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Immunotherapy – breaching the barriers for cancer treatment

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Summary

The great ambition to treat cancer through harnessing a patient's own immune responses has started to become reality. Clinical trials have shown impressive results and some patients reaching the end of existing treatment options have achieved full remission. Yet the response rate even within the most promising trials remain at just 30-40% of patients. To date, the focus of immunotherapy research has been to identify tumour antigens, and to enhance activation of effector lymphocytes. Yet this is only the first step to effective immunotherapy for a broader range of patients. Activated cytotoxic T cells can only act on their tumour cell targets if they have free and easy access to all tumour regions. Solid tumours are complex, heterogeneous environments which vary greatly in their physical properties. We must now focus our efforts on understanding how factors such as the composition, density, and geometry of tumour extracellular matrix (ECM) acts to impede or promote immune cell infiltration and activation, and work to design novel pharmacological interventions which restore and enhance leukocyte trafficking within solid tumours.

Introduction

An early pioneer study in the 1890's by William Coley treating cancer patients with live bacteria, is one of the first examples demonstrating that boosting the patient's immune response against transformed cells was a possibility to cure cancer [1]. Many years later it is now widely demonstrated that the immune system can control tumour growth and that evading this specific immune response is a hallmark of cancer [2]. Over the last decades scientists have developed a number of therapeutic avenues to exploit the possibility of harnessing immune responses to treat cancer.

Therapeutic cancer vaccines include inoculation of cancer cell lysates, isolated tumor associated antigens and neoantigens, and autologous dendritic cells loaded with these same tumour antigens. Normally triggered by an adjuvant of choice, this approach generates an adaptive immune response against the tumour [3-6]. Inoculation of specifically-designed chimeric antigen receptor (CAR) T cells and NK cells could be considered a more targeted version of cancer vaccination [7,8]. Alternatively, spontaneous anti-tumour responses in cancer patients can be exploited by adoptive transfer of expanded popoluations of autologous tumor-infiltrating lymphocytes. Adjuvant treatment is still used today, and immunotherapy is the most successful therapy for non-muscle-invasive bladder cancer [9]. Last but not least, immune checkpoint blockade therapy neutralises molecules that cancer cells and associated stromal cells use to dampen the immune response. The most popular and effective targets are CTLA-4 and PD-1 used alone, together, or in combination with other therapies [10].

The enormous potential of these therapies is backed up by a large number of studies using animal models. Nevertheless, efficacy in humans is not as good as expected. In order to work, all immunotherapy approaches have specific requirements to meet. One of these is the ability of effector immune cells to access the whole tumour. Here we review the importance of the tumour stroma in shaping the tumour microenvironment and how this impacts the effectiveness of immunotherapy. We focus particularly in the extracellular matrix composition and organisation, how it creates both physical and signalling niches around tumours and its impact on immunological anti-tumoural responses.

The generation of the extracellular matrix in the tumour microenvironment

The extracellular matrix (ECM) is composed of a network of macromolecules including fibrillar proteins, proteoglycans and glycoproteins that serve both biophysical and biochemical functions. It acts as a physical scaffold to maintain the structure and mechanical integrity of tissues, as well as an active signalling constituent through the sequestration and release of growth factors and cytokines [11]. The composition, anisotropy and biomechanics of the ECM is uniquely tailored to the specific function of the tissue.

The primary mediators of ECM deposition and maintenance are fibroblasts. In pathological contexts such as wound healing and fibrosis, fibroblasts are activated by soluble mediators like Transforming growth factor beta (TGF-β) to increase ECM production and remodelling. In cancer, fibroblasts are chronically activated like a 'wound that does not heal' resulting in severe desmoplasia, as well as dramatic changes in ECM composition and topography. The tumour microenvironment is typically enriched in fibrillar collagens, fibronectin, periostin, tenascin C, hyaluronan, and versican among others, and their upregulation is associated with poor prognosis [12-17]. At the structural level upregulation of the lysyl oxidase (LOX) family of enzymes elevates ECM cross-linking, and there is a progressive transition to ECM anisotropy or alignment which requires both cell intrinsic factors such as polarity and actomyosin contractility, but also external factors such as the physical forces exerted by the growing tumour [18-20].

