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Targeting stroma to treat cancers

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Abstract

All cancers depend on stroma for support of growth. Leukemias, solid tumors, cancer cells causing effusions, metastases as well as micro-disseminated cancer cells release factors that stimulate stromal cells, which in turn produce ligands that stimulate cancer cells. Therefore, elimination of stromal support by destroying the stromal cells or by inhibiting feedback stimulation of cancer growth is in the focus of many evolving therapies. A stringent evaluation of the efficacy of stromal targeting requires testing in animal models. Most current studies emphasize the successes of stromal targeting rather than deciphering its limitations. Here we show that many of the stromal targeting approaches, while often reducing tumor growth rates, are rarely curative. Therefore, we will also discuss conditions where stromal targeting can eradicate large established tumors. Finally, we will examine still unanswered questions of this promising and exciting area of cancer research.

Keywords

tumor stroma; tumor promotion; cancer therapy; relapse; eradication

1. Abuses of terminology

Without adherence to proper terminology, scientific results are easily misread. There is an enormous thirst for high impact factors of journals and for translational bench-to bedside protocols by agencies and by the public. This situation fosters highly misleading titles that have become fashionable to "increase visibility", i.e., inflate the impact of papers. For example: "*Eradication*" does not mean just a reduction of tumor growth, it should mean destroying a tumor completely like tearing a tree out with its roots so it cannot re-grow. Experimental microscopic subcutaneous cancers treated on day 6 or day 9 are not "*established cancers*". "*Tumor*" means to lay and professional people a swelling, which is what the word means also in Latin; therefore, you cannot treat a "*tumor-bearing mouse*" before a tumor becomes visible or palpable. There also is a very strong bias to use the word "*therapeutic*" for treating small lesions of only a few mm in diameter, which is of unknown relevance. Clinically most tumors are not detected until they are 0.5 to 1 cm in diameter and contain 10^9 cells [1]. Individuals, mice or people, can be treated, e.g. by vaccination, to

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prevent cancer, but *"therapy"* implies treatment of existing disease. Therefore, humans are treated when they have diagnosable cancer, but it is inappropriate in experimental models to call therapy as being effective few days or so after cancer cell inoculation when the tumors are microscopic. Oncogene-transgenic cancer models in animals are widely referred to as "*spontaneous"* implying these cancers closely mimic human cancers that are mostly sporadic. However, the term *"spontaneous cancer"* is clearly reserved for cancers arising in the absence of any experimental manipulation [2]. These kinds of abuses mean that the words eventually lose their original meaning, and that loss is no longer even noticed. Abuse of terminology does not serve either scientists or patients well.

2. Tumor promoting stroma

2.1 Tumor formation depends on reciprocal induction between cancer cells and stroma

Rudolf Virchow believed that compression of the growing cancer cells induced a structural fibroblastic framework in which the cancer cells grew. He thought that cancer cells and stroma both developed from the same primitive precursors. This concept changed with Paul Ehrlich stating clearly that the host provided the stroma of solid tumors [3]. But it was Borst in 1924 [4], who was the first to clearly point out the essential mutual relationship between cancer cells and tumor stroma by stating: "With regards to the question of whether the epithelium or the connective tissue has the leading role in carcinogenesis, we think that asking "*either/or*" is bad." In other words, stroma of tumors is dependent on the presence of cancer cells, and cancer cells are dependent on stromal cells (reviewed in [5]). Thus, cancer cells release factors that attract stromal precursor cells, and stromal cells in turn produce factors that support cancer cell growth. Once the cancer cells are destroyed, the stroma that supported the tumor will vanish within weeks unless scars have formed during the regression process. Unfortunately, it is usually omitted that the reverse is often not true: destruction of stroma does not necessarily lead to the death of all cancer cells (see section 8).

