Tumor Associated Immune Cells- On the One Hand and on the other hand?

Terrence P McGarty
White Paper No 167
October, 2019

The immune system is a powerful protective part of human homeostasis. Invaders, external and internal, can be identified, tagged, attacked and disposed of. On the other hand, the immune cells often take part in protecting and enhancing the viability of such invaders as cancer cells. We examine this dual role of elements of the immune system based on several approaches to the current understandings.

Notice

This document represents the personal opinion of the author and is not meant to be in any way the offering of medical advice or otherwise. It represents solely an analysis by the author of certain data which is generally available. The author furthermore makes no representations that the data available in the referenced papers is free from error. The Author also does not represent in any manner or fashion that the documents and information contained herein can be used other than for expressing the opinions of the Author. Any use made and actions resulting directly or otherwise from any of the documents, information, analyses, or data or otherwise is the sole responsibility of the user and The Author expressly takes no liability for any direct or indirect losses, harm, damage or otherwise resulting from the use or reliance upon any of the Author's opinions as herein expressed. There is no representation by The Author, express or otherwise, that the materials contained herein are investment advice, business advice, legal advice, medical advice or in any way should be relied upon by anyone for any purpose. The Author does not provide any financial, investment, medical, legal or similar advice in this document or in its publications on any related Internet sites.

Furthermore, this document contains references to and quotes from papers and documents under the premise of Fair Use in order to present ideas and understandings in context. The Author has attempted to make any and all references to such material separate from those of the author per se and has referenced the source expressly in all cases. These documents are for the dissemination of ideas and have no commercial intent. The Author would appreciate any communications relating to these documents and these should be sent to:

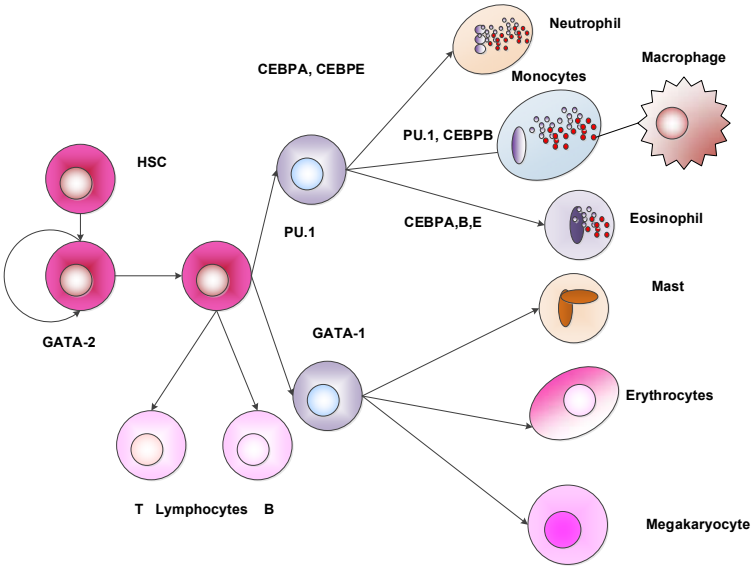
tmcgarty@telmarc.com.

Contents

1 INTRODUCTION

The immune system is a powerful protective part of human homeostasis. Invaders, external and internal, can be identified, tagged, attacked and disposed of. On the other hand, the immune cells often take part in protecting and enhancing the viability of such invaders as cancer cells. We examine this dual role of elements of the immune system based on several approaches to the current understandings.

Several of the elements of the immune system we focus upon are the macrophages, the neutrophils and the mast cells. They are all part of the innate immune system and all are in a sense part of the first barrier of defense to intruders of homeostasis. We reflect upon these as below.



Tumor activate macrophages (TAMs) have been known as cells which have protected and supported tumor cells. TAMs have been examined by many researchers and there seems to be a slow but advancing opportunity to address them and eliminate their influence[1].

From a recent paper by Etzerodt et al[2]:

Tumor-associated macrophages (TAMs) play critical roles in tumor progression but are also capable of contributing to antitumor immunity. Recent studies have revealed an unprecedented

[1] https://www.researchgate.net/publication/334959399_Immunotherapy_Possible_Directions

[2] Etzerodt et al, Specific targeting of CD163+ TAMs mobilizes inflammatory monocytes and promotes T cell–mediated tumor regression, Jrl Experimental Medicine, August 2019

heterogeneity among TAMs in both human cancer and experimental models. Nevertheless, we still understand little about the contribution of different TAM subsets to tumor progression.

Here, we demonstrate that CD163-expressing TAMs specifically maintain immune suppression in an experimental model of melanoma that is resistant to anti–PD-1 checkpoint therapy. Specific depletion of the CD163+ macrophages results in a massive infiltration of activated T cells and tumor regression. Importantly, the infiltration of cytotoxic T cells was accompanied by the mobilization of inflammatory monocytes that significantly contributed to tumor regression.

Thus, the specific targeting of CD163+ TAMs reeducates the tumor immune microenvironment and promotes both myeloid and T cell–mediated antitumor immunity, illustrating the importance of selective targeting of tumor-associated myeloid cells in a therapeutic context.

This is a powerful observation and adds greatly to how these protective macrophages can be handled. From Eureka[3]:

…new form of immunotherapy that has so far been tested on mice makes it probable, that oncologists in the future may be able to treat some of the patients who are not responding to existing types of immunotherapy. Instead of attacking the cancer cells directly, the new technique target and remove a subtype of immune cells known as macrophages, after which the immune system itself begins to attack the cancer.

This is shown by a new study published in the Journal of Experimental Medicine in which researchers from Aarhus University, Denmark, have collaborated with colleagues in France, the UK and USA, to show that the serious form of skin cancer 'malignant melanoma' can be defeated by using the new method. This variant of skin cancer accounts for eighty per cent of all deaths from melanomas.

"We've studied what happens to the tumour when it is exposed to targeted treatment that removes precisely ten per cent of the macrophages that are supporting the cancer tumour instead of fighting it," says Anders Etzerodt, PhD and assistant professor in cancer immunology at the Department of Biomedicine at Aarhus University…"The most important result is that the depletion of this specific type of macrophage causes the tumour to shrink, which is triggered by a subsequent mobilisation of new macrophages and, ultimately, also an activation of the so-called T cells which attack the tumour," says Anders Etzerodt.

The type of macrophages which the researchers have removed express a specific receptor, CD163, on the cell surface. Unlike other macrophages, these are known to have an undesirable effect in connection with cancer. Instead of recognising cancer cells as unwanted tissue, the macrophage sees the tumours as normal tissue that needs help with regeneration. It is also

[3] https://www.eurekalert.org/pub_releases/2019-08/au-rda082719.php

widely recognised that survival rates are worse if there are many macrophages, which express the CD163 receptor in the tumour.

This capability may be significant in addressing a wide variety of cancers.

As Molina-Cerrillo et al have noted:

It is important to point out that not all cases of macrophage tumoral infiltration have the same characteristics. This is due to the fact that the tumor is able to promote an immunotolerant M2 macrophage phenotype. In addition, this polarization leads to the secretion of immunosuppressive cytokines such as transforming growth factor β or interleukin 10 (IL-10) together with CXCL2, which avoid the final effective activity of the cytotoxic T lymphocyte, despite immunotherapeutic treatment.6 Moreover, other cells of the innate immune system also regulate the polarization and activity of the effector lymphocyte.

In this sense, it is important to consider the role of the fibroblast by its ability to generate stroma around the tumor and promote angiogenesis; the mast cell by its degranulation via immunoglobulin E that promotes the secretion of anti-inflammatory cytokines and angiogenesis and prevents the CD4+ T-lymphocyte polarization to type 1 T helper, increasing the regulatory T-cell response; and, finally, the neutrophil by its ability to attract myeloid-derived suppressor cells with immunosuppressive and tumor immune evasion properties via secretion of IL-10[4], nitric oxide, IL-1β, or indolamine 2,3 dioxygenase (IDO). In the end, all these mechanisms lead to tumor progression and invasiveness.

Indeed, other immunosuppressive mechanisms are conducted by the macrophage not only by cytokine release, but by the direct expression of proteins of the B7 family ligands, such as programmed death-ligand. It is interesting to note that the macrophage may have a role that differs from that of the tumor promoter. Even if the majority of tumors have a predominant M2 phenotype, some others have an initial predominant M1 phenotype, demonstrating better local tumor control.9 If the macrophage is "educated" toward an M1 phenotype, characterized by the expression of genes such as INHBA, CCR2, SERPINE1, and MMP12, its activity may change completely. This M1 macrophage is able to produce interferon γ and IL-12, as well as other inflammatory cytokines, which finally have the ability to induce CD4+ T polarization toward a type 1 T helper phenotype and therefore stimulate CD8+ T cells as well as natural killer lymphocytes.

For the most part, those immune changes are directed toward an effective antitumor immune response. Immunotherapy, or even its combination with other drugs, may be able to modify these immune responses induced by the lymphocyte in certain patients.

[4] From Abbas et al I*L-10 is a cytokine that is produced by and inhibits activation of macrophages and DCs. IL-10 inhibits the production of various inflammatory cytokines by activated macrophages and DCs, including IL-1, TNF, and IL-12. Because it is both produced by macrophages and DCs and inhibits the functions of these cells, IL-10 is an excellent example of a negative feedback regulator. Alternatively activated macrophages make more IL-10 than classically activated macrophages. IL-10 is produced by some nonlymphoid cell types (e.g., keratinocytes). IL-10 is also produced by regulatory T cells, and we will discuss the details of IL-10 in this context in Chapter 15. Loss-of-function mutations in the IL-10 receptor result in severe colitis developing in infancy.*

As Quaranta and Schmid note:

Despite the incredible clinical benefits obtained by the use of immune checkpoint blockers (ICBs), resistance is still common for many types of cancer. Central for ICBs to work is activation and infiltration of cytotoxic CD8+ T cells following tumour-antigen recognition. However, it is now accepted that even in the case of immunogenic tumours, the efector functions of CD8+ T cells are highly compromised by the presence of an immunosuppressive tumour microenvironment (TME) at the tumour site. Tumour-associated macrophages (TAMs) are among the most abundant non-malignant stromal cell types within the TME and they are crucial drivers of tumour progression, metastasis and resistance to therapy. TAMs are able to regulate either directly or indirectly various aspects of tumour immunity, including T cell recruitment and functions. In this review we discuss the mechanisms by which TAMs subvert CD8+ T cell immune surveillance and how their targeting in combination with ICBs represents a very powerful therapeutic strategy.

They continue:

Despite the enormous clinical benefits given by the use of ICBs, only patients with certain tumour types, particularly melanoma, non-small-cell lung and renal cell cancers, respond positively to this kind of therapy. In other cancer types, including pancreatic, colorectal and ovarian cancer, patients are largely refractory or only a small fraction of patients shows a positive response . It became clear that the presence of an active CD8+ T cell response within the tumour is the key factor for prediction of anti- PD-1/PD-L1 therapeutic efficacy [36,37]. The induction of CD8+ T cell-mediated anti-tumour activity critically depends on tumour-mutational load and tumour- associated neo-antigens. However, it is now accepted that in addition to tumour-intrinsic factors (Reviewed in) anti-tumour immune defence, and in consequence the therapeutic activity of ICB, are strictly correlated with the presence of a complex immunosuppressive network within the TME . Moreover, in many solid cancer types the physical exclusion of CD8+ T cells from the tumour site has been demonstrated to be a critical limiting factor for ICB approaches.

A dense fibrotic stroma, hypoxia, and abnormal blood vessel architecture can impede the efficient infiltration of CD8+ T cells into the tumour thereby prohibiting CD8+ T cell–cancer cell interaction. In this scenario, CD8+ T cells are no longer capable to physically interact with and kill cancer cells [40–44]. Thus, overcoming the physical barrier restrictions imposed by the TME is necessary for successful ICB therapies. In the light of these observations, the identification of key immunosuppressive factors within the TME and their combined targeting with ICB provides an attractive treatment strategy to enhance the efficacy of ICB therapies in cancer.

2 PROLIFERATION

Proliferation of malignant cells requires various growth processes and amongst them the vascular growth is often the most important. We discuss briefly several growth factors that lead to such proliferation. As Fouad and Aanel have noted:

Vascularization

Tumors cannot grow beyond 2-3 mm nor metastasize without new vasculature. Although angiogenesis is the most discussed, various other modes of tumor vascularization exist with redundancy in usage, partly explaining resistance towards antagonizing a single mode. There exists a confusion in the literature where on occasions "angiogenesis" encompasses all forms of neo-vascularization, while on others it refers to the classic vascular sprouting with other modes treated as separate entities. We choose to adopt the latter terminology.

We have argued previously that such small tumors, without activated growth via vascularization may be carcinoma in situ or even a non-malignant growth. The assertion regarding 3mm and smaller lesions, I would argue even up to 5mm often shown no vascularization and this represent at most a transition phase and at least a growth which may never materialize. The authors continue:

Angiogenesis

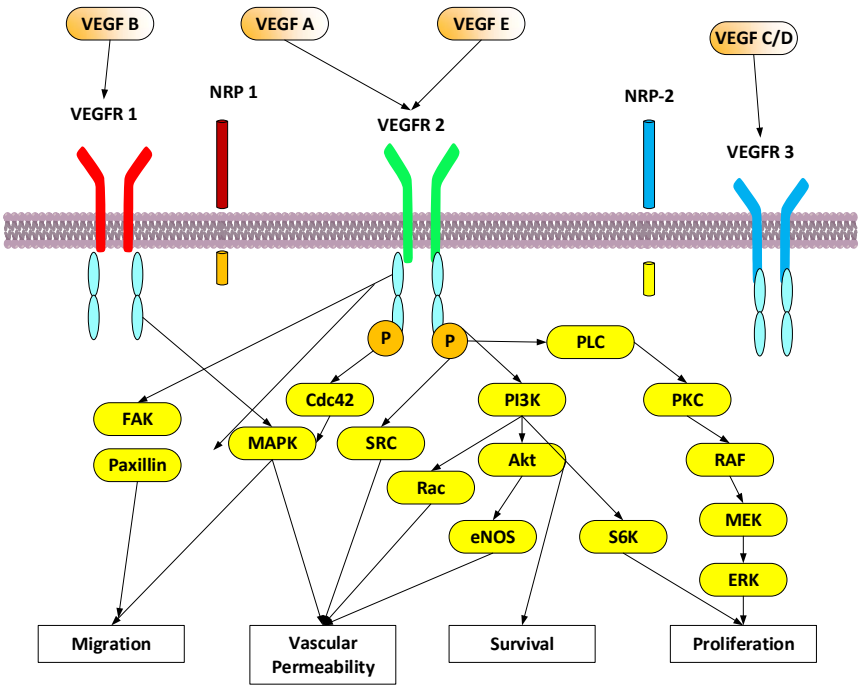
Angiogenesis is the process of sprouting, cell division, migration and assembly of endothelial cells (ECs) from pre-existing vessels. It is utilized during embryogenesis for expansion and remodeling of primitive vascular networks, and is part of postnatal events including wound healing, the female reproductive cycle, and chronic inflammation. In these events, however, angiogenesis is turned off or may be prolonged but self-limiting, unlike in case of malignancies where the process is continuously activated. Regulation of angiogenesis involves pro- and anti-angiogenic factors; their balance determines the status of the "angiogenic switch". Only when a trigger tips the balance towards pro-angiogenic factors (as in case of malignancy) is the switch turned on and do vascularuiescent tissues show signs of angiogenesis.

The most important trigger of angiogenesis is hypoxia. ECs possess a number of oxygen- sensing mechanisms, chiefly those interfacing with the hypoxia-inducible transcription factor (HIF) family, regulating the expression of a multitude of genes not only involved in angiogenesis, but in cell survival, metabolism, and inflammation as well. Responding to hypoxia, stabilized HIF initiates an adaptive transcriptional response, many products of which are factors involved in turning on the angiogenic switch. With hypoxia being a feature of tumors, it is not surprising that HIF levels are higher in many cancers, correlating with poor clinical pro- gnosis. Other angiogenic switch triggers in tumors include metabolic rewiring of ECs creating an acidic TME, alterations in genes control- ling production of angiogenic regulators, mechanical stress, and inflammatory cell infiltrate.

These triggers may be tumor and tissue specific and may alternate during various stages of tumor development. The effectors include a plethora of pro-angio- genic molecules, the VEGF signaling pathway is the most potent of which. Controlled by HIF activity, and also directly by growth signaling, VEGF is overexpressed in a multitude of malignancies and its activated signaling leads to EC proliferation, survival, migration and differentiation, and mediation of vascular permeability.

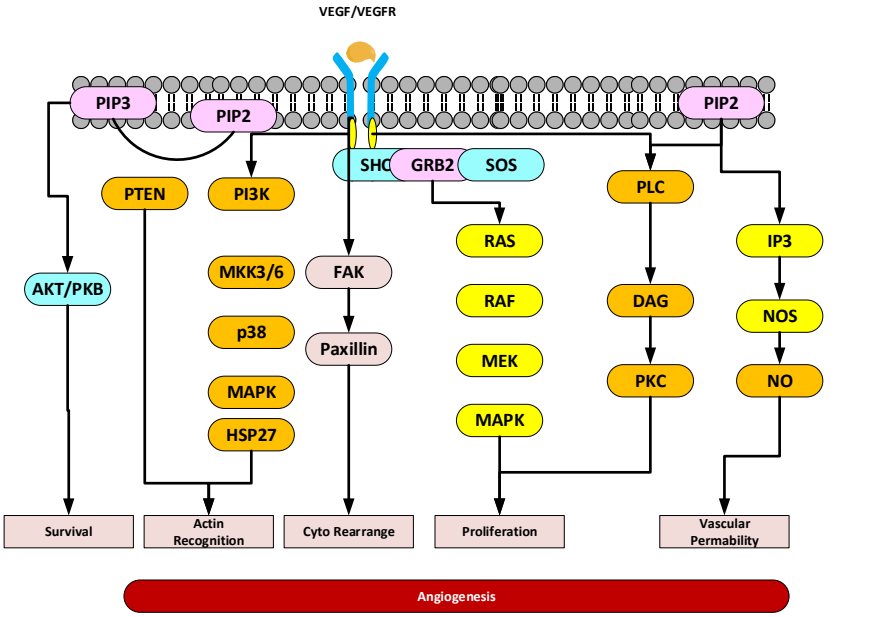
A number of VEGF independent effectors exist; those may work complementarily, independently, or compensatively for VEGF signaling. A growing list of opposing anti-angiogenic factors stand on the other end of the angiogenic balance, susceptible to sabotage in tumor settings. The sprouting tumor vasculature differs from normal one. Dilated and tortuous vessels with ECs not forming regular monolayers, and resting on a basement membrane of variable thickness, and pericytes forming abnormally loose associations with ECs, all lead to leakiness. The blood flow is chaotic with resulting areas of hypoxia and acidosis; these stressful conditions have a number of effects including potentiating angiogenesis, lowering therapeutic effective and allowing resistant clonal expansion.

The details of VEGF signalling are shown below:



The above is for the three types of VEGF. Further details are shown below:

http://www.sinobiological.com/VEGF-Signaling-a-1395.html?utm_source=journals&utm_medium=ntent&utm_campaign=VEGF-Signaling



3 IMMUNE SYSTEM

We initially focus on three of the immune system cells; macrophage, neutrophil and mast cells. Each has a classic role to play from antigen-presenting, to attack and destroy. However the simplicity of the initial understanding gets distorted once we understand that each of these players can act and be acted upon. Their action can destroy inappropriate cells and elements and at the same time protect and nourish putative cancer cells.
l

3.1 MACROPHAGES

We start with the macrophages. As Abbas et al describe them:

Phagocytes, including neutrophils and macrophages*, are cells whose primary function is to ingest and destroy microbes and remove damaged tissues. The functional responses of phagocytes in host defense consist of sequential steps: recruitment of the cells to the sites of infection, recognition of and activation by microbes, ingestion of the microbes by the process of phagocytosis, and destruction of ingested microbes. In addition, through direct contact and by secreting cytokines, phagocytes communicate with other cells in ways that promote or regulate immune responses. Blood neutrophils and monocytes are both produced in the bone marrow, circulate in the blood, and are recruited to sites of inflammation. Although both are actively phagocytic, they differ in significant ways.*

(i) The neutrophil response is more rapid and the lifespan of these cells is short, whereas monocytes become macrophages in the tissues, can live for long periods, and so

(ii) the macrophage response may last for a prolonged time. Neutrophils mainly use cytoskeletal rearrangements and enzyme assembly to mount rapid, transient responses, whereas macrophages rely mostly on new gene transcription.

A major function of macrophages in host defense is to ingest microbes by the process of phagocytosis and then to kill the ingested microbes. The mechanisms of phagocytosis and killing, … include formation of cytoplasmic membrane–bound organelles that contain the microbes, the fusion of these organelles with lysosomes, the enzymatic generation of reactive oxygen and nitrogen species in the lysosome that are toxic to microbes, and digestion of microbial proteins by proteolytic enzymes. In addition to ingesting microbes, macrophages ingest necrotic host cells, including cells that die in tissues because of the effects of toxins, trauma or interrupted blood supply, and neutrophils that die after accumulating at sites of infection. This is part of the cleaning up process after infection or sterile tissue injury.

Macrophages also recognize and engulf apoptotic cells before the dead cells can release their contents and induce inflammatory responses. Throughout the body and throughout the life of an individual, unwanted cells die by apoptosis as part of many physiologic processes, such as development, growth, and renewal of healthy tissues, and the dead cells are eliminated by macrophages. Macrophages are activated by microbial substances to secrete several different

cytokines that act on endothelial cells lining blood vessels to enhance the recruitment of more monocytes and other leukocytes from the blood into sites of infections, thereby amplifying the protective response against the microbes. Other cytokines act on leukocytes and stimulate their migration to tissue sites of infection or damage.

Macrophages serve as antigen-presenting cells (APCs) that display fragments of protein antigens to and activate T lymphocytes. This function is important in the effector phase of T cell–mediated immune responses. Macrophages promote the repair of damaged tissues by stimulating new blood vessel growth (angiogenesis) and synthesis of collagen-rich extracellular matrix (fibrosis). These functions are mediated by cytokines secreted by the macrophages that act on various tissue cells.

Macrophages can acquire distinct functional capabilities, depending on the types of activating stimuli they are exposed to. The clearest example of this is the response of macrophages to different cytokines made by subsets of T cells.

Some of these cytokines activate macrophages to become efficient at killing microbes, called classical activation, and these cells are called M1 macrophages.

Other cytokines activate macrophages to promote tissue remodeling and repair, called alternative activation, and these cells are called M2 macrophages.

These different pathways of activation and the cytokines involved. The relationship between blood monocyte subsets, discussed earlier, and macrophage subsets is not well understood, but classical (inflammatory) monocytes and M1 macrophages share functional properties. Macrophages may also assume different morphologic forms after activation by external stimuli, such as microbes. Some develop abundant cytoplasm and are called epithelioid cells because of their resemblance to epithelial cells of the skin. Activated macrophages can fuse to form multinucleated giant cells, which occurs frequently in certain types of microbial infections, such as with mycobacteria, and in response to indigestible foreign bodies.

From Salinas et al further delineate the M1 and M2 distinctions:

Macrophages can be divided schematically into two main classes in line with the Th1/Th2 dichotomy.

1. M1 macrophages (classically activated cells) originate upon encounter with IFN[5]-and microbial stimuli such as LPS and are characterized by IL-12high and IL-23 production and consequent activation of polarized type I T cell response, cytotoxic activity against phagocytozed microorganisms and neoplastic cells, expression of high levels of ROI, and good capability as APCs.

[5] From Abbas et al, *IFN-γ activates macrophages to kill phagocytosed microbes. Macrophage activation resulting in increased microbicidal activity is called classical macrophage activation, to be contrasted with an alternative activation pathway that is induced by Th2 cytokines; these types of macrophage activation are described in more detail later.*

In general, M1 macrophages act as soldiers: they defend the host from viral and microbial infections, fight against tumors, produce high amounts of inflammatory cytokines, and activate the immune response.

2. On the other hand, distinct types of M2 cells differentiate when monocytes are stimulated with IL-4 and IL-13 (M2a), with immune complexes/TLR ligands (M2b), or with IL-10 and glucocorticoids (M2c).

Hallmarks of M2 macrophages are IL-10 high IL-12 low IL-1ra high IL-1 decoyRhigh production, CCL17 and CCL22 secretion, high expression of mannose, scavenger and galactose-type receptors, poor antigen-presenting capability and wound-healing promotion.

Further, M2 express specific change in some metabolic pathways: arginine metabolism is oriented toward the production of ornitine and polyamine instead of citrulline and NO.

M2 cells are workers of the host: they promote scavenging of debris, angiogenesis, remodeling and repair of wounded/damaged tissues. Of note, M2 cells control the inflammatory response by down-regulating M1-mediated functions.

In addition, M2 macrophages are competent effector cells against parasitic infections . The loss of equilibrium of M1 and M2 cell number may lead to pathological events: an M1 excess could induce chronic inflammatory diseases, whereas an uncontrolled number of M2 could promote severe immune suppression.

As Quaranta and Schmid note:

*Macrophages originate from t**hree different developmental pathways**.*

*All tissue embryonic macrophages derive from macrophage precursors in the yolk sac and fetal liver. During adulthood, fetal macrophages are replaced gradually by macrophages derived from bone marrow **hematopoietic stem cell (HSCs)**.*

*Some types of tissue resident macrophages, including bone osteoclasts, **epidermal Langerhans** cells, lung alveolar macrophages, microglia and **liver Kupffer** cells develop from **embryonic macrophages** and persist in adult tissues independently of replenishment by Ly6Chigh monocytes originated from HSCs during adulthood.*

*Instead, other types of tissue macrophages such as intestine, dermis, heart and pancreas macrophages u**ndergo a continuous turnover in adulthood by recruitment of circulating monocytes which differentiate into macrophages upon tissue infiltration**.*

Infiltrating monocytes derived from HSCs are also the main source of macrophage replenishment into inflamed and remodeling tissues, and this process is driven by cytokines and chemokines, such as C-C motif chemokine ligand (CCL) 2, CCL5 and macrophage colony stimulating factor-1 (CSF-1).

*There are different markers to identify monocyte-macrophages diversity in human and mouse. In human, c**irculating monocytes, which originate from the bone marrow, can be classified in two subsets**:*

(i) CD14+ CD16neg 'inflammatory' or 'classical' and

(ii) CD14+ CD16+ 'patrolling' or 'non-classical' monocytes.

In the same way, mouse 'inflammatory' monocytes are classified as CD11b+ Ly6Chigh CCR2high CX3CR1low, in contrast 'patrolling' monocytes are CD11b+ Ly6Glow CCR2low CX3CR1high.

***Patrolling monocytes monitor the microvasculature under steady-state** conditions and rarely extravasate into tissue. However, they can rapidly accumulate in lung metastatic tissue and inhibit cancer cell seeding and growth by exerting anti-tumour functions through recruitment of NKs. Macrophages are a population of heterogeneous and plastic cells.*

Once resident in tissues, macrophages acquire a distinctive phenotype in response to different signals present in the immediate microenvironment. Environmental stimuli, like IFN or microbial products, like the lipopolysaccharide

From Ruffell and Coussens:

Macrophages produce an array of cytokines, chemokines, polypeptide growth factors, hormones, matrix-remodeling proteases, and metabolites, many of which possess tumor-promoting activities. A caveat to some of these reported activities is that many findings originate from cell culture studies utilizing neoplastic myeloid cell lines or bone marrow-derived macrophages and, therefore, cannot account for the complex milieu of polarization signals to which macrophages would be exposed in vivo. This includes the aforementioned CSF-1 and CCL2, prostaglandin E2 (PGE2), and damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 protein (HMGB1), extracellular ATP, and degraded extracellular matrix components …

Macrophages are well described regulators of tumor angiogenesis, with supporting evidence derived from both clinical and experimental studies in which much of their capability is associated with vascular endothelial growth factor (VEGF) signaling. This includes macrophage production of VEGF-A, production of VEGF homologs such as placental growth factor, enhancement of VEGF-A bioavailability through matrix metalloproteinase (MMP)-9 activity, and induction of VEGF-A production by endothelial cells via WNT7B expression. VEGF-A drives the formation of abnormal vasculature in tumors, consisting of excessive branching, dead-end vessels, and vessel leakiness, that, together, impact tumor hemodynamics and drug delivery.

VEGF antagonists induce vascular normalization, and several studies have reported increased uptake of chemotherapeutics associated with this process, likely because of reduced vessel leakiness and interstitial fluid pressure. Although macrophages are not necessarily a dominant

source of VEGF-A in all tumor tissues, specific deletion of VEGF-A in macrophages via lysozyme Mpromoter-driven Cre recombinase revealed their role in driving abnormal vascular phenotypes in tumors.

Importantly, similar to the use of VEGF antagonists, tumors in these mice were more sensitive to chemotherapy, although, unexpectedly, they also grew at a faster rate because of improved tissue perfusion and reduced hypoxia in the absence of therapeutic intervention…

3.2 NEUTROPHILS

Neutrophils are the garbage collectors of the immune system leaving behind the classic collection of pus. However, they are much more complex than that. As Gregory and Houghton note:

The tumor microenvironment is composed of numerous immune and nonimmune cell types in addition to tumor cells themselves. Controlled experiments have shown important roles for many of these cells, including lymphocytes, natural killer (NK) cells, macrophages, fibroblasts, endothelial cells, and pericytes.

Although commonly encountered within the tumor microenvironment, neutrophils have not been traditionally considered anything more than a casual observer, and certainly not a disease modifying entity. This view likely reflects disbelief that such a short-lived cell could impact a chronic, progressive disease.

However, upregulation of polymorphonuclear leukocyte (PMN)–chemotactic substances ensures the constant replenishment of tumor-associated neutrophils (TAN), which are fully capable of modifying tumor growth and invasiveness. Neutrophils, or PMNs, exist to defend the host from invading microorganisms and to assist in wound healing. Invading pathogens elicit an inflammatory response that recruits neutrophils to sites of infection.

Once there, neutrophils engulf and eliminate microorganisms using an arsenal of cytotoxic substances.

Activated neutrophils also release proteinases into the extracellular environment, leading to damage of surrounding host tissue.

