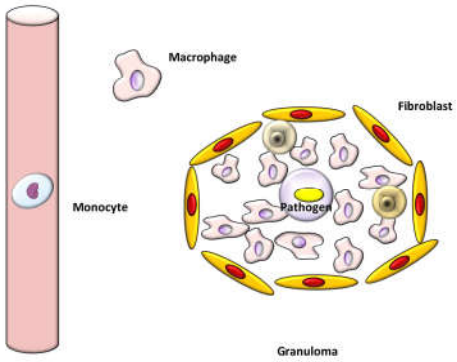

GRANULOMAS AND LONG HAUL DISEASES: HIDING IN PLAIN SIGHT

TLC 193

ABSTRACT

Granulomas are common in many pathogen infections such as TB and sarcoidosis. In cancers, we see TME, tumor micro environments, which have similar characteristics. We examine both extremes and then suggest that such may be the case in disease with recurrence such as COVID and UTIs.

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November 2022

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Our approach herein is to take elements of what is recent in the literature focused on a specific topic and attempt to develop a tapestry image of these connectable elements. We do not provide any new or fundamental results but merely attempt to assemble elements in a systematic manner.

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Contents

1 INTRODUCTION

Granulomas have been known for well over a century. Basically they are a pathogen surrounded by both immune and somatic cells in a protective fashion. The granulomas are protective encasements of a pathogen. In this Note we examine granulomas in a counterpoint to tumor micro environments, TME, in cancer cells. Namely the protective interaction between a pathogen and normally aggressive immune environments and ancillary cells.

1.1 THE GRANULOMA

Let us begin with the basic granuloma. As Williams and Williams have noted:

The granulomatous inflammatory response is ubiquitous in pathology, being a manifestation of many infective, toxic, allergic, autoimmune and neoplastic diseases and also conditions of unknown aetiology. Schistosomiasis, tuberculosis and leprosy, all infective granulomatous diseases, together affect more than 200 million people worldwide, and granulomatous reactions to other irritants are a regular occurrence in everyday clinical histopathology. A knowledge of the basic pathophysiology of this distinctive tissue reaction is therefore of fundamental importance in the understanding of many disease processes.

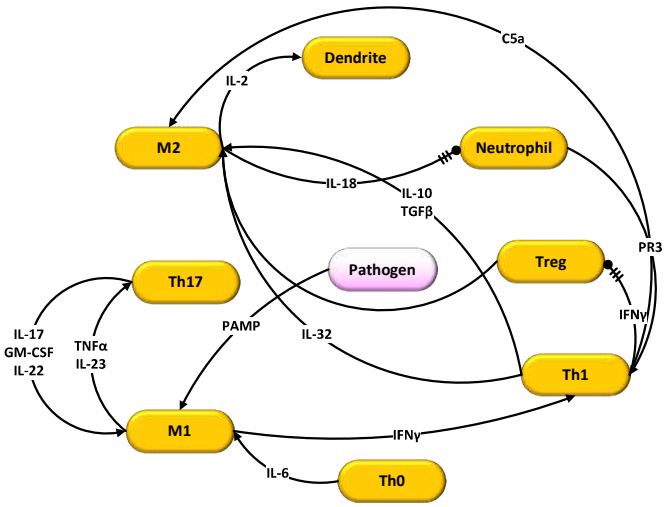
Granulomatous inflammation is best defined as a special variety of chronic inflammation in which cells of the mononuclear phagocyte system are predominant and take the form of macrophages, epithelioid cells and multinucleated giant cells. In most instances these cells are aggregated into well demarcated focal lesions called granulomas, although a looser, more diffuse arrangement may be found. In addition there is usually an admixture of other cells, especially lymphocytes, plasma cells and fibroblasts

Granulomas have been observed in a variety of pathologic conditions such as TB and sarcoid. However their presence may be more common in a variety of other pathogen based diseases. Shah et al note in a more recent article:

Granulomatous inflammation is a histologic pattern of tissue reaction which appears following cell injury. Granulomatous inflammation is caused by a variety of conditions including infection, autoimmune, toxic, allergic, drug, and neoplastic conditions. The tissue reaction pattern narrows the pathologic and clinical differential diagnosis and subsequent clinical management. Common reaction patterns include necrotizing granulomas, non-necrotizing granulomas, suppurative granulomas, diffuse granulomatous inflammation, and foreign body giant cell reaction. Prototypical examples of necrotizing granulomas are seen with mycobacterial infections and non-necrotizing granulomas with sarcoidosis.

However, broad differential diagnoses exist within each category. Using a pattern based algorithmic approach, identification of the etiology becomes apparent when taken with clinical context. The pulmonary system is one of the most commonly affected sites to encounter granulomatous inflammation. Infectious causes of granuloma are most prevalent with mycobacteria and dimorphic fungi leading the differential diagnoses. Unlike the lung, skin can

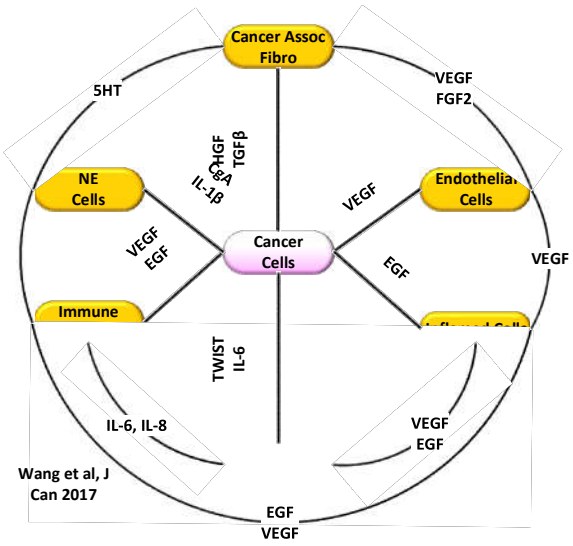
be affected by several routes, including direct inoculation, endogenous sources, and hematogenous spread. This broad basis of involvement introduces a variety of infectious agents, which can present as necrotizing or non-necrotizing granulomatous inflammation. Non-infectious etiologies require a thorough clinicopathologic review to narrow the scope of the pathogenesis which include: foreign body reaction, autoimmune, neoplastic, and drug related etiologies. Granulomatous inflammation of the kidney, often referred to as granulomatous interstitial nephritis (GIN) is unlike organ systems such as the skin or lungs. The differential diagnosis of GIN is more frequently due to drugs and sarcoidosis as compared to infections (fungal and mycobacterial)



1.2 THE TME

The tumor micro environment, TME, is the collection of all elements that comprise the environment that surrounds the cancer cell. This includes all other cells, immune and supportive, stroma and otherwise, and we would also include all signalling elements that support that environment.

We use the example of Wang et al to demonstrate these elements as shown below:



The TME is currently a well-accepted paradigm for the protection of a malignant cells from attack and destruction by a variety of immune cells. Namely the malignant cell manages to recruit other cells and turn their normally offensive tactic into defensive one, all to insure survival of the malignant cell. We shall review this construct since it allows us to construct a similar paradigm for granulomas.

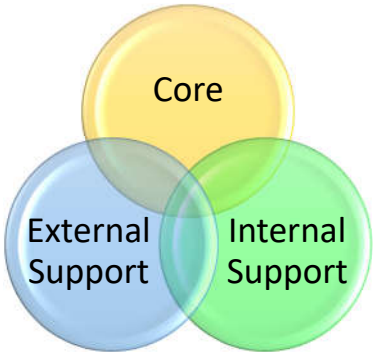
1.3 Overview

We attempt to establish a paradigm parallelism between granulomas and TME. Namely as shown below:

TME	Granuloma

• Core cancer cell(s)
• External Support
  • M1 and M2 macrophage
  • Fibroblasts
• Internal Support
  • mTOR
  • VEGF
  • RGF
  • IL-6, IL-8
	• Core pathogen
	• External support
	  • M1 and M2 macrophage
	  • Fibroblasts
	• Internal Support
	  • VEGF
	  • mTOR

Thus we see a balance between three elements. The Core of the central initiating element, the Internal elements and these are pathways and related cytokines, chemokines and the like, and the External elements or the participating cells that combine their functionality in a granuloma to assure persistence.