2.2 Tumor promoting anti-apoptotic stroma

Lack of tumor stroma drastically reduces tumorigenicity [6-8]. Inoculated cancer cells embedded in tumor stroma are 10 to 100-fold more tumorigenic than stroma-free suspensions of cancer cells [6, 7]. It had long been known that certain cancers would only grow in mice when transplanted as tumor fragments. This erroneously was thought to be due to more cancer cells being inoculated with fragments [9]. Later analyses revealed fragment inoculations contained fewer cancer cells than injected cell suspensions [6, 7]. Another erroneous explanation was that the stroma of fragments provided a physical barrier preventing cancer cells from migrating to draining lymph nodes and priming a protective T cell response [7, 10]. It is more likely, however, that cancer cells simply remain more viable when embedded in tumor stroma (by preventing anoikis [11]) and therefore, less antigen is available for release and T cell stimulation. In any case, as long as cancer cells express sufficient levels of antigen, professional antigen presenting cells in the tumor stroma pick up the antigen and travel to the draining lymph nodes where they present the antigen to naïve T cells [12].

Stroma promotes tumor growth by two distinct mechanisms: (i) stimulation of vasculature and connective tissue by inflammation-induced attraction of the various myeloid and lymphoid derived cell types and (ii) the suppression of an immune response. Vasculature provides oxygenation and nutrients; connective tissue and the extracellular matrix (ECM) are needed for adherence, structure, and the binding/release of cytokines, chemokines and growth factors that signal as part of a paracrine loop to the cancer cells [13-16]. The tight link between inflammation, leukocyte infiltration and cancer is being studied intensively. It is the topic of many reviews and therefore is not covered in depth here [17-21]. In short, it

has been observed that tumor infiltrating T cells and other leukocytes secrete cytokines and growth factors that activate the stroma and accelerate tumor growth [14, 15, 22, 23]. Angiogenesis is a fundamental necessity for tumor growth. Myeloid cells including mast cells, eosinophils, and tumor-associated macrophages (TAM) have been shown to promote tumor angiogenesis (reviewed in [24]). However, also cancer cells themselves and tumor-associated fibroblasts (TAF) can produce pro-angiogenic as well as growth stimulatory factors such as vascular endothelial growth factor (VEGF) (reviewed in [25]).

2.3 Cancers, solid or leukemic, depend on paracrine stimulatory loops involving stroma

For growth, leukemias, solid tumors, malignant effusions, metastases as well as micro-disseminated cancer cells depend on reciprocal cancer cell-stroma interactions. This loop seems to depend on oncogenic mutations in the cancer cells that cause them to release molecules, which induce non-malignant stromal cells of the host to make factors that promote cancer cell growth [26]. An early example is the oncogenic fusion protein RET/PTC3 (RP3) in thyroid cancer [27, 28]. Stromal cells then produce other growth factors and alter the ECM so that both are conductive to growth and invasion. Two-stage carcinogenesis involves an initiating mutational event followed by repeated applications of a nonmutagenic pro-inflammatory promoter (typically the phorbol ester PMA (phorbol 12-myristate 13-acetate)) [29]. The critical step in cancer development is the acquisition by the cancer cell to release its own stimuli to the stroma, so the neoplastic cells become promoter-independent, and thus truly neoplastic.

2.4 Paracrine loop mismatch persists in “humanized” mice

Many paracrine factors have been identified. Interruption of paracrine loops by antibodies, receptor traps or drugs are important new therapeutic avenues to explore for more effective cancer therapy (see below). However, despite all what we have learned about chemokines, cytokines their receptors and signaling cascades, we do not have adequate procedures for culturing cancer cells from the most common human cancers: breast, prostate, lung, colon and pancreas. Eventually use of the right combination of factors and surfaces for growth will probably be found. The search may be aided by growing human cancer in immuno-incompetent mice and culturing the cancer cells in a three dimensional matrix ([30] and reviewed in [11, 31]). Only some human cancers can be grown in immuno-incompetent mice and can be serially transferred in these mice. But it is unclear at present whether, and for which human cancers “humanizing” immunodeficient mice with human stem cells must be used. Both human and fibroblastic precursors may be critical for success. Generating artificial extracellular matrixes may also mimic cancer-stroma interactions in humans. It must be remembered, however, that there are major hurdles: e.g. human and murine IFN-γ do not work on the reciprocal receptors [32] and thus human IFN-γ will not affect fibroblasts of the non-bone-marrow derived stroma in “humanized mice”. There are many papers claiming growth of human cancer xenografts, which is too often based on transient inflammatory reactions. Reports of “prevention” or “reduced growth” of such transplants after treatment has, therefore, questionable significance and reports on the effects of therapies targeting stroma may be misleading because of lack of interaction between human cancer cells and the non-BM derived stroma of mouse origin.