Extracellular Matrix structures define tumour microenvironments

These pathological changes in ECM abundance, cross-linking and architecture modify the mechanics of tumour tissue, increasing tumour stiffness and ECM engagement. Integrin and FAK dependant adhesions in turn stimulate proliferative signalling and inhibition of growth suppression and apoptosis in transformed cells [21-23]. The tumour-associated ECM also generates alignotactic, haptotactic and durotactic gradients that enhance invasion and metastasis. During the initial phase of metastasis tumour cells must depart the primary site and navigate toward blood and lymphatic vessels, and aligned collagen and fibronectin bundles generate permissive 'highways' directing their migration and intravasation [20,24-27]. Stiffness and fibronectin gradients have also been shown to provide guidance cues to migrating normal and transformed breast epithelial cells [28-31]. Metastatic dissemination is also favoured by ECM rigidity by driving an epithelial to mesenchymal transition [32,33]. Tumour-associated desmoplasia could be explained as a foreign body response, a 'walling off' of transformed cells through the generation of an obstructive barrier parallel to the invasive front.

Conceivably this would act in a tumour suppressive manner, preventing tumour cell escape and inducing cell cycle arrest through elevated compressive stress [34]. However, during tumour progression the re-orientation of ECM bundles perpendicular to the tumour front is likely to counteract these initial effects [20]. Understanding the different functions of ECM in tumour progression and the balance between tumour suppressive versus tumour promoting functions will be necessary to designing therapeutic interventions.

Extracellular matrix and immune infiltration

Just as the composition of the ECM determines architecture, and compartmentalisation of healthy tissues, the newly generated ECM around tumours also impacts tumour composition; including the spread of blood and lymphatic vessels and infiltration of immune cells. Many studies have also shown the relevance of the ECM in the regulation of the immune response in different pathological processes. ECM is a range of complex structures that can both provide a route through tissues, and a physical barrier to cell migration. This depends greatly on the patterns of ECM fibres, since T cells actively migrate along matrix fibres meaning that directionality of ECM fibres dictates leukocyte migration [35]. Additionally, ECM components can bind specific immune receptors, affecting leukocyte proliferation, polarisation/differentiation and trafficking. For example, glucosaminoglycans and proteoglycans can act as functional ligands directly regulating recruitment and activation of innate and adaptive immune cells [36,37]. The duality of ECM to be either protective or tumour promoting means that we need a nuanced and carefully studied approach to ECM as a target for enhancing immunotherapy, but the potential benefits of getting this right are immense.

Extracellular matrix control of angiogenesis and lymphangiogenesis

For immune infiltration, there must be an adequate blood supply surrounding the tumour for leukocytes to be recruited from. These vessels are the major routes of traffic for immune cells infiltrating the tumour site. Their abundance and intrinsic properties of the tumour vasculature conditions leukocyte infiltration [38-40] ECM components regulate angiogenesis by both binding angiogenic factors such VEGFs [41], and by affecting the elasticity of tissues. Stiffer ECM promotes angiogenesis via increased expression of VEGFR2 in endothelial cells (Figure 1), positively regulated by p190RhoGAP/GATA2 [42]. Furthermore, mechanosensing counteracts the antiproliferative role of IL-1b on endothelial cells, suggesting that stiff tissues dictates angiogenesis also under inflammatory stress [43]. Interestingly, lymphatic vessel development seems to respond in a reverse manner. Soft

tissues (0.2-0.3 kPa) induce GATA2 expression in lymphatic endothelial cell (LEC) precursors, enhances their response to VEGF-C, promoting LEC migration and vessel sprouting [44]. Atomic force microscopy on human breast cancer has shown that stiffness gradients are formed in solid tumours, being generally stiffer than surrounding tissue (0.4kPa healthy vs 1.2kPa tumour), with the invasive front being the stiffest [45,46]. These mechanical gradients influence where and when new vessels form during tumour progression (Figure 1), and therefore the access routes for immune infiltrate. Fankhauser et al. recently demonstrated that VEGF-C treatment potentiates immunotherapy by attracting naïve T cells, which are locally activated upon immunotherapy-induced tumour cell killing [47]. Interestingly, targeting the tumour vasculature can also improve immune therapy [39,48]. Overall, careful characterisation of tumour vasculature remodelling will determine the value of combined therapies.