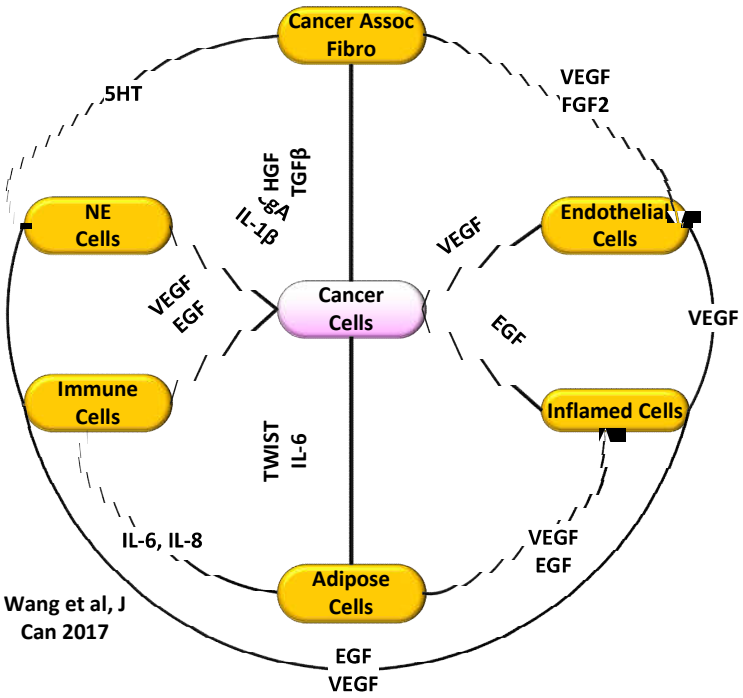


Now it is important to recognize that granulomas are still works in progress and that some are well understood and others less so. The processes that allow them to survive and regenerate still demand increased understanding. As such one aspect we examine is that ol senescence on granuloma cells. Rather than dying off such as in autophagy they seem to remain dormant until reactivated. The classic case if TB. Granulomas also do not metastasize. They also do not appear to genetically change in time or location.

2 TME, AN ESTABLISHED PARADIGM

The tumor micro-environment, TME, is a well-studied example of a pathogen, in this case a cancer cell, co-opting the immune system and other cells to create a protective environment for the invader. To better understand a granuloma it is worthwhile to understand the dynamics of the TME because similar effects are occurring.

An example of the complexity of the TME is shown below based upon Wang et al as modified. Here is shown the participants in such an environment as well as the cytokines, chemokines and other activating and activated control mechanisms. As a notice, we will attempt to build a homology between the TME and granulomas and thus this graphic does drive home a significant point.



One of the more significant ones is the VEGF family members which we shall examine later. We also find IL-6 as a major factor as well.

As Galvez et al have noted:

Effective responses to immunotherapy drive de novo peripheral immune responses. …

At baseline, conventional dendritic cells (cDCs) in the TME take up tumour antigen and travel to the draining lymph node (dLN), where they can then transfer antigen to resident cDCs through the formation of direct synapses.

T cells in the TME reach states of terminal exhaustion due to chronic stimulation, the harsh environment and immunosuppressive cues.

Dysfunctional intratumoural T cells accumulate structurally damaged mitochondria, and upregulate CD103 and CD38 coinciding with irreversible epigenetic remodelling. Thus, effective antitumour responses driven by therapy must rely on another source of functional effector T cells. …

Immunotherapeutic intervention through PD1 and PDL1 checkpoint blockade increases the interaction between cDCs and naive T cells in the dLN, and, alongside CD28 co-stimulation, facilitates the priming and rapid expansion of new T cell clones with new antigen specificities.

Checkpoint blockade also leads to the proliferation of existing T cell clones in circulation. These expanding peripheral T cells ultimately infiltrate the TME, and express markers indicative of antigen-specific activation and demonstrate functional cytotoxicity.

Productive de novo immune responses can also be achieved through CD40 agonism, which can drive cDC activation in settings resistant to checkpoint blockade and initiate these new T cell responses to replace exhausted intratumoural clones

They continue:

Beyond excessive production of monocytic and neutrophilic cells through aberrant hematopoiesis, perturbations in dendritic cells have been observed in the periphery of tumour-burdened hosts.

This has important implications for the development of antitumour immune responses, as dendritic cells are critical orchestrators of CD8+ and CD4+ T cell priming, differentiation and proliferation in many contexts, including cancer.

The frequencies of dendritic cell subsets are decreased in peripheral blood of human ovarian, prostate, breast, lung and renal cancers as well as head and neck squamous cell carcinoma and melanoma when compared with healthy control donors.

In patients with pancreatic or breast cancer and in mouse models of these cancer types, a decrease in the frequency of peripheral type 1 conventional dendritic cells (cDC1s) was driven through tumour-derived G-CSF, which caused a downregulation of IRF8 in dendritic cell precursors, reducing the differentiation of mature dendritic cells.

Similarly, tumour-derived vascular endothelial growth factor (VEGF) has been shown to inhibit the maturation of dendritic cell precursors.

An alternative mechanism for dendritic cell paucity in a mouse model of pancreatic cancer was shown to be mediated by serum IL-6 driving increased dendritic cell apoptosis.

In patients with pancreatic cancer and mouse models of pancreatic cancer, peripheral dendritic cells differentiate into a semi-mature state characterized by moderate increases in co-stimulatory and co-inhibitory receptors.

Bulk transcriptomic analyses of these peripheral dendritic cells from mice bearing pancreatic tumours revealed that these semi-mature dendritic cells showed upregulation of genes involved in proteasomal degradation but did not show upregulation of T cell polarizing cytokines, suggesting that, similar to semi-mature dendritic cells in other contexts, they only partially possess the capacity to provide stimulation to T cells. Substantially less is known about the organization of other major immune lineages in the tumour macroenvironment.

Lymphopenia is common in patients with breast cancer, lymphoma and sarcoma. Interestingly, circulating T cells in patients with breast, lung and cervical45 cancers have decreased diversity in the repertoire of ***T cell receptors (TCRs)****. As greater TCR diversity is associated with better tumour control in patients with melanoma, an improved understanding of TCR repertoire fluctuations driven by cancer is warranted. Furthermore, as a decreased TCR repertoire in humans is associated with age as well as other prior immunological exposures such as chronic infection48, these changes may also be a cause for malignant outgrowth. The causal relationship between TCR diversity and cancer has yet to be determined.*

Peripheral T cells are also functionally perturbed, as polyclonal memory CD4+ and CD8+ T cells from peripheral blood have decreased capacity to produce IL-2 and IFNγ in response to stimulation with PMA and ionomycin in human patients with breast cancer. Peripheral naive CD4+ T cells also exhibited decreased responses to IL-6 stimulation as measured by phosphorylation of STAT1 and STAT3 in patients with breast cancer…

Giraldo et al note:

The highly complex and heterogenous ecosystem of a tumour not only contains malignant cells, but also interacting cells from the host such as endothelial cells, stromal fibroblasts, and a variety of immune cells that control tumour growth and invasion. It is well established that anti-tumour immunity is a critical hurdle that must be overcome for tumours to initiate, grow and spread and that anti-tumour immunity can be modulated using current immunotherapies to achieve meaningful anti-tumour clinical responses. Pioneering studies in melanoma, ovarian and colorectal cancer have demonstrated that certain features of the tumour immune microenvironment (TME)—in particular, the degree of tumour infiltration by cytotoxic T cells—can predict a patient's clinical outcome. More recently, studies in renal cell cancer have highlighted the importance of assessing the phenotype of the infiltrating T cells to predict early relapse. Furthermore, intricate interactions with non-immune cellular players such as endothelial cells and fibroblasts modulate the clinical impact of immune cells in the TME. Here, we review the critical components of the TME in solid tumours and how they shape the immune cell contexture, and we summarise numerous studies evaluating its clinical significance from a prognostic and theranostic perspective

They continue:

First, a portion of mutated peptide epitopes resulting from either driver or passenger mutations can be presented on tumour cell class I MHC molecules and be recognised by CD8+ T cells, which, together with T-cell-attracting chemokines such as CXCL9 and CXCL10, promotes a brisk infiltration by cytotoxic T lymphocytes.

The mutations that create tumour-associated neoantigens have become more appreciated with recent advances in sequencing and computational techniques, leading to the concept that a tumour mass is often composed of different subclones of cells with different immunogenic potentials .....

Second, some driver or passenger mutations can induce molecular pathways that shape tumour infiltration by immune cells independently of their neoantigenic potential.

For example, the mutation-driven activation of the Wnt-β-catenin pathway in melanoma, colorectal cancer, and hepatocellular carcinoma24 limit the accumulation of cytotoxic T cells and DCs. In addition, models of lung adenocarcinoma suggest that mutations in the Myc and Ras pathways cooperate to establish an immunosuppressive microenvironment by driving expression of the chemokine CCL9—which recruits immunosuppressive and angiogenic macrophages – and interleukin (IL)-23, and prevents the accumulation of cytotoxic NK cells and T cells in the tumour ....

Third, mutations in tumour cells can alter immune cell functions once they are recruited within the tumour mass.

For example, ... found that oncogenic RAS can drive tumour cell intrinsic upregulation of the programmed cell death ligand 1 (PDL1).30 By binding to programmed cell death protein 1 (PD-1) expressed on activated T cells, PD-L1 suppresses the effector activity of T cells that might otherwise confer cytotoxicity.....

These three mechanisms shape the composition of immune cells within the tumour significantly. In some tumour types, such as lung adenocarcinoma, the extent of T cell infiltration is tightly correlated with neoantigen frequency.31 However, no such correlation has been observed in melanoma patients.

