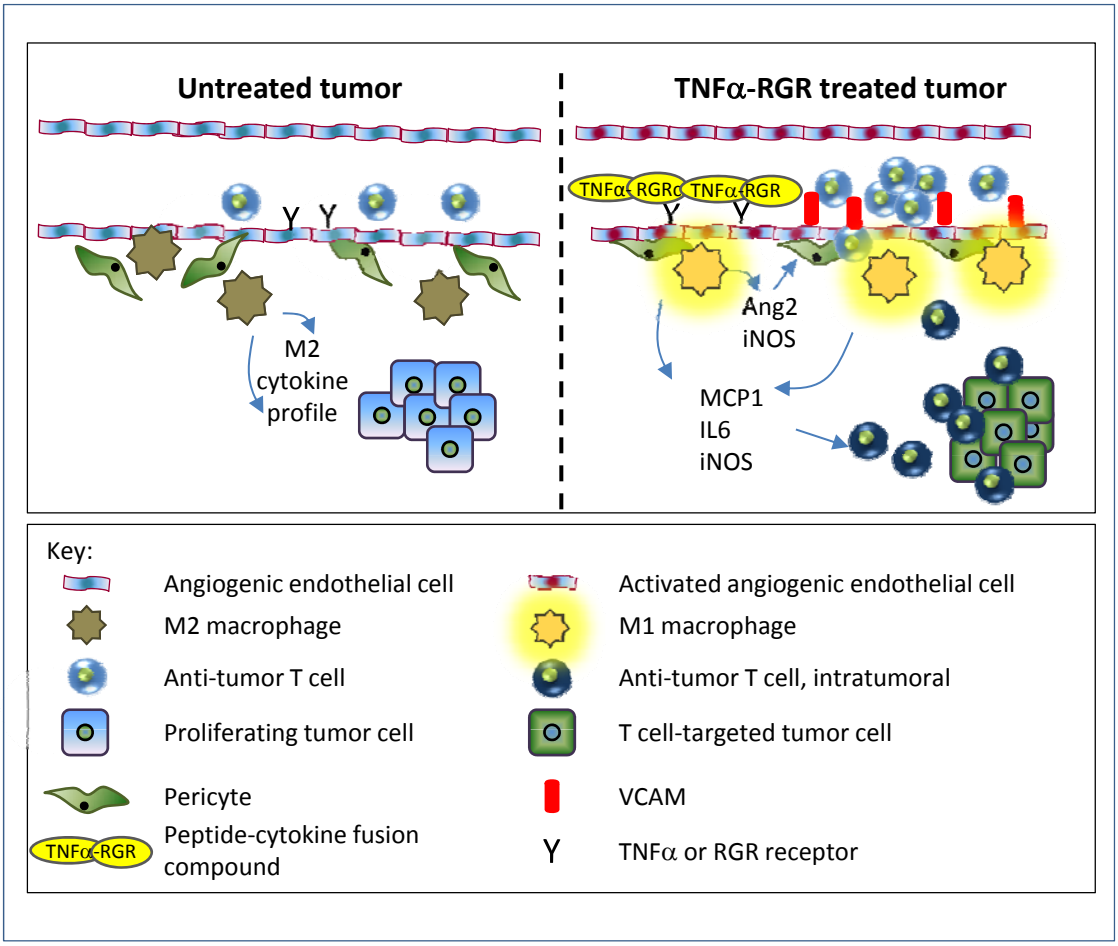
Untreated tumor
TNFα-RGR treated tumor
M2
cytokine
profile
TNFα
RGRα
TNFα-RGR
Ang2
iNOS
MCP1
IL6
iNOS
Key:
Angiogenic endothelial cell
Activated angiogenic endothelial cell
M2 macrophage
M1 macrophage
Anti-tumor T cell
Anti-tumor T cell, intratumoral
Proliferating tumor cell
T cell-targeted tumor cell
Pericyte
VCAM
TNFα-RGR
Peptide-cytokine fusion compound
TNFα or RGR receptor

Table 1: Vessel targeting and anti-tumor cytotoxicity[a]