Immune filtration determined by extracellular matrix structure

The presence of capillaries in the tumour microenvironment does not necessarily ensure intratumoural blood flow, since high interstitial pressure and solid-stress causes anomalous hydrodynamic blood flow [49], and ECM structures can accumulate to form physical barriers [35]. For example, hyalunoranic acid (HA), which plays essential roles in tumour growth [50], and is associated with poor prognosis, also increases the tumour interstitial fluid pressure (tIFP) impairing vascular function and hindering access of drugs and immune cells (Figure 2) [51]. Targeting hyalunoran increases efficacy of immunotherapy by increasing infiltration of cytotoxic T cells [52]. Both cancer cells and CAFs are considered sources of HA and studies have shown that contact between both cell types promotes high HA production [53,54]. A relatively large number of secreted factors induce HA synthesis, such as platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), transforming growth factor beta (TGFβ), cytokines and some chemokines [55]. Extracellular ATP and UTP also upregulates hyaluronic acid synthase 2 (HAS2) in human epidermal keratinocytes [56,57]. On the other hand, other secreted factors lower HA production in fibroblasts, such as IL-10 and IFN alpha [58]. Many of these signalling molecules are produced by leukocytes [59], however the complex interplay between leukocytes and fibroblasts, and the inflammatory microenvironment is still not fully understood.

Physical constraints are not the only mechanism by which abnormal ECM impedes leukocyte recruitment. ECM-affiliated proteins [60] can sequester growth factors and chemoattractants leading to defects in leukocyte extravasation (Figure 2). For example, secretion of galectin-3 by tumour cells

binds the glycans of glycoproteins and forms lattices by oligomerization. These lattices sequester other glycosylated molecules such as IFN gamma, inhibiting formation of a functional gradient, and blocking T cell recruitment [61]. Galectin-3 targeting augments the efficacy of T cell therapy, also demonstrating the impact of this mechanism. These sink-like structures may apply to other glycosylated proteins such as chemokines, affecting tumour infiltration of other leukocytes. Chemokine availability is also influenced by the glycocalyx, which retains glycosylated proteins on the surface of cells, essential for the establishment of chemokine gradients [62]. Oligomerization of chemokines can drive glycocalyx cross-linking, establishing a mechanism that can alter the physical properties of cells and ECM [62,63]. In an *in vitro* system, lung tumour cell-derived TNF alpha, disrupted the endothelial glycocalyx via activation of endothelial heparanase [64] affecting its capacity to present chemokines [65]. It is therefore important to assess the glycocalyx status of tumour vasculature in order to maximize recruitment of immune cells for immunotherapy.

Matrix-immune response feedback

With 275 protein-coding genes (195 glycoproteins, 36 proteoglycans and 44 collagens), elements of the core matrisome [66] there exists an immense array of ligand domains for specific receptors expressed infiltrating immune cells. A wealth of studies has shown how these ECM-ligands regulate the adaptive immune response, with pathogen recognition receptors and adhesion molecules as key regulators [67]. Apart from acting as ligands, the ECM scaffolding and mechanoproperties can directly modulate the anti-tumour immune response. High substrate stiffness induces expression of the immune suppressor molecule PD-L1 in a number of tumour cells, which is blocked when actin polymerization is inhibited [68]. Inhibitory PD-1 ligands are also expressed by tumour stromal cells, including CAFs [69]. Although the mechanism is not characterised, these finding sheds light on the regulation of PD-L1 expression by the ECM, relevant for immune evasion and selective depletion of tumour-specific CD8+ cytotoxic cells.