2.5 “Pseudo-Stroma” resulting from epithelial-to-mesenchymal transition (EMT) cannot replace host-derived stroma

The less differentiated, more fibroblastic appearance of carcinoma cells adjacent to the stroma is associated with the acquisition of genes such as snail, slug and vimentin, prominently expressed by mobile embryonic fibroblastic cells [33, 34]. While these changes may help cancer cells to acquire an increased metastatic and invasive potential, there is no

evidence that they make cancer growth self-sufficient and can replace the need of cancer cells to establish a paracrine stimulatory loop with nonmalignant stroma [35].

3. The four components of tumor stroma

Stroma consists of connective tissue and extracellular matrix, vasculature and infiltrating hematopoietic cells. The latter comprise myeloid and lymphoid lineage cells, both derived from hematopoietic stem cells (HSC) in the bone marrow.

3.1 Connective tissue and vasculature

R.A. Willis in a careful survey of his own studies and published literature [36] subdivided tumor stroma into just two major components, connective tissue - which usually represents the bulk of stroma - and vasculature which is usually a smaller fraction. Fibroblasts are a prominent cell type in tumor stroma as well as in healing wounds and embryonic connective tissues. Unfortunately, we still lack reliable fibroblast-specific immunological markers for these cells despite repeated assertions to the contrary [37, 38]. For example, fibroblast–specific protein 1 (FSP-1), also called S100A4, has been found on various cell types including epithelial cancer cells and leukocytes [39-43]. Therefore, fibroblasts are still mostly defined by morphology and function [44-46]. Characteristically, they synthesize, secrete and modulate proteins of the fibrous ECM, particularly collagen [47-49]. ECM is a major reservoir for growth factors and cytokines [50]. Furthermore, the ECM is gaining increased attention due to its role in providing a scaffold but also growth signals, thereby preventing cell death due to anoikis (reviewed in [11, 31]). Interestingly, fibroblastic cells have been reported to present antigen and stimulate lymphocytes under certain experimental conditions [51, 52]. Stromal fibroblasts in cancers are metabolically active and the degree of activation correlates with aggressiveness of the cancer and it correlates inversely with survival of patients [53, 54]. The resting less active fibroblasts that are usually referred to *as fibrocytes*, are rarely observed in stroma of malignant tumors. Unfortunately the term *fibrocyte* has also recently been used to designate a cell population in the peripheral blood that can enter sites of inflammation and produce collagen [55]. Fibrocytes are resting cells surrounded by collagen fibers and extracellular matrix and are the prominent cell types in mature adult connective tissues and some malignancies, e.g. breast cancers [48, 49, 56-58].

3.2 Sources of progenitor cells

Mitoses in tumor stromal cells seem too sparse to explain the dense cellularity of stroma, as reported over a century ago [59]. Most likely, the cells in the stroma come from progenitor cells entering the site of tumor growth either via the blood circulation or from adjacent normal tissues. The relative contribution of these two sources is still hotly debated [60]. However, recent studies using non-myeloablative conditions have shown convincingly that precursors for tumor vasculature come primarily from local, i.e., adjacent normal tissues, not from the circulating, bone-marrow-derived precursor pool [61-64]. Indeed, recent and older studies demonstrate major progenitor reservoirs not only in the bone marrow but also in the perivascular spaces of all other organs [65-67]. Thus, tissues adjacent to the neoplastic lesion, wherever they may occur throughout the body, should have these progenitors. It is not entirely clear how these mesenchymal progenitor cells relate to the so-called pericytes [68] that are also thought to be pluripotent and that build sleeves around, and are essential for, endothelial cells to form capillaries. We are lacking conclusive evidence determining whether adjacent tissues are also a major source of fibroblasts in tumor stroma. In wound healing, however, there is clear evidence for adjacent normal tissues being the main source of fibroblast progenitors based on extensive and careful studies using parabiosis and labeling techniques [69-71]. Other studies suggest that precursors circulating in the blood can enter sites of inflammation, injury and tumors to generate stromal fibroblasts. The differentiation