Additionally, neutrophils produce cytokines and chemokines, which can impact inflammatory cell recruitment, altering the immune response. This process of PMN recruitment and activation, observed in infection, is recapitulated within the tumor microenvironment; however, accumulating evidence suggests that, in this context, PMNs act to the detriment of the host. … The crosstalk between immune cells and tumor cells that leads to phenotypic alterations in tumor biology has been broadly termed immunosculpting or immunoediting.

Concrete examples of tumor-mediated signals eliciting protumor responses from neutrophils have been found.

For instance, granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by breast cancer cells has been shown to elicit the production of the IL-6–like cytokine oncostatin M by neutrophils in coculture experiments.

In turn, oncostatin M–stimulated breast cancer cells exhibited increased VEGF production and increased invasiveness in Matrigel invasion assays. Another study revealed that a factor present in the conditioned media obtained from hepatocellular carcinoma cells induced the production of hepatocyte growth factor (HGF) from neutrophils, which promoted increased invasiveness by the tumor cells.

These findings suggest that, although neutrophils release a paucity of cytokine when compared with macrophages and other cells within the tumor microenvironment, the temporal-spatial elaboration of these substances by PMN plays unique roles.

Thus neutrophils play a much more complex role than often understood. They communicate via cytokines and other paracrine factors to the tumor mass often protecting and enhancing the mass itself.

3.3 MAST CELLS

Mast cells are the third of the cells of interest. From Metcalfe,

Mast cells have a rather unique position among cells of the immune response. Their progenitors are bone marrow derived, yet under normal conditions appear in the mature state only within vascularized tissues, where they are long-lived.

Mast cells appear historically ancient, yet their roles in mammalian biology, including disease pathogeneses and host defense mechanisms, often remain speculative and based on in vitro studies and animal models2-4 with 2 primary exceptions—IgE-mediated immediate hypersensitivity reactions and mastocytosis.

Complicating the understanding of the role of mast cells in human biology is that while other normal human immune cell functions often become more obvious in the absence of a specific cell type, such as with agranulocytosis, or in the absence of normal function of a specific pathway, as in autoimmune lymphoproliferative syndrome (ALPS) associated with defective lymphocyte Fas-mediated apoptosis, the single similar situation involving mast cells and human disease characterized to date is mastocytosis, resulting from disturbed control of mast cell proliferation.

Mast cell research initially relied upon observation on mast cell appearance and numbers in tissue biopsies, sometimes correlated with tissue histamine levels. With time, methods were developed to obtain and study mast cells ex vivo. The most common protocols relied on obtaining mast cells from the peritoneal cavity of rodents, or enrichment of mast cells from tissue digests.

These approaches initiated modern mast cell biology with the first work on histamine, slow-reacting substance of anaphylaxis (SRS-A), and other mast cell–derived mediators including proteases, and the early studies on the mechanisms of mast cell signal transduction. In more recent years, and with the identification of key mast cell growth factors, investigators have discovered how to culture mast cells in vitro from pluripotential precursors. This development has facilitated the further study of human mast cell gene expression, signal transduction, and production of mediators relevant to inflammation. …

Mast cells and IgE have long been associated with the pathogenesis of the acute manifestations of the immediate hypersensitivity reaction, the pathophysiologic hallmark of allergic rhinitis, allergic asthma, and anaphylaxis. The central role of mast cells in these disorders is widely accepted.

In 1921, Prausnitz and Kustner demonstrated that serum transferred into the skin of a normal recipient induced a local allergic reaction upon contacting the antigen to which the donor was sensitive. This came to be known as the PK test.66 The sensitizing factor was termed "reagin." In the late 1960s, reagin was isolated from normal serum … and from a myeloma … and found to be a unique class of immunoglobulin designated IgE. IgE is now known to bind with high affinity to FcεRI, and the expression of this receptor is related to the serum IgE concentration.

High "constitutive" levels of FcεRI expression are restricted to mast cells and basophils, and this feature helps explain the unique role of mast cells as tissue-based effector cells in allergic inflammation. In humans, low levels of expression are detected in Langerhans cells, peripheral blood dendritic cells, and monocytes. In mast cells and basophils, FcεRI has a tetrameric structure composed of a single IgE-binding ε chain, a single ε chain, and 2 identical disulfide-linked chains. All 3 subunits must be present for efficient cell surface expression in rodents, but human cells can express FcεRI in the absence of the ε chain.

In humans, the FcεRI expressed by hematopoietic cells other than mast cells and basophils consists of only the ε2 form. The aggregation of FcεRI that is occupied by IgE is sufficient for initiating downstream signal transduction events involving tyrosine phosphorylation that activate the mast cells or basophils to degranulate and to secrete lipid mediators and cytokines

… The FcεRI ε chain functions as an amplifier of signaling through FcεRI. After degranulation, mast cells are believed to survive and regranulate. This may contribute to the increase in mast cells in association with chronic inflammation

As Varricchi et al note:

Mast cells were first identified in human tumors and named by Paul Ehrlich. These cells are present in all classes of vertebrates, and it has been estimated that they have emerged >500 million years ago, long before the development of adaptive immunity (3). Mast cells are distributed throughout nearly all human tissues and often in close proximity to epithelia, fibroblasts, blood and lymphatic vessels, and nerves. Human mast cells form a heterogeneous

population of cells with differences in their ultrastructure, morphology, mediator content, and surface receptors. Human mast cells derive from CD34+, CD117+ pluripotent hematopoietic stem cells, which arise in the bone marrow.

Mast cell progenitors enter the circulation and subsequently complete their maturation in tissues. These cells store and release upon activation a wide spectrum of biologically active mediators that individually have been shown to have potential positive or negative effects on various target cells. Increasing evidence indicates that mast cells act as sentinels of the surrounding environment, with the capacity to rapidly perceive tissue insults and initiate biochemical programs of inflammation or repair.

Mast cells are activated not only by IgE, specific antigens, and superallergens, the main mechanisms which account for their function in allergic disorders, but also by a plethora of immunologic and non-immunologic stimuli.

Mast cells and their mediators have been canonically associated with a detrimental role in allergic diseases, but these cells can induce a protective immune response of the host against noxious substances, viral and microbial pathogens. Interestingly, epidemiological and experimental studies indicate an inverse association between IgE-mediated allergies and cancer, implying tumor-protective effect of IgE.

The initiation and progression of cancer are multistep processes characterized by the accumulation of a variable number of genetic and epigenetic alterations. The immunosurveillance system recognizes and eliminates mutant cells constantly generated.

However, immune-resistant cancer cells can slip through this system and proceed to develop tumors. Normal microenvironment [immune cells, fibroblasts, blood and lymphatic vessels, and interstitial extracellular matrix (ECM)] plays a central role in maintaining tissue homeostasis and is a barrier to tumorigenesis. Incorrect signals (chemokines, cytokines, reactive oxygen species, lipid mediators, etc.) from an aberrant microenvironment alter tissue homeostasis and initiate/ promote tumor growth. Thus, the multiple interactions between stromal and tumor cells are crucial for the initial phases of tumor development.

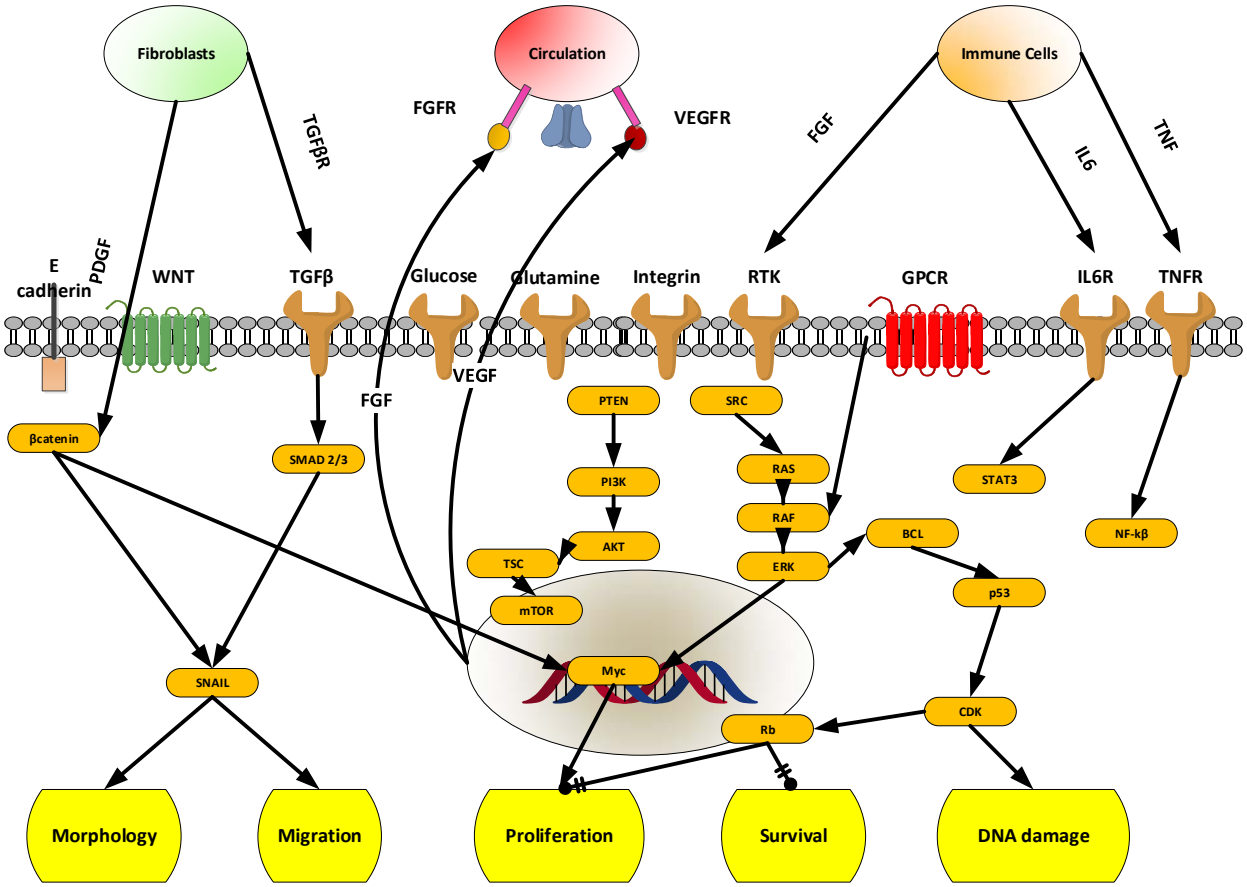
Prolonged low-grade inflammation or smoldering inflammation is a hallmark of cancer. Several cells of the innate and adaptive immune system (macrophages, mast cells, lymphocytes, neutrophils, NK, and NK T cells) are stromal components of the inflammatory microenvironment that can promote the development of experimental and human tumors.

Genetic Instability	• ROS
Angiogenesis	• VEGF-A,B CXCL8, IL-6, FGF-2
Lymphamgiogenesis	• VEGF-C, VEGF-D
Tissue Remodel	• MMR-9, Tryptase
EMT	• CXCL8. IL-8
Stemness	• CXCL8, IL-8
Activate STAT-3	• IL-6
EMT Immunosuppress	• TGFβ
Proliferation	• PAF
Suppress Adaptive System	• IL-13

4 TUMMOR ASSOCIATED IMNMUNE CELLS

We briefly examine the tumor associated immune cells which we focused upon earlier. Again we look at macrophages, mast cells and neutrophils. As with many immune cells they can sense the state of cells they come in contact with, react to stimuli from other cells and send out stimuli to those in their environment.

The tumor micro environment, TME is complex. One may look at it as follows:



The above is a simplified attempt to demonstrate the complexity of the cell, the extracellular matrix[6], the immune system[7] and the circulatory system[8].

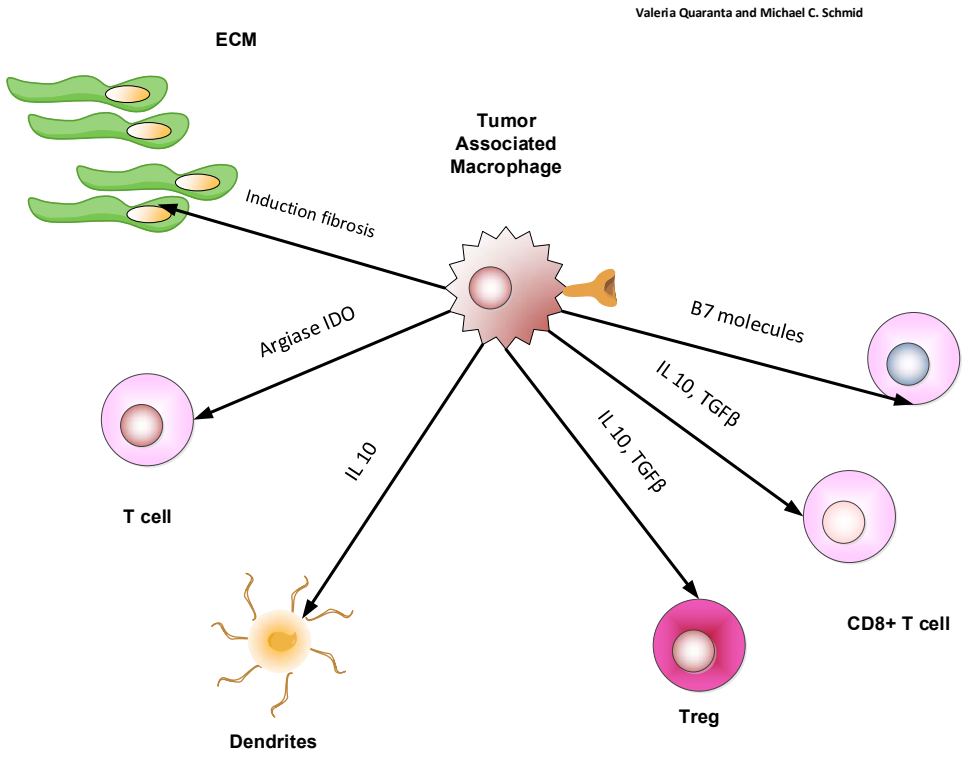
4.1 TUMOR ASSOCIATED MACROPHAGES

[6] https://www.researchgate.net/publication/315374581_Extracellular_Matrix_vs_Intracellular_Pathways

[7] https://www.researchgate.net/publication/314090163_Cancer_Immunotherapy_A_Systems_Approach

[8] See Cantley et al p 419 as modified.

Let us begin with macrophages. To the beginning student of the immune system one often sees the macrophage as that wandering cell that sense invaders and then sends out signals as to their presence. In a simple sense this is the case. But then again as with all immune system elements it is always more than that.



Grivennikov et al note:

The most frequently found immune cells within the tumor microenvironment are tumor-associated macrophages (TAMs) and T cells. TAMs mostly promote tumor growth and may be obligatory for angiogenesis, invasion, and metastasis, and high TAM content generally correlates with poor prognosis.

As DeVita et al have noted[9]:

For example, tumor-associated macrophages (TAMs) can comprise a large proportion of tumor bulk. TAMs are often found at points of basement membrane breakdown and at the invasive front. By producing uPA, MMP7, and MMP9, TAMs help tumors degrade extracellular proteins.

The numerous growth factors that TAMs produce:

[9] DeVita et al p 124

FGF, fibroblast growth factor

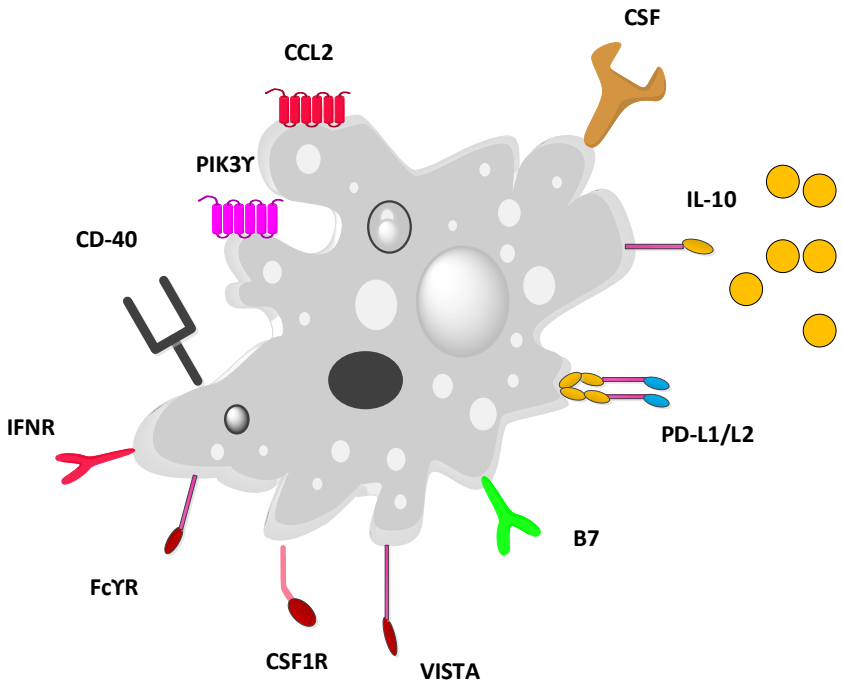
EGF, epidermal growth factor receptor ligands, and

PDGF, platelet derived growth factor, stimulates tumor cell growth and motility.

As in normal wound healing, these growth factors secreted by the TAMs or the tumors themselves activate fibroblasts.

These carcinoma-associated fibroblasts (CAFs) promote primary tumor growth by secreting stromal cell-derived factor 1 (SDF-1 or CXCL12), the ligand for CXCR4 on tumor cells. Angiogenesis is also aided by the action of CAFs through recruitment of endothelial progenitor cells by CXCL12 ***and by the action of TAMs that are recruited to areas of hypoxia to produce VEGF****. To ensure the loyalty of TAMs in promoting tumor growth, the tumor microenvironment can contain immunomodulatory factors like* ***TGF-β****, cyclooxygenase-2 (COX2),* ***CSF-1*** *(macrophage growth factor, colony-stimulating factor-1), IL-10, and IL-6, which inhibits maturation of dendritic cells and promotes TAMs that are immunosuppressed*

The TAM appears as below in terms of its receptors.



We shall examine these surface proteins in some detail as they apply to the development of a malignancy. Now as DiNardo and Ruffel note:

The presence of tumour-associated macrophages (TAMs) is generally associated with a poor prognosis in solid tumours. This has been shown in studies performed on individual tumour types

using traditional immunohistochemistry techniques to quantify cellular density and in more recent analyses that infer the presence of macrophages across malignancies using gene expression profiles. These findings are consistent with the established role of macrophages in promoting multiple aspects of tumorigenesis in experimental models, from initiation through to angiogenesis and systemic dissemination.

Most relevant for patients, TAMs are known to suppress responses to standard-of-care therapeutics, including chemotherapy, irradiation and angiogenic inhibitors. Although this includes direct regulation of survival and cell death pathways in tumour cells in vivo modelling indicates that improved efficacy following macrophage depletion is often dependent upon enhanced recruitment or function of cytotoxic CD8+ T cells.

Perhaps not surprisingly, macrophage antagonists demonstrate combinatorial efficacy when combined with immunotherapy, including checkpoint blockade. Clinical trials examining these combinations are now ongoing. In this Review, we discuss how macrophages are induced into becoming immunosuppressive, the mechanisms by which they suppress antitumour immunity and how this information is being utilized to develop therapeutics and design clinical trials.

From Wilke et al in Curiel we have:

TAMs (tumor associated macrophages) form the major APC subset (by number) in solid human epithelial cancers. Several years ago, our group discovered that both tumor cells and microenvironmental macrophages in ovarian cancer expressed CCL22, a chemokine instrumental in attracting Tregs to the tumor environment.

Interestingly, because the presence of Tregs predicts poorer survival and is associated with a high death hazard in ovarian cancer patients, TAMs may contribute to their prognoses. Indeed, we subsequently demonstrated that although they are highly B7-H4 positive, ovarian cancer cells do not directly mediate antitumor T cell suppression. However, B7-H4+ macrophages from the human ovarian tumor microenvironment are powerful suppressors of tumor-associated antigen-specific T cell immunity. B7-H4 blockade restored the stimulatory capacity of macrophages and mediated ovarian tumor regression in vivo in NOD/SCID mice. Both IL-10 and IL-6, often found in high concentrations in the tumor environment, can induce B7-H4 expression on macrophages.

Contrastingly, two cytokines minimally expressed in the same environment—GM-CSF and IL-4—inhibit B7-H4 expression. Interestingly, forced expression of B7-H4 in macrophages from healthy donors conferred a suppressive phenotype on the cells. As for the prognostic significance of B7-H4+ macrophages in ovarian cancer, we documented an inverse relationship between the intensity of B7-H4 expression on macrophages and patient survival. Importantly, Tregs, typically predictors of poor prognoses in cancer patients, could induce B7-H4 expression on myeloid APCs (including macrophages) and were positively associated with B7-H4+ macrophage presence in ovarian tumors.

A later observation of Wan and colleagues showed that the mean density of TAMs is significantly higher in ovarian cancer than in benign ovarian lesions and that the average 5-year survival

rate in patients with low densities of TAM was significantly higher than in patients with larger TAM populations, agreeing well with our observations. Multivariate analysis demonstrated that TAM infiltration status serves as an independent negative predictor for overall survival of patients with ovarian cancer. The presence of CCL17+ or CCL22+ cells in CD14+ monocytes and macrophages within gastric tumors correlated directly with Treg cell presence. Tregs were also shown to migrate toward CCL17 and CCL22

Kundu and Surh note:

Tumor-associated macrophages, mast cells and neutrophils play an important role in tumor angiogenesis by secreting VEGF, IL-8, TNFa, MMPs and other factors that increase vascular permeability.

Thus, chronic inflammation-driven tumor angiogenesis and a sustained 'inflammation-cancer-inflammation' loop proves Dvorak's early proposition that tumors are wounds that never heal. The role of various proinflammatory mediators in tumor angiogenesis will be discussed further.

Poh and Ernst note a more differentiated characterization of M1 and M2, separating M2 into four subsets as follows:

Tumor-associated macrophage heterogeneity is not only dependent on the nature of their monocytic precursor, but also on their functional diversity. To coordinate complex processes to promote immunity, while also minimizing damage to tissues where these responses occur, macrophages can reversibly alter their endotype in response to environmental cues.

These environmental cues include stimuli derived from pathogens, parenchymal, and immune cells, as well as the extracellular matrix. Similar to the Th1/Th2 T-cell dichotomy, macrophages may be broadly classified into two groups, referred to as:

(i) ***"classically activated M1" (CAM) or***

(ii) ***"alternatively activated M2" (AAM) endotypes.***

Much our understanding of macrophage polarization has relied on ***in vitro techniques****, whereby macrophages are stimulated with M1- or M2-polarizing signals.*

(i) ***For M1 this typically involves stimulation with IFNγ or lipopolysaccharide (LPS),***

(ii) ***while M2 polarization usually involves stimulation with IL4 or IL13.***

Changes in gene expression, cell-surface markers and signaling pathways have subsequently been used to distinguish the various activation states, and the contribution of some of these factors in mediating CAM/AAM characteristics has been validated in genetically engineered mouse models.

However, given the heterogeneity of tissues, macrophage polarization should be regarded as a complex process that occurs over a continuum. The current classification of CAM or M1 macrophages is in part based on their response to stimulation with bacterial LPS, TNFα, and/or IFNγ. TNFα is produced by antigen presenting cells upon recognition of pathogenic signals, while IFNγ is produced by innate and adaptive immune cells such as natural killer (NK) and Th1 cells. Once activated, CAMs secrete pro-inflammatory cytokines (IL1, IL6, and TNFα) and effector molecules (including reactive nitrogen intermediates) and express chemokines such as CXCL9 and CXCL0.

These molecules exert and amplify antimicrobial and tumoricidal activities alongside increased Th1 adaptive immune responses through enhanced antigen presentation. Because these cytokines play an important role in immune defense, their inappropriate release can result in chronic inflammation and extensive tissue damage.

***Alternatively activated M2 macrophages are broadly characterized by their anti-inflammatory and wound-healing endotype**. While these functional outputs are important for the maintenance of tissue homeostasis, aberrant AAM activation can trigger allergic reactions, promote tumor growth, and delay immune responses toward pathogens.*

Among the most important activators of AAMs are IL4, IL10, and IL13; however, several other stimuli and signaling pathways can also induce AAM polarization.

Thus, AAMs can be further divided into M2a, M2b, M2c, and M2d. The M2a subtype is stimulated in response to IL4, IL13, as well as fungal and helminth infections.

M2a macrophages express high levels of mannose receptor (CD206) and secrete large amounts of pro-fibrotic factors including fibronectin, insulin-like growth factor and TGFβ, which are all involved in wound healing and tissue repair.

***M2b macrophages are stimulated by immune complexes and bacterial LPS and exhibit upregulated expression of CD206 and the MER receptor tyrosine kinase.** They primarily produce IL10, IL1β, IL6, and TNFα, which exert anti-inflammatory effects.*

***M2c macrophages** are activated by IL10, TGFβ, and glucocorticoids and are also generally thought to be anti-inflammatory in nature. Finally, differentiation of*

***M2d macrophages** occurs in response to co-stimulation with **TLR ligands and adenosine.** M2d macrophages express low levels of CD206 but are high producers of **IL10 and VEGF**. In light of these findings, it is now appreciated that the "AAM" terminology encompasses a functionally diverse group of macrophages that share the functional outputs of tumor progression by stimulating immunosuppression and angiogenesis.*

We summarize the above in the following table.

Type	*Activated by*	*Produce*

M1	*stimulation with IFNγ or lipopolysaccharide (LPS)*	
M2a	*stimulation with IL4 or IL13*	*mannose receptor (CD206) and secrete large amounts of pro-fibrotic factors including fibronectin, insulin-like growth factor and TGFβ,*
M2b	*by immune complexes and bacterial LPS*	*upregulated expression of CD206 and the MER receptor tyrosine kinase.*
M2c	*activated by IL10, TGFβ, and glucocorticoids*	
M2d	*co-stimulation with TLR ligands and adenosine*	*CD206 but are high producers of IL10 and VEGF.*

From Laviron and Boissonnas we have an interesting reconfiguration of this M1 and M2 fabric. They authors present a somewhat alternative view as follows:

Tumor-associated macrophages (TAM) represent a major component of the tumor microenvironment (TME) that has been extensively studied in the past decades. They play a major role in tumor growth, metastatic dissemination, and therapy failure. Countless reports have described that TAMs can promote angiogenesis, inhibit the anti-tumor immune response, in particular T-cell-mediated cytotoxicity, support tumor growth, and secrete different factors involved in extracellular matrix (ECM) remodeling thus facilitating tumor cell motility and intravasation. High TAM infiltration is generally correlated with poor outcomes in several types of cancer, such as breast, ovarian, and lung cancer.

However, in some indications TAM can be associated with enhanced anti-tumor immunity. Although macrophages were originally described as arising exclusively from circulating monocyte precursors, it was shown in the recent years that several organs harbor embryonic-derived populations of ***resident macrophages (ResMac)*** *that maintain and self-renew throughout adulthood.*

This new concept ***challenges the dogma of TAM origin and questions their relative function. TAM subsets were originally classified as tumoricidal vs. tumor-promoting, often referred as M1/M2 macrophages,*** *based on the expression of specific markers. However, the wide diversity of TAM cannot be covered by this nomenclature and many subsets express overlapping markers of the M1/M2 polarization.*

Whether TAM heterogeneity originates from their high plasticity or rather from independent specific lineages giving rise to multiple populations is still unclear. Although cellular ontogeny can recapitulate parts of the heterogeneity, it appears that environmental cues are also major determinants in cell education. Macrophage diversity would then be the result not only of ontogeny but also of niche- specific signaling events of tumor immunity.

One can thus wonder whether the origin of TAM dictates their role in tumor development and is associated with various functions. This represent a key issue for anti-cancer therapies as these subsets might be differentially targeted regarding their role in tumor development. …

Although the precise origin of ResMac is still under debate, fate-mapping models highlighted a differential origin of tissue macrophages deriving either from an embryonic precursor (yolk sac, fetal liver) or a monocyte precursor from adult hematopoiesis origin.

***These precursors seed the tissues in different waves during development and adulthood giving rise to different ResMac**. The dynamics of these waves vary between organs, age, and macrophage subsets.*

In some organs, such as the brain, the lung and the liver,

*(i) some **embryonic-derived ResMac (named here EmD-ResMac)** maintain by self-renewal in adults whereas in the gut, the skin, the heart, and the pancreas*

*(ii) most subsets are progressively replaced through the dierentiation of monocyte precursors from adult hematopoiesis into **monocyte-derived ResMac (named here MoD-ResMac**) with different turnover rates.*

The ability of newly recruited macrophages to self-maintain in the tissue and become a ResMac per se is proposed to be tightly regulated by space availability and competition for growth factors in the niche. This turnover appears to be variable among subsets in a given organ and could be induced by exposure to homeostatic environmental cues (e.g., mechanical, metabolic) specific of distinct sub-tissular regions.

In the gut, long-lived macrophages with precise sub-tissular localization are key regulators of physiological functions. In the lungs, alveolar macrophages (AM) originate almost exclusively from yolk-sac derived macrophages and self-maintain throughout adulthood, whereas lung interstitial macrophages follow a more complex regulation, unveiling further heterogeneity in this subset. While some of these interstitial macrophages have an embryonic origin , others differentiate from distinct monocyte precursors according to the sub-tissular niche they colonize, thus becoming the dominant population during adulthood. …

The common characterization of TAM subsets relies on the M1/M2 polarization model induced by different in vitro stimuli. This model rapidly finds limitation in complex environments (in vivo) in which M1 and M2 stimuli can be present and generate very dynamic microanatomical niches.

Tumors should be considered as an evolving tissue in which space availability and growth factors expression are changing over time and where inflammatory signals are generated by the loss of tissue integrity and immune cell infiltration.

It is thus not surprising to find a wide range of activation profiles in the TME. No typical M1/M2-associated marker defined one or the other TAM subset in lung unveiling heterogeneity among each subset.

No direct link between TAM origin and the commonly described pro- or anti-tumor profile could be achieved in this study. One could expect that macrophage ontogeny and their anatomic localization define specific niches dictating their polarization toward a specific phenotype and function.

