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# Intratumoral TNFα improves immunotherapy

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**Abbreviations:** Ang2, angiopoietin 2; HGR, histidine-rich glycoprotein; IFNγ, interferonγ; IL-6, interleukin 6; iNOS, inducible nitric oxide; MCP1, monocyte chemotactic protein 1; Tag, SV40 large T antigen; RIP, rat insulin gene promoter; TNFα, tumor necrosis factorα; VCAM, vascular cell adhesion molecule

Solid tumors are frequently resistant to immunotherapy. We demonstrated that low-dose tumor necrosis factorα (TNFα), when directly targeted to the tumor environment, exerts dual effects by improving vessel functionality and activating immune cells. This vascular remodeling in an inflammatory context enhances active immunotherapy and promotes tumor regression.

Tumor growth relies on interactions with stromal cells, which can also contribute to immune evasion and limit the efficacy of immunotherapy. For instance, due to ongoing angiogenesis, solid tumors develop abnormal and leaky blood vessels, which increase hypoxia and interstitial fluid pressure, two parameters known to interfere with anticancer therapy.[1] However, the tumor stroma is highly dynamic in nature and recent publications have highlighted that reversing abnormal features of stromal cells can largely improve the outcome of immunotherapy.[2,3] In this context, we became interested in tumor necrosis factor α (TNFα) as it is highly upregulated in tumors with normalized vessels undergoing immune regression, suggesting a local immunomodulatory function.[4] TNFα is a pleiotropic inflammatory cytokine best known for its capacity to induce tumor and endothelial cell death. However, high-dose TNFα is toxic for normal tissue, which restricts its clinical applications. Tumor-targeting strategies such as conjugating TNFα with vessel homing peptides have been shown to prevent systemic toxicity, and also improve the efficacy of chemotherapy.[5] Synergism between intratumoral TNFα and chemotherapy has been attributed to increased vascular permeability, but analyses of stromal effects in vivo have so far been limited.[6] Moreover, the role of TNFα as an adjuvant to immunotherapy has not been explored until recently.[7]

Our work demonstrated that tumor-targeted TNFα has profound effects on the tumor microenvironment by stabilizing blood vessels and potentiating immunotherapy.[8] TNFα was conjugated to a vascular homing peptide which specifically binds to angiogenic tumor vessels in a murine model of pancreatic endocrine tumors (RIPTag, expression of the SV40 Large T antigen by the rat insulin gene promoter). Peptide-coupled TNFα accumulates around tumor vessels, attracts T cells into the tumor microenvironment and primes an endogenous antitumor $CD8^+$ T cell-dependent immune response, ultimately enhancing overall survival.[8] Considering the immunostimulatory properties of tumor-targeted TNFα monotherapy, we hypothesized that it could also function as adjuvant in conjunction with active immunotherapy. Indeed, intratumoral TNFα "opens" tumors to the influx of adoptively transferred, pre-activated effector cells (**Fig. 1**). This is remarkable since fully activated effector cells are per se unable to penetrate into insulinomas in RIPTag mice. Under TNFα therapy, however, transgenic T cells specific for the model tumor antigen Tag accumulate and proliferate in the tumor, leading to very significant improvements in survival.

These results raised the question of how local TNFα renders the tumor microenvironment permissive for anti-tumor immune responses. Our results clearly show that low-dose TNFα does not compromise barrier function or destroy vessels. Instead, it induces a regular vascular network with small vessel calibers surrounded by stabilizing mural cells. Overall, vessels are less leaky and tumor perfusion is improved. This is an important finding in the field of tumor immunology as it demonstrates that a functional vasculature and an improved tumor perfusion greatly enhance tumor-specific immune responses. This is further supported by our observation that tumor-targeting of another inflammatory cytokine, interferon γ (IFNγ), which predominantly induces endothelial cell death fails to support immune cell infiltration into tumors.[8] Also, repetitive, low-dose TNFα infusion into tumors ultimately induces endothelial cell death and hence limits the influx of effector cells. Interestingly, reduction of vascular leakiness by pharmacological or genetic normalization of the tumor vasculature also enhances adoptive T-cell therapy.[2,9] In contrast, destruction of tumor