Other tumours, such as pancreatic adenocarcinoma and renal cell carcinoma (RCC), have a lower frequency of neoantigens but might still exhibit a high degree of infiltration by T cells. Thus, although the mutational load is tightly correlated with the degree of tumour inflammation, the nature of the driver mutations and additional neoantigenic mutations that accumulate critically influence the nature and function of the immune cell composition.

2.1 TUMOR ASSOCIATED MACROPHAGES

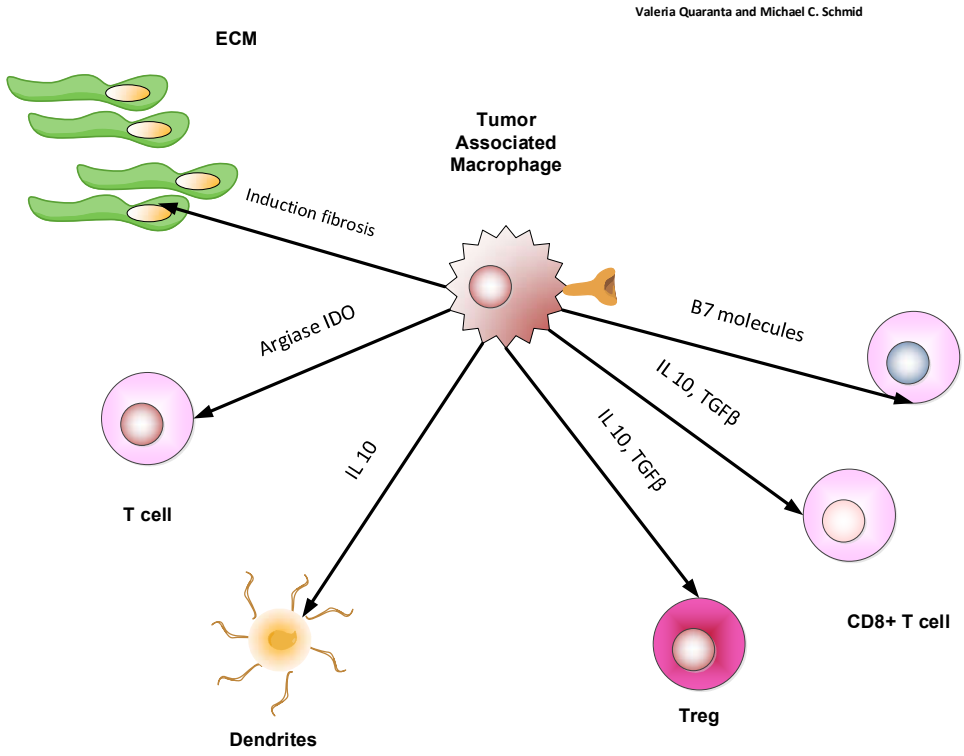
Let us begin with macrophages. To the beginning student of the immune system one often sees the macrophage as that wandering cell that sense invaders and then sends out signals as to their presence. In a simple sense this is the case. But then again as with all immune system elements it is always more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

The TAMs have been found to play a significant role in facilitating cancer cell proliferation. M1 and M2 macrophages can counter one another as well as transform from one to the other. The author notes:

Macrophages are classified into M1- and M2-polarized subtypes. The M1-subtype secretes inflammatory cytokines and reactive oxygen intermediates and presents antigen to tumor suppressive T cells.

However, the M2-subtypes, which are tumor promoting, induce T cell anergy, produce extracellular matrix components, repair damaged tissues, and induce angiogenesis. Although the origins of macrophages in many cancers remain uncertain, most of the macrophages recruited to the tumor microenvironment, known as the TAMs, become the tumor supportive M2 subtype. In glioblastoma, glioma CSCs activate the STAT3 pathway to produce cytokines, which recruit and polarize macrophages to become M2-like.

After recruitment, TAMs, in turn, serve as a CSC niche to support CSC growth. For example, in breast cancer, the physical interaction between TAMs and CSCs activates the EphA4 receptor on CSCs and the downstream Src and NF- κB pathways, which promote self-renewal.



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[1]:

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:

FGF, fibroblast growth factor

EGF, epidermal growth factor receptor ligands, and

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

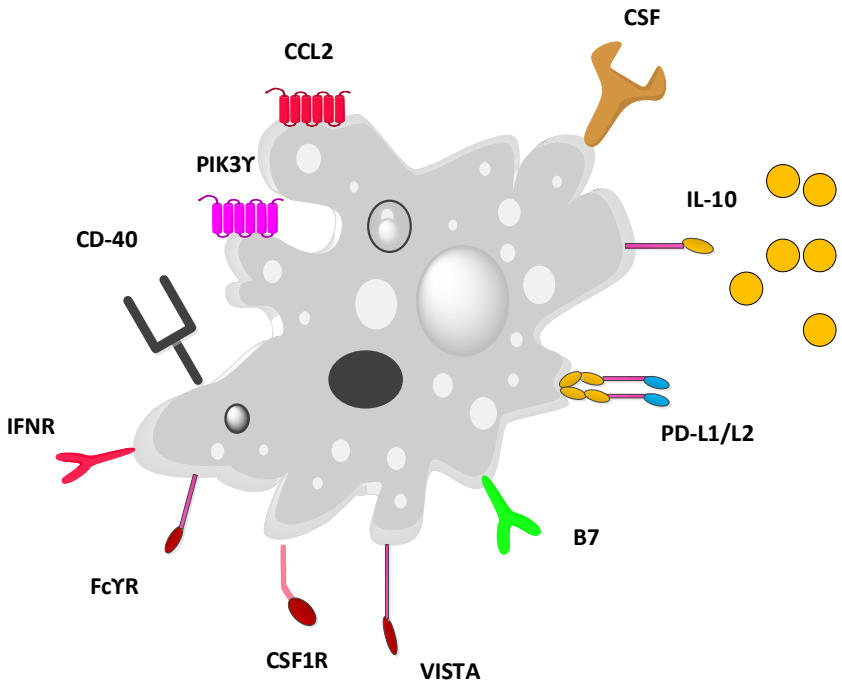
As in normal wound healing, these growth factors secreted by the TAMs or the tumors themselves activate fibroblasts.

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.

[1] DeVita et al p 124



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. .... 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.

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.

2.2 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 …

2.3 TUMOR ASSOCIATED FIBROBLASTS

Fibroblasts are common cells that generally do not form any specific functioning collection of cells. The fibroblast is resident in the stroma of most organs.

As Lau et al have noted:

Cancer-associated fibroblasts (CAFs) are the major components of the tumor stroma. Recent studies have revealed that CAFs are a heterogeneous population, most of which acquire the activated phenotype with increased contractile force, proliferative activity, and enhanced secretion of ECM, proteases, and growth factors. CAFs emerge from multiple origins that widely vary among different cancer types. Several studies have shown that cancer cells could actually secrete signaling molecules, such as basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), and interleukin IL-6 to "educate" resting fibroblasts to become CAFs, and in turn, CAFs promote tumor growth and sustain the stemness property of CSCs in a paracrine manner.

Through the secretion of hepatocyte growth factor (HGF), CAFs from colon cancer were demonstrated to support CSC properties through the induction of Wnt/β-catenin signaling. More interestingly, the paracrine activation of Wnt/β-catenin signaling by CAFs could restore the stem-like features of non-CSCs, thereby expanding the pool of these cells.

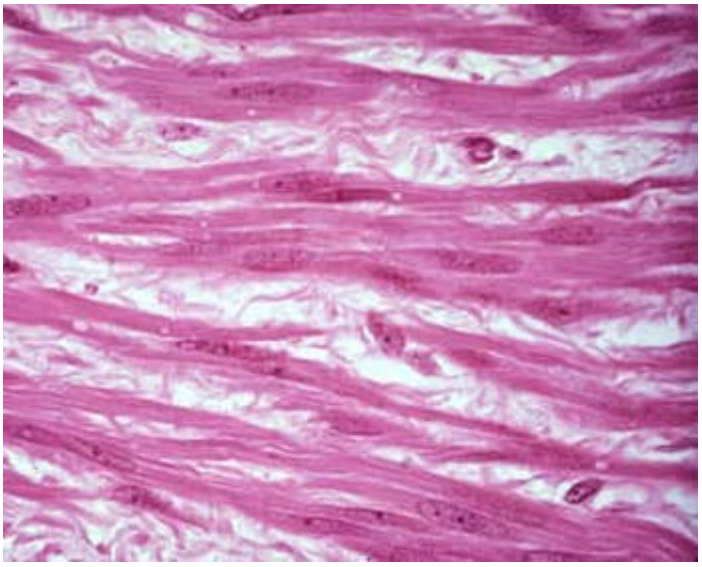
Using conditioned media from CAFs, we showed that CAFs from liver cancer promote cancer stemness through the noncanonical induction of the Notch signaling effector HEY-1 mediated by HGF. A recent study also demonstrated that CAFs in lung cancer induce the expression of the NANOG transcription network through paracrine insulin-like growth factor II (IGF-II)/IGF- 1R signaling. EMT is the process where cancer cells acquire a mesenchymal trait and become more invasive and metastatic.