Thus one may conclude that the TAMs are of varying types activating and being activated in a multiplicity of ways.

4.2 TUMOR ASSOCIATED NEUTROPHILS

Wu et al note:

The mobilization of neutrophils from bone marrow to tumor sites occurs in three phases including expansion and maturation of pre-mature neutrophils in the bone marrow, intravasation to circulation through attachment to endothelial cells, and the chemotactic movement of neutrophils to tumor sites . The pre-mature neutrophils are derived from hematopoietic stem cells.

The proliferation and maturation of neutrophils require the regulation of granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF). The neutrophil maturation also includes the nuclear morphology change—the original round-shape nucleus finalizes to a segmented shaped nucleus and surface antigen expression changes including CD 65 and CD16 .

The release of neutrophils in bone marrow mainly depends on the interplay between CXCR4 and CXCR2 and its ligands. These two receptors belong to the CXC chemokine receptor family as G-protein coupled receptors. CXCR4 and CXCR2 are expressed on the surface of the neutrophil and span seven times the neutrophil membrane . The role of CXCR4 is for neutrophil homing in the bone marrow. Higher levels of CXCR4 and its ligands (for instance, CXCL12) will restrain the neutrophils mobility. An initial step for neutrophil movement is the disruption of CXCR4 and its ligand expression by factors including G-CSF. ...

Tumor-associated neutrophils are generally considered a pro-tumor factor in multiple tumor types, including breast cancer.

Using over 5000 cases of 25 different cancer types, Gentles et al. indicated that higher polymorpho-nuclear cell (PMN, including neutrophils) infiltration would lead to the lowest overall survival for those cancer patients compared to other leukocytes .

Additionally, the higher neutrophil to lymphocyte ratio (NLR) indicates a worse prognosis for those patients. There are also studies regarding neutrophils establishing a pre-metastatic niche

for the malignant tumor cells. These studies indicate the overall pro-tumor functions of neutrophils in multiple cancer types.

4.3 TUMOR ASSOCIATED MAST CELLS

From Visciano et al:

Mast cells (MCs) originate in the bone marrow, enter the circulation as immature precursors, and reside in virtually all vascularized tissues. Once settled into a tissue, they undergo maturation, taking on characteristics specific for that tissue. The c-kit receptor ligand, Stem Cell Factor (SCF), is the most relevant factor for human MC maturation and differentiation.

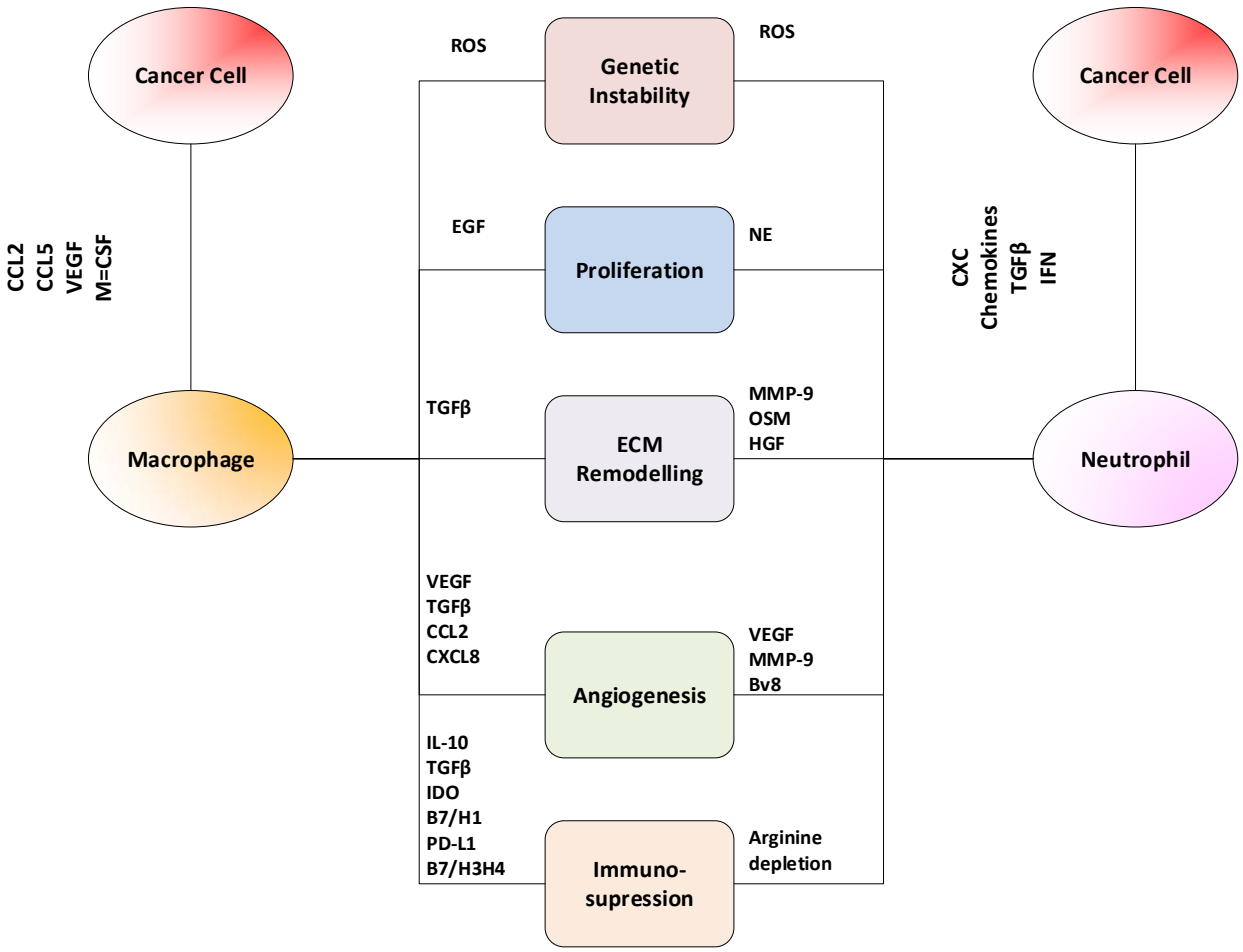
MCs are involved in both the innate and the adaptive arms of immunity and represent versatile cells that can have effector or immunomodulatory functions. MCs are important effector cells in antigen-induced anaphylaxis and other acute IgE-dependent allergic reactions. MCs can be activated by immunologic or nonimmunologic stimuli and, depending on the type of activation, release a specific profile of mediators.

Cross-linking of IgE bound to FcεRI expressed on the plasma membrane of MCs induces the activation of downstream events leading to the secretion of biologically active molecules implicated in allergic reactions. Non-IgE-mediated stimuli can also activate MCs (e.g., cytokines, chemokines, and endogenous danger signals). MC activation results in the release of several proinflammatory factors including preformed mediators (histamine, tryptase, chymase, carboxypeptidase A, and proteoglycans) stored in secretory granules and de novo synthesized lipid mediators …

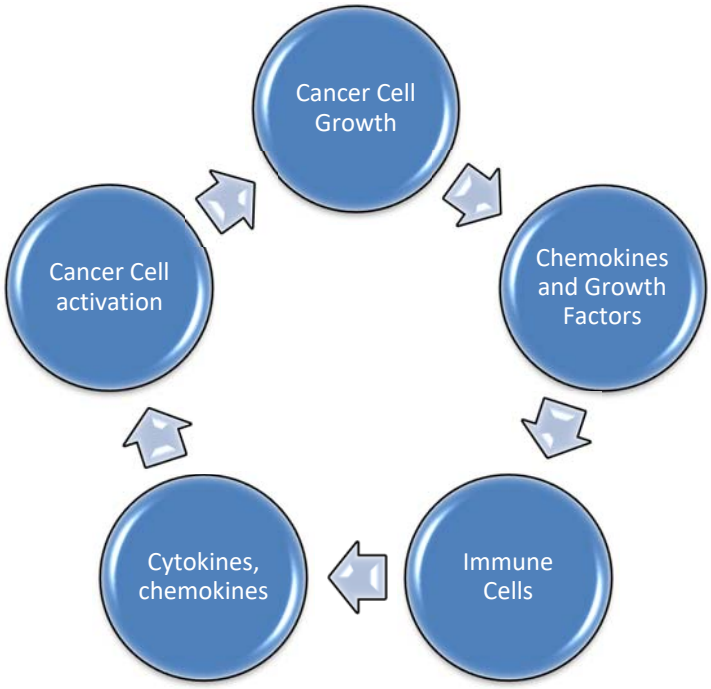
4.4 SUMMARY

We present below a diagram reflecting the interactions between the cancer cells and macrophages and neutrophils and the effectors related to each[10].

[10] See Leonard and Schreiber, p 420, by Galderio et al.



The five actions involved are impacted by the macrophages and neutrophils which in turn impact the cancer cell progression. The figure below depicts this positive feedback set of effects as demonstrated above.



Thus any attempt at a therapeutic must address all of these steps in the process. Namely we have a combined effort of cytokines, chemokines and growth factors[11].

[11] See Appendix for a summary of the growth factors as well as https://www.researchgate.net/publication/329702571_Growth_Factors_Pathways_and_Cancers

5 APPLICATIONS

We now examine immune cells impacts on a multiplicity of solid tumors. As is all too often the case these immune cells are less than protective against the invades and they actually assist in the invasion.

5.1 PROSTATE CANCER

Prostate cancer is a complex cancer wherein most forms are relatively indolent while a few others are markedly aggressive[12]. Assessing which is which has been a challenge. Perhaps via TAMs and the like one may find additional markers to assess virulence.

Taichman et al note:

Selected therapies in development for prostate cancer directed against prostate cancer and host cell interaction in the bone microenvironment. Tumor cells alter the bone microenvironment by stimulating osteoclasts parathyroid hormone–related protein (PTHRP), IL-6, IL-1, and TNF-a and by stimulating osteoblasts endothelin-1 (ET-1), FGF, PDGF, IGFs, TGF-β, and bone morphogenic proteins (BMPs). Osteolysis, in turn, releases TGF-β, TNF-α, and EGF, stimulating cancer cell proliferation. Receptor activator of NF-κB ligand (RANKL) expression by osteoblasts binds to the RANK receptor, promoting osteoclast formation and function. …

Immunotherapy approaches include inhibiting the infiltration of tumor-associated macrophages by inhibiting CCL2, prolonging T cell response by inhibiting the inhibitory receptor CTL-associated antigen–4 (CTLA-4) using the antibody ipilimumab (MDX-010), and stimulating antigen-presenting cells through vaccines such as GVAX and Sipuleucel-T

Prostate cancer mimicry of HSC/progenitor cell homing mechanisms. The metastatic process of prostate cancer cells (PCa cells) is functionally similar to the migrational, or homing, behavior of HSCs to the bone marrow. Numerous molecules have been implicated in regulating HSC homing, participating as both chemo-attractants and regulators of cell growth. Endothelial cell–derived factors such as CCL2 act as chemo-attractants and growth factors for HSCs, tumor-associated macrophages, and prostate cancer cells.

What is interesting is that Sipuleucel is a dendrite activated therapeutic with some reasonable span of application. Now histologically one can examine the sections removed in a prostate biopsy and the process is generally focused upon the changed in the prostate cells. There generally is limited amounts of tissues available to determine the pathological structure of the immune cells in the ECM. Now Silvestri et al note:

[12] https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach

Generally, a dense infiltration of lymphocytes has been correlated with longer patient survival, whereas a significant reduction of T cells is detected in high grade prostatic adenocarcinomas compared to benign nodular prostatic hyperplasia, suggesting that tumor progression may be associated with alterations in cell-mediated immune responses.

Furthermore, ***high density of M2-polarized tumor-associated macrophages (TAM) is observed in both epithelial and stromal compartments and is statistically associated to poorer prognosis.*** *TAMs are a* ***significant component of the inflammatory infiltrates in PCa.*** *Moreover, increased TAMs levels in biopsy are predictive of worse recurrence free survival in men treated with primary ADT. An inverse correlation between total macrophage density and time to recurrence has also been reported from different analysis.*

Inflammation has always been a major concern in PCa[13]. We have examined this before in cases of HGPIN. However normal path results recording HGPIN often fail to provide the details of any inflammatory process. The author continues:

Similar results were observed for Tregs. Another negative prognostic factor is represented by increasing myeloid-derived suppressor cells (MDSC) detected both at tumor site as well in the peripheral blood of patients. The increase of these cells correlated with other negative prognostic factors, such as lactate dehydrogenase, alkaline phosphatase, PSA, and anemia in PCa. Finally, a strong correlation between the DCs and PCa characteristics has also been observed.

Patients with metastatic disease showed fewer circulating myeloid DCs than their age-matched controls and a lower number of DCs was parallel with a higher Gleason score while DCs are elevated in low risk cancer. These results indicate that, in PCa patients, monocytes do not develop into myeloid DCs as efficiently as they do in healthy individuals. This idea is also supported by observations that serum of PCa patients inhibited monocytes differentiation into DCs and that the degree of inhibition correlated with higher PSA levels

Thus, assessing prognostic markers should include measure of various immune cells.

5.2 THYROID

Thyroid cancers present an interesting counter example. Unlike the prostate, bladder, colon, or skin, for the most part the thyroid is free from any form of inflammation except for auto-immune disease or excess radiation exposure[14]. On the one hand, thyroid cancers are generally indolent and slow growing, as contrast to say the pancreas, yet there is a small percentage of the

[13] https://www.researchgate.net/publication/325047485_PROSTATIC_INTRAEPITHELIAL_NEOPLASIA_PROGRESSION_REGRESSION_A_MODEL_FOR_PROSTATE_CANCER

[14] https://www.researchgate.net/publication/331935614_Thyroid_Cancer_Seek_and_Ye_Shall_Find

anaplastic type which is more aggressive than any of the varieties we discuss[15]. From Visciano et al we have a discussion regarding mast cells (MC) and thyroid cancer (TC):

There is compelling evidence that the tumor microenvironment plays a major role in mediating aggressive features of cancer cells, including invasive capacity and resistance to conventional and novel therapies. Among the different cell populations that infiltrate cancer stroma, mast cells (MCs) can influence several aspects of tumor biology, including tumor development and progression, angiogenesis, lymphangiogenesis, and tissue remodelling.

Thyroid cancer (TC), the most frequent neoplasia of the endocrine system, is characterized by a MC infiltrate, whose density correlates with extrathyroidal extension and invasiveness. Recent evidence suggests the occurrence of epithelial-to-mesenchymal transition (EMT) and stemness in human TC. The precise role of immune cells and their mediators responsible for these features in TC remains unknown. ...

The relationship between MCs (mast cells) and thyroid cancer (TC) was investigated for the first time by our group. Normal thyroid tissues stained essentially negative for tryptase, a specific MC marker, whereas in 95% of PTC samples we found a MC infiltration whose extent correlated with extrathyroidal extension of tumors. We also interrogated, by IHC, a limited number of PDTCs and ATCs for MC presence. We found that MCs are present in PDTC and ATC, and their density correlates with tumor invasiveness.

Taken together these preliminary ***data indicate that MCs play a role in aggressive TC****. Proietti et al. evaluated the presence and distribution of MCs in follicular variant of PTC (FVPTC) and follicular adenoma. MCs were significantly more abundant in the intratumoral and peritumoral areas of FVPTC compared to adenoma. Thus, MC density could be used to distinguish between benign and malignant forms of follicular thyroid lesions.*

The above demonstrates the need for detailed studies of the presence of immune cells since they can often indicate a more aggressive form of malignancy. Here the authors examine mast cells, whereas macrophages are examined by Kim et al who remark as follows:

This study demonstrated the presence of TAM in a canopy structure over thyroid tumor cells in primary tumor tissues from 36 patients with PTC with LN metastasis. In addition, the primary tumors were significantly larger in the group with high TAM density. More LNs had metastases with a higher TAM density, although the difference was not statistically significant. Based on these findings, TAM density is considered to be related to PTC stage. ...

demonstrated a higher TAM density in poorly differentiated PTC and anaplastic thyroid cancer than in well-differentiated PTC, by using CD68 and CD163 immunohistochemistry. ...

reported a high TAM density in well-differentiated PTC, and the TAM density was significantly higher in tumors with TNM staging over stage III. The results from these studies demonstrated

[15] https://www.researchgate.net/publication/335404502_Thyroid_Cancer_and_Genetic_Differentiation

that a high TAM density is present in poorly differentiated PTC and is related to poor prognosis, which indicates TAMs may support PTC growth. However, the role of TAMs in thyroid cancer remains controversial. …

using CD68 immunohistochemistry, (we) found that TAM prevented metastasis in some patients. Of 121 patients with PTC, 15% had TAMs (CD68+) that appeared to have a phagocytic function on cancer cells. These patients had significantly less blood vessel invasion and remote metastasis and largely accompanying invasions of lymphocytes and dendritic cells, than patients without an CD68+ cells. These results suggest that these cells may activate the host immune response to cancer cells. However, in our study, 56% of patients had TAMs without evidence of phagocytosis, and their clinical characteristics were similar to those without TAMs.

The above notes that macrophages can do both; inhibit and excite a malignancy. Again, in pathology reports the focus is often on the nuclear structures to ascertain papillary thyroid structure and generally no discussion of the ECM or immune elements.

Now regarding TAMs, Ryder et al note that they play a significant role:

Thyroid cancers are infiltrated with tumor-associated macrophages (TAMs), yet their role in cancer progression is not known.

The objectives of this study were to characterize the density of TAMs in ***well-differentiated (WDTC)****,* ***poorly differentiated (PDTC)****, and* ***anaplastic thyroid cancers (ATC)*** *and to correlate TAM density with clinicopathologic parameters.*

Immunohistochemistry was performed on tissue microarray sections from WDTC, PDTC, and ATC using macrophage-specific markers. Electronic medical records were used to gather clinical and pathologic data. Follow-up information of PDTC patients was available for 0–12 years. In total, 9 out of 33 WDTC (27%), 20 out of 37 PDTC (54%), and 19 out of 20 ATC (95%) had an increased density of CD68C TAMs (R10 per 0.28 mm2; WDTC versus PDTC, PZ0.03; WDTC versus ATC, P!0.0001; PDTC versus ATC, P!0.002).

Increased TAMs in PDTC was associated with capsular invasion*, extrathyroidal extension, and decreased cancer related survival compared with PDTC with a low density of TAMs. In conclusion, the density of TAMs is increased in advanced thyroid cancers. The presence of a high density of TAMs in PDTC correlates with invasion and decreased cancer-related survival. These results suggest that TAMs may facilitate tumor progression. As novel therapies directed against thyroid tumor cell-specific targets are being tested, the potential role of TAMs as potential modulators of the thyroid cancer behavior will need to be considered*

In the above we have what appears to be a clear set of evidence that TAMs have strong prognostic value. As such they should be considered in any and all pathology results. Others have noted significant TAMs in anaplastic TC. However, ATC is one of the most aggressive and deadly forms of cancer and often result from existing inflammatory or immune system responses in the thyroid. The question may be; are the TAMs the result of the inflammation, pre-existing, a result of the malignant transformation, or a process leading to the malignant transformation?

As Lee et al have noted in a small-scale study in Korea:

Macrophages are one of the components of bone marrow cells *and play roles in innate immunity. Classically, they circulate in the blood and migrate into the inflammatory tissues such as infectious diseases in response to specific, endogenous immune signal. Recently their alternative* ***roles in anti-inflammation, cell clearance and tissue regenerations have also been established especially in inflammatory metabolic disorders including obesity and diabetes.*** *Therefore, macrophages have* ***been categorized into two subtypes—M1 and M2—depending on their distinctive roles.***

(i) M1 macrophages play proinflammatory functions in response to TH1 cytokines and

(ii) M2 macrophages play anti-inflammatory and immunosuppressive activities in response to Th2 cytokines.

However, macrophages are not fixed to a single M1 or M2 subtype but suspected to rapidly differentiate and redifferentiate in various microenvironments. *Tumor-associated macrophages (TAMs), macrophages existing tumor microenvironment, were first described in the early 1980s. Till now, TAMs have been found to play dual functions, both positively or negatively affect tumor growth through interactions with the microenvironment, and these actions are tissue specific.*

Several clinical studies showed that high density TAMs were present in more advanced stages of cancer with a worse prognosis in breast, lung, and bladder cancer. Although a high TAM density has been reported in poorly differentiated papillary thyroid carcinoma (PTC) or anaplastic thyroid cancer, the distribution and function of TAMs in the most common PTC have not been studied yet. In this study, we have demonstrated the existence of TAMs in PTC tissue with lymph node (LN) metastasis and also used expression distribution and morphological properties to investigate the relationship between TAMs and the frequency of extrathyroidal invasion in PTC …

Studies on TAM in thyroid cancer are underway, but clinical data and studies on the mechanisms of TAM action are lacking. ***Thyroid cancer has a relatively favorable prognosis because its growth rate is slow.*** *Therefore, general cancer pathophysiology does not apply to the role of TAMs.*

Currently the role of TAMs is only known in relatively invasive breast, lung, and pancreatic cancers, but the results of this study indicate that ***TAMs also exist in PTC, which is generally well differentiated with a favorable prognosis.*** *Their role in cancer development is exciting; especially, the in light of the animal study targeting TAM that found possible inhibition of PTC. Expectations are high for treating patients with thyroid cancer since the BRAF mutation rate is higher among the Korean population than other ethnic groups.*

The above discussion is compelling since the Korean population has such a high incidence of TC as well as BRAF mutations. The results from this small Korean study go assert the complex role of MC in TAMs.

5.3 MELANOMA

Melanoma is the aggressive form of skin cancer[16]. It can start with a simple melanoma in situ change where the melanocytes loose basal layer stability and start migrating upward through the basal layer. This is a pre-malignant state and progression may follow if the melanocytes start proliferating and move below the basal layer. Also one can see many immune cells in superficial spreading melanoma. Now Salmi et al have noted:

The role of tumor-associated macrophages (TAMs) in cutaneous melanoma is controversial. TAMs include immunogenic and immunosuppressive subtypes, and have distinct functions according to their microanatomical localization. Our aim was to investigate TAMs in benign, premalignant, and malignant melanocytic lesions to determine possible associations with tumor progression and clinicopathological characteristics.

In total, 184 tissue samples, including benign and dysplastic nevi, in-situ melanomas, superficial (Breslow's depth <1mm), and deep (Breslow's depth >4mm) invasive melanomas and lymph node metastases, were analyzed for macrophage content. Samples were stained immunohistochemically for CD68 and CD163, representing all TAMs and M2-macrophages, respectively.

Macrophages were counted by hotspot analysis, and assessed semi-quantitatively from the tumor cell nests and stromal component of malignant cases. CD68+ and CD163+ TAMs were more abundant in invasive melanomas compared with benign nevi. The proportion of TAMs in the tumor nests was higher in deep melanomas and lymph node metastases compared with superficially invasive melanomas.

High amounts of CD68+ macrophages in tumor cell nests were associated with recurrence, whereas low CD163+ macrophage proportion in tumor stroma was associated with recurrence and in primary melanomas also with poor overall survival. TAMs seem to promote tumor progression in cutaneous melanoma. In particular, CD68+ TAMs and their abundance in tumor nests were associated with poor prognostic factors. However, the correlation of low stromal CD163+ TAM proportion with a poor prognosis indicates that the role of TAMs depends on their subtype and microanatomical localization.

Thus, there appears to be sets of CD markers which may be prognostic. Such surface protein markers are generally not assessed in standard pathology reports.

Fujimura et al have noted:

Tumor-associated macrophages (TAMs) and regulatory T cells (Tregs) are significant components of the microenvironment of solid tumors in the majority of cancers. TAMs sequentially develop from monocytes into functional macrophages.

[16] https://www.researchgate.net/publication/264960157_Melanoma_Genomics

In each differentiation stage, TAMs obtain various immunosuppressive functions to maintain the tumor microenvironment (e.g., expression of immune checkpoint molecules, production of Treg-related chemokines and cytokines, production of arginase I).

Although the main population of TAMs is immunosuppressive M2 macrophages, TAMs can be modulated into M1-type macrophages in each differential stage, leading to the suppression of tumor growth. Because the administration of certain drugs or stromal factors can stimulate TAMs to produce specific chemokines, leading to the recruitment of various tumor- infiltrating lymphocytes, TAMs can serve as targets for cancer immunotherapy….

It is interesting to see that the dominant TAMs are M2, namely from the marrow rather than resident. Thus it may be postulated that the tumor establishes some attractant to collect them. As such, perhaps this may be blocked via a MAb or the like. They continue:

Tumor-associated macrophages are characterized by their heterogeneity and plasticity, as they can be functionally reprogrammed to polarized phenotypes by exposure to cancer-related factors, stromal factors, infections, or even drug interventions.

Because TAMs sequentially differentiate from monocytes into functional macrophages through multiple steps, they have heterogeneity and plasticity in cancer. Monocytes recruited from the circulation differentiate into tissue macrophages by macrophage colony-stimulating factor (M-CSF), and are primed with several cytokines such as interferon gamma (IFN-γ), interleukin 4 (IL-4), and IL-13. Thereafter, macrophages change their functional phenotype in response to environmental factors or even tumor-derived protein stimulation.

The priming by IL-4 and IL-13 as well as the IFN may be suppressed.

In skin cancer, for example, targeting the M-CSF receptor with anti-CSF short interfering RNA (siCD115) in TAMs led to modulation of the TIL profile, resulting in growth suppression of B16 melanoma in vivo. In the second phase of priming, type I IFN (IFN-α, IFN-β) and type II IFN (IFN-γ) modulate the production of chemokines from TAMs, suggesting that these cytokines repolarize TAMs in several skin cancers. Cancer stromal factors such as soluble receptor activator of nuclear factor kappa-B ligand (RANKL) derived from cancer cells could be a third mode of stimulation that activates mature M2 macrophages to produce a series of chemokines that recruit immunosuppressive cells such as Tregs and Th2, leading to maintenance of the tumor microenvironment.

These reports suggest that each of these three differentiation steps could serve as a target for immunotherapies. Chemokines from TAMs determine the immunological microenvironment in Tumors Chemokines play crucial roles in determining the profiles of TILs in the tumor microenvironment, and the profiles of chemokines from TAMs are determined by stromal factors of each skin cancer. For example, immune cells in the tumor microenvironment determine the aggressiveness of melanoma.

In metastatic melanoma, periostin (POSTN) is expressed in the region surrounding melanoma cell nests in metastatic melanoma lesions that develop at the wound site. In addition, TAMs are prominent in the tumor stroma in melanoma, and POSTN stimulates CD163+ macrophages to produce several specific cytokines including Treg-related chemokines [chemokine ligand 17 (CCL17), CCL22][17].

Because CCL17 and CCL22 from TAMs attracts Tregs to the tumor site in melanoma, repolarization of TAMs by immunomodulatory reagents such as IFN-β and imiquimod are useful for suppressing tumor growth in melanoma.

The downregulation of CCL22 production was also observed in B16F10 melanoma mouse treated with classical cytotoxic anti-melanoma drugs such as dacarbazine, nimustine hydrochloride, and vincristine, all of which have been used in the adjuvant setting for advanced melanoma for the last 30 years. Other reports have suggested that a series of chemokines (CCL17, CXCL10[18], CCL4[19], and IL-8) in cerebrospinal fluid may be useful for predicting brain metastasis in melanoma patients.

5.4 BLADDER

Bladder cancer is a common cancer that is often seen in smokers and heavy drinkers. The bladder can be under assault by a large number of proto-carcinogens and as such it has the tendency to have carcinogenic transformations. As Kang et al have noted:

Tumor-associated macrophages (TAMs) are the major inflammatory component of the stroma of many tumors and affect different aspects of neoplastic tissue. Macrophages are phagocytic cells that play pivotal roles in inflammation, wound healing, and tissue repair. Exposure to different molecular signals can induce two types of phenotypic differentiation: M1 ("classically activated") and M2 ("alternatively activated"). M1 macrophages respond to cytokines such as interferon-γ, and inhibit tumor progression by expressing proinflammatory and immunostimulatory cytokines.

***However, during tumor progression in the presence of IL-4, IL-10, and IL-13[20], a phenotypic switch occurs and macrophages differentiate into the M2 phenotype, which secretes IL-4, IL-5, and IL-6[21] and increases angiogenesis, matrix remodeling, and immune suppression**. Large*

[17] CCL17 is a chemokine that recruits T cells to the location. CCL22 is a chemokine that recruits both T cells and NK cells.

[18] CXCL10 is a chemokine which is an effector for T cell recruitment.

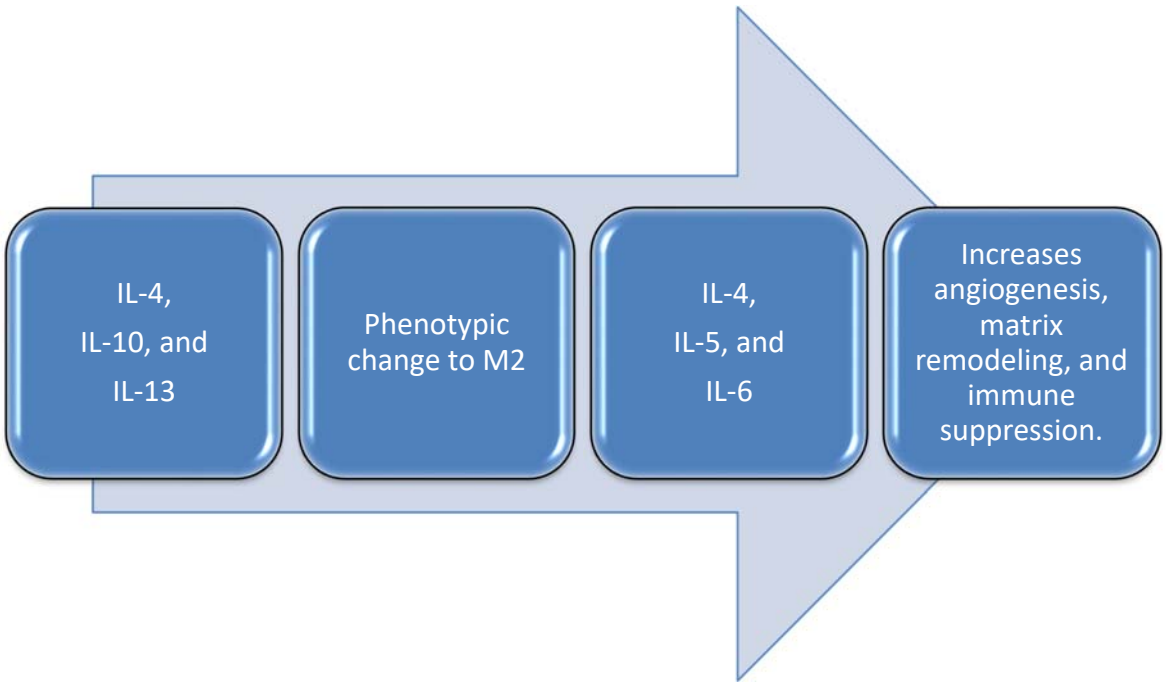
[19] CCL4 is a chemokine which does T cell, dendritic cell, monocyte, and NK recruitment. It is also an HIV coreceptor

[20] IL-4 does B cells: isotype switching to IgE T cells: Th2 differentiation, proliferation Macrophages: alternative activation and inhibition of IFN-γ–mediated classical activation, IL-10 does Macrophages, dendritic cells: inhibition of expression of IL-12, costimulators, and class II MHC, IL-13 does B cells: isotype switching to IgE Epithelial cells: increased mucus production Macrophages: alternative activation.