Tumour-draining lymph nodes (TDLN) represent an important immunological barrier against cancer, being privileged sites for generating tumour-specific immune responses [70]. Leukocyte-fibroblastic stroma interactions in LNs also provides a model system to study the signalling between leukocytes and fibroblastic stroma within tumours and how these influence ECM remodelling. TDLNs often present an immunosuppressive profile characterised by overrepresentation of regulatory CD4+ T cells [71,72]. This inhibitory profile of TDLNs can be reverted by TDLN-targeted adjuvant treatment, which

induces Th1 responses and results in higher frequencies of intratumoural CD8+ cells, slowing down tumour growth in the murine B16–F10 melanoma model [73].

Evidence shows that abnormal ECM composition in TDLNs may affect anti-tumour immune response. In breast cancer, metastatic TDLNs present with accumulation of subcapsular collagen I and III [74] and fibrosis in metastatic LNs is also strongly correlated with poor prognosis in colorectal cancer [75]. More specifically, increased levels of collagen and hyaluronic acid in non-metastatic TDLNs correlated with high bulk tissue elasticity and viscoelasticity, and with elevated intranodal pressures [76]. In pre-metastatic TDLNs, the lymphoid stromal population of fibroblastic reticular cells (FRCs) is increased in number and gradually reprogrammed towards a CAF-like phenotype in response to tumour factors. Importantly, TDLN FRCs present differential regulation of ECM genes and lower expression of Il7 and CCL21, key factors in T cell homeostasis [77-79]. In these studies, loss of IL-7 correlated with low numbers of LN T cells, which may lead to poor anti-tumour responses. It is therefore important to study which cellular interactions might be inducing fibrosis in TDLNs and whether the fibrotic status of TDLNs may affect the response to immunotherapy. These mechanisms may be similar to those controlling ECM production within and surrounding the primary tumours.

Therapeutic opportunities

Given the contribution of ECM to tumour progression, many have reasonably hypothesised that targeting the fibroblastic stroma might offer some therapeutic benefit [80,81]. Targeting the fibroblast-activation protein (FAP) with the neutralising antibody sibrotuzumab has unfortunately failed to show efficacy in a phase II trials for the treatment of metastatic colorectal cancer [82]. More promisingly, the anti-fibrotic agent pirfenidone inhibits tumour promoting actions of CAFs and increases vascular functionality and perfusion, improving doxorubicin chemotherapy treatment in two different cancer models [83,84]. In a landmark study by Olive et al, targeting tumour stroma cross talk using Sonic HedgeHog (SHH) inhibitors improved drug delivery and response in murine PDAC [85]. Despite this promise, phase II clinical trials of a SHH inhibitor have so far been ineffective. More recent studies have enabled a more nuanced picture. Pharmacological and genetic ablation of fibroblast SHH signalling transiently stabilised tumours but ultimately accelerated disease progression [86]. Similarly, genetic depletion of activated fibroblasts gave rise to tumours that were less differentiated, more invasive and overall more aggressive [87]. These studies and others have highlighted the context-dependent role of the stroma and associated ECM, seemingly acting in both tumour promoting and tumour suppressive roles.

As an alternative to targeting the cancer-associated fibroblasts themselves, a number of therapies have focused on the ECM directly, targeting either specific ECM components or structural modifications like cross-linking. Most attention has focused on the latter using a monoclonal antibody against LOXL2 (GS-6624/Simtuzumab) or small molecule inhibitor of transglutaminase 2. Whilst pre-clinical investigations were promising, phase II clinical trials with Simtuzumab in both cancer and fibrosis have so far displayed no clear benefit for patients. A study using a PEGylated enzyme against hyaluronan in pancreatic adenocarcinoma may provide some hope [88]. A recent Phase II study demonstrated a significant increase in objective response and a 3-month extension in median overall survival in patients with high hyaluronan [89].