of blood leukocytes into fibroblasts has repeatedly been described *in vitro* and *in vivo* [72-74] and circulating mononuclear progenitor cells for TAFs have been isolated from venous blood in resected tumors of lung cancer patients [75]. These isolated cells differentiated into fibroblasts in xenografts of human lung cancer in NOD/SCID mice. Similar experiments have shown the differentiation of bone marrow-derived progenitors into TAFs [76, 77]. It remains unclear, however, whether the transferred progenitor cells are of hematopoietic or mesenchymal lineage. A study by LaRue and colleagues does suggest that TAFs and pericytes originate from HSCs [78]. So far, it remains unclear and controversial: (i) what percentage of TAF infiltrate is from bone marrow or surrounding tissue origin [79], (ii) what percentage of bone marrow-derived fibroblasts originate from hematopoietic versus mesenchymal lineage, and (iii) whether the origin of stromal fibroblast results in functionally different subtypes or not.

3.3 Differentiation of MDSC into tumor associated macrophages

Tumor-induced myeloid derived suppressor cells (MDSC) are the focus of a large body of literature (reviewed in [20, 80]). This heterogeneous population of largely immature cells of myeloid origin (Gr-1 and CD11b-positive in mice) suppresses adaptive and innate immunity, which is believed to be an important arm of tumor-induced immune-evasion. The suppression is achieved through several mechanisms including the production of nitric oxide and reactive oxygen species, the nitration of T cell receptors (TCR), arginine and cysteine deprivation, and the induction of regulatory T cells (T_{regs}). Interestingly, several reports show the same immune suppressive capacities of MDSC from naïve and tumor-bearing mice [81, 82] and splenic and tumor-derived MDSC were recently shown to exert their immunosuppressive activity through different mechanisms and onto different T cell subsets [83]. The same and older reports describe the rapid differentiation of MDSCs into suppressive TAMs in tumors [83-85]. In fact, the majority of hematopoietic cells in the tumor are macrophages [84, 86]. A recent review by Mandruzzato and colleagues argues that under certain conditions *in vitro*, depending on T cell numbers and their activation status, the suppression by MDSC may be overcome [87]. Taken together, the impact of the extensive literature on MDSCs, mainly studying circulating or splenic cells, on immune suppression within the tumor is unclear, and will need to be analyzed more carefully.

4. Can malignancy be “reversed” by normal and “induced” by cancerous stroma?

The question is old and contentious and not really answerable. The problems are that cancer and stromal cells can have abnormal karyotypes, which may or may not mean the cells are transformed to be malignant. Also, epithelial cells in tissues surrounding a cancer can contain cells that are morphologically abnormal but not certifiable as cancer cells either. In classic models of two-stage carcinogenesis pre-malignant lesions develop before cancer. These are dependent on promoting factors such as present in wound healing or chemicals that result in stromal stimulation (conditional neoplasms). To become promotion-independent, pre-malignant cells may acquire pathways that induce the stroma to provide a favorable environment [29, 88, 89]. Alternatively, the pre-malignant cells may loose their susceptibility to inhibitory effects in normal stroma that counteract cancer development [90-93]. Studies on mammary cancers show a strong cancer promoting effect of cancerous stroma through the expression of ECM-degrading metalloproteinase stromelysin-1 [94, 95]. However, the influences of and requirement for the stromal microenvironment to support an established cancer must be clearly distinguished from the often substantially different requirements of the stromal microenvironment during tumor initiation and development. For the induction of some epithelial cancers, exposure of stroma to carcinogen may be crucial [38, 96, 97] and result in mutations in stromal cells. But exposure of the stroma to non-

carcinogenic, non-mutagenic agents such as PMA, usually seem to suffice to promote the development of epithelial cells carrying cancer-initiating mutations into an invasive cancer. Teratocarcinoma cells keep their malignant potential and do not genetically return to "normal" even when surrounded by non-cancerous stroma while completely normal cells are unlikely to develop true invasive serially transplantable tumors even when brought into the vicinity of cancer-induced stroma. Many factors such as diet, microbial flora, chemicals and wounding influence inflammation have strong potential impact on cancer induction and development by acting on the stromal microenvironment; while these factors have usually very little if any effect once cancer has developed. This also means that strategies to prevent or most likely cure cancer should be fundamentally different. Yet recent reviews do not make this distinction [11, 98].