[21] IL-5 does Eosinophils: activation, increased generation and IL-6 does Liver: synthesis of acute-phase protein

numbers of studies have focused on identifying the prognostic value of TAMs in solid tumors, and most suggest that TAMs are beneficial for tumor growth and are therefore associated with a poor prognosis. The presence of TAMs correlates positively with increased vascularity and metastasis, and with decreased relapse-free and overall survival rates in breast cancer and non-small cell lung cancer …

The above is a clarifying description of the changes resulting in the development of TAMs. We demonstrate the putative relationship below.



The authors revealed that increased infiltration of TAMs is associated with poor prognosis of bladder carcinoma in situ after intravesical BCG instillation.

Similar to myeloid macrophages, neutrophils contain a subpopulation of neutrophils named tumor-associated neutrophils (TANs). The literature reveals a dual role for neutrophils in tumor biology. Activated leukocytes kill tumor cells, thereby playing a beneficial, protective role in the host. By contrast, TANs promote malignancy in certain situations, e.g., by releasing growth-stimulating signals, matrix-degrading proteases, and mediators of angiogenesis. Recently, … provided evidence for the existence of N1 (antitumoral) and N2 (protumoral) TANs, which are analogous to M1 and M2 macrophages, respectively. This neutrophil plasticity is regulated by molecules within the TME.

The immunosuppressive cytokine TGF-β induces neutrophils to acquire an N2 protumoral phenotype, and the presence of TGF-β prevents generation of antitumorigenic N1 neutrophils. The antitumoral activity of N1 neutrophils includes increased expression of immuno-activating

B cells: proliferation of antibody-producing cells T cells: Th17 differentiation

cytokines and chemokines, lower levels of arginase, a greater capacity to kill tumor cells, and activation of cytotoxic T lymphocytes (CTLs).

Therefore, augmentation of N1 neutrophil numbers and activity might be therapeutically beneficial. For instance, switching off angiogenesis while increasing the local production of TGF-β may skew N2 cells towards an N1 phenotype (97). One of the major mechanisms by which cancer cells block antitumor immune responses involves a specific class of T cells called tumor-infiltrating Tregs. In the vast majority of cases, these cells express the Forkhead box P3 (FOXP3) transcription factor.

Such FOXP3+ Tregs accumulate within neoplastic lesions via several distinct mechanisms, including increased infiltration, local expansion, survival advantage, and de novo development from conventional CD4+ cells (98). Whereas Tregs in healthy peripheral organs constitute approximately 10% of total CD4+ T cells, this proportion is consistently increased in the TME, in which Tregs can account for 30–50% of CD4+ T cells. The phenotype of intratumoral Tregs appears to differ from that of circulating Tregs, and the former are also thought to promote tumor angiogenesis, thereby favoring tumor growth via immuneindependent mechanisms.

Various studies report that tumor infiltration by Tregs has a negative prognostic value, although this seems to be strongly influenced by other clinical and biological parameters such as tumor type, location, and stage, and presence of other immune effector cells, notably CD8+ CTLs.

Tumor-infiltrating lymphocytes (TILs) are host lymphocytes that appear at tumor sites; presumably, they migrate to the tumor to combat the growing malignant cells. They comprise activated T cells, natural killer cells, and non-T or non-B lymphocytes. Evidence suggests that multiple variables contribute to immune escape, including regulatory cells; inhibitory ligands on tumor cells, such as PD-L1

As Zhao et al noted they have performed more extensive studies reflects as follows:

In the present study, we aimed to investigate the influence of lactate shuttling on the functional polarization and spatial distribution of ***transitional cell carcinoma of the bladder (TCCB)*** *cells and macrophages. … We confirmed that* ***TCCB cells reprogrammed macrophages into an M2 phenotype****. Moreover, lactate inhibited M1 polarization and induced M2 polarization of macrophages, but blockade of cancer cell-macrophage lactate flux significantly inhibited the re-education of macrophages by TCCB cells.*

In addition, lactate diffused faster and deeper than large signaling proteins in the microfluidic tumor microenvironment. Furthermore, lactate alone induced the migration of macrophages, and M1, but not M2, macrophages reduced the motility of TCCB cells.

Conclusions: TCCB cells reprogrammed macrophages into an M2 phenotype in a manner that depended on cancer cell-TAM lactate flux. Furthermore, the lactate shuttle may be a determinant of the density of TAMs in tumor tissue*. …*

This discussion again reflects and supports the ability of the malignant cells to transform the macrophage and make it a participant in its attempts to metastasize. They continue:

In the present study, we used a microfluidic coculture chip to study the cancer cell-macrophage interactions in the bladder cancer microenvironment. We confirmed that TCCB cells reprogrammed macrophages into an M2 phenotype in a manner that depended on cancer cell-TAM lactate flux. Therefore, the lactate shuttle is one of the major causes of immunosuppression within the TCCB microenvironment, and MCTs may be a new treatment target for TCCB. Furthermore, the lactate shuttle may be a determinant of the density of TAMs in tumor tissue, although further research is necessary to elucidate the mechanism underlying the chemotactic effect of lactate shuttling.

The above results are significant. The represent a system with multiple control and effector points which in themselves can be targeted by therapeutics. Along those lines we have what Varricchi et al have noted:

Several therapeutic strategies have been envisioned to limit tumor growth by targeting mast cells and their mediators. Mast cells play a pro-tumorigenic role in human bladder cancer through stimulating estrogen receptor β (ERβ). In a murine model of bladder cancer, these authors showed that a selective ERβ antagonist inhibited mast cell-promoted tumor growth. It has been found that mast cells can promote the proliferation of colon cancer in vivo Injection of Fcε-PE40 chimeric toxin, which induced mast cell apoptosis, inhibited colon tumor development in vivo.

Now from a prognostic point of view we have from Hanada et al the following contribution:

We determined the tumor-associated macrophage (TAM) count to investigate its importance in predicting clinical outcome or prognosis in patients with bladder cancer. The TAM count and microvessel count (MVC) were determined immunohistochemically in 63 patients with bladder cancer, including 40 superficial bladder cancers and 23 invasive bladder cancers. To examine the relationship between TAM count and clinical outcome or prognosis in bladder cancer, cystectomy rates, distant metastasis rates, vascular invasion rates and 5 year survival rates were compared between patients with low (< 67) and high (> or = 67) TAM counts. Our results suggest that determination of TAM count in bladder cancer tissues is of value to predict the clinical outcome or prognosis and to select appropriate treatment strategies in patients with bladder cancer.
As Cheah et al have noted:

Tumor-associated macrophages (TAMs) consistently display an alternatively activated phenotype (M2) commonly found in sites of wound healing. These macrophages promote tumor growth while suppressing the host immune response locally.

Polarization and subversion of tumor-infiltrating macrophages is accomplished via immune mediators in the tumor microenvironment. Adding to the complexity of solid tumors is the heterogeneity of the cancer cells. Tumor cells of varying differentiation states and different characteristics coexist within a tumor. However, the different roles of each tumor cell subset during cancer progression remain undefined.

Bladder cancer (BC) represents a growing number of solid tumors characterized by the infiltration of a significant number of myeloid cells in the neoplastic lesion. We have previously determined that keratin 14 (KRT14) expression marks the most primitive differentiation state in BC cells. KRT14 expression is significantly associated with poor overall patient survival. However, the mechanisms used by KRT14-expressing cells to promote tumor growth remain unclear. In the current study, we found that KRT14+ basal BC cells also express higher levels of CD14.

Here, we investigate the strategies used by KRT14+ CD14-high BC cells to promote tumor growth. Results KRT14+ Basal BC Cells Express Higher Levels of CD14. We have previously identified KRT14 expression as a marker specific for the primitive/basal differentiation state in BC. We further determined that CD90+ cells, which express higher levels of KRT14, represent the tumorigenic subpopulation in primary patient basal BC. Interestingly, macrophage-associated markers including CD14 were also enriched in KRT14+ BC. Flow cytometry and histology of patient BC samples indicate that BC cells expressing the epithelial lineage marker EpCAM coexpress CD14.

Quantitative real-time PCR also revealed that CD90+ basal BC cells express higher levels of CD14. ...

Tumor-associated macrophages are a major component of TPI (tumor-promoting inflammation). TAMs promote tumor growth by producing soluble factors that activate neo-angiogeneis and stimulate tumor proliferation. In our study, we observed that CD14-high tumor factor–polarized monocytes and macrophages are more immune-suppressive and are impaired in their ability to stimulate T-cell proliferation.

Inflammation factors secreted by CD14-high BC cells are consistently more efficient at downregulating MHC II on monocytes and polarizing macrophages toward an M2-like phenotype.

Thus we are of the opinion that TAMs and their control mechanisms may be a viable target for a variety of therapeutics.

5.5 KIDNEY

Kidney cancer is another malignancy wherein such proto-inflammatory conditions as Type 2 diabetes and obesity play a role. As Chevrier et al note:

Tumor-associated macrophages (TAMs) are another key immune population in the TME that can either block or facilitate tumor growth. Distinct TAM subsets can induce or repress anti-tumor immunity, angiogenesis, and cell migration. TAM phenotypes are highly plastic, and recent reports show that the model distinguishing between classically polarized anti-tumor M1 and alternatively polarized pro-tumor M2 subtypes incompletely accounts for the phenotypic

diversity in vivo. T cell and TAM phenotypes are attractive biomarkers, and both T cells and TAMs show promise as therapeutic targets.

Treatment with anti-PD-1 and anti-CTLA-4 antibodies can overcome T cell exhaustion in different cancer types, and clinical trials are investigating the effect of depletion of TAMs and the repolarization of pro-tumor TAMs into anti-tumor TAMs.

Broader use of T cells and macrophages as biomarkers and drug targets has been hindered by the fact that human TAM and T cell phenotypes and relationships among them in the TME have not been comprehensively characterized.

Characterization of TAMs in the TME has focused on a handful of markers, and gene expression profiling has been done in bulk tissues or total macrophage populations We performed a large-scale mass cytometry analysis of 73 tumor samples from patients with all grades of ccRCC and five healthy matched kidney samples. We stained cells with two antibody panels created for this study.

Since TAM phenotypes are little characterized in human, the TAM panel originated from an antibody screen, whereas the T cell panel was designed to identify different populations of naive, memory, effector, regulatory, and exhausted T cells. Comparison of mass cytometry to flow cytometry showed an average correlation of 0.88, confirming the reliability of the mass cytometry data. Both panels included markers for the identification of B cells, natural killer cells, plasma cells, granulocytes, and myeloid cells. The samples were barcoded, and standards analyzed on each plate demonstrated that the data were highly consistent across plates …

To generate a comprehensive view of the immune ecosystem of each tumor, we generated two-dimensional maps of the data using the dimensionality reduction algorithm t-SNE. This analysis showed a strong overlap between tumors of all grades. …

T cells were the main immune cell population in the ccRCC TME, with a mean of 51% across samples. The mean frequencies of myeloid cells, natural killer cells, and B cells were 31%, 9%, and 4%, respectively. Granulocytes were present at very low levels in all but one sample. Plasma cells constituted a minor fraction in most samples. A double-positive CD8+/CD4+ population was observed in many samples, reaching up to 25% of the T cell compartment in some samples ….

TAM populations were characterized by more subtle differences in marker expression than T cell populations and were more challenging to categorize. To ensure a robust set of clusters This phenotype suggests that the M-15 cluster corresponds to circulating CD14+ monocytes from the tumor vasculature. Consistent with this hypothesis, this cluster was present in healthy samples and across all tumor grades.

This first branch expands along diffusion component one (DC1) to include clusters M-1 and M-14. Cells along this component were characterized by a progressive loss of CD36 and CD11b and increases in HLA-DR, CD4, CD68, and CD64, a trend culminating with cluster M-5, which showed one of the highest DC1 values. This marker regulation is consistent with changes

observed during the monocyte to macrophage transition that occurs upon migration into tissue. Our data also suggest that CD54 and CD81 are associated with this transition.

The second branch was almost exclusively formed by cells of the M-16 cluster. The M-16 phenotype is consistent with that of inflammatory CD16+ circulating monocytes. This cluster was present in healthy samples and across all tumor grades.

The third branch has a complex structure. One grouping involves clusters M-5, M-11, M-12, and M-13. These clusters displayed the highest levels of HLA-DR, CD68, and CD64 of all clusters and no expression of CD11b or CD36, suggesting that the cells were mature. Consistent with this, these clusters were only present in tumors of grade II and higher.

These clusters were characterized by a previously unrecognized diversity of combinations of pro- (CD163, CD204, and CD206) and anti-tumor (CD169) TAM markers. M-5 and M-13 clusters expressed only CD204, the M-12 cluster was positive for CD204 and CD206, and the M-11 cluster was positive for all four markers. The M-5 cells also expressed high levels of CD38, a marker exclusively found upon M1 polarization in murine macrophages.

Thus, macrophages in the TME can co-express anti-tumor and pro-tumor markers. Another group in the third branch involves M-0, M-10, and M-7 clusters. M-0 and M-10 clusters had high levels of CD206 expression. M-0 cells expressed low levels of CD163, CD169, and CD204, whereas M-10 cells were negative for these markers. M-0 and M-10 clusters were present in healthy tissues, suggesting that these clusters correspond to tissue-resident macrophages.

As Kovaleva et al have noted:

Tumor associated macrophages (TAMs) are an important element of tumor stroma. They originate from blood monocytes attracted by chemokines and cytokines produced by tumor cells and, being instructed by tumor microenvironment, develop into potent tumor-supporting cell population. TAMs were demonstrated to directly stimulate tumor cell proliferation and to promote angiogenesis.

Further TAMs provide for efficient immune escape by producing immunosuppressive cytokines and facilitate tumor dissemination by producing extracellular matrix remodeling enzymes. In renal cell carcinoma (RCC), numerous studies were performed for elucidation of the role of TAM in tumor progression. Using pan-macrophages marker CD68 and type 2 macrophage (M2) markers CD163 and CD206, it was demonstrated that increased density of TAMs is associated with poor survival of patients.

Although most of the studies are focused on M2 population in RCC, several markers rather typical for type 1 macrophages (M1) were also characterized. Macrophages isolated from RCC tumors were shown to produce proinflammatory cytokines TNFα, IL-1β, IL-6, and CCL2. It can be concluded that RCC is an excellent example of a tumor with hybrid phenotype of TAMs that share both M1 and M2 properties. Moreover, TAMs seem to be an attractive therapeutic target

as well. Further investigations are needed for identification of RCC-specific TAM markers with high predictive capacity and/or suitable for therapeutic targeting. ...

TAM presence and association of its amount and markers with ccRCC prognosis were demonstrated using histological techniques .... .

The authors used CD68 as a general macrophage marker and CD163 and CD204 for the identification of macrophage phenotype. They demonstrate that most of CD68+ TAMs in ccRCC also express CD204 which indicates that these are type 2 macrophages. Also CD68+ cells expressed CD163. In the same study, the authors demonstrate that direct coculture of macrophages with RCC cells induces type 2 macrophage phenotype.

This is explained by the expression of membranetype M-CSF on the surface of RCC cells . Another study, performed on TAM population isolated from RCC, showed expression of CD68 and CD163, but not CD206, which may be a result of the different methodology . In the latter study, the authors demonstrated that TAMs isolated from ccRCC produce significant amounts of CCL2— a CC-chemokine that attracts monocytes to tumor site. At the same time, these macrophages produced high amounts of immunosuppressive IL-10. Notably, the authors demonstrated that TAMs from larger tumors produce higher amounts of IL-10 .

This finding may indicate that either larger tumors are more potent in generating conditions favorable for M2 programming or stronger immunosuppressive M2 provide for faster tumor growth. In the same study by Daurkin et al., macrophages isolated from ccRCC showed enhanced eicosanoid production via activated 15- lipoxygenase-2 pathway , which is typical for activation of macrophages by TGFβ. Though unexpected, another specific property of TAMs in RCC is expression of CCR8 associated with higher activity of Stat3-mediated signaling, which is rather typical for inflammatory phenotype. These cells are considered to be capable of stimulating FoxP3 expression in T-cells and have proangiogenic activity.

Proinflammatory properties of TAMs in RCC were demonstrated on cultures of macrophages isolated from primary tumors. Production of high amounts of IL-6, TNFα, and IL-1β was demonstrated. In contrast, primary monocyte derived macrophages from the same patients did not produce these cytokines without LPS stimulation. In the same study, TAMs were demonstrated to stimulate proliferation of established RCC cell lines and short-term established RCC cell lines. IL-1β is an important factor for tumor angiogenesis and for stimulation of tumor invasiveness. It is capable of inducing matrix metalloproteinases MMP-1, MMP-3, MMP-10, and MT1-MMP in RCC cell lines and stimulates invasiveness of RCC as demonstrated in a mouse model.

IL-1R dependent mechanism was shown to be important for the development of protumor macrophage population in a mouse model. Another macrophage derived proinflammatory cytokine TNF was demonstrated to be important for the induction of cancer stem cell marker CD44 overexpression on ccRCC tumor cells.

The observation below seems to reflect the driver for the M2 conversion, namely the putative M2 marker, CD44[22].

At the same time, the density of macrophages expressing M2 marker CD163 appears to be more closely related to CD44 expressing cancer cells. *There are also markers of TAMs that are not classified to M1 or M2 phenotype. One of these markers is T-cell immunoglobulin and mucin domain-containing molecule- 3 (TIM-3). High amounts of TIM-3+ TAMs in ccRCC were associated with poor prognosis of the disease.*

The authors again demonstrate the balance or evolution of M1 and M2 prevalence. This may potentially be a marker regarding the aggressiveness of the lesion.

Accumulated data allow us to conclude that TAMs in RCC show a mixed M1/M2 phenotype. Analysis of the balance between M1 and M2 …on a cohort of 185 RCC patients using histological techniques. *The authors selected CD68 as a general macrophage marker, CD11c as a marker of M1, and CD206 as a marker of M2. Statistical analysis revealed that CD68 alone has a poor predictive value, while low CD11+ and high CD206+ as single variables correlated with reduced survival. At the same time, combined analysis of CD11c and CD206 showed the best predictive value. Patients had best survival prognosis if CD11c+ density was high and CD206+ density was low. Interestingly, CD68 was not needed in this analysis. This study is a very good example of the importance of a complex analysis, using markers for both M1 and M2 phenotypes. It would be interesting, however, to see whether there are 2 independent populations of macrophages in RCC or the same cells show a mixed M1/M2 phenotype.*

The authors then end with a discussion of the development of angiogenesis. VEGF is a significant factor here as we have discussed above. Again one may consider some form of blockage of VEGF functioning in the intermediation process.

Another highly important feature of TAMs is induction of angiogenesis*. In most of the tumors, TAMs are considered to be the source of VEGF which leads to increased microvessel density. RCC is not an exception. In a study performed on a cohort of 51 RCC patients, it was found that high CD68+ TAM density correlates with high microvessel density. Also the levels of VEGF determined by ELISA were found* ***to be higher in RCC compared to normal kidney****. The level of VEGF correlated well with diagnostic chance (symptomatic), growth type (interrapid), angiography findings (hypervascular), and tumor size (≥7 cm). These data are supported by the study, demonstrating that VEGFR1 knockdown leads to a reduced macrophage infiltration in the tumor.*

5.6 COLON

[22] From Abbas et al we note: *Activation also increases the expression of CD44, a receptor for the extracellular matrix molecule hyaluronan. Binding of CD44 to its ligand helps retain effector T cells in the tissues at sites of infection and tissue damage.* CD44 is a response to inflammation and its action enhances the immune response.

Colon cancer is quite common. The cells are exposed to many exogenous factors yet are generally quite well protected. Colon cancer is a slow growing process and for most patients if a colonoscopy is performed say every 5 years the chance of colon cancer death is de minimis[23]. Cai et al have recently related TAM to the EMT transitions in colon cancer as follows:

Given the potential role on clinical treatment, a better understanding of the mechanism of distant metastases in colorectal cancer is critical. Increasing evidences reveals cancer metastasis depends on various curial factors, including up-regulation of integrins and E-cadherin, epithelial–mesenchymal transition (EMT) progression of tumor cells, hypoxia, tumor microenvironment and so on. EMT was initially reported as a tightly regulated lineage change during gastrulation, neural crest delamination, heart valve formation, and embryonic development.

Recently, increasing attention has been attracted for the important role of EMT in cancer metastasis. A lot of molecules are responsible for the EMT in the tumor cells, including inflammatory cytokines, growth factors and numerous transcription factors.

In recent years, the tumor microenvironment has received attention as an important determination of cancer cells metastasis, among which tumor-associated macro-phages (TAMs) have been reported to play a prominent functional role in cancer behaviors.

TAMs are the major inflammatory component in many tumor microenvironments, which affect different aspects of tumor behaviors, including participation in tumor growth in several ways, such as transforming growth factor-β (TGF-β) secretion, remodeling of tumor microenvironment, inflammation suppression and so on. TGF-β is one of the major endogenous regulators of cell growth, which shows enhanced expression in various human cancers, including breast cancer, colorectal cancer, pancreatic cancer and so on. TGF-β participates in multifarious tumor progress, including potent immunosuppressive effects, EMT progression of tumor cells.

Increasing evidences demonstrated that TAMs facilitate distant metastasis in various tumors , including lung cancers, breast cancer and so on. However, the specific mechanism of metastasis induced by TAMs is still unclear and it is a challenge to inhibit the metastasis of colorectal cancer in clinic therapy. In our study, our aim is to reveal the expressional profile of TAMs in the colorectal cancers and investigate its potential role in the metastatic progress. Moreover, finding the under- lying mechanism involved in the colorectal cancer metastasis may lay an important foundation for the clinical treatment. We examined enriched TAMs in tumor tissues from colorectal patients with high metastasis, which was proved to facilitate tumor cells migration and neoplasm metastasis.

Furthermore, our study reveals that TAMs promote the progression of EMT in colorectal cancer cells through the secretion of TGF-β, resulting in the lung metastasis. TGF-β regulates the EMT progression through activation of Smad2,3-4/ Snail/E-cadherin signaling pathway. Blockade of

[23] https://www.cancer.gov/types/colorectal/hp/colorectal-prevention-pdq#_937

TGF-β / TGF-β receptor synapse with TGF-β receptor inhibitor sup- presses colorectal cancer cells lung metastasis.

Cai et al present summary results as follows:

1. TAMs facilitate colorectal cancer cells metastasis:

Recently, TAMs accumulation level has been shown to be of significant in cancer cells metastasis in a variety of cancers. To test the infiltration level of TAMs in colorectal cancer, we analyzed CD68+ cells in CD45+ cells in metastatic and non-metastatic colorectal cancer samples from clinical patients by flow cytometry. Interestingly, we found that metastatic patients have more TAMs accumulated than non-metastatic patients in tumor tissues. Moreover, we verified this by IHC and the CD68 IHC intensity was correlated with flow cytometry analysis. It has been reported that the accumulated TAMs in breast cancer and lung cancer could induce the metastasis.

Our initial results suggested that colorectal cancer tissues have TAMs accumulation and the infiltration level was much higher in metastatic patients tissues than non-metastatic patients. These results indicate that TAMs could promote the colorectal cancer cells lung metastatic seeding.

To further determine whether TAMs were involved in colorectal cancer lung metastasis, we used clodronate liposomes to deplete the macrophages in tumor-bearing mice. We found that macrophages depletion significantly reduced the colorectal cancer cells lung metastasis, which was supported that the macrophages depletion group has the lighter lung weight, fewer pulmonary tumor nodules and less lung metastasis than PBS group. Moreover, we used anti-CCL2 and anti-CSF1R (CD115) to target macrophages in CT26-bearing mice, both the lung weight and pulmonary tumor nodules were reduced. Together, our data suggest that TAMs could promote colorectal cancer lung metastasis.

2. TAMs promote colorectal cancer cells metastasis via the secretion of TGF-β

Our previous data showed that TAMs was involved in colorectal cancer cells lung metastasis. Next, we were wondering that how TAMs promote colorectal cancer cells meta- stasis. On one hand, we performed the … experiments and found that the invasion cells were significantly increased in CT26 and HCT116 with TAMs conditioned medium treatment. On the other hand, the cell migration was enhanced in TAMs conditioned medium treated CT26 and HCT116 by wound healing assay.

3. TGF-β induces the colorectal cancer cells metastasis through the EMT progression

We have found that TGF-β could induce colorectal cancer cells invasion and migration and it has been verified that TGF-β could initiate the EMT to enhance tumor cell invasion and migration in a variety of cancers, which was characterized as loss of E-cadherin and elevated vimentin expression.

We were wondering that whether TGF-β could trigger EMT in colorectal cancer cells. In order to visually capture the morphological change of EMT in vitro, we conducted the cell imaging of CT26 and HCT116 cells with TGF-β or conditioned medium from TAMs treatment. We observed that treating with TGF-β and conditioned medium from TAMs could significantly induce epithelioid CT26 and HCT- 116 cells transition into mesenchymal phenotype, while adding anti–TGF-β antibody reversed the effect.

Moreover, E-cadherin, a hallmark of epithelial cells, was significantly decreased with TGF-β and conditioned medium from TAMs treatment in CT26 and HCT116 cells. Concomitantly, vimentin, one of the mesenchymal cytoskeletal proteins, was remarkably increased with TGF-β and conditioned medium from TAMs treatment

4. TGF-β mediates colorectal cancer cells EMT via Smad2, 3-4/Snail/E-cadherin pathway

Next, we want to figure out how TGF-β endowed the EMT of colorectal cancer cells and to explore potential therapeutic target in clinic. The signaling mechanisms of TGF-β induced EMT has been largely conducted in a variety of cancer cells. In response to TGF-β, the cellular Smad signaling would be activated by phosphorylated TGF-β receptors. To test whether the Smad signaling involved in the colorectal cancer cells EMT, we detected the activation of Smads with TGF-β or conditioned medium from TAMs in CT26 colorectal cancer cells. We found that TGF-β or conditioned medium from TAMs increased the phosphorylation of Smad2/3, and significantly enhanced the expression of Smad4.

Also, we observed that the Smad2/3 and Smad4 complex was translocated into nucleus. Moreover, Snail, the downstream transcription factor of Smad, was increased by TGF-β and conditioned medium from TAMs treatment. While pre-treatment with TGF-β neutralizing anti-body, reversed the effect. …

In summary, our data provided evidence to support that accumulated TAMs in colorectal cancers contributed to distant metastasis through secreting TGF-β which induced EMT by activating Smad2,3-4/Snail pathway. *Blocking TGF-β signaling remarkably reduced the EMT which in turn resulted in decreased metastasis. Our data laid an important foundation for potential application of TGF-β inhibition in clinical treatment as anti-metastatic therapy for colorectal cancer patients.*

The above is an extensive summary of TAMs in colon cancer. Clearly they play a role as seen in the others we have discussed.

5.7 BREAST

Breast cancer is somewhat pervasive and it has various presentations from the DCIS variety which may in fact not even be reflective of a malignancy to full blow metastatic disease. Thus breast cancer has many presentations. Some early work by Lin et al had reported on murine models of breast cancer and TEM:

Tumor progression is characterized by an initial ‘‘avascular phase’’ when the tumors are small and usually dormant with diffusion being the major way to support their metabolic needs. In the subsequent ‘‘vascular phase,’’ the development of a unique tumor vasculature is required for the increased metabolic demand of tumors that have grown beyond a certain size. The induction of this vasculature, termed the ‘‘angiogenic switch’’ , can occur at various stages of tumor progression, depending on the tumor type and the environment.

However, it is clear that malignant tumors require its development as it has been shown that the initiation of revascularization in dormant lesions allows them to progress. The stroma of solid tumors are replete with many leukocytic cells of which macrophages represent a major component.

This is very common is most breast cancer path exams. Generally, the presence if macrophages was considered secondary at best. However, with the recognition of TAMs they become of greater note. The authors continue:

Recent clinical and experimental studies have indicated that these tumor-associated macrophages promote the progression to malignancy. In human breast cancers, macrophages cluster in ‘‘hotspots’’ in avascular areas in human breast cancer samples, which correlates with a high level of angiogenesis and with decreased relapse-free and overall survival of the patients . Macrophages play a crucial role in regulating angiogenesis in wound healing .

They produce many proangiogenic factors including vascular endothelial growth factor (VEGF), tumor necrosis factor a, granulocyte macrophage colony-stimulating factor, interleukin (IL)-1, IL-6, and other factors including matrix metalloproteinases (MMP) and nitric oxide that also have the potential to regulate angiogenesis. Parallels have been drawn between the microenvironment of wound-induced inflammation and that of tumors, as proposed in the hypothesis that tumors are ‘‘wounds that never heal’’ .

However, whether tumor-associated macrophages are able to promote angiogenesis is still not clear. *We have reported in the mouse model of breast cancer caused by the mammary epithelial cell restricted expression of the Polyoma middle T oncoprotein (PyMT mice) that the infiltration of macrophages in primary mammary tumors was positively associated with tumor progression to malignancy. Depletion of macrophages in this model severely delayed tumor progression and dramatically reduced metastasis whereas an increase in macro- phage infiltration by transgenic means remarkably accelerated these processes.*

The above may be questioned regarding angiogenesis promotion. As we have discussed previously there are multiple paths that lead to such a process.