Another approach has been to target ECM associated mechano-signalling in cancer cells directly using ligand mimetics or blocking antibodies against integrins. Cilengitide, a small peptide targeting $\alpha_v\beta_3$ showed promise in Phase II trials in patients with glioblastoma, but unfortunately demonstrated limited efficacy in Phase III [90]. An antibody against $\alpha_v\beta_6$ has also been trialled in idiopathic lung fibrosis to prevent integrin mediated release of TGF-β1, however the results of the Phase II study (NCT01371305) are yet to be published. Signalling nodes downstream of integrins also offer additional points of therapeutic intervention. The tyrosine kinase FAK is activated upon ECM engagement by integrins, and works primarily through Src and downstream Rho/ROCK, ERK, PI3K and YAP to promote further ECM deposition, cell contractility, growth and survival. Small molecule inhibitors of FAK have been developed to disrupt its kinase function through either direct inhibition of the ATP-binding site or allosteric interference. Two of these (PF-04554878/VS-6063 and GSK2256098) are currently in early stage clinical trials. Like other kinase inhibitors, these drugs are challenged by the structural ubiquity of the catalytic domain which confers undesirable cross-reactivity. Another recent approach has been to target specific scaffolding interactions of the kinase target which should give rise to greater selectivity.

Concluding remarks

The key role that the ECM plays in tumour progression is undisputed. Nevertheless, targeting the ECM is yet to prove therapeutic benefit. While combined therapies might be the future, we need to increase our understanding of ECM composition and structure that impacts the efficacy of immunotherapy. Furthermore, arrival of immune cells to the TME will cause changes to stromal cell

behaviour, in turn, feeding back to the immune response. A better understanding of these complex reciprocal interactions will be essential in order to design new effective therapeutic approaches.

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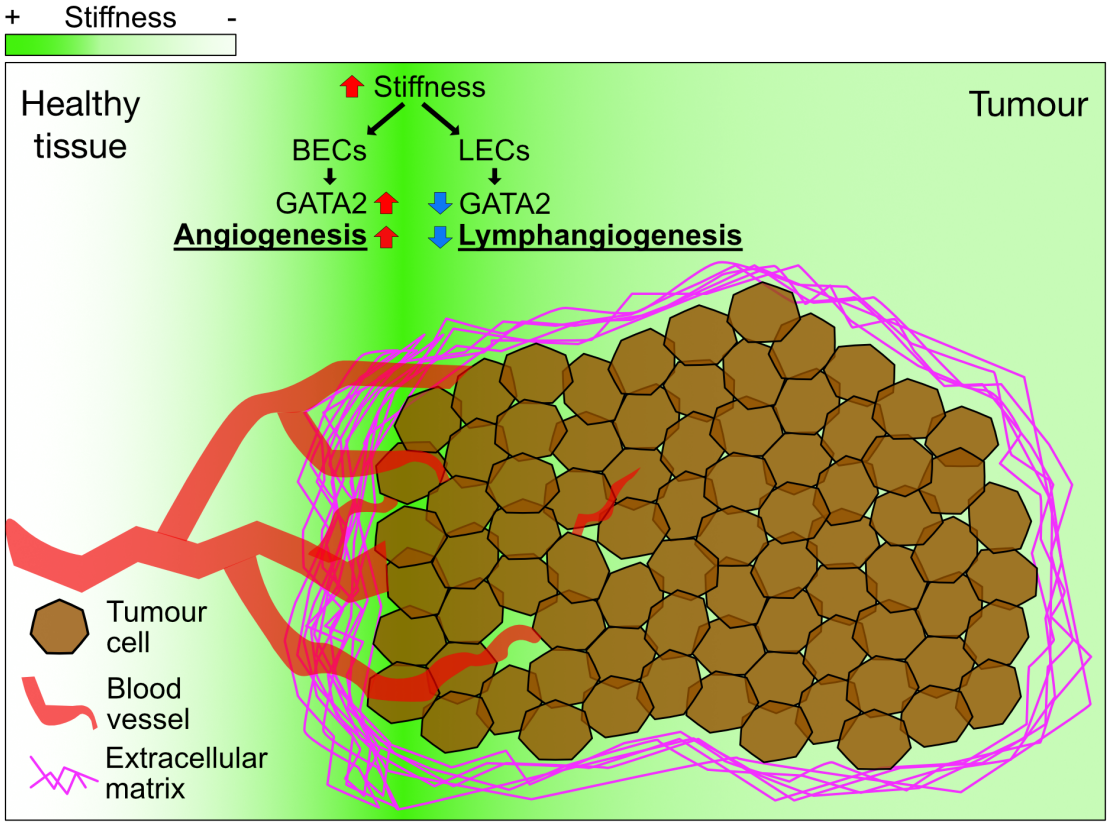