5. Cancers induce local and systemic stromal changes

It is often assumed that the factors released by the cancer cells only act in the immediate vicinity of the cancer cells and this may often be true. However, it is also clear from many studies that cancer cells release factors such as granulocyte-, macrophage- and granulocyte macrophage colony-stimulating factor (G-CSF, M-CSF and GM-CSF) that have systemic effects. Thus in a way, the cancer may use distant organs such as the bone marrow to make its "stromal bed". Whether "systemic stroma" can substitute for local stroma is unclear but in certain cancers such as leukemias the "systemic stroma" apparently suffices to cause progressive growth. For example, chronic lymphocytic leukemia cells release microvesicles that induce bone marrow stromal cells to release VEGF, a survival factor for chronic lymphocytic leukemia [99].

About 30% of patients with solid tumors have elevated numbers of granulocytes in their peripheral blood (>8000/μl); granulocytosis is also common in tumor-bearing mice [100-108]. Growth of autochthonous or transplanted murine tumors is accompanied by splenic enlargement (Schreiber, K. and Schreiber, H. unpublished results and [109]). Enlargement is mostly caused by increased myeloid hemopoiesis with macrophages and neutrophils usually accounting for more than half of the splenocytes [110]. G-CSF [111], GM-CSF and/or IL-6 [108, 112] released by cancer cells may be of particularly important in inducing these systemic effects.

Certain primary tumors can facilitate the outgrowth of a second tumor at a distant site [113, 114], even if the second tumor would never grow (or only grow very slowly) when injected alone. Using human tumor cell lines in an immune compromised host, it was recently found that an "instigating" tumor not only recruits murine stromal cells very effectively to support its own growth but also helps the incorporation of bone marrow-derived cells into the second "responding" tumor [115]. Osteopontin, which is over-expressed by many human and murine cancers [116, 117], and is required for recruitment and migration of neutrophils [118] appears to be required but not sufficient for instigating the growth of the second tumor at a distant site [115]. Systemic facilitation of stroma formation by mobilization of bone marrow-derived hemopoietic progenitors may also be the basis for the development of "metastatic niches". These niches are thought to represent focal changes in the stroma of organs such as lungs caused by circulating factors released from cancer cells of the primary tumor; these changes are thought to provide a bed conducive for circulating cancer cells to settle down, vascularize and form a metastatic tumor [119, 120].

Furthermore, many studies have shown that growing tumors incorporate myeloid-derived $Gr\text{-}1^+$ $CD11b^+$ cells that are proangiogenic [121, 122] and cause increased TGF-β production in tumors [123]. Proteases released from stromal inflammatory cells such as neutrophils and macrophages are likely to be critical in locally activating latent TGF-β

bound to ECM or to cells such as resting T_{regs}. Active TGF-β will then (i) orchestrate attraction and activation of fibroblasts to become SDF1-expressing myofibroblasts, which attract more stromal progenitors [124-126] and (ii) polarize naïve T cells towards T_{regs} thereby antagonizing adaptive immune responses [127-130]. Furthermore, neutrophil-derived elastase attenuated T lymphocyte transmigration by deactivating endothelial-bound SDF-1 [131]. In addition, tumor growth can induce systemic T cells to produce high levels of VEGF and matrix metalloproteinase-9 (MMP-9) and these T cells may help tumor growth at distant sites [23]. Another subset of T cells, which is induced by tumors, is the regulatory type [132]. These T_{regs} alter DC function [133] and convert naïve T cells to become T_{regs} themselves [127-130] thereby preventing the generation of a destructive T cell response. Despite the systemic effects of tumor growth described above, tumor-bearing individuals usually do not seem to be immunosuppressed systemically since they do not suffer from increased opportunistic infections except at very late stages [110].

6. Aggressive cancers induce stroma more effectively

During the continued growth human and experimental cancers become more malignant measured by rate of growth or capacity to metastasize. This is due to the generation of heritable variants, a process referred to as “tumor progression” [88, 134].