Cancer cells that have undergone EMT typically acquire an increased stemness property because some of the EMT-mediating transcription factors, such as Snail and ZEB1, are essential for self-renewal. Several studies have also shown that the activation of EMT could induce the generation of the CSC population.

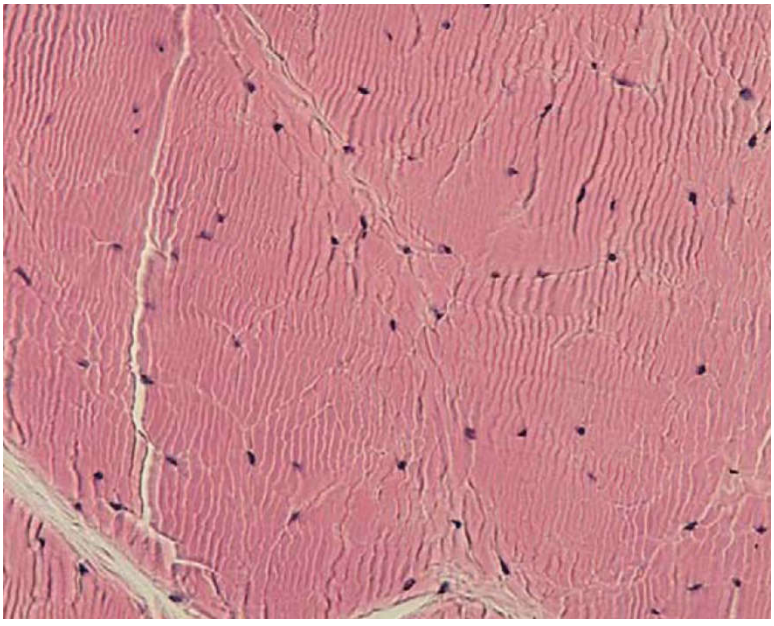
In prostate cancer, CAFs can elicit EMT and increase the stemness properties of cancer cells through the secretion of MMPs. Furthermore, CAFs from breast cancer have been reported to promote the EMT of cancer cells via the secretion of stromal-derived factor 1 (SDF-1) and TGF-β1, providing additional support, suggesting that CAFs play a crucial role in promoting cancer stemness.

2.3.1 Histology

We start by examining the fibroblasts histologically. Fibroblasts seem to be almost universal and part of the vast connective matrix. An example is shown below[2]:

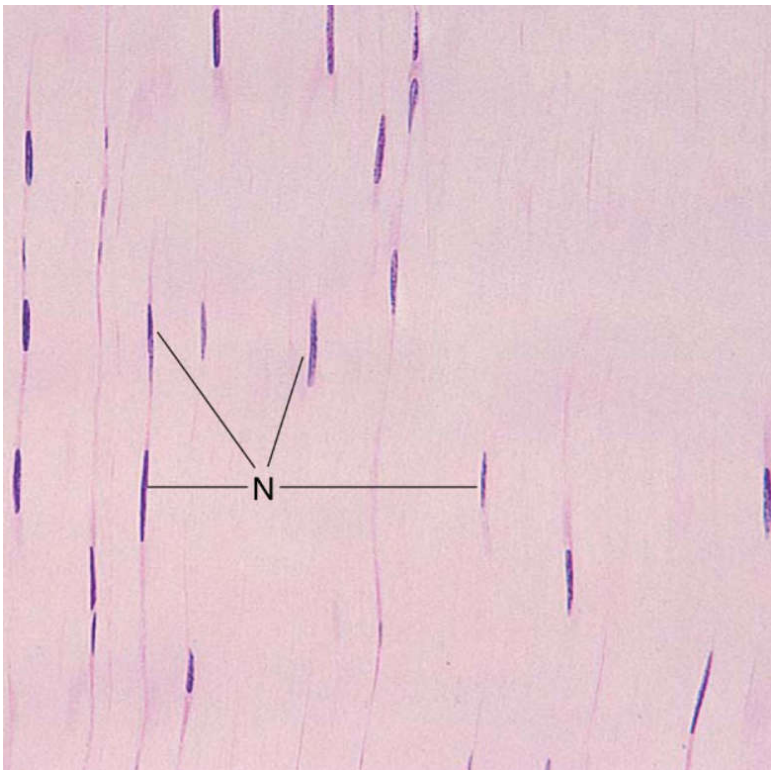


The following are from Gartner and Hiatt:



[2] http://www.meddean.luc.edu/lumen/MedEd/Histo/frames/h_frame7.html

and the following is another example from the same source.



In both of the above cases the fibroblasts are elongated, prominent nuclei and somewhat clear protoplasm. All of the above fibroblasts and long tear shaped cells with prominent nuclei. They generally are unorganized and have a clear cytoplasm.

2.3.2 Fibroblast Functions

From NCBI we have[3]:

The fibroblast is one of the most abundant cell types present in the stroma. It has a variety of functions and composes the basic framework for tissues and organs. Under homeostasis, this cell is responsible for maintaining the extracellular matrix (ECM). During stress, fibroblasts adapt to their environment and have the ability to respond and send local signals. In times of injury, the fibroblast can transform phenotypes and synthesize the building blocks necessary to replace wounded tissue. During pathologic states, the extracellular matrix gets generated in excessive quantities, and collagen is deposited in a dysregulated manner often causing irreversible organ dysfunction or disfiguring appearance….

Fibroblasts are the most common cell type represented in connective tissue. These cells produce a diverse group of products including collagen type I, III, and IV, proteoglycans, fibronectin,

[3] https://www.ncbi.nlm.nih.gov/books/NBK541065/

laminins, glycosaminoglycans, metalloproteinases, and even prostaglandins. In the adult body, fibroblasts remain in a quiescent form until stimuli activate protein synthesis and contractile mechanisms.

These cells synthesize reorganize the ECM found in the skin, lung, heart, kidney, liver, eye, and other organs. The ECM is in constant communication with the surrounding cells as fibroblasts can secrete and respond to both autocrine and paracrine signals. Matrix reorganization occurs through a process of degradation and crosslinking enzymes, produced by fibroblasts, that are activated and regulated by pro-inflammatory cytokines and growth factors. Transcription growth factor-alpha and beta (TGF-A and TGF-B), platelet-derived growth factor (PDGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), epidermal growth factor (EGF), and tumor necrosis factor (TNF) all have implications in fibroblast regulation.

The relationship with the ECM is an important factor especially when we examine its role in various cancers. They continue:

Fibroblasts are a diverse group of cells. Within one organ system, there can be a great variety of functions. Within the integument, dermal fibroblasts in different locations have separate roles. The superficially located lineage involves the formation of the hair follicle and is responsible for reepithelization during wound healing; the deeper lineage is responsible for ECM generation.

Fibroblasts are known for their plasticity; adipocytes, pericytes, endothelial and epithelial cells, otherwise known as terminally differentiated cells, can de-differentiate into fibroblasts.

Stimulation of fibroblasts further increases susceptibility to epigenetic modifications. The ability of fibroblasts to transform is partly due to the variety of cell-surface adhesion receptors (integrins, syndecans, cadherins) that facilitate the communication of fibroblasts with their surroundings. One of these well-described fibroblast transformations is the transformation of fibroblast into the myofibroblast.

Myofibroblasts are present in both healthy and pathologic tissues and contain features of fibroblasts and smooth muscle cells. These cells work in conjunction with vascular endothelial cells to form granulation tissue during times of wound healing.

In the following, many of the cancer related involvements of fibroblasts will focus on the transitioned myofibroblast.

2.3.3 Scars and Markers

Identifying fibroblasts are generally done histologically by visible inspection but they also can be further classified by surface markers. Now this is discussed in Ziani et al who note:

Fibroblasts are spindle-shaped, non-epithelial (cytokeratin negative, E-cadherin negative), non-endothelial (CD31 negative) and non-immune (CD45 negative) cells of a mesenchymal lineage origin (vimentin+). In normal tissue, fibroblasts are usually considered as resting/

quiescent cells with negligible metabolic and transcriptional activities, but with the ability to respond to growth factors to become activated.

This is an exceptionally short and clear description. The fibroblasts are cells somewhat on their own and are interstitial to organ focused cells. The lack of E cadherin allows them to have substantial mobility.

During this activation process, fibroblasts exhibit contractile activity, exert physical forces to modify tissue architecture, acquire proliferation and migration properties and become transcriptionally active leading to the secretion of several factors (cytokines, chemokines, etc.) and ECM components.

The ability of resting fibroblasts to become activated was first observed in the context of wound healing and subsequently in pathologic conditions such as acute or chronic inflammation or tissue fibrosis (a chronic wound healing response).

This chronic tissue repair response also occurs in the context of cancer, considered as a "wound that never heals"*.*

This concept of wound healing is a significant driver of understanding how fibroblasts play such a role in cancers. Wound healing is the process in humans of repairing damaged organs and in turn cells. It is a tissue repair attempt, albeit one poorly accomplished, yet its ultimate protective result allows and facilitates a malignant growth.