To identify the mechanism(s) that macro- phages use to promote tumor progression, we have tested the hypothesis that tumor-associated macrophages stimulate the development of tumor vasculature. Our results indicate that tumor- associated macrophages were actively involved in promoting the angiogenic switch during the malignant transition as well as in the maintenance and/or remodeling of an established vessel network in malignant tumors.

Needless to say this characterization of the existence and influence of TAMs and their TAIs is just the beginning since they play a role in a multiplicity of malignancies.

6 OBSERVATIONS

We now summarize with several observations relating to putative extensions of what we have presented above.

6.1 THERAPEUTIC TARGETS

There appears to be a putative multiplicity of targets for therapeutic applications. As Pathria et al have recently stated:

TAMs express cytokines and enzymes that can suppress T cell recruitment and activation, thereby promoting resistance to immune checkpoint inhibition. Bone-marrow-derived and tissue-resident TAMs each contribute to TAM overall content, and both can promote tumor immunosuppression. In preclinical mouse models, inhibitory targeting of myeloid cell surface receptors (PD-L1, CD47/SIRP1α, CCR2, CSF1R, and integrin α4β1), signaling components (PI3Kγ, mTORC1, BTK, and PDE5), transcription factors (KLF6, STAT3, TWIST, ZEB1, and NFAT1), metabolic pathways (arginine metabolism), and others, can prevent tumor immunosuppression and synergize with immune checkpoint inhibitors to improve antitumor responses.

Epigenetic regulation of macrophage polarization – as with Class IIa HDAC inhibitors – may protect from cancer immunosuppression by stimulating macrophage proinflammatory gene expression, and thus activating cytotoxic T cell antitumor responses. Antagonists of several targets, including CSF1R, CCR2, CD47/SIRP1a, PI3Kγ, BTK, and HDACs, as well as agonists of TLRs are currently under clinical investigation as putative cancer therapies for various malignancies. Macrophages are phagocytes that serve as a first line of defense against pathogenic insults to tissues. These innate immune cells mount proinflammatory responses to pathogens and repair damaged tissues.

However, tumor-associated macrophages (TAMs) express cytokines and chemokines that can suppress antitumor immunity and promote tumor progression. Preclinical studies have identified crucial pathways regulating the recruitment, polarization, and metabolism of TAMs during tumor progression.

Moreover, novel therapeutics targeting these pathways can indirectly stimulate cytotoxic T cell activation and recruitment, and synergize with checkpoint inhibitors, chemotherapy and/or radiation therapy in preclinical studies. Thus, clinical trials with therapeutic agents that promote phagocytosis or suppress survival, proliferation, trafficking, or polarization of TAMs are currently underway. These early results offer the promise of improved cancer outcomes.

Also, as we have seen certain growth factors exacerbate the development of metastatic processes. VEGF is one. In a paper by Holash et al they had noted:

Vascular endothelial growth factor (VEGF) plays a critical role during normal embryonic angiogenesis and also in the pathological angiogenesis that occurs in a number of diseases, including cancer. Initial attempts to block VEGF by using a humanized monoclonal antibody are beginning to show promise in human cancer patients, underscoring the importance of optimizing VEGF blockade. Previous studies have found that one of the most effective ways to block the VEGF-signaling pathway is to prevent VEGF from binding to its normal receptors by administering decoy-soluble receptors.

The highest-affinity VEGF blocker described to date is a soluble decoy receptor created by fusing the first three Ig domains of VEGF receptor 1 to an Ig constant region; however, this fusion protein has very poor in vivo pharmacokinetic properties. By determining the requirements to maintain high affinity while extending in vivo half-life, we were able to engineer a very potent high-affinity VEGF blocker that has markedly enhanced pharmacokinetic properties. This VEGF-Trap effectively suppresses tumor growth and vascularization in vivo, resulting in stunted and almost completely avascular tumors.

VEGF-Trap-mediated blockade may be superior to that achieved by other agents, such as monoclonal antibodies targeted against the VEGF receptor.

We have noted this previously based on other studies. As with many immune control mechanisms one must always look at the downside and controlling VEGF broadly can have significant morbidity. However, if one can have multiple markers then perhaps specific targeting is possible.

Recently Ngambenjawong et al have reported:

With deeper understanding of cancer immunology, diverse strategies for modulation of TAMs are being uncovered and explored for therapeutic applications. Due to the complexity of tumors, combination therapy is typically needed to maximize an anti-tumor response.

Thus, a clear understanding of the modes of drug action as well as mechanisms of resistance is needed in order to design an efficacious combination therapy that minimizes antagonistic effects. For example, identification of PI3k up-regulation in tumor as a resistance mechanism for CSF-1R kinase inhibitor in recurrent glioma suggests that a combination therapy between PI3k and CSF-1R inhibitors could be more beneficial. Conversely, the therapeutics aiming to block macrophage recruitment signals (e.g. CCL2 or CXCL12 inhibition) may not be compatible with the ones that require the presence of macrophages for anti-tumor actions (e.g. anti-CD40 antibody).

In congruence with the well appreciated immunosuppressive roles of TAMs, a consensus was observed regarding the potential benefits of TAM-targeted therapies in potentiating immune checkpoint blockade therapies (anti PD-1/PD-L1/CTLA-4 antibodies) as evidenced in the race among pharmaceutical companies to investigate such combination therapies (Table 2). Improvement in gene sequencing and analysis technologies greatly facilitates the adoption of precision medicine where patients could be examined for genetic makeup and matched with appropriate therapeutic regimens.

Documenting patients' genetic profile and the corresponding therapeutic outcome are also beneficial in correlation studies to better predict patient response as well as in refining drug development.

In the case of CSF-1R inhibition therapy, certain single nucleotide polymorphisms (SNPs) in CSF-1R have been identified that reduce the potency of emactuzumab. Nonetheless, the study may help in the future design of the next-generation CSF-1R blockade therapy. To reap the benefit of a steady rise in molecularly targeted therapies that are promising for clinical translation, it is more than ever important to be resource-efficient. This may be possible through careful validation of pre-clinical studies and innovative design of clinical trials as seen, for example, in the I-SPY 2 trial (NCT01042379).

The above comment regarding SNP variability and interference draws the issue regarding inter-patient genetic variability. However one suspects that sequencing the genome is not necessarily the answer. The SNP variability may be more prevalent than thought but hidden perhaps by epigenetic factors such as methylation of histone compression. They continue:

With numerous therapeutic targets being identified and drug candidates being explored for modulation of TAMs, drug delivery technologies will soon come into play to further enhance therapeutic efficacy of these drugs, for example, by improving pharmacokinetics, stability, selectivity, or intracellular delivery while limiting systemic toxicity.

Together with advancement in gene-editing technology, effective silencing of genes that promote pro-tumoral functions of TAMs (e.g. STAT3, SIRPα, PI3k, or Gpr132) may one day be a practicable therapeutic option. Finally, a cost barrier is another factor that could impede the clinical translation especially when multiple antibodies are used in a combination therapy as currently investigated in several trials.

The problem may be alleviated with improvement in manufacturing efficiency or development of cheaper alternatives such as small molecule drugs or peptide analogs.

Indeed, the potential for a pan-tumor-environment therapeutic regime has potential but only after a better and complete understanding of the TME. Furthermore the plasticity of the TME may become a challenge itself.

6.2 PROGNOSTIC MARKERS

There is an ongoing need for non-invasive markers for various malignancies. Most of these markers would be blood borne such as PSA albeit with greater specificity and sensitivity. One generally tries to obtain AOC (area under the curve) performance as high as possible. From Bilen et al we have such a discussion regarding the markers available from immune counts as follows:

Optimal prognostic and predictive biomarkers for patients with advanced-stage cancer patients who received immunotherapy (IO) are lacking. Inflammatory markers, such as the neutrophil-to-

lymphocyte ratio (NLR), the monocyte-to-lymphocyte ratio (MLR), and the platelet-to-lymphocyte ratio (PLR), are readily available.

The authors investigated the association between these markers and clinical outcomes of patients with advanced-stage cancer who received IO. …

Baseline and early changes in NLR, MLR, and PLR values were strongly associated with clinical outcomes in patients who received IO-based treatment regimens on phase 1 trials. Confirmation in a homogenous patient population treated on late-stage trials or outside of trial settings is warranted. These values may warrant consideration for inclusion when risk stratifying patients enrolled onto phase 1 clinical trials of IO agents. …

Elevated baseline and early increases in NLR, MLR, and PLR values are strongly associated with poor clinical outcomes in this cohort of patients treated on IO-based phase 1 clinical trials. These values may warrant consideration when risk-stratifying patients who are being enrolled onto phase 1 clinical trials. Given the high concordance among these variables, any of these markers may be used in future analysis. Future studies investigating the relation between changes in these values and the tumor microenvironment are crucial to elucidate the underlying biologic explanation for the prognostic and predictive value of these markers

6.3 ADJUNCTS TO PATHOLOGICAL STAGING

Generally, the pathological analysis of tumors addresses the malignant cells with minimal recognition of the presence of the various immune cells and other elements of the ECM. The question then is; can we use the information on the tumor associated immune (TAI) cells to further diagnose and stage the lesion? We have addressed this question obliquely elsewhere when discussing just what we mean by cancers[24].

Namely, there are certain lesions where the cells are clearly aberrant yet they are not proliferating and the immune ECM involvement portends a benign progression. Thus perhaps adding details on path reports so that a larger study may be performed would have value.

6.4 MACROPHAGES AND THEIR PLASTICITY

Macrophages such as the M1 and M2 are not necessarily fixed but exhibit great plasticity, moving from one extreme to another. As De Palma and Lewis have noted[25]:

Once resident in tissues, macrophages acquire a distinct, tissue-specific phenotype in response to signals present within individual microenvironments. The exact combination of such tissue-specific cues dictates both the differentiation and activation status of these cells.

[24] https://www.researchgate.net/publication/334947163_What_is_Meant_by_Cancer

[25] https://www.cell.com/cancer-cell/fulltext/S1535-6108(13)00069-X

Two extreme forms of the latter are generally referred to as "classical" (or M1) and "alternative" (or M2) activation, which parallel Th1/Th2 programming of adaptive immune cells.

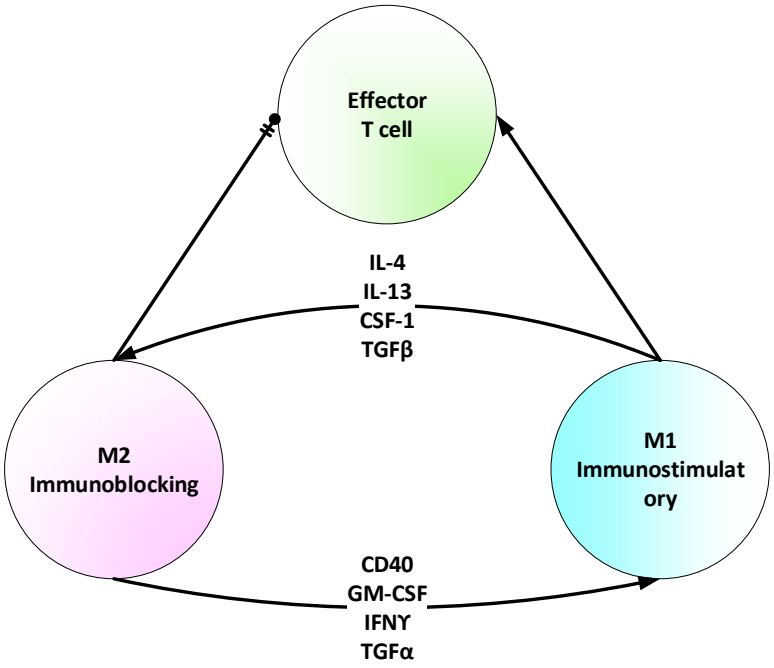
During acute inflammation, macrophages are M1-activated by toll-like receptor (TLR) agonists and Th1 cytokines (e.g., interferon [IFN]-γ). This enhances their ability to kill and phagocytose pathogens, upregulate proinflammatory cytokines (e.g., interleukin [IL]-1β, IL-12, and tumor necrosis factor-α [TNF-α]) and reactive molecular species, and present antigens via major histocompatibility complex (MHC) class II molecules.

Alternatively, Th2 cytokines, like IL-4 and 13, stimulate monocytes/macrophages to express an M2 activation state. This is characterized by higher production of the anti-inflammatory cytokine, IL-10; lower expression of proinflammatory cytokines; amplification of metabolic pathways that can suppress adaptive immune responses; and the upregulation of cell-surface scavenger receptors, such as mannose receptor (MRC1/CD206) and hemoglobin/aptoglobin scavenger receptor (CD163).

As such, M2 macrophage activation may facilitate the resolution of inflammation and promote tissue repair (including angiogenesis) after the acute inflammatory phase. In healthy tissues, macrophages often express a mixed M1/M2 phenotype; hence "M1" and "M2" polarization should be regarded as extreme ends of a continuum of activation states, with their exact point on the scale depending on the precise mix of local signals present in a given microenvironment.

Thus the M1 state is the attack and kill state and the M2 state is the protect and grow state of the macrophage colony. The M2 state does this perforce of the plasticity of the macrophage which initially sees the "invader" and attacks and then sees the result and tries to "heal" the wound. In doing the latter is in effect facilitates the cancer cells growth. Perhaps, therefore, turning back from M2 to M1 would allow the attack to continue towards some resolution. Yet as know, there can be a multiplicity of unintended consequences.

Some of the plasticity effectors and processes are shown below based upon the work of Noy and Pollard:



6.5 IN VITRO VS IN VIVO

Understanding the role of TAMs, and the TME, we can see that in vitro models suffer greatly due to the lack of the putative protective and plastic environment surrounding cancer cells. The challenge to those doing such work is to reflect the true holistic environment of the tumor. De Palma and Lewis have noted:

Mouse tumor models, including genetically engineered mouse models (GEMMs), are being used extensively to study mechanisms underlying tumor (and TAM) responses to anticancer therapies. However, even sophisticated GEMMs of cancer cannot simulate the endless variations in TAM abundance, distribution, and phenotypes between and within different types and subtypes of human cancer. Nor do they necessarily model the ability of such tissues to recruit monocytes during therapy.

Future work should therefore aim to define the identities and molecular profiles of distinct TAM subtypes in human cancer biopsies before, during, and after therapy. Specific TAM signatures could then be used to stratify patients carrying defined genetic lesions in order to explore how such signatures correlate with the response of individual patients to chemo-, radio-, or targeted therapies, and/or the emergence of secondary resistance. If such studies demonstrate the predictive value of specific TAM subtypes for individual tumor responses, then their further characterization in mouse tumor models could help develop more effective cancer therapies. Undeniably, such clinical approaches should consider the biological complexities on a tumor (sub)type and individual patient basis and harness them to design effective personalized therapies.

Indeed, one must be cautious regarding the ability to project from one controlled environment to the human species. Perhaps that is one reason why we all too often see but a 30-40% response rate to many immunotherapeutic approaches, namely the complex variability and plasticity of the TME.

7 REFERENCES

1. Aaronson, Growth Factors and Cancer, Science, Vol 254 Nov 1991.
2. Abbas et al, Cellular and Molecular Immunology, Elsevier, 2018
3. Bao et al, TGF-β2 induces proliferation and inhibits apoptosis of human Tenon capsule fibroblast by miR-26 and its targeting of CTGF, Biomedicine & Pharmacotherapy, Volume 104, August 2018, Pages 558-565, open access
4. Bercovivi et al, The Remarkable Plasticity of Macrophages: A Chance to Fight Cancer, Front in Immunology, July 2019
5. Bilen et al, The prognostic and predictive impact of inflammatory biomarkers in patients who have advanced-stage cancer treated with immunotherapy, Cancer. 2019 Jan 1;125(1):127-134
6. Botehlo et al, Vascular Endothelial Growth Factor (VEGF) and Prostate Pathology, International Braz J Urol Vol. 36 (4): 430-438, July - August, 2010
7. Cai et al, Tumor-Associated Macrophages Derived TGF-β Induced Epithelial to Mesenchymal Transition in Colorectal Cancer Cells through Smad2,3-4/Snail Signaling Pathway, Cancer Res Treat. 2019;51(1):252-266
8. Cantley et al, Signal Transduction, CSHL Books, 2014
9. Carpenter and Cohen, Epidermal Growth Factor, The Journal Of Biological Chemistry, Vol. 265, No. 14, Issue of May 15, pp. 7709-7712.1990
10. Chanmee et al, Tumor-Associated Macrophages as Major Players in the Tumor Microenvironment, Cancers 2014, 6, 1670-1690
11. Cheah et al, CD14-expressing cancer cells establish the inflammatory and proliferative tumor microenvironment in bladder cancer, PNAS, April 14, 2015, vol. 112, no. 15, 4725–4730
12. Chen et al, PDGF-D promotes cell growth, aggressiveness, angiogenesis and EMT transformation of colorectal cancer by activation of Notch1/Twist1 pathway, Oncotarget, 2017, Vol. 8, (No. 6), pp: 9961-9973
13. Chevier et al, An Immune Atlas of Clear Cell Renal Cell Carcinoma, Cell 169, 736–749, May 4, 2017
14. Cortez-Retamozo et al, Origins of tumor-associated macrophages and neutrophils, PNAS | February 14, 2012 | vol. 109 | no. 7
15. Curiel (ed.), Cancer Immunotherapy, Springer Science and Business Media, New York, 2013
16. De Palma and Lewis, Macrophage Regulation of Tumor Responses to Anticancer Therapies, Cancer Cell, March 18, 2013
17. DeVita, Hellman, and Rosenberg, Cancer: Principles & Practice of Oncology, 8th Ed, LWW, 2008
18. DiNardo and Ruffel, Macrophages as regulators of tumour immunity and immunotherapy, Nature Reviews Immunology, volume 19, pages369–382 (2019)

19. Doug, Targeting Tumor-Associated Macrophages (Tams) Reprogramming for Cancer Metastasis Therapy, J Pharm Pharmaceutics 6(1) 2019
20. Eisel et al, Cognate Interaction with CD4+ T Cells Instructs Tumor-Associated Macrophages to Acquire M1-Like Phenotype, Frontiers in Immuno, Feb 2019
21. Etzerodt et al, Specific targeting of CD163+ TAMs mobilizes inflammatory monocytes and promotes T cell–mediated tumor regression, Jrl Experimental Medicine, August 2019
22. Fouad and Aanel, Revisiting the hallmarks of cancer, Am J Cancer Res 2017;7(5):1016-1036
23. Frank, Diabetic Retinopathy, NEJM, 2004;350:48-58
24. Fridlender and Albelda, Tumor-associated neutrophils: friend or foe?, Carcinogenesis vol.33 no.5 pp.949–955, 2012
25. Fryczkowski et al, Circulating Levels of Omentin, Leptin, VEGF, and HGF and Their Clinical Relevance with PSA Marker in Prostate Cancer, Disease Markers Volume 2018, Article ID 3852401, 9 pages
26. Fujimura et al, Tumor-Associated Macrophages: Therapeutic Targets for Skin Cancer, Front in Oncology, January 2018 | Volume 8 | Article 3
27. Galluzzi et al, Classification of current anticancer immunotherapies, Oncotarget, Vol. 5, No. 24, 2014
28. Green, Cell Death, CSHL Press (New York) 2018.
29. Gregory and Houghton, Tumor-Associated Neutrophils: New Targets for Cancer Therapy, Cancer Res; 71(7) April 1, 2011
30. Grivennikov et al, Immunity, Inflammation, and Cancer, Cell 140, 883–899, March 19, 2010
31. Gupta and Massagué, Cancer Metastasis: Building a Framework, Cell 127, November 17, 2006
32. Hanada et al, Prognostic value of tumor-associated macrophage count in human bladder cancer., Int J Urol. 2000 Jul;7(7):263-9.
33. Hanahan and Coussens, Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment, Cancer Cell 21, March 20, 2012
34. Hayden and Ghosh, NF-κB in immunobiology, Cell Research (2011) 21:223-244
35. Holash et al, VEGF-Trap: A VEGF blocker with potent antitumor effects, PNAS August 20, 2002 vol. 99 no. 17 11393–11398
36. Hosel and Schmid, The complexity of NF-κB signaling in inflammation and cancer, Molecular Cancer 2013, 12:86
37. Huang et al, Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy, PNAS | October 23, 2012 | vol. 109 | no. 43
38. Joyce and Feron, T cell exclusion, immune privilege, and the tumor microenvironment, Science, 3 April 2015 • Vol 348 Issue 6230

39. Kang et al, The role of the tumor microenvironment in bladder cancer development and progression, Transl Cancer Res 2017;6 (Suppl 4)
40. Karaman et al, Vascular endothelial growth factor signaling in development and disease, Development (2018) 145
41. Karp et al, Handbook of Targeted Cancer Therapy and Immunotherapy, 2nd Ed, Lippincott, 2018
42. Kerbel, Tumor Angiogenesis, NEJM, May 2008
43. Kim et al, The Expression of Tumor-Associated Macrophages in Papillary Thyroid Carcinoma, Endocrinol Metab 2013;28:192-198
44. Kovaleva et al, Tumor Associated Macrophages in Kidney Cancer, Analytical Cellular Pathology, Volume 2016, Article ID 9307549
45. Kundu and Surh, Inflammation: Gearing the journey to cancer, Mutation Research 659 (2008) 15–30
46. Landskron et al, Chronic Inflammation and Cytokines in the Tumor Microenvironment, Jrl Immuno Research, 2014
47. Larionova et al, Interaction of tumor-associated macrophages and cancer chemotherapy, Oncoimmunology, 2019, VOL. 8, NO. 7,
48. Laviron and Boissonnas, Ontogeny of Tumor-Associated Macrophages, Frton in Immun, July 2019
49. Lecis et al, Immune Checkpoint Ligand Reverse Signaling: Looking Back to Go Forward in Cancer Therapy, Cancers 2019,11, 624
50. Lee et al, The Expression of Tumor-Associated Macrophages in Papillary Thyroid Carcinoma, Endocrinol Metab 2013;28:192-198
51. Leonard and Schreiber, Cytokines, CSHL, 2018
52. Lin et al, Macrophages Regulate the Angiogenic Switch in a Mouse Model of Breast Cancer, Cancer Res 2006; 66: . December 1, 2006
53. Linehan and Ricketts, The Metabolic Basis of Kidney Cancer, Semin Cancer Biol. 2013 February ; 23(1): 46-55.
54. Linehan et al, The genetic basis of kidney cancer: a metabolic disease, Nat Rev Urol. 2010 May ; 7(5): 277–285
55. Litwack, Human Biochemistry, Academic (New York) 2018, pp 515-543
56. Mendelsohn and Baselga, The EGF receptor family as targets for cancer therapy, Oncogene (2000) 19, 6550 – 6565
57. Metcalfe, Mast cells and mastocytosis, Blood, 2008 112: 946-956
58. Molina-Cerrillo et al, Tumor-Associated Macrophages: “Good Cop-Bad Cop”, Cancer · February 2019

59. Mora et al, Strategies to Interfere with Tumor Metabolism through the Interplay of Innate and Adaptive Immunity, Cells 2019, 8, 445

60. Morgan, Cell Cycle, Sinauer (San Francisco) 2007.

61. Mowers et al, Autophagy in cancer metastasis, Oncogene (2017) 36, 1619–1630

62. Murekatete et al, Targeting Insulin-Like Growth Factor-I and Extracellular Matrix Interactions in Melanoma Progression, Scientific Reports, 2018

63. Murgaski et al, Unleashing Tumour-Dendritic Cells to Fight Cancer by Tackling Their Three A's: Abundance, Activation and Antigen-Delivery, Cancers 2019, 11, 670

64. Ngambenjawong et al, Progress in tumor-associated macrophage (TAM)-targeted therapeutics, Adv Drug Deliv Rev. 2017 May 15; 114: 206–221

65. Noy and Pollard, Tumor-Associated Macrophages: From Mechanisms to Therapy, Immunity 41, July 17, 2014

66. Nyberg et al, Tumor microenvironment and angiogenesis, Frontiers in Bioscience 13, 6537-6553, May 1, 2008

67. Olsen et al, Fibroblast Growth Factor (FGF) Homologous Factors Share Structural but Not Functional Homology with FGFs, The Journal Of Biological Chemistry Vol. 278, No. 36, Issue of September 5, pp. 34226–34236, 2003

68. Ornitz and Ito, The Fibroblast Growth Factor signaling pathway, WIREs Dev Biol 2015, 4:215–266

69. Ornitz and Itoh, Fibroblast growth factors, Genome Biology 2001, 2(3):reviews3005.1–3005.12, March 2001

70. Page et al, Immune Modulation in Cancer with Antibodies, Immune Modulation in Cancer, https://www.annualreviews.org/ October 2013

71. Patel et al, Neuroendocrine Differentiation of Prostate Cancer, An Intriguing Example of Tumor Evolution at Play, Cancers 2019, 11, 1405

72. Pathria et al, Targeting Tumor-Associated Macrophages in Cancer, Trends in Immunology, March 2019

73. Peruzzi B, Bottaro DP. Targeting the c-Met signaling pathway in cancer. Clin Cancer Res 2006 Jun 15;12(12):3657–60

74. Pezella et al, Cancer Biology, Oxford, 2019

75. Pietras et al, PDGF receptors as cancer drug targets, CANCER CELL : MAY 2003 • VOL. 3

76. Poh and Ernst, Targeting Macrophages in Cancer: From Bench to Bedside, Front in Oncology, 12 March 2018

77. Prendergast and Jaffee, Cancer Immunotherapy, Academic, 2013

78. Purushoth et al, Cancer Progression Related with Tumor-associated Macrophages, Cancer and Oncology Research 4(4): 53-63, 2016

79. Quaranta and Schmid, Macrophage-Mediated Subversion of Anti-Tumour Immunity, Cells 2019, 8, 747

80. Rahim et al, Molecular Regulation of Bone Marrow Metastasis in Prostate and Breast Cancer, Bone Marrow Research, Volume 2014, Article ID 405920, 12 pages

81. Riabov, et al, Role of tumor associated macrophages in tumorangiogenesis and lymphangiogenesis, Front in Physiology, March 2014

82. Roma-Rodrigues et al, Targeting Tumor Microenvironment for Cancer Therapy, Int. J. Mol. Sci. 2019, 20, 840

83. Rucci and Angelucci, Prostate Cancer and Bone: The Elective Affinities, BioMed Research International, Volume 2014, Article ID 167035, 14 pages

84. Ruffell and Coussens, Macrophages and Therapeutic Resistance in Cancer, Cancer Cell, April 2015

85. Ruffell1 and Coussens, Macrophages and Therapeutic Resistance in Cancer, Cancer Cell, April 2015

86. Ryder et al, Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer, Endocrine-Related Cancer (2008) 15 1069–1074

87. Salinero and Rafii, Endothelial cell adaptation in regeneration, Science, Dec 2018

88. Salmi et al, The number and localization of CD68+ and CD163+ macrophages in different stages of cutaneous melanoma, Melanoma Research 2018, Vol 00 No 00

89. Sengupta et al, Regulation of the mTOR Complex 1 Pathway by Nutrients, Growth Factors, and Stress, Molecular Cell 40, October 22, 2010

90. Shen and Rubin, Prostate Cancer, CSHL, 2019

91. Sherbet, Growth actors and Their Receptors, Elsevier (New York) 2011

92. Silvestri et al, A Perspective of Immunotherapy for Prostate Cancer, Cancers 2016

93. Singh et al, EGF receptor ligands: recent advances, F1000 Research 2016.

94. Solinas et al, Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation, Volume 86, November 2009 Journal of Leukocyte Biology

95. Spravchikov et al, Glucose Effects on Skin Keratinocytes, Diabetes, Vol. 50, July 2001

96. Steidl et al, Tumor-Associated Macrophages and Survival in Classic Hodgkin's Lymphoma NEJM, March 10, 2010

97. Suarez-Carmona et al, EMT and inflammation: inseparable actors of cancer progression, Molecular Oncology 11 (2017) 805–823

98. Taichman et al, The evolving biology and treatment of prostate cancer, The Journal of Clinical Investigation http://www.jci.org Volume 117 Number 9 September 2007

99. Takayama et al., Increased infiltration of tumor associated macrophages is associated with poor prognosis of bladder carcinoma in situ after intravesical bacillus Calmette-Guerin instillation., J Urol. 2009 Apr;181(4):1894-900

100. Takeuchi and Ito, Receptor Tyrosine Kinases and Targeted Cancer Therapeutics, Biol. Pharm. Bull. 34(12) 1774—1780 (2011)

101. Tukan et al, Analysis of Circulating Tumor Cells in Patients with Non-Metastatic High-Risk Prostate Cancer before and after Radiotherapy Using Three Different Enumeration Assays, Cancers 2019 V 11 N 802

102. Varricchi et al, Are Mast Cells MASTers in Cancer?, Front. in Immuno, 2 April 2017

103. Venere et al, Growth Factor Receptors Define Cancer Hierarchies, Cancer Cell 23, February 11, 2013

104. Visciano et al, Tumor-Associated Mast Cells in Thyroid Cancer, International Journal of Endocrinology, Volume 2015

105. Wesche et al, Fibroblast growth factors and their receptors in cancer, Biochem. J. (2011) 437, 199–213

106. Witsch et al, Roles for Growth Factors in Cancer Progression, PHYSIOLOGY 25: 85–101, 2010

107. Wu et al, Tumor-Associated Neutrophils in Cancer: Going Pro, Tumor-Associated Neutrophils in Cancer: Going Pro

108. Yang et al, Growth Factors and their receptors in cancer metastases, Frontiers in Bioscience 16, 531-538, January 1, 2011

109. Zhang et al, Regulation of epithelial-mesenchymal transition by tumor-associated macrophages in cancer, Am J Transl Res 2015;7(10):1699-1711

110. Zhang, et al, Prognostic Significance of Tumor-Associated Macrophages in Solid Tumor: A Meta-Analysis of the Literature, PLOS One, Dec 2012

111. Zhao et al, Bladder cancer cells re-educate TAMs through lactate shuttling in the microfluidic cancer microenvironment, Oncotarget, Vol. 6, No. 36 2015

112. Zhu et al, Epithelial derived CTGF promotes breast tumor progression via inducing EMT and collagen I fibers deposition, Oncotarget, Vol. 6, No. 28, 2015

113. Zingales et al, Metformin: A Bridge between Diabetes and Prostate Cancer, Frontiers in Oncology, October 2017, Volume 7, Article 243

114.