The most dramatic example may well be the progression of UV-induced regressor tumors to progressor variants: regressors are rejected in fully syngeneic naïve mice at any testable dose, while progressors grow. Since tumor stroma increases the viability and growth of cancer cells and has been shown to suppress the immune system, it is not surprising that cancer cell variants capable of inducing tumor stroma escape immune destruction. Evidence for this comes from studying heritable variants that escaped immune destruction in T cell-competent mice inoculated with regressor tumors. Regressor tumors can be induced in T cell-deficient mice [135-139]; when transplanted into naïve immunocompetent syngeneic mice, these tumors are regularly destroyed by a T cell response after transient growth. Occasionally, progressor variants escape this immune destruction, and most of these variants show more rapid and effective stromal induction and activation but no loss of antigen [14, 15]. It is likely that a systemic innate $Gr\text{-}1^+$ response is critical. Systemic depletion of neutrophils and possibly other $Gr\text{-}1^+$ cells by treatment with anti-Gr-1 antibodies not only reduces the growth rate of malignant cells in naïve T cell-deficient mice [15] but also leads to destruction of inoculated cancer cells in T cell competent mice [13]. While antigen loss can be found in progressor variants evolving from some regressors it is not mandatory. Instead, all progressor variants we have analyzed so far induce stroma much more effectively by more effective paracrine loops and most (but not all) retain the same immunogenicity as regressors [14, 140, 141].

7. Stroma as a target for cancer therapy

Because of the important role of tumor stroma in tumor progression and the suppression of the immune system, it has become a much-investigated target for cancer therapies. For targeting tumor stroma effectively, it is important to know what its components are and what the turnover of these components is (see 3). MDSCs, and more importantly TAMs, are attractive targets, as these cell types have been shown to both promote growth and suppress effective immunotherapy. Several targeting strategies are being explored, often to improve the usual failure of cancer vaccines. One of the approaches is to force differentiation of MDSC to mature myeloid cells such as dendritic cells, macrophages, or granulocytes. A treatment with all *trans*-retinoic acid (ATRA) and vaccination showed the hypothesized effect on MDSC and T cells and subsequently led to slower tumor growth [142]. A model of autochthonous pancreatic tumor shows regression in 30% of mice after activating

macrophages to become tumoricidal [143]: the treatment with the CD40 agonist induced a T cell independent shrinkage of the tumors; it did not however lead to their eradication. In a model of IL-4 over-expression by transplanted tumors, the tumoricidal potency of neutrophils was demonstrated [144]. In an oncogene-transgenic mouse model of autochthonous mammary tumors and transplanted metastasis it was shown that a skewing of TAMs toward a M1 phenotype (in $STAT6^{-/-}$ mice) enhanced the host's anti-cancer immune response [82, 145]. The anti-tumor response was dependent on IFN-γ and $CD8^{+}$ T cells and was accompanied by a decrease in circulating MDSC. To what extent this finding can be translated to therapy, for example by using STAT6 siRNA, remains to be shown. Nevertheless, the effect might be useful for post-surgical treatment of minimal residual disease after removal of the primary tumor.

Using the same model, it was demonstrated that MDCS were killed by T cells via Fas-FasL interactions [81]. FasL-deficient mice showed higher numbers of circulating MDCS and higher incidence of metastasis. However, as discussed above, MDSC are a small population of myeloid stromal cells compared to TAMs, which do express Fas but could not be killed through this receptor [81]. Therefore, the general therapeutic importance of this latter finding is not clear. Another strategy is to eliminate MDSCs with drugs such as gemcitabine [146]. The numbers of MDSCs were reduced and T and NK cell activity was augmented, which resulted in slight regression and then slower growth of large established tumors. Depletion of MDSCs with anti-Gr-1 antibody reduced but did not prevent growth of UV-induced cancer cells in athymic nude mice [14, 15]. In euthymic mice, T cells were not capable of controlling cancer cell inoculums of progressor variants [14] but treatment of the challenged mice with anti-Gr-1 antibody prevented outgrowth of the tumor [13], indicating a synergistic effect between T cells and the depletion of cancer-promoting stromal cells. Interestingly, the approach of combining MDSC depletion by cyclophosphamide and inducing inflammatory monocytes and neutrophils by IL-12 led to T cell dependent eradication of 70% of established MC38 colon carcinoma tumors [147]. In another model, an immune response against TAMs was induced. Legumain-specific T cells reduced metastasis after immunization and reduced death of mice, which were vaccinated shortly after cancer cell inoculation [148].