Indeed, emergence and/or accumulation of cancer cells in a given tissue represent a tissue injury, imitating a chronic wound healing response toward the tumor cells, also known as tumor fibrosis or desmoplastic reaction.

Consequently, major players in tumor fibrotic microenvironment include activated fibroblasts, termed cancer-associated fibroblasts (CAFs), which represent one of the most abundant stromal cell types of several carcinomas including breast, prostate, pancreatic, esophageal, and colon cancers while CAFs are less abundant, but still present, in other neoplasias including ovarian, melanoma, or renal tumors. For example, in pancreatic cancer, 60–70% of the tumor tissue is composed of a desmoplastic stroma characterized by extensive collagen deposition and activated CAFs.

Now it is the CAF that we will focus upon. However, the key issue to note is that the fibroblasts play a significant role in wound repair. As Kumar et al note:

Several cell types proliferate during tissue repair. These include the remnants of the injured tissue (which attempt to restore normal structure), vascular endothelial cells (to create new vessels that provide the nutrients needed for the repair process), and ***fibroblasts (the source of the fibrous tissue that forms the scar to fill defects that cannot be corrected by regeneration).***

The ability of tissues to repair themselves is determined, in part, by their intrinsic proliferative capacity. In some tissues (sometimes called labile tissues), cells are constantly being lost and

must be continually replaced by new cells that are derived from tissue stem cells and rapidly proliferating immature progenitors. These types of tissues include hematopoietic cells in the bone marrow and many surface epithelia, such as the basal layers of the squamous epithelia of the skin, oral cavity, vagina, and cervix; the cuboidal epithelia of the ducts draining exocrine organs (e.g., salivary glands, pancreas, biliary tract); the columnar epithelium of the gastrointestinal tract, uterus, and fallopian tubes; and the transitional epithelium of the urinary tract. These tissues can readily regenerate after injury as long as the pool of stem cells is preserved.

Other tissues (called stable tissues) are made up of cells that are normally in the G0 stage of the cell cycle and hence not proliferating, but they are capable of dividing in response to injury or loss of tissue mass. These tissues include the parenchyma of most solid organs, such as liver, kidney, and pancreas. ***Endothelial cells, fibroblasts, and smooth muscle cells are also normally quiescent but can proliferate in response to growth factors, a reaction that is particularly important in wound healing.***

Now they continue on the process of developing a scar, or scar tissue as follows:

1. Within minutes after injury, a hemostatic plug comprised of platelets is formed, which stops bleeding and provides a scaffold for infiltrating inflammatory cells.

2. Inflammation. This step is comprised of the typical acute and chronic inflammatory responses. Breakdown products of complement activation, chemokines released from activated platelets, and other mediators produced at the site of injury function as chemotactic agents to recruit neutrophils and then monocytes during the next 6 to 48 hours. As described earlier, these inflammatory cells eliminate the offending agents, such as microbes that may have entered through the wound, and clear the debris. Macrophages are the central cellular players in the repair process—M1 macrophages clear microbes and necrotic tissue and promote inflammation in a positive feedback loop, and M2 macrophages produce growth factors that stimulate the proliferation of many cell types in the next stage of repair. As the injurious agents and necrotic cells are cleared, the inflammation resolves; how this inflammatory flame is extinguished in most situations of injury is still not well defined.

3. Cell proliferation. In the next stage, which takes up to 10 days, several cell types, including epithelial cells, endothelial and other vascular cells, and fibroblasts, proliferate and migrate to close the now-clean wound. Each cell type serves unique functions.

a. *Epithelial cells respond to locally produced growth factors and migrate over the wound to cover it.*

b. *Endothelial and other vascular cells proliferate to form new blood vessels, a process known as angiogenesis. Because of the importance of this process in physiologic host responses and in many pathologic conditions, it is described in more detail later.*

c. ***Fibroblasts proliferate and migrate into the site of injury and lay down collagen fibers that form the scar.***

d. The combination of proliferating fibroblasts, loose connective tissue, new blood vessels and scattered chronic inflammatory cells, forms a type of tissue that is unique to healing wounds and is called granulation tissue. This term derives from its pink, soft, granular gross appearance, such as that seen beneath the scab of a skin wound.

4. Remodeling. The connective tissue that has been deposited by fibroblasts is reorganized to produce the stable fibrous scar. This process begins 2 to 3 weeks after injury and may continue for months or years.

Now as we noted earlier, this process seems to occur with the introduction of malignant cells as well. Unlike a normal benign scar, However, a malignant scar or tumor, uses the same elements but it does so in a manner to protect itself. It uses the fibroblasts as a tool for protection.

2.4 Other Detailed Elements of TME

From Lau et al the other stromal cells in the TME consist of the following elements:

2.4.1 Adipocytes.

Obesity is a well-recognized risk factor of several common human malignancies, including breast cancer, colon cancer, and liver cancer.

In addition to its epidemic significance, emerging studies have uncovered the functional role of adipose tissues in carcinogenesis and cancer progression, particularly in cancers with adipose tissue constituting a major part of the tumor microenvironment.

Adipose tissue primarily comprises adipocytes and a variety of cells that make up the stromal vascular fraction. In addition to its lipid storage function, adipocytes can actively secrete multiple adipokines and cytokines, such as leptin, adiponectin, IL-6, MCP-1, and TNF-α, during excessive adiposity. In addition to its role in lipid homeostasis, many of these adipokines and cytokines are proinflammatory, which attract the infiltration of inflammatory cells, particularly macrophages, causing chronic inflammation to promote cancer growth and metastasis.

Furthermore, some of these adipocyte-secreted adipokines/cytokines were directly involved in regulating CSCs. In breast cancer, the expression of leptin receptor is highly upregulated in tumor tissue, particularly in the CSC subpopulation, as driven by the selfrenewal associated transcription factors OCT-4 and SOX-2. The secretion of leptin by adipocytes activates the STAT3 signaling in CSCs and induces the expression of OCT-4 and SOX-2, in turn stimulating the expression of leptin receptor, which maintains a self-reinforcing signaling cascade to expand the CSC population and promote tumor growth.

Another study showed that the coculture of adipocytes and breast cancer cells stimulates the production of various cytokines that promote cancer stemness through the Src/SOX-2/miR-302b signaling pathway. In prostate cancer, where obesity is associated with a more aggressive

phenotype, adipocytes produce cathepsin B (CTSB) upon coculture with prostate cancer cells to support the selfrenewal of CSCs. Adipocytes from colorectal cancer are also demonstrated to enhance cancer stemness, and their oncogenic function can be impaired by grape seed extract, a well proven agent with anticolorectal cancer activity, through inducing the "browning" of adipocytes.

2.4.2 Perivascular Cells.

Angiogenesis is essential for tumor growth and metastasis. With the excessive production of proangiogenic factors by cancer cells, tumors typically develop disorganized and rich blood vessel networks to meet the high demand on oxygen and nutrients required for tumor outgrowth. CSCs promote tumor angiogenesis.

For example, in brain, skin, pancreatic, and liver cancer, the CD133+ CSC populations produce higher levels of proangiogenic factors, such as vascular endothelial growth factor (VEGF) and SDF-1, recruit more endothelial cells, and stimulate more tube formation compared with their differentiated CD133− counterparts. Intriguingly, glioblastoma stem cells, which reside in the perivascular niche, undergo differentiation to generate vascular pericytes and endothelial cells to expand tumor vascularization.

Indeed, a mean of approximately 60% of endothelial cells in glioblastoma are derived from neoplastic cells. In turn, CSCs reside in close proximity to the perivascular niche, which provides functional support. Strong evidence suggests that vascular endothelial cells play a key role in maintaining CSCs. In the context of glioblastoma, endothelial cells provide Notch ligands to neighboring CSCs, activating Notch signaling and promoting CSCs self-renewal. In another study, perivascular endothelial cells were demonstrated to activate Notch signaling in glioma stem cells through another soluble factor, nitric oxide.

A similar observation was also made in colon cancer, suggesting that endothelial cells secrete the Notch ligand Jagged-1 to promote colon CSC phenotype. A recent study on head and neck cancer also highlighted a role for endothelial cells in regulating CSCs, in which endothelial cells were shown to secrete epidermal growth factor (EGF) to induce EMT and promote cancer stemness. Together, these findings reveal an intriguing reciprocal interaction between CSCs and perivascular cells.

2.4.3 CSCs and Immune Evasion.

Tumor immune escape is a fundamental step for tumor development and the major reason for the failure in cancer immunotherapy. Cancer cells evade the infiltration and the cytotoxic function of natural killer (NK) T cells and CD8+ cytotoxic T cells through various strategies, including the active attraction of immunesuppressive cells, production of immune-suppressive factors, and the activation of "immune checkpoints" that induce anergy or apoptosis in T lymphocytes to downmodulate immune functions.