Figure 1: Differential tissue stiffness in cancer impacts angiogenesis.
Tissue stiffness varies dramatically from healthy (0.4kPa) to tumoral (1.2kPa) tissue, with an increase in stromal stiffness heterogeneity in the invasive region. ECM rigidity induces blood vessel sprouting via upregulation of GATA2 and increased VEGFR2/VEGF-A signalling in blood endothelial cells (BECs). On the contrary, ECM rigidity might suppress lymphangiogenesis in a similar manner, since lymphatic endothelial cells (LECs) present lower levels of GATA2 and decreased VEGFR3/VEGF-C response in stiffer substrates. This may lead to angiogenic hot spots across the tumour tissue.

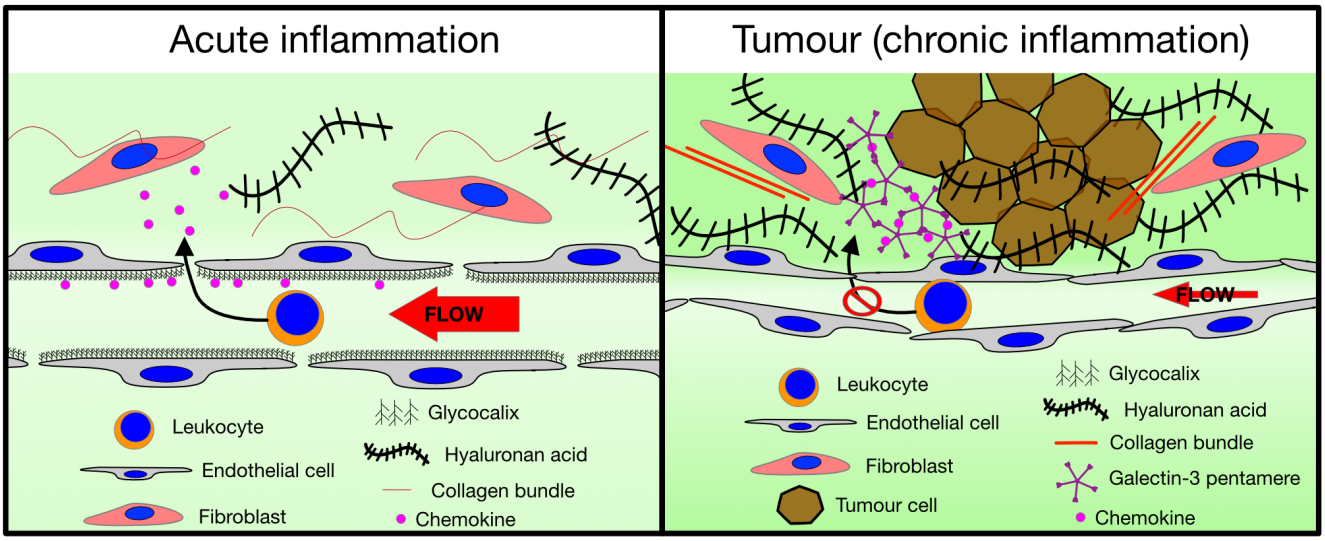

Figure 2: Immune infiltration. Similar osmotic pressures between fenestrated small capillaries and adjacent tissue enables normal exchange of small molecules in organs. During acute inflammation, this allows diffusion and gradient formation of chemoattractants that are partially trapped by the luminal glycocalyx of the endothelium, assisting leukocyte recruitment into the inflammation site. Cancer development represents a chronic inflammatory response in which vasculature is affected in a number of ways. Tumour growth and excess of ECM components such as collagens and hyaluronan acid increases interstitial fluid pressure that hinders molecule exchange. Blood vessels become tortuous, impeding normal flow and extravasation of leukocytes. Furthermore, cancer cells can induce loss of the luminal glycocalyx in endothelial cells, impeding the formation of chemoattractant gradients, which are retain within the tissue bound to tumour-derived galectin-3 lattices.