An alternative approach to differentiating and depleting MDSCs is to inhibit expansion or function of these cells. For example, the administration of MMP-9 inhibitors led to a delay in outgrowth of autochthonous mammary tumors by inhibiting MDSC expansion and macrophage infiltration into tumor stroma [149]. Also the inhibition of arginase 1 expression induced strengthened T cells responses and slowed tumor growth by inhibiting the suppressive effects exerted by MDSCs [150].

Macrophages and MDSC are not the only stromal cells that are targeted in attempts to cure cancer. In a transgenic mouse model fibroblast activation protein-α (FAP)-expressing cells were depleted in 12 day-old tumors, leading to a significant reduction of tumor growth when the cancer cells expressed a tumor-specific antigen (OVA_{257}) [151]. Again, the effect on tumor growth was dependent on the adaptive immune system, as it was abrogated by ablation of IFN-γ and TNF-α and the effect was not existent in T and B cell-deficient mice. Similar results were obtained in two studies where T cells, which were induced by a FAP-DNA vaccine, targeted FAP-expressing cells [152, 153]. Another stromal target are pericytes. Targeting the pericytes-associated high molecular weight-melanoma associated antigen (HMW-MAA) with antibodies or T cells prevented tumor growth (reviewed in [154]).

Most studies targeting tumor stroma in one way or the other show the hypothesized effects on the stromal cells, such as their depletion and activation of the adaptive and/or innate

immune systems. However, the effect on tumor growth is often marginal (slower growth), especially when treatment was initiated at later times. Most studies show the need for T cells for reduced tumor growth, thus the addition of these cells by adoptive transfer may lead to better outcomes (see below).

8. Cancer cells harbor clonal cancer-specific mutations but stroma does not

Stroma recruiting cancer variants arise because of the remarkable genetic instability of cancer cells. By contrast, stromal cells are non-malignant and generally genetically stable. Although chromosomal abnormalities occur [155-162] these changes are rare and do not show the clonality that is characteristic of cancer cells [163]. There are however studies that do describe the same leukemia-associated mutations to be present in a part of the myeloid stem cells and the malignant cells of patients [164]. Epigenetic changes in stromal cells can contribute to their tumor-promoting phenotype [165, 166], but when stromal cells are targeted for destruction by chemo-, radiation-, and/or immunotherapy, there is no escape of mutant stromal cells, and thus there is no loss of the targeted gene(s), antigen(s) or antigen-presenting MHC molecules [35].

9. Loading of stroma with cancer antigens

9.1 Cancer cell derived membrane vesicles as carriers of antigen

Modulation of tumor stroma is not only induced by soluble factors but also by small vesicles released by cancer cells. This includes pro-angiogenic effects by microvesicles released from ovarian cancer [167] and vesicles from B cell leukemia cells that activate bone-marrow stem cells [99]. Most cells release membrane vesicles of various sizes and origin such as microvesicles that have budded from the plasma membrane and exosomes, which are derived from intracellular compartments (reviewed in [168] and [169]). These vesicles contain hydrophilic compartments, and can therefore transport membrane bound proteins and also molecules of cytoplasmatic or luminal origin. Accordingly, microvesicles and exosomes can transfer transmembrane peptide-MHC molecules of cancer cells [170, 171] and antigenic material from the cytoplasm. Exosomes obtained from cancer cells cultured *in vitro* [172] or from patients' ascites [173] have been shown to contain tumor antigens capable of stimulating T cells. However, these vesicles not only transport antigen, but can also influence the immune system in other ways, for example inhibit [174] or stimulate immune responses [175, 176], the latter after exposing the cancer cells to stress conditions such as a heat shock [177, 178].

9.2 Stroma has no cancer specific-antigens except for those absorbed from adjacent cancer cells

Antigens originating from cancer cells can be cross-presented by stromal cells, thereby making stromal cells targets of cancer-specific cytotoxic T cells. Combined $CD8^+$ T cell attack on cancer and stromal cells leads to tumor eradication [179, 180]. In a model where only stroma can be targeted by $CD8^+$ T cells because on MHC restriction, growth arrest and long term “equilibrium” of tumor is observed [35]. In this model, the T cells cannot target the cancer cells directly, but the cross-presenting tumor stroma is destroyed [35, 179]. These tumors become completely necrotic inside while cancer cells continue to proliferate at the margins of the tumors. This is in accordance with the notion that targeting stroma alone usually does not eradicate tumors (see section 8). In another model, where the stroma cannot be targeted because of low antigen amounts expressed by the cancer, antigen loss variants take over and eventually kill the tumor-bearer [179].