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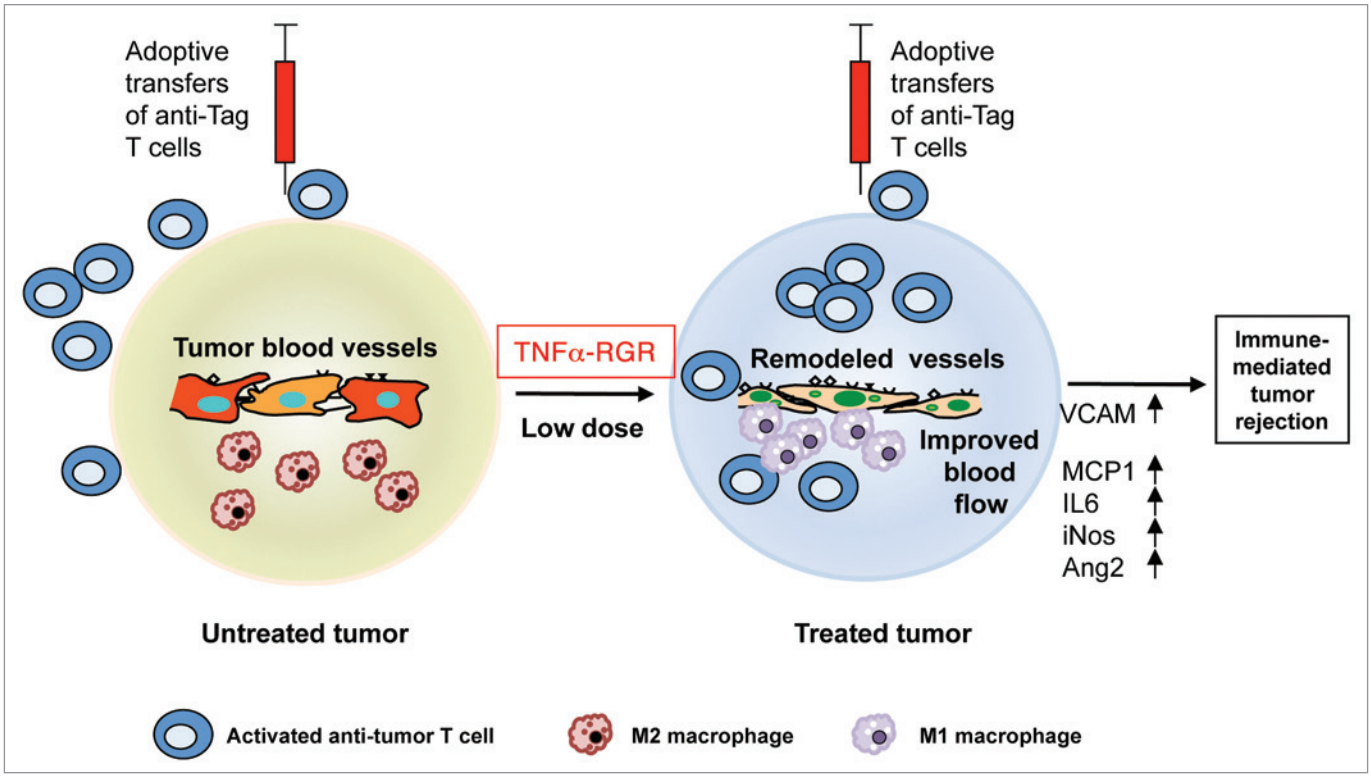


**Figure 1.** Intratumoral low-dose tumor necrosis factorα (TNFα-RGR, TNFα conjugated with vascular homing peptide, injected i.v.) increases tumor vessel stability and vascular perfusion. Remodeled vessels are highly activated and express vascular cell adhesion molecule (VCAM). Tumor resident macrophages switch from a M2 to a M1 phenotype, express high levels of VCAM, monocyte chemotactic protein 1 (MCP1), interleukin 6 (IL-6), inducible nitric oxide synthetase (iNOS) and angiopoietin 2 (Ang2), and cluster around tumor vessels. Adoptively transferred antitumor T cells are unable to penetrate into untreated tumors, but infiltrate tumors after "pre-conditioning" with TNFα, which leads to tumor regression.

blood vessels, for instance by vasculature-disrupting agents that stimulate production of high TNFα levels does not support active T-cell immunotherapy.[10]

Besides vascular remodeling, intratumoral TNFα elicits widespread stromal activation and elevated expression of the vascular cell adhesion molecule (VCAM) on endothelial cells, fibroblasts and macrophages. We demonstrated that macrophages play an important role in amplifying vessel activation by secreting angiopoietin 2 (Ang2), a tie2 tyrosine kinase receptor ligand that—in conjunction with TNFα—upregulates the expression of endothelial adhesion molecules. Endothelial activation my in turn facilitate leukocyte extravasation. Once tumor-specific effector cells have reached the tumor site, they encounter a favorable inflammatory environment since low-dose TNFα also relieves iummunosuppression by tumor-resident macrophages. Thus, TNFα acts on multiple stromal cells to improve tumor perfusion, leukocyte extravasation and immune stimulation. Along similar lines, the histidine-rich glycoprotein (HRG) has recently been shown to polarize macrophages to create an immunostimulatory tumor environment that also normalizes blood vessels.[3]

Collectively, our study reveals that low-dose TNFα targeted into solid tumors is a promising adjuvant that improves vessel function and antitumor immunity. This can be exploited in the context of active and passive immunotherapy. Our findings also encourage further development of combination therapies that simultaneously alter tumor-associated stroma and activate antitumor immune responses.

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