8 APPENDIX: GROWTH FACTORS

We summarize here several key growth factors that play to the TAM and TAI areas. TGF and VEGF are significant factors.

8.1 TGF

The Transforming Growth Factor, TGF, has several forms. They control the SMAD signalling pathway which in turn controls transcriptional actions. We will outline some of these herein. Some of these gene activations control the S phase as discussed above.

From NCBI we list the following:

TGFB1[26]*: This gene encodes a secreted ligand of the TGF-beta (transforming growth factor-beta) superfamily of proteins. Ligands of this family bind various TGF-beta receptors leading to recruitment and activation of SMAD family transcription factors that regulate gene expression. The encoded preproprotein is proteolytically processed to generate a latency-associated peptide (LAP) and a mature peptide, and is found in either a latent form composed of a mature peptide homodimer, a LAP homodimer, and a latent TGF-beta binding protein, or in an active form consisting solely of the mature peptide homodimer.*

The mature peptide may also form heterodimers with other TGFB family members. This encoded protein regulates cell proliferation, differentiation and growth, and can modulate expression and activation of other growth factors including interferon gamma and tumor necrosis factor alpha. This gene is frequently upregulated in tumor cells, and mutations in this gene result in Camurati-Engelmann disease

TGFB2[27]*: he protein encoded by this gene is a transmembrane protein that has a protein kinase domain, forms a heterodimeric complex with TGF-beta receptor type-1, and binds TGF-beta. This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of genes related to cell proliferation, cell cycle arrest, wound healing, immunosuppression, and tumorigenesis. Mutations in this gene have been associated with Marfan Syndrome, Loeys-Deitz Aortic Aneurysm Syndrome, and the development of various types of tumors.*

As Yang et al note:

TGF-β is a member of a growth factor family that regulates cellular proliferation, differentiation, apoptosis and extracellular matrix formation. TGF- β usually serves as a tumour suppressor in the normal tissues by inhibiting cell proliferation and inducing apoptosis, but it

[26] https://www.ncbi.nlm.nih.gov/gene/1950

[27] https://www.ncbi.nlm.nih.gov/gene/7048

promotes tumour progression and invasion if the tumour cells overcome its cytostatic and apoptotic effects.

Thus TGF is a powerful GF and can effect a multiplicity of changes. It appears to be a regulator of excessive cell proliferation and suppression can result in proliferation and uncontrolled cellular activity. The author continues:

In fact TGF-β is one of the most potent inducers of EMT both in cultured cells and animal models. It initiates carcinogenic EMT in different systems in vitro and in vivo, inhibits the growth of epithelial cells and promotes the growth of mesenchymal cells.

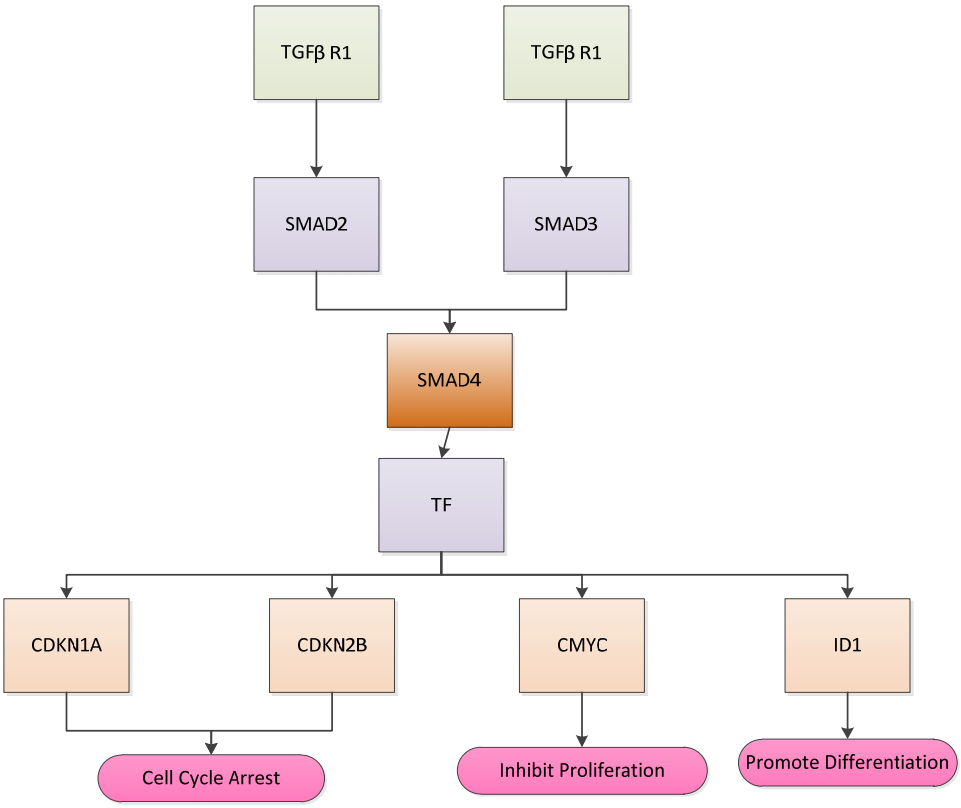
TGF-β signals through three cell surface receptors: the type I (TβRI), type II (TβRII) and type III (TβRIII) receptors. TβRIII can bind all TGF-β isoforms and presents them to TβRII. After binding with ligand, TβRII recruits and phosphorylates TβRI to activate its kinase activity. TβRI then phosphorylates and activates Smad2/3, which bind to Smad4, and the complex accumulates in the nucleus and interacts with other transcription factors to regulate the expression of a multitude of target genes

Thus TGF should be considered one of the more critical GF to be examined.

8.1.1 TGF, SMAD4 and Signalling

SMAD4 is an element in the TGF-β signalling chain. TGF is a cytokine, specifically a transforming growth factor cytokine. Like the Wnt-Apc pathway, the TGF pathway links defective development to cancer. The pathway is shown in part below (from Bunz p 199). Normal TGF signalling down-regulates the growth of most normal cells. Several of the genes in the TGF/SMAD pathway activation suppress growth. Specifically the genes CDKN1A and CDKN2B encode the cyclin dependent kinase inhibitors which suppress growth. Activated SMAD pathways also appear to suppress the transcription of other genes including c-Myc.

We show some of the TGF SMAD signalling below. We will elaborate this later.



SMAD4 controls the G1 to S transition. As stated in NCBI[28]:

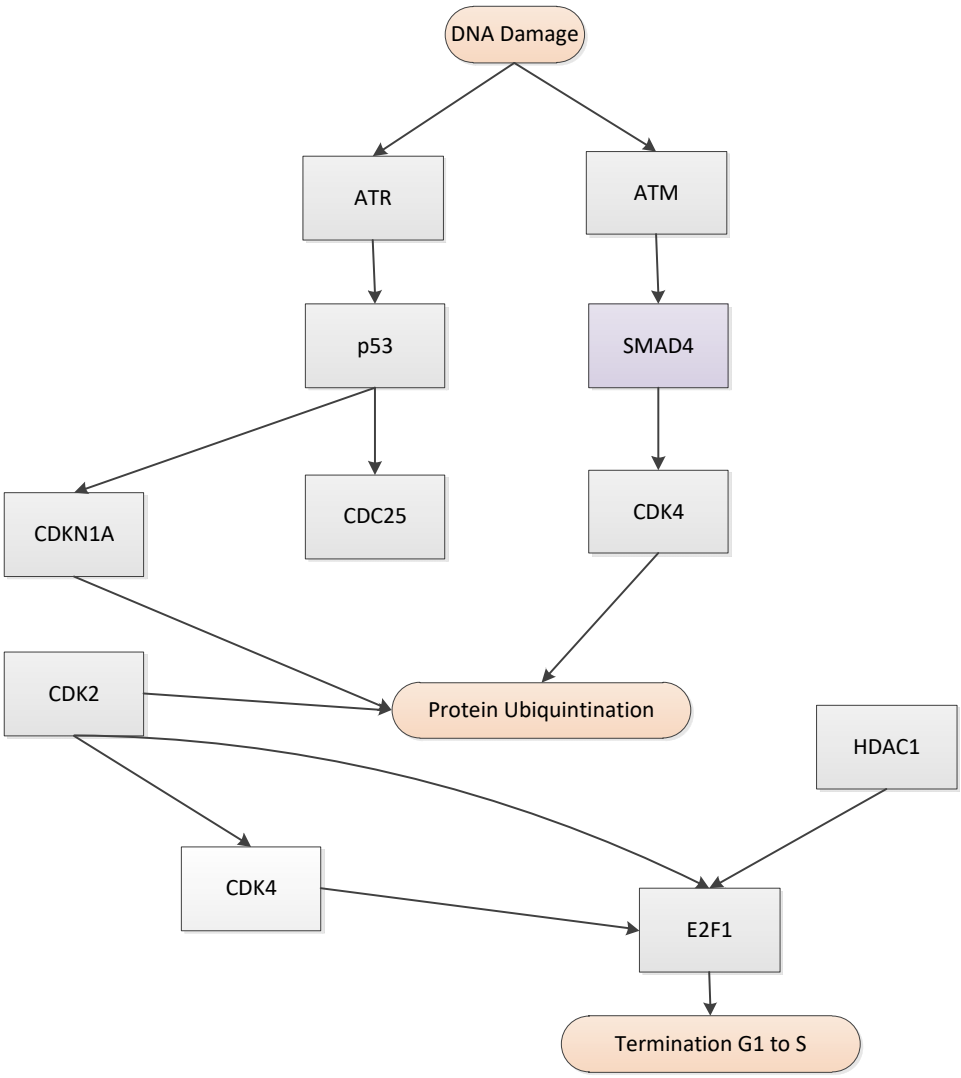
This gene encodes a member of the Smad family of signal transduction proteins. Smad proteins are phosphorylated and activated by transmembrane serine-threonine receptor kinases in response to TGF-beta signaling. The product of this gene forms homomeric complexes and heteromeric complexes with other activated Smad proteins, which then accumulate in the nucleus and regulate the transcription of target genes.

This protein binds to DNA and recognizes an 8-bp palindromic sequence (GTCTAGAC) called the Smad-binding element (SBE). The Smad proteins are subject to complex regulation by post-translational modifications. Mutations or deletions in this gene have been shown to result in pancreatic cancer, juvenile polyposis syndrome, and hereditary hemorrhagic telangiectasia syndrome.

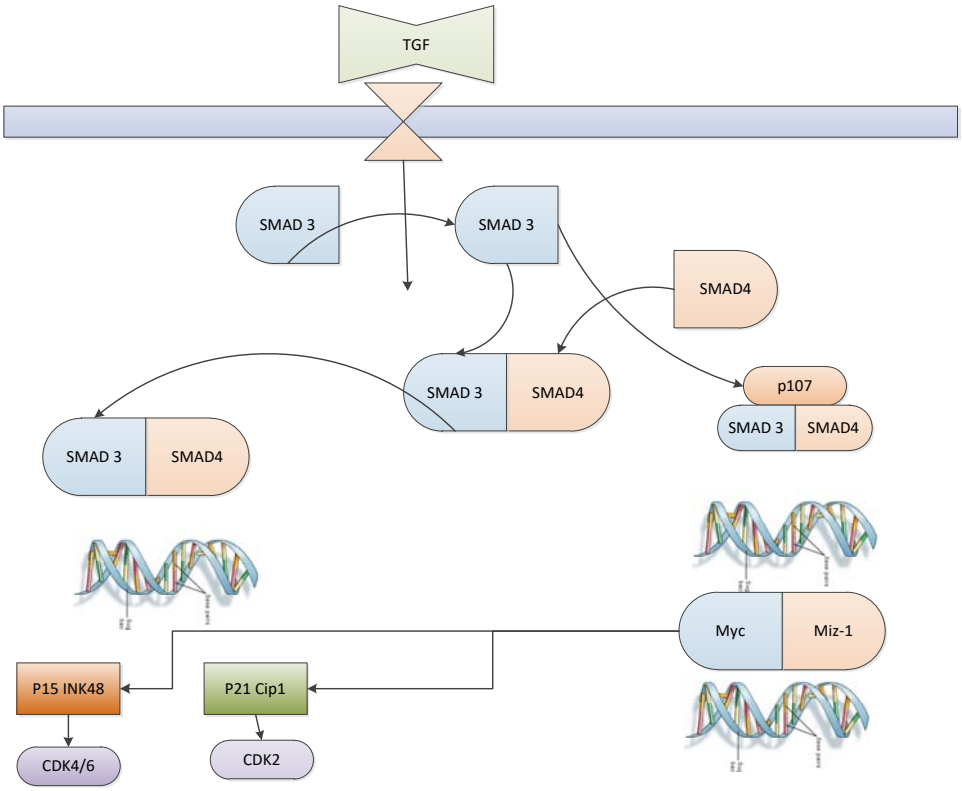
We use the NCI data set for its pathway[29]:

[28] http://www.ncbi.nlm.nih.gov/gene/4089

[29] http://pid.nci.nih.gov/search/pathway_landing.shtml?pathway_id=100160&source=BioCarta&genes_a=4089&genes_b=&what=graphic&jpg=on&ppage=1



The SMAD pathway is also detailed by NCI and one is referred to that source for further detail. From Weinberg (p 291) we also have the SMAD4 pathway showing its immediate control of the DNA transcription.



As Weinberg states (p 292):

"… Half of all pancreatic carcinomas and more than a quarter of all colon carcinomas carry mutant inactivated Smad4 proteins. Without the presence of Smad4 neither Smad2-Smad4 norr Smad3-Smad4 complexes can form. These two complexes are the chief agents dispatched by the TGF-β receptor to the nucleus with the important assignment to shut down proliferation."

This control mechanism is shown above.

8.1.2 TGF Cancers

In the thesis of Schlegel, the author states:[30]

We contend that melanoma cells switch between two defined gene expression signatures, each underlying a distinct cell phenotype, which together drive disease progression. Presented in this thesis are the in vitro and in vivo experimental validations for this model, the investigation of the role of TGF-ß-like signalling, predominantly its role in growth inhibition, and the identification of Id2 as a gene involved in TGF-ß-induced growth inhibition response. After a literature review of genes identified to have phenotype-specific expression, we identified Wnt and TGF-ß signalling as drivers of the identified transcriptional signatures. By in vitro characterization of phenotypically opposed cells, we identified the two phenotypes as proliferative and invasive. As

[30] http://e-collection.ethbib.ethz.ch/eserv/eth:30488/eth-30488-01.pdf

well as showing divergent proliferative and invasive behavior, cell types could be discriminated based on their growth susceptibility to TGF-ß and their capacity for vasculogenic mimicry.

Reduced susceptibility to the growth inhibiting effects of TGF-ß and the capacity for vasculogenic mimicry have both been associated with increased invasive and metastatic properties of melanoma cells. Our model suggests that both proliferative and invasive transcriptional signatures are important in disease progression and that each melanoma cell retains the capacity to express either signature given appropriate signalling. Our model also accounts for much observed gene expression heterogeneity in melanoma tumours.

This heterogeneity and reversibility of transcription programs were also shown in vivo using a xenograft mouse model. We also investigated the motive forces behind differential TGF-ß signalling. Smad activation was present in all melanoma cultures irrespective of the presence of a TGF-ß signature, which suggested Smad-independent TGF-ß signalling. The TGF-ß Smad-dependent pathway has long been considered as being central to TGF-ß signalling but it is now recognized that TGF-ß signals via crosstalk with alternative pathways.

We investigated alternative pathways but could identify no link between the activation status of several MAPK pathways and the TGF-ß signature. TGF-ß is a multifunctional cytokine which controls aspects of cell proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis, immune surveillance, and survival. TGF-ß was initially defined as a transforming cytokine but it is now understood that TGF-ß has dual roles both as tumor suppressor and tumor promoter.

To better understand the regulation behind the expression of these opposite behaviors, we studied TGF-ß's cytostatic effect, which plays an important role in its tumor suppressing function and which is lost as melanoma cells become more invasive and metastatic. We identified the Id2 gene as differentially regulated by TGF-ß and link the loss of its regulation to acquired resistance to TGF-ß in invasive phenotype cells.

We show that TGF-ß induces cell cycle arrest through induction ofpI5fnk4b and repression of Id2. Furthermore, Id2 overexpression in proliferative phenotype cells counteracts pI5fnk4b induction and consequently protects melanoma cells from TGF-ß-mediated inhibition of proliferation.

Treating tumours comprised of cells with variably expressing transcription signatures presents a difficult challenge. This is because specific therapies have targeted factors we identify here as being subject to repeated changes in regulation. It is therefore of primary importance we recognize that the existing paradigm for melanoma progression is insufficient for the design of effective therapies.

The above work is a recent and reasonable summary of TGF status.

8.2 VEGF

The vascular endothelial growth factor is a critical GF associated with vascular growth and ultimately metastasis.

As NCBI notes:

VEGFA[31]*: This gene is a member of the PDGF/VEGF growth factor family. It encodes a heparin-binding protein, which exists as a disulfide-linked homodimer. This growth factor induces proliferation and migration of vascular endothelial cells, and is essential for both physiological and pathological angiogenesis. Disruption of this gene in mice resulted in abnormal embryonic blood vessel formation. This gene is upregulated in many known tumors and its expression is correlated with tumor stage and progression. Elevated levels of this protein are found in patients with POEMS syndrome, also known as Crow-Fukase syndrome. Allelic variants of this gene have been associated with microvascular complications of diabetes 1 (MVCD1) and atherosclerosis. Alternatively spliced transcript variants encoding different isoforms have been described. There is also evidence for alternative translation initiation from upstream non-AUG (CUG) codons resulting in additional isoforms. A recent study showed that a C-terminally extended isoform is produced by use of an alternative in-frame translation termination codon via a stop codon readthrough mechanism, and that this isoform is antiangiogenic. Expression of some isoforms derived from the AUG start codon is regulated by a small upstream open reading frame, which is located within an internal ribosome entry site.*

VEGFB[32]*: his gene encodes a member of the PDGF (platelet-derived growth factor)/VEGF (vascular endothelial growth factor) family. The VEGF family members regulate the formation of blood vessels and are involved in endothelial cell physiology. This member is a ligand for VEGFR-1 (vascular endothelial growth factor receptor 1) and NRP-1 (neuropilin-1). Studies in mice showed that this gene was co-expressed with nuclear-encoded mitochondrial genes and the encoded protein specifically controlled endothelial uptake of fatty acids. Alternatively spliced transcript variants encoding distinct isoforms have been identified.*

In general we would focus on the A type.

8.2.1 VEGF Functioning

We now consider the functions associated with VEGF. It is primarily related to vascular growth and as such becomes a key element in any cancer proliferation. As Botehlo et al note:

Vascular Endothelial Growth Factor (VEGF) is a growth factor involved in the promotion of endothelial cell proliferation, vascular permeability and angiogenesis, which are critical stages

[31] https://www.ncbi.nlm.nih.gov/gene/7422

[32] https://www.ncbi.nlm.nih.gov/gene/7423

for tumor growth and development, namely prostate cancer. It is synthesized by adenocarcinoma cells, and in prostatic cancer patients the prostatic gland contributes considerably to circulating VEGF levels. Elevated plasma VEGF levels could reflect prostatic VEGF production, making VEGF a potentially interesting tumor marker to support the decision of submitting a patient to prostatic biopsy.

Previous studies on this topic are conflicting. Some authors have found higher levels of VEGF in prostatic cancer patients, while others found no differences between subjects with benign prostatic hyperplasia (BPH) and those with malignant disease, or increased values only in patients with metastatic prostatic cancer or hormone-refractory disease. However, most previous studies evaluated relatively small samples and all suffered from limited-challenge bias, as prostatitis, which may interfere with the diagnostic value of VEGF, was not evaluated separately in any of the studies and in many studies the control group only included subjects with no suspicion of prostatic cancer.

We attempted to evaluate VEGF as a diagnostic tool for prostatic cancer, comparing its serum levels across groups of patients with suspected prostate cancer, presenting different prostatic pathologies (including BPH, prostatitis, high grade prostate intraepithelial neoplasia (HGPIN) and prostate cancer)….

VEGF levels are higher in subjects with prostatitis and prostatic cancer compared to patients at high prostate cancer risk but whose prostatic biopsy only revealed normal or hyperplasic tissue. However, in this consecutive series of patients eligible for prostatic biopsy there were no overall differences in VEGF serum levels between subjects with benign prostatic disease and prostate cancer cases….

Our results contribute to explain the heterogeneity observed in the literature on this topic. Prostatitis is an inflammatory condition associated with angiogenesis that raises VEGF levels, similar to the observed in prostate cancer, and may be highly prevalent in patients with increased tPSA levels.

Reports of prostatitis prevalence range from 10% to 63% , and was 16.1% in our series. We observed no relevant difference in VEGF circulating levels between patients with benign prostatic histology and cancer, when patients with prostatitis were also considered in the latter group. The two previous studies that evaluated participants with high risk of prostate cancer also observed no significant associations between cancer and VEGF levels….Other studies showed higher VEGF levels in patients with prostate cancer when compared with healthy controls or subjects with benign prostatic hypertrophy.

Such comparisons however, are not clinically relevant since elevated tPSA is the most frequent indication for prostatic biopsy, and reflect limited-challenge-bias. A diagnostic test must be evaluated in a clinically relevant population, preferably in a consecutive series of individuals in whom the target condition is suspected. Studies using healthy controls, not representing the whole spectrum of potential diagnosis alternative to prostate cancer which are able to generate false-positive results, namely when prostatitis is present, produce inflated estimates of diagnostic accuracy.

We have noted before two of the VEGF isoforms. It is worth noting the others as well. As Yang et al note:

VEGF is the key regulator in tumour angiogenesis and expressed in all solid tumours studied. Its expression has been regarded as a risk factor for metastases from colon and breast cancer. VEGF belongs to a family of angiogenic factors and consists of several isoforms termed as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PIG-F.

Among these factors VEGF-A plays an essential role in angiogenesis which binds to two tyrosine kinase receptors, named VEGFR-1 and VEGFR- 2. VEGF-A have six isoforms with different lengths of amino acid residues resultant from alternative splicing. VEGFA is involved in every stage of vascular development due to its potential of inducing endothelial cell proliferation and survival. The capability of VEGF in promotion of vascular ***endothelial cell (EC)*** *proliferation has been well documented in in vitro and in vivo models, its pro-survival effect on EC has been suggested by a study in which the ablation of VEGF has significantly increased apoptosis of EC.*

VEGF is also a vascular permeability factor which enhances vascular leakage and permeability. Induction of vascular permeability is an essential early step in angiogenesis which results in leakage of plasma proteins, including fibrinogen and other clotting proteins. The clotting system is rapidly activated by tissue factors and results in the deposition of extravascular fibrin in tumour stroma; the fibrin can transform the antiangiogenic stroma into a provisional stroma that is strongly pro-angiogenic. Consistent with a role in the regulation of vascular permeability, VEGF also induces endothelial fenestration in some vascular beds

Recently Karaman et al have noted:

The vascular circulatory system evolved to enable the shuttling of nutrients, oxygen or waste products between various tissues, employing networks of blood vessels and lymphatic vessels that arise by the processes of angiogenesis and lymphangiogenesis, respectively.

Over the past few decades, vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) have emerged as the principal drivers of angiogenesis and lymphangiogenesis, and hence the development and maintenance of both of these vascular systems.

The field of VEGF/VEGFR signaling was established by seminal papers describing the functional role of VEGFA (which was initially named as VPF, for vascular permeability factor) and the identification of VEGFA as an endothelial growth factor These discoveries were followed by the identification of the receptor tyrosine kinases VEGFR1 (FLT1), VEGFR2 (KDR/FLK1) and VEGFR3 (FLT4), which were later shown to bind to VEGFs.

Since then, a multitude of studies has provided insights into the mechanisms and regulation of VEGF/VEGFR signaling. As organ development and function relies heavily on the parallel development and maintenance of organ-specific vascular systems, understanding the role and

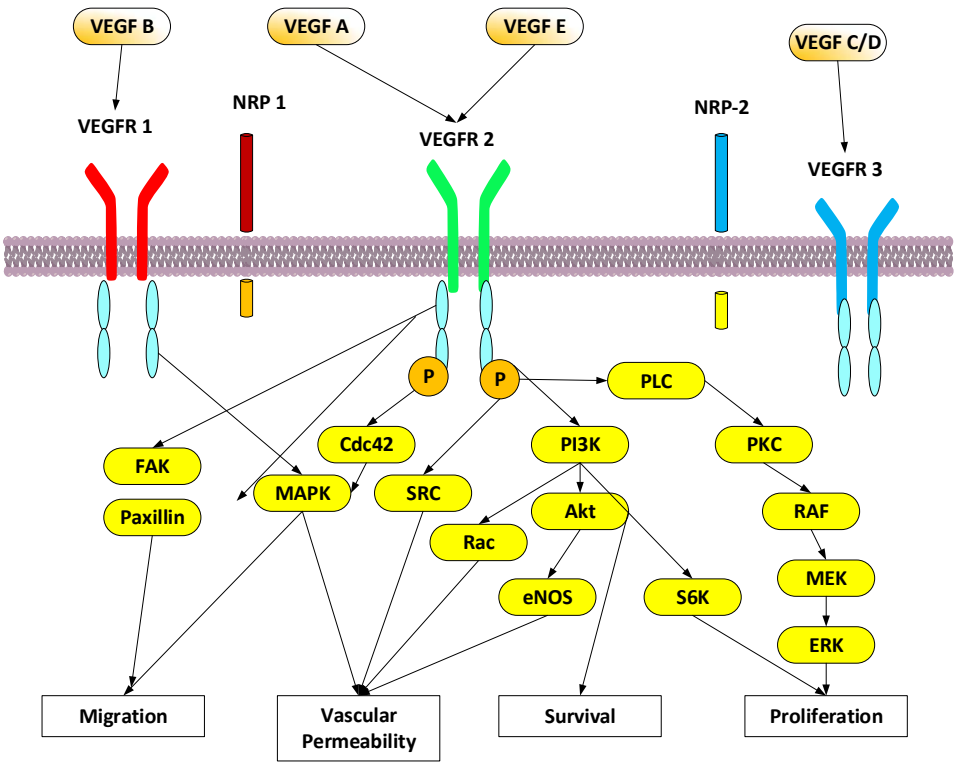
contribution of VEGF/VEGFR signaling to these processes is essential for furthering our understanding of development.

In addition, it is now clear that VEGF signaling is essential for the physiological function of many tissues and plays important roles in the pathogenesis of diseases such as cardiovascular disease, ***cancer*** *and ocular disease….*

During the past few decades, it has been established that angiogenesis is essential for embryonic development and homeostasis in adults, as well as for the progression of cancer and other diseases. More recently, lymphangiogenesis was also shown to be essential for embryonic development, and to be involved in many pathological processes such as lymphedema, inflammatory diseases and ***tumor metastasis****.*

Our knowledge of the circulatory system in general and of the molecular mechanisms controlling angiogenesis and lymphangiogenesis has improved considerably due to progress in the identification of regulatory molecules and markers specific to the blood vascular and lymphatic endothelium.

The signalling pathways for VEGF are shown below:



8.2.2 VEGF and Cancers

We have noted the impact of VEGF in PCa but it is a player in a broad base of cancers. As Fryczkowski et al note:

VEGF secreting tumors are able to grow rapidly and metastasize. Solid tumors require consistent angiogenesis together with tumor growth to supply them with nutrients and oxygen. High concentration of VEGF in plasma of patients with tumor results in poor prognosis. Hepatocyte growth factor (HGF) is another molecule inducing tumor angiogenesis. It was initially identified as a potent hepatotrophic factor responsible for liver regeneration, but now its other functions like mediating tumor-stromal interaction with morpho-, moto-, and mitogenic activities have become known.

We shall discuss HGF next but the complex interaction between various GF/GFR is an essential observation. The complexity of these interactions has not yet been fully understood. The authors continue:

Moreover, HGF intensifies the potential angiogenic activity in vascular endothelial cells [40]; it also acts as a paracrine factor responsible for morphogenesis, cell growth, and cell motility. It has been shown that cancer-associated fibroblasts promote cell scattering, epithelial-mesenchymal transition (EMT), and migration of cancer cells in an HGF-dependent manner. Both HGF and c-Met are upregulated in different types of human cancers such as breast, lung, colorectal, gastric, and oesophageal cancer

Now VEGF errors can affect a wide variety of cell aberrations. For example in retinopathy:

The increased expression of VEGF has become a focal point of current research on the pathogenesis of diabetic retinopathy, as well as other retinal and choroidal vascular diseases. The VEGFs are a family of peptides produced from a single gene by alternative splicing. VEGF isoforms are specifically mitogenic for vascular endothelial cells and also increase permeability at blood–tissue barriers — hence the original name, vascular permeability factor. VEGF is essential for the formation of the fetal vascular system; targeted disruption (knockout) of the VEGF gene in mice leads to impaired vasculogenesis and death in utero.

Normally, VEGF expression decreases substantially after birth, but some cells constitutively secrete picomolar amounts; cells in the neural retina secrete 15 to 20 pg per milligram of protein, and cells in the combined choroid and retinal pigment epithelium secrete 50 pg per milligram of protein. Constitutive VEGF secretion from the retinal pigment epithelium is asymmetric, occurring primarily from the basal surface of these cells, and perhaps accounts in part for the richly vascular choriocapillaris, which lies opposite the basal surface of the retinal pigment epithelium.

The choriocapillary endothelium is itself asymmetrical, with a thin, fenestrated inner portion facing the retinal pigment epithelium and a thick, nonfenestrated outer portion facing the deeper layers of the choroid. In vitro experimentation has shown that VEGF appears to induce endothelial fenestrations in cultured capillary endothelial cells that are derived from bovine adrenal cortex. Endothelial fenestrations are thought to increase vascular permeability. VEGF expression is enhanced by hypoxia, which is a major stimulus for retinal neovascularization. Reduced retinal blood flow and accompanying hypoxia may be present even before the early signs of retinopathy, such as loss of capillary pericytes and endothelial cells, are identified, and

these changes are likely to be accompanied by an increase in the synthesis and secretion of VEGF.