Several studies have revealed that the activation of prosurvival pathways, such as PI3K/AKT, in CSCs not only facilitates escape from conventional chemotherapies but also confers immune

evasion. The expression of MHC-I and MHC-II proteins, required for recognition by T lymphocytes to elicit immune responses, is also downregulated in CSCs. In head and neck cancer, the programmed death-ligand 1 (PD-L1), which binds to the programmed death 1 (PD-1) receptor on T cells to suppress its function, is selectively expressed on CD44+ CSCs [42]. Furthermore, it has been well documented that CSCs actively recruit immune-suppressive cells into the tumor microenvironment.

In addition to functions in modulating immune cells, these tumor-associated immune-suppressive cells, which mainly include tumor-associated macrophages myeloid-derived suppressor cells (MDSCs), T-regulatory (Treg) cells, and NK cells, have been widely demonstrated to support CSCs through multiple pathways.

2.4.4 Myeloid-Derived Suppressor Cells.

MDSCs are a heterogeneous population of myeloid-originated progenitor cells. ...As the name indicates, the main feature of MDSCs is their function on immunosuppression. MDSCs suppress immune function primarily through multiple mechanisms, including the production of arginase, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), cyclooxygenase-2 (COX-2), and TGF-β, which together inhibit the proliferation and function of T cells.

Recent studies have demonstrated that MDSCs are actively recruited into tumors and these tumor-associated MDSCs play an important role in tumor progression. The recruitment of MDSCs into tumor sites is primarily mediated by various cancer cells that produce chemokines, including CCL2, CCL15, CXCL5, and CXCL12. MDSCs are implicated in multiple stages of tumor progression, particularly the regulation of CSCs. In ovarian cancer, coculture with MDSCs stimulates the expression of miR-101 in cancer cells, which regulates CtBP2 to control the expression of stemness genes, such as NANOG, OCT-4, and SOX-2.

In syngeneic mammary tumor models, CSCs displayed the elevated production of granulocyte colony-stimulating factor (G-CSF), which stimulates the recruitment of MDSCs into the tumor microenvironment. MDSCs reciprocally enhance CSC properties through the activation of Notch signaling. Furthermore, tumor-infiltrated MDSCs, which showed the activation of STAT3 signaling, can enhance the stemness of pancreatic cancer cells through the induction of EMT, with a concomitant increase in the expression of stemness genes, including Snail, Slug, ZEB1, NANOG, and OCT-4.

As Cronan et al noted about macrophages and granulomas:

Mycobacterium tuberculosis infection in humans triggers formation of granulomas, which are tightly organized immune cell aggregates that are the central structure of tuberculosis. Infected and uninfected macrophages interdigitate, assuming an altered, flattened appearance. Although pathologists have described these changes for over a century, the molecular and cellular programs underlying this transition are unclear. Here, using the zebrafish-Mycobacterium marinum model, we found that mycobacterial granuloma formation is accompanied by

macrophage induction of canonical epithelial molecules and structures. We identified fundamental macrophage reprogramming events that parallel E-cadherin-dependent mesenchymal-epithelial transitions. Macrophage-specific disruption of E-cadherin function resulted in disordered granuloma formation, enhanced immune cell access, decreased bacterial burden, and increased host survival, suggesting that the granuloma can also serve a bacteria-protective role. Granuloma macrophages in humans with tuberculosis were similarly transformed. Thus, during mycobacterial infection, granuloma macrophages are broadly reprogrammed by epithelial modules, and this reprogramming alters the trajectory of infection and the associated immune response … Granulomas are macrophage-derived structures, which later recruit other immune cells, and can form in response to a variety of persistent inflammatory or non-inflammatory stimuli, including foreign bodies, schistosome eggs, and pathogenic mycobacteria among others. In schistosomiasis and TB, aggregating macrophages undergo a series of stereotyped morphological changes; their cytoplasm expands, and zipper-like interdigitations form between the apposed membranes of neighboring. Histological characterization of this transformation led pathologists to term these macrophages ''epithelioid'' as far back as at least 1888, due to their abundant cytoplasm, pale, oval nuclei, and close interdigitation with neighboring cells. Although they resemble epithelial cells by histology, the epithelioid cells that form the central scaffold of tuberculous granulomas are thought to be macrophage derived.

2.4.5 T-Regulatory Cells.

As Lau et al noted :

The fine cross talk between CSCs and immunosuppressive cells also involves Treg cells. Treg cells are defined by the CD4+CD25+FOXP3+ T cell subpopulation, with FOXP3 as an important transcriptional regulator of Treg cell development and function. Treg cell-mediated immunosuppression primarily occurs through the production of various cytokines, such as IL-10, IL-35, and TGF-β, direct cell-cell contact via gap junctions, or metabolic disruption in which CD39 and CD73, expressed on Treg cells, facilitate the conversion of ATP to adenosine, which suppresses cytotoxic T cell and/or NK cell activity.

In tumors, Treg cells are accumulated by various mechanisms, primarily involving chemokine attractions. For example, the chemokines CCL22 and CCL28 are produced by tumor cells to attract CCR4- and CCR10-expressing Treg cells, respectively, leading to the accumulation of Treg cells in various human cancers. Indeed, the number of Treg cells inside the tumor microenvironment is associated with clinical outcome. The higher number of Treg cells within the tumor is correlated with poor prognosis in a wide array of cancers, including gastric, esophageal, pancreatic, liver, and breast cancers. In addition to its immune-suppressive role, the functional importance of tumor-infiltrating Treg cells in regulating CSCs is starting to emerge.

A recent report demonstrated that, under hypoxia, FOXP3+ Treg cells are induced to express IL-17, which drives the expansion of CSCs through the activation of Akt and MAPK signaling

pathways in colorectal cancer, evidenced by the increase in the expression of colorectal CSC markers, including CD133, CD44s, and EpCAM. Furthermore, Treg cells produce and secrete prostaglandin (PGE2) for immunosuppression, and PGE2 has been implicated in the regulation of CSC properties in colorectal cancer through NF-κB.

2.4.6 Natural Killer Cells.

NK cells are often the first to attack aberrant intruders including cancer cells. As part of the innate immune system they can be effect first remediation players. However NK cells can be co-opted as are other immune elements. Lau et al note:

The ability of natural killer (NK) cells to kill or spare depends on their expression of activating (mostly stress-induced proteins) and inhibitory (in particular MHC class I molecules) ligands on the surface of target cells*.*

Approximately 95% of peripheral blood NK cells are CD56dim CD16+ which exerts strong cytotoxic activity. The remaining 5% of peripheral blood NK cells are CD56bright CD16− and show cytotoxicity through strong cytokine production. CD133+ glioblastoma stem cells that are able to express high levels of the activating DNAM-1 ligands PVR and Nectin-2 and low levels of MHC class I molecules have been reported to be poorly recognized and lysed by NK cells. Their cytotoxic activity was revamped following IL-2 or IL-15 activation.

Breast cancer CSCs have also been reported to fail to express detectable levels of NK ligands, which is consistent with metastatic spread. In melanoma and GBM, CSCs are highly resistant to NK cells and become susceptible to NK cytotoxicity only following stimulation with IL-2. However, the preferential resistance of CSC to NK cells is not the rule, as colon CSCs express lower MHC class I and higher levels of NK-activating ligands, including NKp30L and Nkp44L as compared to differentiated cells, which are responsible for the CSC susceptibility to NK cell killing.

Another mechanism by which cancer cells may evade from the cytotoxic effect of NK cells is the induction of apoptosis in microenvironmental immune cells through the interaction of CD95 (Apo1/Fas) with its ligand (CD95L). Interestingly, CD95R/L regulates CSC plasticity and its blockade reduces CSC in different tumor cell models, while activation of CD95R/L increases CSC number and is responsible for CSC reduced sensitivity to CD95-mediated apoptosis.

Collectively, CSCs are more refractory to the cytotoxic effect of NK cells in a variety of cancer types.

2.4.7 Other Stromal Cells.

Also Lau et al note:

There is increasing evidence that ***mast cells*** *(MCs) and their mediators are involved in the remodeling of the tumor microenvironment. Recent evidence has showed that MC regulates*

stemness of thyroid cancer through IL-8-Akt-Slug pathway. In prostate cancer, MC increased stem/progenitor cell population via altering LncRNA-HOTAIR/PRC2-androgen receptor- (AR-) MMP9 signals. In addition, neutrophils were found to play a crucial role in regulation of CSC populations. ...

Hypoxia Hypoxic microenvironments in tumors result from the rapid growth of cancer cells, which exceeds the limit of blood supply. In response to the hypoxia, the hypoxia-related gene expression is driven through the activated hypoxiainducible factor (HIF) and transcription factors HIF-1α and HIF-2α that bind to the hypoxia-regulated element (HRE) gene promoters. The capacity of HIFs to promote cancer cell stemness has been well documented. Studies have shown that HIFs can increase the expression of stem cell markers in breast cancer.