Ligand	Target	Receptor	Active compound (directly conjugated to ligand)	Combination therapy	Tumors	Refs
Preclinical models						
RGD and analogues	TEC	α_v integrins	TNFα	chemotherapy	lymphoma	[80]
			IL12	none	neuroblastoma	[81]
			^{90}Y (β-emitter)	none	ovarian cancer xenograft	[82]
			TRAIL	chemotherapy	colon cancer xenografts	[20]
			$(KLAKLAK)_2$	none	breast cancer xenograft	[21]
NGR	TEC/TPC	CD13	TNFα	chemotherapy	melanoma, lymphoma xenografts and transgenic prostate tumor model	[27] [37] [38] [83]
			doxorubicin	none	breast cancer xenograft	[23]
			tTF	none	lung carcinoma, fibrosarcoma, melanoma xenotransplants	[84] [85]
L19 or F8	TEC	ED-B fibronectin	TNFα	chemotherapy	fibrosarcoma, colon carcinoma	[86] [87]
			IL2	chemotherapy	lymphoma, melanoma	[80]
			IL12	chemotherapy	teratocarcinoma, lymphoma, colon carcinoma	[88] [89]
Clinical trials						
NGR	TEC/TPC	CD13	TNFα	none	hepatocellular carcinoma (phase II), colorectal carcinoma (phase II), malignant pleural mesothelioma (phase II)	[40] [90] [39]
			TNFα	capecitabine-oxaliplatin	colorectal carcinoma (phase II)	[91]
			TNFα	doxorubicin	solid tumors (phase Ib), ovarian carcinoma (phase II)	[34-35]
L19	TEC	ED-B fibronectin	TNFα	none	solid tumors (phase I/II)	[92]
			IL2	dacarbazine	metastatic melanoma (phase II)	[93]

[a]Abbreviations: CD13, metalloprotease aminopeptidase N; ED-B, extra domain and B splice variant; F8, antibody fragment directed against a specific splice variant of fibronectin; IL2, interleukin 2; IL12, interleukin 12; $(KLAKLAK)_2$, apoptosis-inducing peptide; L19, antibody fragment directed against a specific splice variant of fibronectin; NGR, peptide containing the NGR motif (Asn-Gly-Arg); RGD, peptide containing the RGD motif (Arg-Gly-Asp); TEC, tumor endothelial cells; TNFα, tumor necrosis factor α; TPC, tumor pericytes; TRAIL, TNF-related apoptosis-inducing ligand; tTF, truncated tissue factor.

Table 2: Vessel targeting and active immunotherapy[a]

Ligand	Target	Receptor	Active compound (directly conjugated to ligand)	Combination therapy	Tumors	Refs
RGR	TEC/TPC	N/A	CD40 agonist antibody and IL2	adoptive T cell transfer	pancreatic neuroendocrine cancer	[43]
			CpG-ODN containing liposomes	adoptive T cell transfer or anti-tumor vaccination	pancreatic neuroendocrine cancer	[44]
			TNFα	adoptive T cell transfer or anti-tumor vaccination	pancreatic neuroendocrine cancer	[45]
NGR	TEC/TPC	CD13	TNFα	anti-tumor vaccination or anti-tumor vaccination/chemotherapy	melanoma	[54]
L19	TEC	anti-ED-B fibronectin	IL2	anti-CTLA-4	teratocarcinoma, colon carcinoma	[98]

Abbreviations: CD13, metalloprotease aminopeptidase N; CD40, TNF receptor superfamily member 5; CpG-ODN, oligodeoxynucleotides with CpG motifs; CTLA4, cytotoxic T-lymphocyte antigen 4; ED-B, extra domain and B splice variant; IL2, interleukin 2; L19, antibody fragment directed against a specific splice variant of fibronectin; NGR, peptide containing the NGR motif (Asn-Gly-Arg); RGR, peptide containing the RGR motif (Arg-Gly- Arg); TEC, tumor endothelial cells; TNFα, tumor necrosis factor α; TPC, tumor pericytes;