Cross-presented antigen can also stimulate naïve T cells. APCs in the tumor pick up antigen, given that the levels are sufficient, travel to the draining lymph nodes and present the antigen to naïve T cells [12]. A different mechanism of T cell activation is the cross-presentation of antigen released by dead cancer cells and transported to the draining lymph nodes via lymphatic flow where it is taken up and presented by professional antigen presenting cells [181].

9.3 Approaches to load the margins with cancer-specific antigens and destroy it: sensitization or exogenous introduction of antigen

The amount of antigen expressed by the cancer cells seems to influence the degree of cross-presentation by the stroma [179]. We have shown, however, that the release of antigen can be enhanced by locally irradiating tumors or by administering the chemotherapeutical drug gemcitabine [182]. This pretreatment led to the eradication of tumors that would under simple adoptive T cell therapy escape as antigen loss variants. These experiments show the importance of cross-presentation for tumor eradication. Cross-presenting stromal cells become targets and once killed there is no support for the growth of remaining loss variants. Tumors are usually detected at a size of one cm in diameter and 10^9 cells [1]. Most of the variants have already evolved at this point. Variants can arise by deletion of the epitope or reduced surface expression/presentation. It is unclear whether the remaining loss variants die through T cell-mediated bystander killing, missing paracrine loop or anoikis, death by loss of attachment through destruction of ECM and fibroblasts [11].

10. Cancer cells survive at tumor margins because of pre-existing stroma and its vasculature

Cancer cells survive at tumor margins where they are supported by preexisting stroma and vasculature, which is different from neovasculature. There are several important implications: (i) Agents that block neangiogenesis such as anti-VEGF antibodies or VEGF-receptor traps arrest the growth of tumors but do not affect preexisting vessels at the tumor margins and therefore fail to be curative; (ii) targeting molecules preferentially expressed on neovasculature has the same limitations; (iii) for tumor eradication, antigens released from cancer cells must be abundant enough to be cross-presented by pre-existing stroma at the tumor margin so the T cells “excise” the tumor mass in the healthy margin which is also the aim of curative surgery.

11. Conclusion

Tumor stroma, consisting of connective tissue and ECM, vasculature and an infiltrate of immune cells is critical for tumor growth. Apart from providing the growing tumor with structure and blood supply, stromal cells are part of a paracrine loop necessary for the survival of cancer cells and also induce immune suppression. The importance of the single aspects of the support of tumor growth are not clear and it remains to be answered whether the cancer promotion effect or the immune suppressive effect supplied by the stroma plays the more important role. Because of the crucial role of stroma in tumor growth, however, various strategies are employed to target stroma and thereby treat cancer. Most approaches do not lead to cancer eradication however. This is probably due to redundancies among stromal cell types and the inability to target stroma just outside of the cancer rim. Surviving cancer cells can recruit new stroma and use preexistent vasculature to support their growth.

Only in cases where T cells can target cancer cells, stromal cells and presumably also the extended rim of the cancer (healthy margin), could established tumors be rejected. So far, this has only been shown using model antigens for tumor-specific antigens like SIYRYYGL,

LCMV gp33 and OVA_{257}, which are transfected into cancer cells [179, 180, 183]. We do not know how these peptides differ from naturally occurring tumor-specific antigens or which kinds of antigens can be effectively cross-presented. Furthermore, up to the present, only TCR-transgenic T cells have been used for adoptive transfer when targeting single epitopes. The evolving field of TCR gene transfer [184, 185] enables us to generate therapeutic quantities of tumor-specific T cells. The use of this approach to target tumor-specific antigens that are efficiently cross-presented on tumor stroma will hopefully lead to the successful results we now find in animal studies.

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Abbreviations

APC	antigen presenting cell
BM	bone marrow
DC	dendritic cell
ECM	extracellular matrix
FAP	fibroblast activation protein-α
HSC	hematopoietic stem cells
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MMP-9	matrix metalloproteinase-9
PMA	phorbol 12-myristate 13-acetate
TAF	tumor-associated fibroblast
TAM	tumor-associated macrophage
TCR	T cell receptors
T_{reg}	regulatory T cell
VEGF	vascular endothelial growth factor