Bae et al. demonstrated that hypoxia can elevate the expression of the stem cell marker SOX2 in prostate cancer cell lines. In addition, the overexpression of HIF-1α has been associated with stem cell marker CD44 in bladder cancer. In addition to HIFs, the hypoxia-mediated overexpression of extracellular carbonic anhydrases, CAIV and CAXII, facilitates cancer cell survival and the maintenance of CSC function. Given that CSC is related to metastasis and cancer cell invasion, the contribution of hypoxia to the enhanced CSC migration has been reported in several studies.

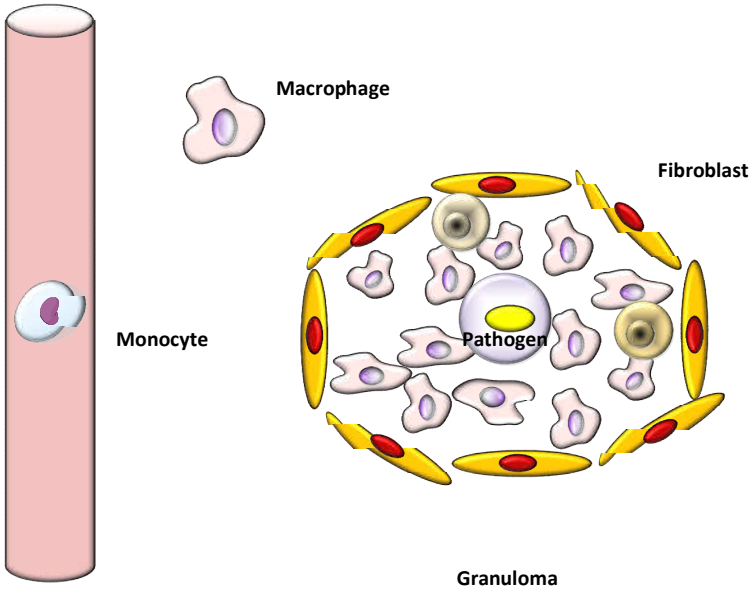
The upregulation of EMT-related gene expression under hypoxic stress can enhance the invasiveness and the stem-like properties of cancer. Maeda et al. showed that HIF-1α is correlated with the EMT and cell migration in CD133+ pancreatic CSCs. In addition to cancer cell invasion, hypoxia contributes to drug resistance by maintaining CSCs in a quiescent state to confer resistance to chemotherapeutics that commonly target actively dividing cancer cells.

Studies have reported that hypoxia promotes SOX-2-mediated drug resistance in ovarian CSCs via Notch signaling.

The downregulation of HIF-1α using a lentivirus-mediated approach can increase the chemosensitivity in triple negative breast cancer. These data demonstrated that hypoxia plays an important role in the CSC niche and is substantially involved in the regulation of cancer cell stemness.

3 GRANULOMAS

We now consider granulomas and attempt to establish a parallelism between a granuloma and the TME we have just summarized. First the granuloma generically appears as shown below. There is a pathogen which attracts monocytes changing into macrophages. Other immune cells are involved such as the M2 macrophages and T cells. It is then generally encased in a fibroblast covering. The pathogen may or may not survive.



3.1 STRUCTURE

Let us now consider the structure in some further detail. As Fatima has indicated:

The following histological features may appear on the examination of granulomas.

Macrophage Morphology:

Macrophages may undergo various changes within the granuloma, resulting in a range of histological appearances. Activated macrophages may undergo transformation resulting in an 'epithelioid' appearance, with flattened cell shape, ovoid nuclei, and membranes that interdigitate with adjacent cells.

The fusion of multiple macrophages together produces multinucleated giant cells (Langhans cells). Macrophages in which oxidized lipids have accumulated are known as foam cells.

Necrosis:

Necrosis develops in a subset of granulomas as they mature. Granulomas of specific etiologies are more likely to undergo necrosis than others, though the reasons behind this are not well-understood.

Tubercular and other infectious granulomas frequently become necrotic, while those caused by sarcoidosis and Crohn disease generally do not, even when large and mature. Tuberculous granulomas are particularly associated with ***caseating granulomas****, in which the necrotic area exhibits a 'cheese-like' macroscopic appearance. Necrosis is generally thought to be a pathological process and is known to be associated with increased morbidity and transmission in tuberculosis.*

Fibrosis:

Fibrosis is a prominent feature of many granulomas, which is unsurprising given the chronic nature of the lesions. Fibrosis may be adaptive, for example, as a method of sequestering parasitic eggs but is more often pathological. Fibrosis can lead to widespread loss of tissue function and may lead, for example, to liver failure in schistosomiasis or reduced lung

Herbath et al note:

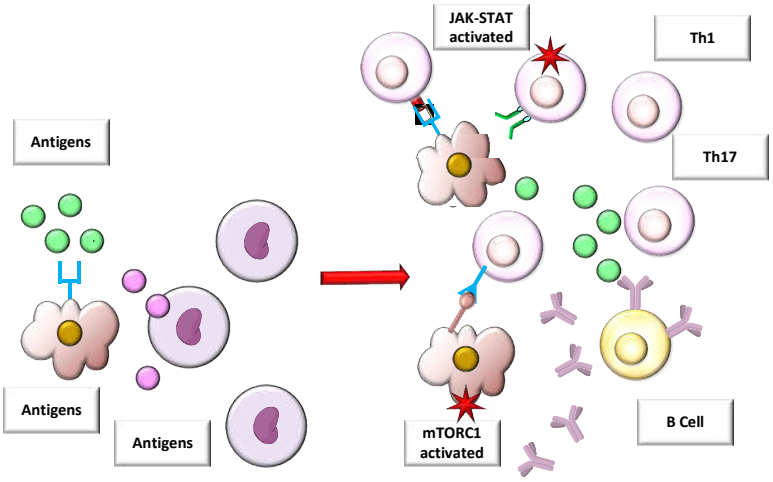
Granulomatous inflammation is a histologic pattern of tissue reaction which appears following cell injury. Granulomatous inflammation is caused by a variety of conditions including infection, autoimmune, toxic, allergic, drug, and neoplastic conditions. The tissue reaction pattern narrows the pathologic and clinical differential diagnosis and subsequent clinical management.

Common reaction patterns include necrotizing granulomas, non-necrotizing granulomas, suppurative granulomas, diffuse granulomatous inflammation, and foreign body giant cell reaction. Prototypical examples of necrotizing granulomas are seen with mycobacterial infections and non-necrotizing granulomas with sarcoidosis.

However, broad differential diagnoses exist within each category. Using a pattern based algorithmic approach, identification of the etiology becomes apparent when taken with clinical context. The pulmonary system is one of the most commonly affected sites to encounter granulomatous inflammation. Infectious causes of granuloma are most prevalent with mycobacteria and dimorphic fungi leading the differential diagnoses. Unlike the lung, skin can be affected by several routes, including direct inoculation, endogenous sources, and hematogenous spread.

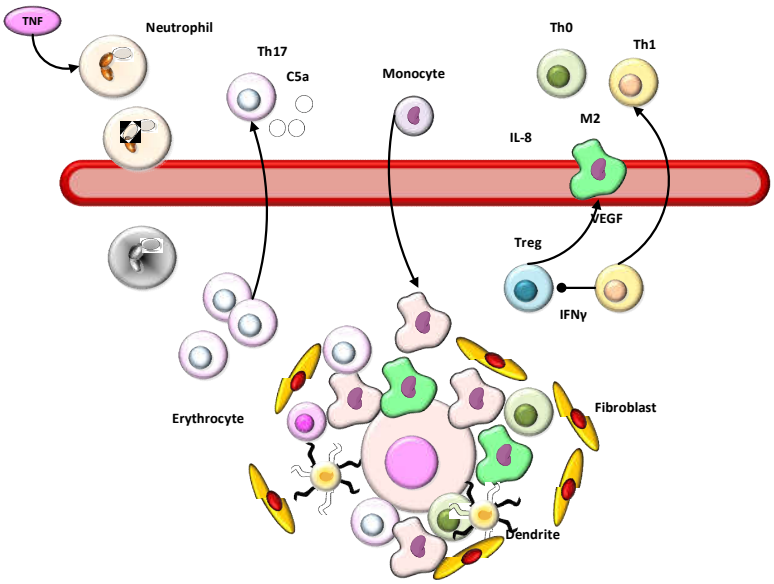
This broad basis of involvement introduces a variety of infectious agents, which can present as necrotizing or non-necrotizing granulomatous inflammation. Non-infectious etiologies require a thorough clinicopathologic review to narrow the scope of the pathogenesis which include: foreign body reaction, autoimmune, neoplastic, and drug related etiologies. Granulomatous inflammation of the kidney, often referred to as granulomatous interstitial nephritis (GIN) is unlike organ systems such as the skin or lungs. The differential diagnosis of GIN is more frequently due to drugs and sarcoidosis as compared to infections (fungal and mycobacterial)

An example of the progression of a granuloma and its drivers is demonstrated below:



Note that we have the external elements clustering about the pathogen and then the internal elements, such as mTORC1, JAK, Th17, Th1 all being activated by the external elements. This is a bit unlike the TME where the cancer cell is itself a major participant. As noted previously the granuloma kernel is the pathogen and its sole or dominant activation is through the PARP.