Indeed, increased VEGF protein has been demonstrated by immunocytochemical analysis of nonvascular cells in the eyes of persons with diabetes even in the absence of retinopathy, supporting the hypothesis that diabetic retinopathy begins as a disease of retinal neurons and glia and only later involves the retinal vasculature.

Thus understanding VEGF and its functions is essential to understanding not only malignancies but a variety of other cellular aberrations.

8.3 HGF

The hepatocyte growth factor, HCF, has already been mentioned in its interactions with VEGF.

As NCBI notes[33]:

This gene encodes a protein that binds to the hepatocyte growth factor receptor to regulate cell growth, cell motility and morphogenesis in numerous cell and tissue types. Alternative splicing results in multiple transcript variants, at least one of which encodes a preproprotein that is proteolytically processed to generate alpha and beta chains, which form the mature heterodimer. This protein is secreted by mesenchymal cells and acts as a multi-functional cytokine on cells of mainly epithelial origin. This protein also plays a role in angiogenesis, tumorogenesis, and tissue regeneration. Although the encoded protein is a member of the peptidase S1 family of serine proteases, it lacks peptidase activity. Mutations in this gene are associated with nonsyndromic hearing loss.

HGF activates the MET pathway and in turn MEK and results in proliferation. As Linehan and Ricketts note:

METencodes the cell surface receptor for the growth factor, hepatocyte growth factor (HGF). Growth factor-dependent activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway increases cell surface expression of nutrient transporters, resulting in increased uptake of glucose, amino acids and other nutrients, and increased growth and proliferation through the up regulation of the PI3K-AKT and PI3K-RAS-Erk pathways.

Additionally, nutrient-stimulated HGF/MET signaling induces phosphorylation of LKB1 on Ser428 through the RAS-Erk1/2-p90RSK pathway in a manner that results in uncoupling it with its low energy sensing partner, 5'AMP -activated protein kinase (AMPK).(38) If this occurred in an uncontrolled manner it would inhibit AMPK activation in the presence of low energy levels and implicates HGF/MET activation in deregulation of the LKB1-AMPK- mTOR nutrient and energy sensing pathway.

Therefore normal growth patterns can be controlled by the growth factor receptor activity levels and the surrounding nutrient levels, whereas the mutated constitutively active HGF/MET can drive uncontrolled growth irrelevant of the surrounding environmental conditions and overcome the negative regulation of AMPK.

A clinical trial has been conducted evaluating the role of a small molecule inhibitor of both HGF/MET and VEGFR2/KDR in patients with papillary kidney cancer either with or without germline METmutations. A partial response to therapy has been seen in patients with papillary kidney cancer, and results in patients with or without germline METmutation is currently being analyzed

[33] https://www.ncbi.nlm.nih.gov/gene/3082

8.3.1 HGF Functions

As noted, HGF functions via the MET pathway. From Yang et al we have the following discussion regarding that function:

HGF is the most potent mitogen for mature hepatocytes in primary culture and acts as a trigger for liver regeneration after partial hepatectomy and liver injury. It is also implicated in the metastatic spread of tumours as a scatter factor and has been proposed as a strong and independent predictor of recurrence in human breast cancer.

Its receptor (cMET) is over-expressed in most human cancers. Colon cancer cell invasion and motility potential is significantly increased following incubation with HGF.

This suggests that HGF plays an important role in cancer metastasis initiation. Membrane ruffling is an early event in cell movement; HGF induces rapid membrane ruffling, formation of microspikes and increased cell motility in colon cancer cells indicating HGF enhances cell motility through induction of cell membrane ruffling. An investigation into the tyrosine phosphorylation and translocation of ruffling proteins in colon cancer cells has found that HGF stimulates the function of the ruffling protein (ezrin) which initiates cancer cell membrane ruffling and other early signals for cancer cells to move and invade.

The mechanisms which trigger cancer cell membrane ruffling are largely unestablished. A further study indicated that cyctosolic free Ca2+ may be involved in the mechanism (64). In addition to acting as a cancer cell motility and invasion stimulator, HGF also enhances cancer angiogenesis by increasing Vascular Endothelial Growth Factor (VEGF) promoter activity and inducing hypoxia inducible factor-1 (HIF- 1) expression. The pivotal role and the comprehensive function of HGF and its receptor (cMET) in cancer metastasis initiation and development...

The HGF impact is across a wide variety of cell types. As Peruzzi and Bottaro note:

On binding to the cell surface receptor tyrosine kinase (TK) known as c-Met, hepatocyte growth factor (HGF) stimulates mitogenesis, motogenesis, and morphogenesis in a wide range of cellular targets including, epithelial and endothelial cells, hematopoietic cells, neurons, melanocytes, and hepatocytes.

These pleiotropic actions are fundamentally important during development, homeostasis, and tissue regeneration. HGF signaling also contributes to oncogenesis and tumor progression in several human cancers and promotes aggressive cellular invasiveness that is strongly linked to tumor metastasis.

Our present understanding of c-Met oncogenic signaling supports at least three avenues of pathway selective anticancer drug development: antagonism of ligand/receptor interaction, inhibition of TK catalytic activity, and blockade of intracellular receptor/effector interactions.

Potent and selective preclinical drug candidates have been developed using all three strategies, and human clinical trials in two of the three areas are now under way.

The identification of hepatocyte growth factor (HGF) as the natural ligand for the c-Met receptor protein and the identity of scatter factor (SF) and HGF united a collection of findings showing that a single receptor transduced multiple biological activities, including motility, proliferation, survival, and morphogenesis (3–6). Both HGF/SF and c-Met proteins are processed proteolytically from single-chain precursors into mature disulfide-linked heterodimers.

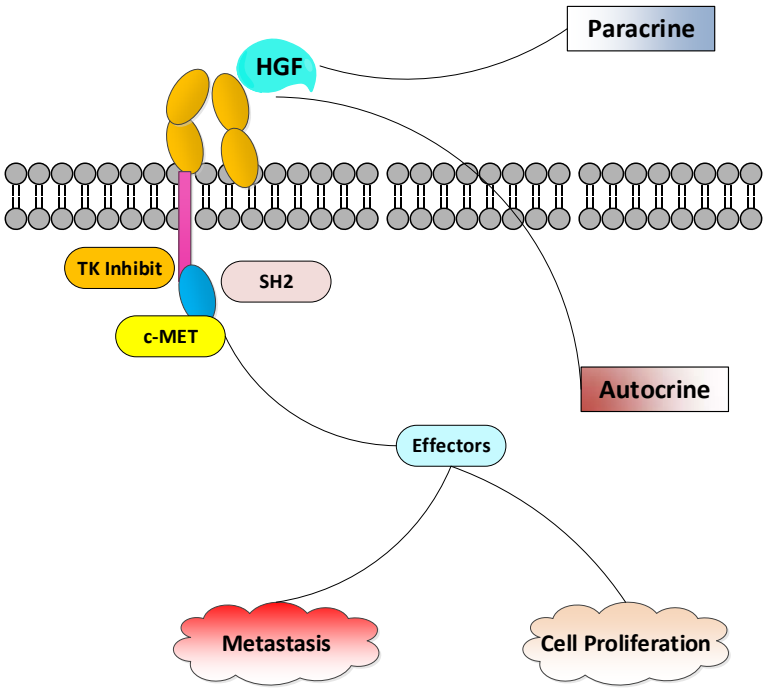
Both are widely expressed early in development; deletion of either gene causes lethal disruptions to embryogenesis; and widespread expression persists throughout adulthood (3, 4, 6).

Both MET and HGF/SF genes are up-regulated after kidney, liver, or heart injury, suggestive of a general homeostatic mechanism of protection against tissue damage and promotion of tissue repair and regeneration (7–11).

Upon HGF/SF binding, c-Met autophosphorylation occurs on two tyrosine residues (Y1234 and Y1235) within the activation loop of the TK domain, which regulate kinase activity.

Phosphorylation on two tyrosine residues near the COOH terminus (Y1349 and Y1356) forms a multifunctional docking site that recruits intracellular adapters via Src homology- 2 domains and other recognition motifs, leading to downstream signaling. An intact multifunctional docking site is required to mediate transformation and induce a metastatic phenotype.

The HGF paths are graphically shown below:



8.3.2 HGF and Cancers

As Linehan et al note in discussing Kidney Cancer:

The proto-oncogene MET (hepatocyte growth factor receptor) was identified as the gene for hereditary papillary renal carcinoma by genetic linkage analysis in families with this inherited renal cancer syndrome. MET encodes the cell surface receptor for ***hepatocyte growth factor (HGF), which is involved in mitogenesis, morphogenesis and motogenesis.***

Activating mutations in the tyrosine kinase domain of MET, have been detected in the germline of affected patients and in a subset of sporadic type 1 papillary kidney cancers.

The histological patterns of hereditary and sporadic type 1 papillary kidney tumors with MET mutations share a distinct morphological phenotype consisting of papillary or tubulo/papillary architecture with slender short papillae containing delicate fibrovascular cores lined by small cells with low grade basophilic nuclei and scant amphophilic cytoplasm.

One effect of growth factor-dependent activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway is increased cell surface expression of nutrient transporters increasing uptake of amino acids, glucose and other nutrients.(4) Nutrient-stimulated HGF/MET signaling induces phosphorylation of serine/threonine-protein kinase 11 [STK11; also referred to as LKB1, the upstream kinase of 5'AMP–activated protein kinase (AMPK)] on Ser428 through the RAS-Erk1/2-p90RSK pathway, implicating MET in the LKB1-AMPK-mTOR nutrient and energy sensing pathway. A clinical trial is currently underway to determine the effect of foretinib, a kinase inhibitor of both MET and VEGF receptors, in patients with papillary kidney cancer (hereditary and sporadic). There is early evidence of efficacy of this agent in patients with germline mutations in the tyrosine kinase domain of MET.

It is possible that there would be response to an agent which has activity against MET in tumors that are characterized by a mutation in the tyrosine kinase domain of MET; it is also possible that such an agent would have activity in tumors which have MET amplification. It is not known if an agent such as foretinib would have activity against kidney tumors that are caused by mutation of other genes such as TSC1 or TSC2.

8.4 PDGF

PDGF is the platelet derived growth factor. There are multiple variants which we detail as follows.

PDGF	***Description***
PDGFA[34]	This gene encodes a member of the protein family comprised of both platelet-derived growth factors (PDGF) and vascular endothelial growth factors (VEGF). The encoded preproprotein is proteolytically processed to generate platelet-derived growth factor subunit A, which can homodimerize, or alternatively, heterodimerize with the related platelet-derived growth factor subunit B. These proteins bind and activate PDGF receptor tyrosine kinases, which play a role in a wide range of developmental processes. Alternative splicing results in multiple transcript variants.
PDGFB[35]	This gene encodes a member of the protein family comprised of both platelet-derived growth factors (PDGF) and vascular endothelial growth factors (VEGF). The encoded preproprotein is proteolytically processed to generate platelet-derived growth factor subunit B, which can homodimerize, or alternatively, heterodimerize with the related platelet-derived growth factor subunit A. These proteins bind and activate PDGF receptor tyrosine kinases, which play a role in a wide range of developmental processes. Mutations in this gene are associated with meningioma. Reciprocal translocations between chromosomes 22 and 17, at sites where this gene and that for collagen type 1, alpha 1 are located, are associated with dermatofibrosarcoma protuberans, a rare skin tumor. Alternative splicing results in multiple transcript variants
PDGFC[36]	The protein encoded by this gene is a member of the platelet-derived growth factor family. The four members of this family are mitogenic factors for cells of mesenchymal origin and are characterized by a core motif of eight cysteines. This gene product appears to form only homodimers. It differs from the platelet-derived growth factor alpha and beta polypeptides in having an unusual N-terminal domain, the CUB domain. Alternatively spliced transcript variants have been found for this gene.

[34] https://www.ncbi.nlm.nih.gov/gene/5154

[35] https://www.ncbi.nlm.nih.gov/gene/5155

[36] https://www.ncbi.nlm.nih.gov/gene/56034

PDGF	***Description***
PDGFD[37]	The protein encoded by this gene is a member of the platelet-derived growth factor family. The four members of this family are mitogenic factors for cells of mesenchymal origin and are characterized by a core motif of eight cysteines, seven of which are found in this factor. This gene product only forms homodimers and, therefore, does not dimerize with the other three family members. It differs from alpha and beta members of this family in having an unusual N-terminal domain, the CUB domain. Two splice variants have been identified for this gene.

8.4.1 PDGF Functions

As Pietras et al note:

Four PDGF polypeptide chains have been identified, which make up five dimeric PDGF isoforms: PDGF-AA, -AB, -BB, -CC, and -DD. The isoforms exert their cellular effects through tyrosine kinase a- and p-receptors. All PDGF isoforms, except PDGF-DD, induce PDGF a-receptor dimerization, whereas PDGF-BB and -DD activate PDGF preceptor dimers. In addition, all isoforms except PDGF-AA activate both receptor types in cells coexpressing the a- and p-receptors.

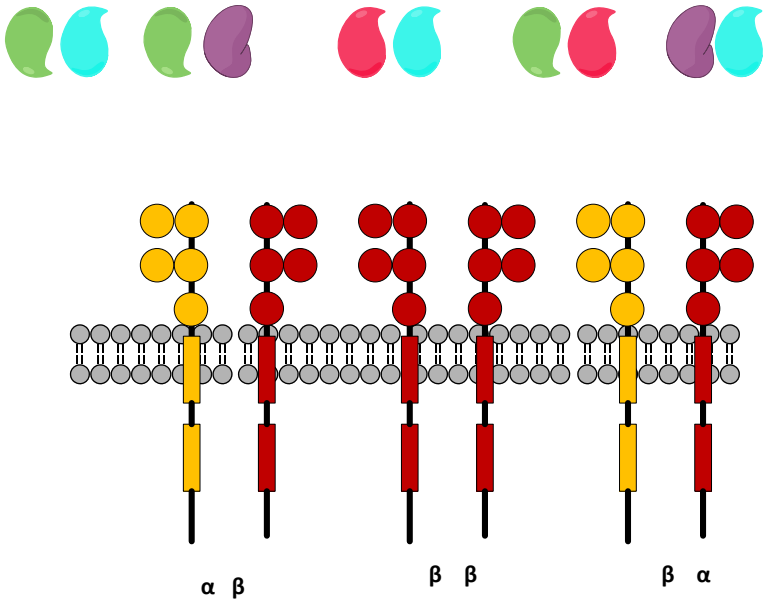
Ligand-induced receptor dimerization causes receptor autophosphorylation, whereafter intracellular signaling pathways are activated by recruitment of SH2 domain-containing signaling molecules (e.g., c-Src, phospholipase C-y, phos- phatidyl-inositol-3'-kinase and the Grb2/Sos complex) to specific phosphorylated tyrosine residues. Activation of these pathways ultimately induces various cellular responses, including cell proliferation, survival, and migration.

Targeting of the genes for PDGF-A and B chains, and for the two receptors, has provided a detailed understanding of the physiological functions of PDGF during development. The selective upregulation of PDGF receptors on endothelial cells in the mouse model of prostate cancer bone metastases should stimulate further study of expression of PDGF receptors on tumor endothelial cells. The frequent expression of PDGF receptors on perivascular cells also suggests as yet unexploited therapeutic opportunities. The well-documented effect of PDGF on pericyte recruitment points to the possibility of combining anti-endothelial agents, like vascular endothelial growth factor receptor inhibitors, with pericyte-targeting PDGF antagonists.

Finally, it has been shown that bone marrow- derived cells contribute to the angiogenic switch in tumors (Coussens et al., 2000). PDGF receptor inhibitors can possibly inhibit this process, since PDGF stimulates migration and proliferation of macrophages

[37] https://www.ncbi.nlm.nih.gov/gene/80310

From Litwak (p528) we have the following 3 PDGFR and five PDGF structures. The PDGFR gave two separate chains, an A and a B, or as shown, an α and β. There are five PDGF, A thru E, but we focus on the first 4 only as noted above.



8.4.2 PDGF and Cancer

Platelet derive growth factors are known to be drivers and facilitators of various cancers. As Chen et al note:

Platelet-derived growth factor-D (PDGF-D) plays a crucial role in the progression of several cancers. However, its role in colorectal cancer (CRC) remains unclear. Our study showed that PDGF-D was highly expressed in CRC tissues and was positively associated with the clinicopathological features. Down-regulation of PDGF-D inhibited the tumor growth, migration and angiogenesis of SW480 cells in vitro and in vivo. Whereas up-regulation of PDGF-D promoted the malignant behaviors of HCT116 cells. Moreover, PDGF-D up-regulated the expression of Notch1 and Twist1 in CRC cells.

In addition, PDGF-D expression promoted Epithelial to mesenchymal transition (EMT), which was accompanied with decreased E-cadherin and increased Vimentin expression. Consistently, PDGF-D, Notch1, and Twist1 are obviously up-regulated in transforming growth factor-beta 1 (TGF-β1) treated HCT116 cells. Since Notch1 and Twist1 play an important role in EMT and tumor progression, we examined whether there is a correlation between Notch1 and Twist1 in EMT status.

Our results showed that up-regulation of Notch1 was able to rescue the effects of PDGF-D down-regulation on Twist1 expression in SW480 cells, whereas down-regulation of Notch1 reduced Twist1 expression in HCT116 cells. Furthermore, we found that Twist1 promoted EMT

and aggressiveness of CRC cells. These results suggest that PDGF-D promotes tumor growth and aggressiveness of CRC, moreover, down-regulation of PDGF-D inactivates Notch1/Twist1 axis, which could reverse EMT and prevent CRC progression.

They further make the following observations regarding PDGF-D as an example:

1. PDGF-D is highly expressed in CRC tissues and cell lines
2. PDGF-D expression promotes cell growth and colony formation in CRC cell lines
3. PDGF-D expression promotes cell cycle distribution, aggressiveness, and angiogenesis, but not apoptosis in CRC cell lines
4. PDGF-D increases the expression of Notch1 in CRC cells
5. PDGF-D induces the EMT profile in CRC cells
6. PDGF-D is significantly increased in TGF-β1 treated HCT116 cells
7. Downregulation of PDGF-D reversed EMT in TGF-β1 treated HCT116 cells
8. PDGF-D promotes cell growth, aggressiveness and EMT transformation of CRC through activation of Notch1/Twist1 pathway
9. PDGF-D promotes tumorigenesis, angiogenesis and EMT profile of CRC cells in vivo

8.5 IGF

The IGF, insulin growth factor, and the IGFR, and its respective sub-elements, are major factors in many malignancies. For example, as noted in NCBI:

IGF1[38]*: The protein encoded by this gene is similar to insulin in function and structure and is a member of a family of proteins involved in mediating growth and development. The encoded protein is processed from a precursor, bound by a specific receptor, and secreted. Defects in this gene are a cause of insulin-like growth factor I deficiency. Alternative splicing results in multiple transcript variants encoding different isoforms that may undergo similar processing to generate mature protein.*

IGF1R[39]*: This receptor binds insulin-like growth factor with a high affinity. It has tyrosine kinase activity. The insulin-like growth factor I receptor plays a critical role in transformation events. Cleavage of the precursor generates alpha and beta subunits. It is highly overexpressed in most malignant tissues where it functions as an anti-apoptotic agent by enhancing cell survival. Alternatively spliced transcript variants encoding distinct isoforms have been found for this gene.*

There has been a great deal of study of the IGF and its constituents[40].

8.5.1 IGF Overview

The insulin growth factor is a key element in glucose control. Spravchikov et al have discussed the impact of poor glucose management on skin keratinocytes. This discussion is critical in trying to understand the role of the IGF and glucose on cancer initiation and progression. Thus it is worth a mild digression to understand their findings.

They note:

Glucose is known to affect insulin action as well by regulating the expression of several genes, including the IGF-I receptor (IGFR) and insulin receptor (IR) genes, at both the transcriptional and translational levels. Moreover, hyperglycemia was shown to inhibit insulin action. This inhibition is thought to be a result of serine phosphorylation through a PKCmediated mechanism as well as by activation of protein tyrosine phosphatases, which deactivates the IR function. In addition to its possible involvement in the development of complications of chronic diabetes, glucose was shown to downregulate its own transport and metabolism. As a result, high glucose levels create a vicious cycle in which even less glucose enters the cells, resulting in increased

[38] https://www.ncbi.nlm.nih.gov/gene/3479

[39] https://www.ncbi.nlm.nih.gov/gene/3480

[40] https://www.sciencedirect.com/topics/neuroscience/insulin-like-growth-factor-1

blood glucose levels, which in turn further disrupt the transport and metabolism of glucose into the cells. It is therefore clear that glucose per se, either directly or via changes in insulin signaling, is an important factor in both the regulation of its own transport and metabolism and in the pathogenesis of chronic complications of diabetes… Glucose inhibits the phosphorylation of the IGFR.

We have shown so far that exposure of keratinocytes to high glucose concentrations, mimicking the hyperglycemic state, has effects on skin cells, resulting in inhibition of proliferation and an abnormal differentiation process. However, in diabetic patients, development of hyperglycemia also results in changes in insulin and IGF-I signaling….

As mentioned earlier, another effect of insulin and IGF-I on keratinocytes is an increase in cellular proliferation. Therefore, we evaluated the proliferation rate of keratinocytes in response to chronic insulin or IGF-I stimulation in the presence of 2 or 20 mmol/l D-glucose. …, both insulin and IGF-I induced an increase in the proliferation rate of the cells (142 and 155% above control, respectively). However, in the presence of high glucose concentrations, the effects of both hormones—but mainly of IGF-I—were reduced (129 and 123% above control, respectively). Glucose effects were specific, as there was no effect on the activity of keratinocyte growth factor on glucose transport…

We have previously shown that in skin keratinocytes, IR and IGFR have different roles in skin proliferation that are mediated via distinct signaling pathways. In addition, we have shown in the present study that high glucose levels, in the absence of any additional perturbation, are associated with decreased cellular proliferation. Thus, glucose inhibits proliferation by both direct effects as well as by reducing the stimulatory effect of IGF-I on proliferation. In conclusion, the consequence of high glucose inhibition on the proliferation of skin keratinocytes and its enhancement of their differentiation is obvious.

By changing the proliferation-differentiation balance, which is one of the essential steps in the healing process, as well as by decreasing other possible local effects of IGF-I on wound healing, high glucose levels might indeed contribute to impaired wound healing in diabetes.

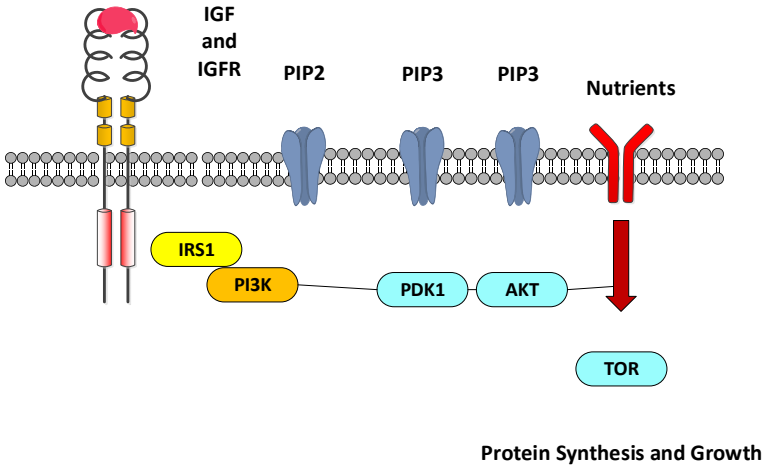
From Yang et al:

The insulin-like growth factor system consists of two ligands (IGF-I and -II), two main receptors (IGF-IR and IGFIIR), six different IGF binding proteins (IGFBP1- 6) and four IGFBP related peptides (IGFBP Rp1-4). The IGF ligands have a short life-span unless they are bound to a binding protein which transports them in the circulation and delivers them to specific tissues. Components of the IGF system are found throughout the body in various fluids and tissues. IGFs act on a variety of mammalian cells in an endocrine, paracrine and autocrine manner to regulate cell proliferation, apoptosis, transformation and differentiation. They influence the growth of normal tissue as well as that of several cancers.

Converging data from clinical and laboratory studies clearly indicate that IGF-I is implicated in cancer cell migration and invasion. IGF-I receptor (IGF-IR) expression is correlated with colorectal cancer venous invasion and liver metastasis, and has been proposed as a predictor of

liver metastasis from colorectal cancer. Blockade of the paracrine action of IGF-I can suppress liver metastases from colorectal cancer. It has been established that IGF-IR and the integrins interact together to form a complex at the colon cell-cell contact sites, whilst addition of IGF-I to this complex causes integrin redistribution within the cell-cell contact site and is associated with an increase in the migration of colorectal cancer cells.

From Morgan we have the putative interaction of IGF with the IGFR and the resulting cell reaction as shown below[41]:



8.5.2 IGF Cancers

Excess activation of IGF has been linked to a variety of cancers. As Murekatete et al have noted regarding melanomas:

Insulin-like growth factor (IGF)-I binds to the ECM protein vitronectin (VN) through IGF binding proteins (IGFBPs) to enhance proliferation and migration of skin keratinocytes and fibroblasts. Although evidence exists for the role of individual components of the complex (IGF-I, IGFBP-3 and VN), the cellular functions stimulated by these proteins together as a complex remains un-investigated in melanoma cells. We report here that the IGF-I:IGFBP-3:VN trimeric complex stimulates a dose dependent increase in the proliferation and migration of WM35 and Sk-MEL28 melanoma cells.

In 3D Matrigel™ and hydrogel cultures, both cell lines formed primary tumor-like spheroids, which increased in size in a dose-dependent manner in response to the trimeric complex. Furthermore, we reveal IGFBP- 3:VN protein complexes in malignant melanoma and squamous cell carcinoma patient tissues, where the IGFBP-3:VN complex was seen to be predominantly tumor cell-associated. Peptide antagonists designed to target the binding of IGF-I:IGFBP-3 to VN were demonstrated to inhibit IGF-I:IGFBP- 3:VN-stimulated cell migration, invasion and

[41] See Morgan, p 216

3D tumor cell growth of melanoma cells. Overall, this study provides new data on IGF:ECM interactions in skin malignancies and demonstrates the potential usefulness of a growth factor:ECM-disrupting strategy for abrogating tumor progression.

They continue:

The high mortality rate of melanoma is associated with the metastasis of malignant melanoma cells to critical organs of the body1. Insulin-like growth factor-I (IGF-I), amongst others, is known to enhance tumor growth and invasion2. IGF-I can act as a paracrine factor that drives malignant cell transformation through the activation of the IGF type-I receptor (IGF-IR)3. All melanocytic cells express the IGF-IR, with increased expression correlated with disease progression4,5.

In addition, growth factor interactions with the extracellular matrix (ECM) play important roles in tumor biology, facilitating tumor cell attachment, proliferation and invasion, and resistance against chemotherapeutic drugs. Proteins in the IGF system have been shown to interact with ECM proteins such as fibronectin (FN), vitronectin (VN), laminins, as well as integrins, which in turn, modulate the function of IGF-I9,10. Previous studies have demonstrated that IGF-I interacts with VN through IGFBPs to form IGF-I:IGFBP:VN trimeric (TRI) complexes. Further, IGFBP:VN complexes have been observed in tumor biopsies from breast cancer patients, associating with the invasive front of tumor clusters and around tumor blood vessels.

This is aligned with the concept that VN is a matricellular protein that functions as a scaffold onto which growth factors, such as IGF-I, are captured, exposing cells to concentrated foci of growth factors available for receptor stimulation. Indeed, complexes of TRI have been shown to promote enhanced cell attachment and migration, as well as protein synthesis, in human keratinocytes14 and breast cancer cell lines.

8.6 CTGF

CTGF is the connective tissue growth factor.

As NCBI notes[42]:

*The protein encoded by this **gene is a mitogen** that is secreted by vascular endothelial cells. The encoded protein plays a role in chondrocyte proliferation and differentiation, cell adhesion in many cell types, and is related to **platelet-derived growth factor**. Certain polymorphisms in this gene have been linked with a higher incidence of systemic sclerosis.*

As with many of the GF, the mitogenic actions are critical in many if not most cancers. Reducing mitosis is reducing metastasis.

8.6.1 CTGF Functions

Bao et al note that down-regulating CTGF has a significant effect on reducing proliferation. They note:

TGF-β2 promoted proliferation and inhibited apoptosis of human Tenon capsule fibroblasts in a dose-dependent manner. TGF-β2 induced down-regulation of miR-26 and up-regulation of CTGF in a dose-dependent manner. CTGF was the target gene of miR-26 and miR-26 had a negative regulatory effect on CTGF expression. miR-26 up-regulation could significantly decrease proliferation and increase apoptosis of human Tenon capsule fibroblasts after induced by TGF-β2 ($P < 0.05$).

Down-regulation of CTGF could markedly decrease proliferation and increase apoptosis of human Tenon capsule fibroblasts after induced by TGF-β2 ($P < 0.05$). miR-26 could inhibit proliferation and promote apoptosis of human Tenon capsule fibroblasts after they were induced by TGF-β2 through suppressing CTGF expression.

8.6.2 CTGF and Cancer

As Zhu et al note:

In this study, we therefore analyzed the expressions of a large panel of cytokines (132 known cytokines and growth factors) in several tumor-stromal, as well as normal-stromal clinical breast cohorts. The results consistently indicated the potential importance of connective tissue growth factor (CTGF) in tumor progression.

In particular, CTGF is preferentially produced in tumor cells, and the elevated CTGF gene expression in tumor cells significantly correlates with poor clinical prognosis in breast tumors.

[42] https://www.ncbi.nlm.nih.gov/gene/1490 also known as CCN2

Furthermore, in our tissue microarray analysis on 84 patient-derived xenograft models, high protein expression of CTGF correlates remarkably with the stroma-rich tumors that have poor clinical prognosis and outcome. For breast cancer, especially the triple-negative subtype, patients with stroma-rich tumors have shown a significant higher risk of poor prognosis and worse outcome compared to those with stroma-poor tumors, which neither currently used clinic-pathological parameters nor molecular profiling techniques are able to categorize this set of patients with respect to prognosis.

CTGF has previously been identified as a fibrogenic cytokine that is highly expressed in wound healing and fibrotic lesions.

In human cancers, the pleiotropic functions of CTGF have been investigated, including the function as an oncoprotein in glioma and melanoma, but a tumor-suppressor in lung cancer and colon cancer. In breast cancer, studies have shown that CTGF cooperates with other genes to mediate osteolytic metastasis, and high expression of CTGF mRNA in the bulk tumor correlated with advanced tumor stages, however, the mechanistic origin of CTGF has rare been explored. Whether the high level of CTGF is from tumor cells or stromal cells, and furthermore, whether CTGF mediates tumor-stroma dialogue and how CTGF regulates tumor progression in the microenvironment have not yet been clearly shown.

Our data show that CTGF in tumor epithelial cells but not stromal cells had significant clinical relevance, and through a series of bioinformatics and biological analyses, we also identified that

1) CTGF facilitated tumor growth and metastasis via promoting the deposition and orientation of collagen I fibers at the primary tumor stroma;

2) CTGF was capable to promote tumor cell migration, invasion and mammosphere formation via inducing epithelial-mesenchymal transition (EMT); and

3) the CTGF-tumor necrosis factor receptor I (TNFR1)-IκB autocrine signaling is the predominant mechanism in CTGF-mediated tumor progression.

Our data provided amble evidence that targeting the CTGF-TMFR1- IκB signaling is a promising strategy to prohibit breast tumor progression.

8.7 EGF

The epidermal growth factor, EGF, is another GF associated with malignancies. As NCBI notes[43]:

This gene encodes a member of the epidermal growth factor superfamily. The encoded preproprotein is proteolytically processed to generate the 53-amino acid epidermal growth factor peptide. This protein acts a potent mitogenic factor that plays an important role in the growth, proliferation and differentiation of numerous cell types. This protein acts by binding with high affinity to the cell surface receptor, epidermal growth factor receptor. Defects in this gene are the cause of hypomagnesemia type 4.

Dysregulation of this gene has been associated with the growth and progression of certain cancers. Alternative splicing results in multiple transcript variants, at least one of which encodes a preproprotein that is proteolytically processed.

8.7.1 EGF Functions

We begin with a simple overview of the EGF functions. As Singh et al note:

EGF is the prototypic and founding member of the EGFR ligand family, first identified from submaxillary gland extracts during nerve growth factor studies. The EGF-EGFR ligand-receptor system has greatly enhanced our understanding of receptor tyrosine kinase signaling, as evidenced by more than 70,000 publications for EGF alone. A recent review has distilled our current understanding of EGF and its actions.

More recently, a study uncovered that EGF-induced EGFR signaling enhances production of intracellular reactive oxygen species (ROS) by dual oxidase 1 (DUOX1) This nicely complements earlier studies in which ROS were shown to enhance EGFR signaling by modulating both positive and negative regulators of EGFR signaling (ADAMs and protein tyrosine phosphatases). In another recent study, urinary EGF has been shown to be an independent risk factor for progression of chronic kidney disease, substantiating earlier findings.

They then note its functioning:

Modes of signaling via epidermal growth factor receptor (EGFR) ligands.

Autocrine signaling occurs when a ligand is released from a cell and binds to EGFR on that same cell.

Paracrine signaling refers to the released ligand acting on a nearby cell, usually a different cell type.

[43] https://www.ncbi.nlm.nih.gov/gene/1950

Juxtacrine signaling occurs when a non-cleaved, transmembrane ligand binds to EGFR on an adjacent cell; this is best documented for heparin-binding epidermal growth factor-like growth factor (HBEGF). Amphiregulin (AREG), transforming growth factor-alpha (TGFA), and HBEGF, as well as EGFR, can be packaged into signaling competent exosomes. Uptake of exosomal AREG by recipient cells is, at least in part, dependent on EGFR, leading to the term exosomal targeted receptor activation (ExTRAcrine).

ExTRAcrine signaling has features of autocrine, paracrine, and juxtacrine signaling as well as possibly endocrine signaling since EGFR and AREG can be detected in human plasma exosome.

8.7.2 EGF and Cancer

Relationships between EGF and cancers are significant. From Yang et al we have the following:

EGF and its receptor (EGFR) have been associated with tumour cell invasion and metastasis initiation.

Dysregulation of EGFR signalling, including receptor over expression and/or activation has been shown to be a significant effector in the progression of human cancers including neoplasms of the brain, lung, breast, ovary, prostate, and pancreas.

A recent study investigated the relationship between EGFR and the adhesion molecule-integrin in human pancreatic carcinoma cells and demonstrated that the crosstalk between EGFR signalling and integrin in the cancer cell membrane is implicated in carcinoma cell invasion and metastasis. Integrins are a family of adhesion proteins that regulate cell migration.

The fact that EGF stimulated integrins-mediated carcinoma cell migration on vitronectin suggests that EGFR regulates cancer cell migration through the adhesion proteins, the integrins. EGFR inhibitors, such as erlotinib, provide clinical benefit in patients with advanced non-small cell lung cancer metastasis which suggests a critical role for EGF and its receptor in the initial steps of cancer metastasis. The mechanism of EGF activation of adhesion proteins in cancer cell remains to be elucidated.

Some studies indicate EGF induces tumour cell invasion and metastasis through de-phosphorylation and downregulation of focal adhesion kinase, while other studies suggest EGFR activates the Src family of kinases (SFK). The fact that activated Src kinase is involved in the rearrangement of the actin cytoskeleton, cell-matrix interactions, and cell-cell adhesion processes that promote cell invasion suggests a role for Src activity in tumour metastasis development.

Added insight is provided by Mendelsohn and Baselga who note:

Human carcinomas frequently express high levels of receptors in the EGF receptor family, and overexpression of at least two of these receptors, the EGF receptor (EGFr) and closely related ErbB2, has been associated with a more aggressive clinical behavior. Further, transfection or

activation of high levels of these two receptors in nonmalignant cell lines can lead to a transformed phenotype. For these reasons therapies directed at preventing the function of these receptors have the potential to be useful anti-cancer treatments. In the last two decades monoclonal antibodies (MAbs) which block activation of the EGFr and ErbB2 have been developed.

These MAbs have shown promising preclinical activity and `chimeric' and `humanized' MAbs have been produced in order to obviate the problem of host immune reactions. Clinical activity with these antibodies has been documented: trastuzumab, a humanized anti-ErbB2 MAb, is active and was recently approved in combination with paclitaxel for the therapy of patients with metastatic ErbB2-overexpressing breast cancer; IMC- C225, a chimeric anti-EGFr MAb, has shown impressive activity when combined with radiation therapy and reverses resistance to chemotherapy. In addition to antibodies, compounds that directly inhibit receptor tyrosine kinases have shown preclinical activity and early clinical activity has been reported. A series of phase III studies with these antibodies and direct tyrosine kinase inhibitors are ongoing or planned, and will further address the role of these active anti-receptor agents in the treatment of patients with cancer.

Finally from Calderon and Prins[44]:

Epidermal growth factor (Egf), a secreted peptide, is produced by the luminal epithelial cells in the prostate, and is found at the highest concentration in human prostatic secretions compared to the rest of the body. Epidermal growth factor exerts its effects by binding to its tyrosine kinase receptor, epidermal growth factor receptor (EgfR).

Upon binding, EgfR can homo- or heterodimerize with erbB2 receptors, causing autophosphorylation of its tyrosine residues that in turn activate the phosphatidylinositol 3'-kinase (PI3K), mitogen activated protein kinase (MAPK), or phospholipase C-γ (PLC-γ) signaling cascades. In the developing murine prostate gland, Egf has been shown to mediate its actions through the PLC-γ signaling pathway.

Furthermore, rat UGS explants treated with exogenous Egf showed stimulation of prostate bud formation in the absence of androgens, thus positively regulating prostatic budding.

[44] https://www.sciencedirect.com/science/article/pii/B9780128126363000055

8.8 FGF

There are about 20 fibroblast growth factors, FGF. They act both as endocrine and paracrine promoters, being secreted and then acting upon FGF receptors on target cells[45]. In addition certain FGF act in an autocrine manner as well as noted below.

Fibroblasts are cells which inhabit and promote the extracellular matrix. The FGF a named for these cells but are generated in a variety of cells throughout the body. They can stimulate various pathways in target cells initiating proliferation as well as angiogenesis.

From NCBI we have listed a few as follows:

FGF	***Description***
FGF7[46]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. This protein is a potent epithelial cell-specific growth factor, whose mitogenic activity is predominantly exhibited in keratinocytes but not in fibroblasts and endothelial cells. Studies of mouse and rat homologs of this gene implicated roles in morphogenesis of epithelium, re-epithelialization of wounds, hair development and early lung organogenesis.
FGF19[47]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes including embryonic development cell growth, morphogenesis, tissue repair, tumor growth and invasion. This growth factor is a high affinity, heparin dependent ligand for FGFR4. Expression of this gene was detected only in fetal but not adult brain tissue. Synergistic interaction of the chick homolog and Wnt-8c has been shown to be required for initiation of inner ear development.

[45] See Sherbet Chapter 15.

[46] https://www.ncbi.nlm.nih.gov/gene/2252

[47] https://www.ncbi.nlm.nih.gov/gene/9965

FGF	*Description*
FGF10[48]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. This protein exhibits mitogenic activity for keratinizing epidermal cells, but essentially no activity for fibroblasts, which is similar to the biological activity of FGF7. Studies of the mouse homolog of suggested that this gene is required for embryonic epidermal morphogenesis including brain development, lung morphogenesis, and initiation of lim bud formation. This gene is also implicated to be a primary factor in the process of wound healing.
FGF4[49]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities and are involved in a variety of biological processes including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. This gene was identified by its oncogenic transforming activity. This gene and FGF3, another oncogenic growth factor, are located closely on chromosome 11. Co-amplification of both genes was found in various kinds of human tumors. Studies on the mouse homolog suggested a function in bone morphogenesis and limb development through the sonic hedgehog (SHH) signaling pathway.
FGF12[50]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth, and invasion. This growth factor lacks the N-terminal signal sequence present in most of the FGF family members, but it contains clusters of basic residues that have been demonstrated to act as a nuclear localization signal. When transfected into mammalian cells, this protein accumulated in the nucleus, but was not secreted. The specific function of this gene has not yet been determined. Two alternatively spliced transcript variants encoding distinct isoforms have been reported.

[48] https://www.ncbi.nlm.nih.gov/gene/2255

[49] https://www.ncbi.nlm.nih.gov/gene/2249

[50] https://www.ncbi.nlm.nih.gov/gene/2257

FGF	*Description*
FGF3[51]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities and are involved in a variety of biological processes including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. This gene was identified by its similarity with mouse fgf3/int-2, a proto-oncogene activated in virally induced mammary tumors in the mouse. Frequent amplification of this gene has been found in human tumors, which may be important for neoplastic transformation and tumor progression. Studies of the similar genes in mouse and chicken suggested the role in inner ear formation
FGF6[52]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. This gene displayed oncogenic transforming activity when transfected into mammalian cells. The mouse homolog of this gene exhibits a restricted expression profile predominantly in the myogenic lineage, which suggested a role in muscle regeneration or differentiation
FGF22[53]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities and are involved in a variety of biological processes including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. The mouse homolog of this gene was found to be preferentially expressed in the inner root sheath of the hair follicle, which suggested a role in hair development. Alternative splicing results in multiple transcript variants
FGF1[54]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. This protein functions as a modifier of endothelial cell migration and proliferation, as well as an angiogenic factor. It acts as a mitogen for a variety of mesoderm- and neuroectoderm-derived cells in vitro, thus is thought to be involved in organogenesis. Multiple alternatively spliced variants encoding different isoforms have been described.

[51] https://www.ncbi.nlm.nih.gov/gene/2248

[52] https://www.ncbi.nlm.nih.gov/gene/2251

[53] https://www.ncbi.nlm.nih.gov/gene/27006

[54] https://www.ncbi.nlm.nih.gov/gene/2246

FGF	*Description*
FGF2[55]	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members bind heparin and possess broad mitogenic and angiogenic activities. This protein has been implicated in diverse biological processes, such as limb and nervous system development, wound healing, and tumor growth. The mRNA for this gene contains multiple polyadenylation sites, and is alternatively translated from non-AUG (CUG) and AUG initiation codons, resulting in five different isoforms with distinct properties. The CUG-initiated isoforms are localized in the nucleus and are responsible for the intracrine effect, whereas, the AUG-initiated form is mostly cytosolic and is responsible for the paracrine and autocrine effects of this FGF

8.8.1 FGF Functions

The fibroblast growth factor is a complex set of growth factors that are also related to multiple malignancies.

As Ornitz and Itoh note:

The Fibroblast Growth Factor (FGF) family is comprised of secreted signaling proteins (secreted FGFs) that signal to receptor tyrosine kinases and intracellular non-signaling proteins (intracellular FGFs (iFGFs)) that serve as cofactors for voltage gated sodium channels and other molecules.

Additionally, secreted FGFs and iFGFs may have direct functions in the nucleus and functional interactions with other cellular proteins. Members of both branches of the FGF family are related by core sequence conservation and structure and are found in vertebrates and invertebrates.

Secreted FGFs are expressed in nearly all tissues and they serve essential roles in the earliest stages of embryonic development, during organogenesis, and in the adult, where they function as homeostatic factors that are important for tissue maintenance, repair, regeneration, and metabolism).

In general, secreted FGFs function as autocrine or paracrine factors (canonical FGFs; also called paracrine FGFs), however, three members of the secreted FGFs have evolved to function as endocrine factors (endocrine FGFs) with essential roles in the adult where they regulate phosphate, bile acid, carbohydrate and lipid metabolism in addition to the canonical FGF functions that control cell proliferation, differentiation and survival. At the cellular level, secreted FGFs regulate fundamental cellular processes that include positive and negative regulation of proliferation, survival, migration, differentiation, and metabolism.

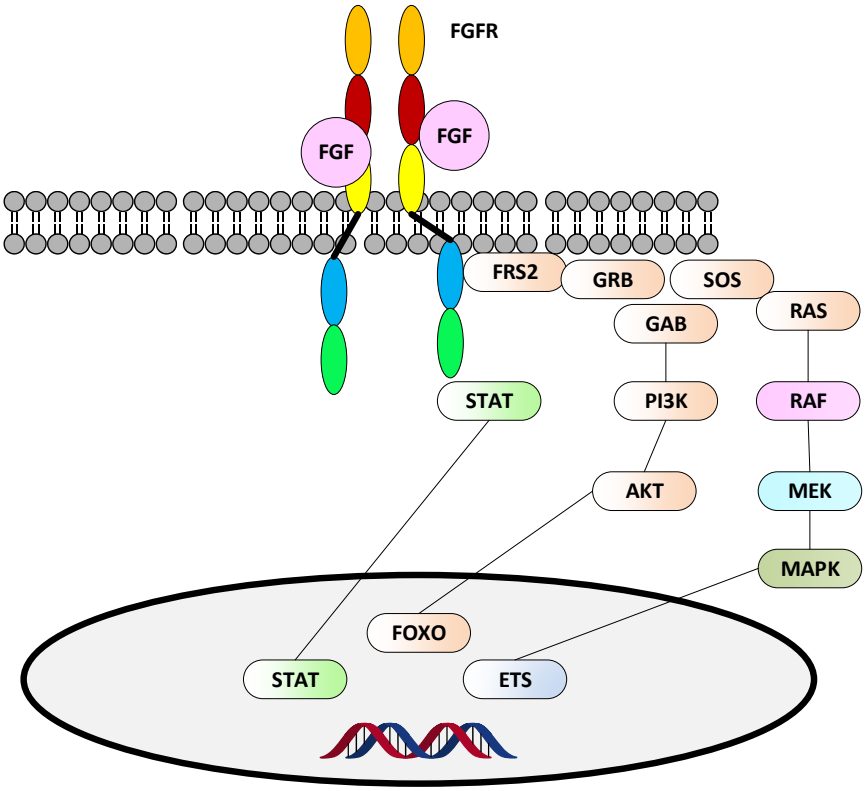
[55] https://www.ncbi.nlm.nih.gov/gene/2247

As Ornitz and Itoh (2001) noted:

Most Fgf genes are found scattered throughout the genome. In human, 22 FGF genes have been identified and the chromosomal locations of all except FGF16 are known. Several human FGF genes are clustered within the genome. FGF3, FGF4 and FGF19 are located on chromosome 11q13 and are separated by only 40 and 10 kb, respectively; FGF6 and FGF23 are located within 55 kb on chromosome 12p13; and FGF17and FGF20 map to chromosome 8p21-p22.

These gene locations indicate that the FGF gene family was generated both by gene and chromosomal duplication and translocation during evolution. Interestingly, a transcriptionally active portion of human FGF7, located on chromosome 15q13-q22, has been amplified to about 16 copies, which are dispersed throughout the human genome.

For the most part FGF1 and FGF2 are predominant as regards to a focus on cancer growth. The cell pathway controls are depicted below. Note they lead to proliferation and growth.



8.8.2 FGF and Cancers

FGF are critical to many cellular proliferations. Thus they like the others are key in many cancers. As Wesche et al state:

FGFs (fibroblast growth factors) and their receptors (FGFRs) play essential roles in tightly regulating cell proliferation, survival, migration and differentiation during development and adult life. Deregulation of FGFR signalling, on the other hand, has been associated with many developmental syndromes, and with human cancer. In cancer, FGFRs have been found to become overactivated by several mechanisms, including gene amplification, chromosomal translocation and mutations. FGFR alterations are detected in a variety of human cancers, such as breast, bladder, prostate, endometrial and lung cancers, as well as haematological malignancies.

Accumulating evidence indicates that FGFs and FGFRs may act in an oncogenic fashion to promote multiple steps of cancer progression by inducing mitogenic and survival signals, as well as promoting epithelial– mesenchymal transition, invasion and tumour angiogenesis. Therapeutic strategies targeting FGFs and FGFRs in human cancer are therefore currently being explored. In the present review we will give an overview of FGF signalling, the main FGFR alterations found in human cancer to date, how they may contribute to specific cancer types and strategies for therapeutic intervention.

FGF signalling is crucial during development, and mutated FGFRs have been found to be the cause of several developmental syndromes. Prominent examples include the germline gain-of-function mutations often found in human skeletal dysplasia. For instance, in achondroplasia, a mutation in the transmembrane helix of FGFR3 (G380R) promotes dimerization and subsequent activation of the tyrosine kinase domain. In the lethal skeletal disorder thanatophoric dysplasia, single mutations generating a new cysteine residue (S249C or Y373C) in the extracellular part of the receptors cause the formation of a disulfide bond, linking two individual receptors. Thus an intermolecular bond forces dimerization in the absence of ligand, resulting in ligand-independent constitutive signalling. Interestingly, the same mutations discovered to be the cause of many developmental disorders are also found mutated in tumour cells.

The mutations found in achondroplasia and thanatophoric dysplasia, which cause dimerization and thereby constitutive activation of FGFR3, are also frequently found in bladder cancer. Yet other mutations in FGFR2 that cause dimer formation are implicated in craniosynostosis syndromes and have also been found in endometrial cancers. A mutation that promotes dimerization is just one mechanism that can increase ligand-independent signalling from FGFRs.

Other mutations located to the kinase domain of FGFRs can change the conformation of the domain to cause permanently active kinases. Mutations in the kinase domain of FGFR4 have been found in the childhood sarcomaRMS(Rhabdomyosarcoma), and these mutations were shown to cause autophosphorylation and constitutive signalling. Some mutations in FGFRs identified in human cancer have also been shown to cause loss-of-function suggesting that, in certain circumstances, FGFRs can act as tumour suppressors. The majority of FGF ligand mutations described in human disease are germline loss-of-function mutations.

9 KEY CYTOKINES

From Abbas et al as modified:

Cytokine and Subunits	Principal Cell Source	Cytokine Receptor and Subunits*	Principal Cellular Targets and Biologic Effects
Type I Cytokine Family Members			
Interleukin-2 (IL-2)	T cells	CD25 (IL-2Rα) CD122 (IL-2Rβ) CD132 (γc)	T cells: proliferation and differentiation into effector and memory cells; promotes regulatory T cell development, survival, and function NK cells: proliferation, activation B cells: proliferation, antibody synthesis (in vitro)
Interleukin-3 (IL-3)	T cells	CD123 (IL-3Rα) CD131 (βc)	Immature hematopoietic progenitors: induced maturation of all hematopoietic lineages
Interleukin-4 (IL-4)	$CD4^+$ T cells (Th2, Tfh), mast cells	CD124 (IL-4Rα) CD132 (γc)	B cells: isotype switching to IgE T cells: Th2 differentiation, proliferation Macrophages: alternative activation and inhibition of IFN-γ–mediated classical activation
Interleukin-5 (IL-5)	$CD4^+$ T cells (Th2), group 2 ILCs	CD125 (IL-5Rα) CD131 (βc)	Eosinophils: activation, increased generation
Interleukin-6 (IL-6)	Macrophages, endothelial cells, T cells	CD126 (IL-6Rα) CD130 (gp130)	Liver: synthesis of acute-phase protein B cells: proliferation of antibody-producing cells T cells: Th17 differentiation
Interleukin-7 (IL-7)	Fibroblasts, bone marrow stromal cells	CD127 (IL-7R) CD132 (γc)	Immature lymphoid progenitors: proliferation of early T and B cell progenitors T lymphocytes: survival of naive and memory cells
Interleukin-9 (IL-9)	$CD4^+$ T cells	CD129 (IL-9R) CD132 (γc)	Mast cells, B cells, T cells, and tissue cells: survival and activation
Interleukin-11 (IL-11)	Bone marrow stromal cells	IL-11Rα CD130 (gp130)	Production of platelets
Interleukin-12 (IL-12): IL-12A (p35) IL-12B (p40)	Macrophages, dendritic cells	CD212 (IL-12Rβ1) IL-12Rβ2	T cells: Th1 differentiation NK cells and T cells: IFN-γ synthesis, increased cytotoxic activity
Interleukin-13 (IL-13)	$CD4^+$ T cells (Th2), NKT cells, group 2 ILCs, mast cells	CD213a1 (IL-13Rα1) CD213a2 (IL-13Rα2) CD132 (γc)	B cells: isotype switching to IgE Epithelial cells: increased mucus production Macrophages: alternative activation
Interleukin-15 (IL-15)	Macrophages, other cell types	IL-15Rα CD122 (IL-2Rβ) CD132 (γc)	NK cells: proliferation T cells: survival and proliferation of memory $CD8^+$ cells

Interleukin-17A (IL-17A) Interleukin-17F (IL-17F)	$CD4^+$ T cells (Th17), group 3 ILCs	CD217 (IL-17RA) IL-17RC	Epithelial cells, macrophages and other cell types: increased chemokine and cytokine production; GM-CSF and G-CSF production
Interleukin-21 (IL-21)	Th2 cells, Th17 cells, Tfh cells	CD360 (IL-21R) CD132 (γc)	B cells: activation, proliferation, differentiation Tfh cells: development Th17 cells: increased generation
Interleukin-23 (IL-23): IL-23A (p19) IL-12B (p40)	Macrophages, dendritic cells	IL-23R CD212 (IL-12Rβ1)	T cells: differentiation and expansion of Th17 cells
Interleukin-25 (IL-25; IL-17E)	T cells, mast cells, eosinophils, macrophages, mucosal epithelial cells	IL-17RB	T cells and various other cell types: expression of IL-4, IL-5, IL-13
Interleukin-27 (IL-27): IL-27 (p28) EBI3 (IL-27B)	Macrophages, dendritic cells	IL-27Rα CD130 (gp130)	T cells: enhancement of Th1 differentiation; inhibition of Th17 differentiation NK cells: IFN-γ synthesis?
Stem cell factor (c-Kit ligand)	Bone marrow stromal cells	CD117 (KIT)	Pluripotent hematopoietic stem cells: induced maturation of all hematopoietic lineages
Granulocyte-monocyte CSF (GM-CSF)	T cells, macrophages, endothelial cells, fibroblasts	CD116 (GM-CSFRα) CD131 (βc)	Immature and committed progenitors, mature macrophages: induced maturation of granulocytes and monocytes, macrophage activation
Monocyte CSF (M-CSF, CSF1)	Macrophages, endothelial cells, bone marrow cells, fibroblasts	CD115 (CSF1R)	Committed hematopoietic progenitors: induced maturation of monocytes
Granulocyte CSF (G-CSF, CSF3)	Macrophages, fibroblasts, endothelial cells	CD114 (CSF3R)	Committed hematopoietic progenitors: induced maturation of granulocytes
Thymic stromal lymphopoietin (TSLP)	Keratinocytes, bronchial epithelial cells, fibroblasts, smooth muscle cells, endothelial cells, mast cells, macrophages, granulocytes and dendritic cells	TSLP-receptor CD127 (IL-7R)	Dendritic cells: activation Eosinophils: activation Mast cells: cytokine production T cells: Th2 differentiation
Type II Cytokine Family Members			
IFN-α (multiple proteins)	Plasmacytoid dendritic cells, macrophages	IFNAR1 CD118 (IFNAR2)	All cells: antiviral state, increased class I MHC expression NK cells: activation
IFN-β	Fibroblasts, plasmacytoid dendritic cells	IFNAR1 CD118 (IFNAR2)	All cells: antiviral state, increased class I MHC expression NK cells: activation
Interferon-γ (IFN-γ)	T cells (Th1, $CD8^+$ T cells), NK cells	CD119 (IFNGR1) IFNGR2	Macrophages: classical activation (increased microbicidal functions) B cells: isotype switching to

			opsonizing and complement-fixing IgG subclasses (established in mice) T cells: Th1 differentiation Various cells: increased expression of class I and class II MHC molecules, increased antigen processing and presentation to T cells
Interleukin-10 (IL-10)	Macrophages, T cells (mainly regulatory T cells)	CD210 (IL-10Rα) IL-10Rβ	Macrophages, dendritic cells: inhibition of expression of IL-12, costimulators, and class II MHC
Interleukin-22 (IL-22)	Th17 cells	IL-22Rα1 *or* IL-22Rα2 IL-10Rβ2	Epithelial cells: production of defensins, increased barrier function Hepatocytes: survival
Interleukin-26 (IL-26)	T cells, monocytes	IL-20R1IL-10R2	Not established
Interferon-λs (type III interferons)	Dendritic cells	IFNLR1 (IL-28Rα) CD210B (IL-10Rβ2)	Epithelial cells: antiviral state
Leukemia inhibitory factor (LIF)	Embryonic trophectoderm, bone marrow stromal cells	CD118 (LIFR) CD130 (gp130)	Stem cells: block in differentiation
Oncostatin M	Bone marrow stromal cells	OSMR CD130 (gp130)	Endothelial cells: regulation of hematopoietic cytokine production Cancer cells: inhibition of proliferation
TNF Superfamily Cytokines[†]			
Tumor necrosis factor (TNF, TNFSF1)	Macrophages, NK cells, T cells	CD120a (TNFRSF1) *or* CD120b (TNFRSF2)	Endothelial cells: activation (inflammation, coagulation) Neutrophils: activation Hypothalamus: fever Muscle, fat: catabolism (cachexia)
Lymphotoxin-α (LTα, TNFSF1)	T cells, B cells	CD120a (TNFRSF1) or CD120b (TNFRSF2)	Same as TNF
Lymphotoxin-αβ (LTαβ)	T cells, NK cells, follicular B cells, lymphoid inducer cells	LTβR	Lymphoid tissue stromal cells and follicular dendritic cells: chemokine expression and lymphoid organogenesis
BAFF (CD257, TNFSF13B)	Dendritic cells, monocytes, follicular dendritic cells, B cells	BAFF-R (TNFRSF13C) *or* TACI (TNFRSF13B) *or* BCMA (TNFRSF17)	B cells: survival, proliferation
APRIL (CD256, TNFSF13)	T cells, dendritic cells, monocytes, follicular dendritic cells	TACI (TNFRSF13B) *or* BCMA (TNFRSF17)	B cells: survival, proliferation
Osteoprotegerin (OPG, TNFRSF11B)	Osteoblasts	RANKL	Osteoclast precursor cells: inhibits osteoclast differentiation
IL-1 Family Cytokines			
Interleukin-1α (IL-1α)	Macrophages, dendritic cells, fibroblasts, endothelial cells, keratinocytes, hepatocytes	CD121a (IL-1R1) IL-1RAP *or* CD121b (IL-1R2)	Endothelial cells: activation (inflammation, coagulation) Hypothalamus: fever

Interleukin-1β (IL-1β)	Macrophages, dendritic cells, fibroblasts, endothelial cells, keratinocyte	CD121a (IL-1R1) IL-1RAP *or* CD121b (IL-1R2)	Endothelial cells: activation (inflammation, coagulation) Hypothalamus: fever Liver: synthesis of acute-phase proteins T cells: Th17 differentiation
Interleukin-1 receptor antagonist (IL-1RA)	Macrophages	CD121a (IL-1R1) IL-1RAP	Various cells: competitive antagonist of IL-1
Interleukin-18 (IL-18)	Monocytes, macrophages, dendritic cells, Kupffer cells, keratinocytes, chondrocytes, synovial fibroblasts, osteoblasts	CD218a (IL-18Rα) CD218b (IL-18Rβ)	NK cells and T cells: IFN-γ synthesis Monocytes: expression of GM-CSF, TNF, IL-1β Neutrophils: activation, cytokine release
Interleukin-33 (IL-33)	Endothelial cells, smooth muscle cells, keratinocytes, fibroblasts	ST2 (IL1RL1) IL-1 Receptor Accessory Protein (IL1RAP)	T cells: Th2 development ILCs: activation of group 2 ILCs
Other Cytokines			
Transforming growth factor-β (TGF-β)	T cells (mainly Tregs), macrophages, other cell types	TGF-β R1 TGF-β R2 TGF-β R3	T cells: inhibition of proliferation and effector functions; differentiation of Th17 and Treg B cells: inhibition of proliferation; IgA production Macrophages: inhibition of activation; stimulation of angiogenic factors Fibroblasts: increased collagen synthesis

10 INDEX