The graphic below demonstrates the activation of a granuloma via the circulatory system:



Clearly there are multiple external and internal elements. There can be an increase in neutrophil entry but then an apoptosis of the neutrophil. Macrophages play a critical role in the structure as do the fibroblasts. Now Hilhorst et al note:

Upon priming neutrophils bring to their surface high levels of proteinase-3 (PR3) and myeloperoxidase (MPO), the autoantigens recognized by ANCA. The priming process is believed to be mediated by TNFa. TNFa production may be induced by a variety of triggers; e.g., S. aureus, silica, etc.

Anti-PR3 or anti-MPO antibodies are then able to bind these enzymes on the cell surface, causing neutrophils to degranulate, bind to endothelial cells, enter the perivascular tissue, and release ROS; thereby damaging the vessel wall.

In the tissue, neutrophils releases so-called neutrophil extracellular traps (NETs), which are networks of fibers and DNA to trap pathogens. Many of the highly activated neutrophils become apoptotic and are then phagocytosed by resident M2 macrophages, a process that has been named efferocytosis and that may be deficient in GPA. During efferocytosis, macrophages release TGF-b, IL-8, and CCL2.

Monocytes may also be activated by circulating ANCA, enhancing their chemotactic responsiveness and enabling them to participate in granuloma formation. The lower panel represents the formation of a sterile granuloma in extravascular tissue. Monocytes and CD4C cells enter the granuloma following a gradient of chemokines and cytokines; a process sufficient to transform monocytes into macrophages.

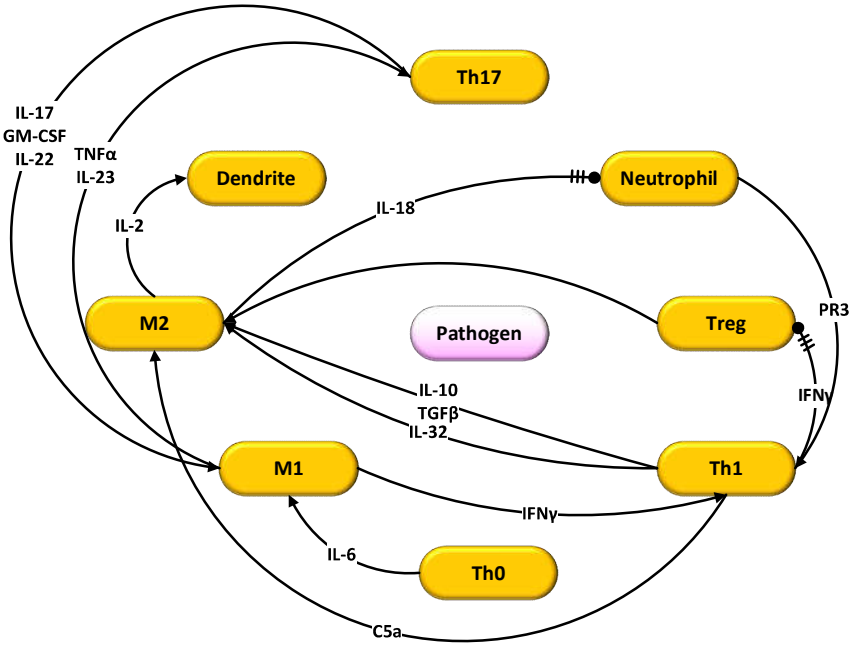
Commitment to the M1 or M2 lineage is dependent on the specific cytokine environment. Neutrophils and eosinophils are commonly found in granulomas in GPA. Also, B cells have been reported in the surroundings of granulomas, where they may undergo further maturation. Multinucleate giant cells are present within the granulomas, resulting from the fusion of either macrophages or dendritic cells. The organized arrangement of the granuloma provides an ideal

platform for macrophage–T cell interaction. CD4C cells coming into contact with IL-6 and TGF-b producing M1 cells are skewed toward the Th17 lineage. M1 also secrete IL-23; sustaining the Th17 population. In turn,

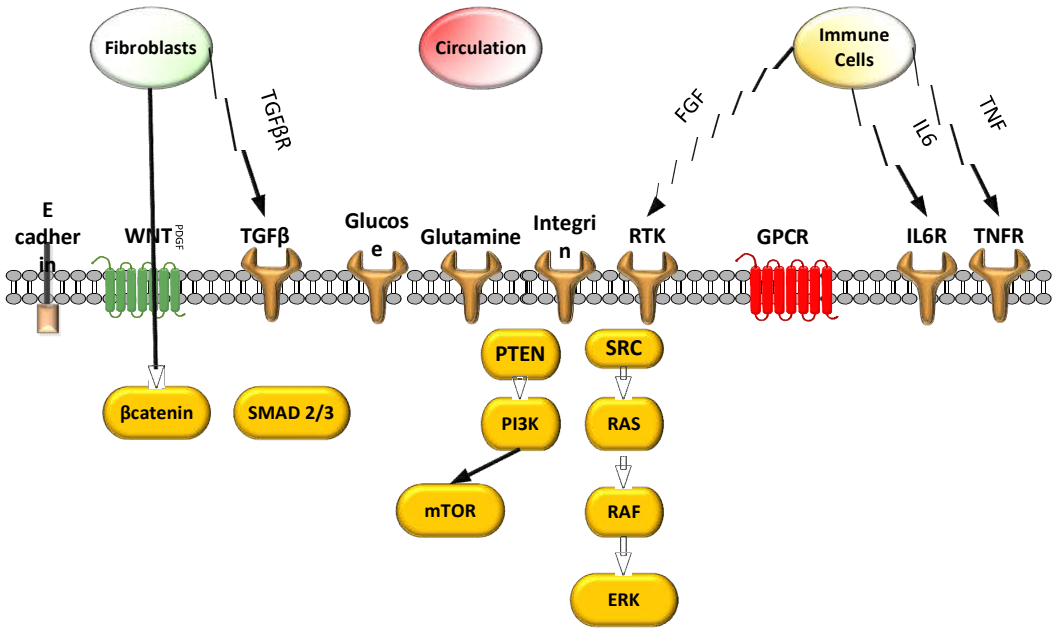
Th17 cells can secrete granulocyte and GM-CSF in addition to IL-17 and IL-22, thus stabilizing M1 differentiation. However, M2 cells are equally represented in granulomas and secrete IL-10 and TGF-b.

M2 cells are a source of vascular endothelial growth factor (VEGF) and support the outgrowth of microvessels, critically important as the granuloma grows in size.

The last statement above shows the criticality of the VEGF in insuring vasculature for survival.



The details of cellular growth and activation factors are shown below. All of these to some degree play a significant role in the granuloma formation and persistence.



3.2 CLASSES

We now summarize the different types of granulomas and from Shah et al we have the following drivers of granuloma formation:

Foreign Body

Talc, starch, suture, hyaluronic acid (and other injectable fillers) Necrotizing Granulomas

Infectious

Coccidioides immitis/C. posadasii, Cryptococcus neoformans/C. gattii, Histoplasma capsulatum, Blastomyces dermatitidis, Aspergillus spp., Mucorales, Mycobacterium tuberculosis, Non-tuberculous mycobacteria, Brucella spp., Nocardia spp., Yersinia spp., Bartonella henselae, Pneumocystis jiroveci, Echinococcus granulosus, xanthogranulomatous pyelonephritis+

***Autoimmune**:*

Rheumatoid nodule, granuloma annulare, necrobiosis lipoidica, granulomatosis with polyangiitis
Non-Necrotizing Granulomas

Infectious:

Candida albicans (hepatosplenic candidiasis), C. immitis/C. posadasii, Coxiella burnetii, cytomegalovirus, M. tuberculosis, non-tuberculous mycobacteria including M. leprae (tuberculoid forms), Schistosoma spp., Toxoplasma gondii, Rickettsia spp., Salmonella typhi, hepatitis A & C virii,

***Autoimmune**:*

Sarcoidosis, Churg Strauss, giant cell arteritis, systemic lupus erythematous, Crohn disease, primary biliary
cirrhosis, orofacial granulomatosis, rosacea, granuloma annulare

Toxic:

actinic granuloma, berylliosis, zirconium, hot tub lung

Drug:

Bacillus Calmette-Guérin, Non-steroidal anti-inflammatory drugs, antibiotics, methotrexate

Other:

Lymphoid interstitial pneumonia, hypersensitivity pneumonitis, chronic lymphocytic leukemia Suppurative Granulomas

Infectious

Actinomyces spp., Dirofilaria spp., Acanthamoeba spp., Balamuthia mandrillaris, B. henselae, B. dermatitidis, Brucella spp., Chlamydia trachomatis (serotypes L1, L2, L3 causing lymphogranuloma venereum), dematiaceous fungi causing chromoblastomycoses and phaeohyphomycosis, non-tuberculous mycobacteria, Francisella tularensis, Prototheca spp., Sporothrix schenckii, Paracoccidioides brasiliensis, Yersinia spp., Enterobius vermicularis Histiocytic response,no granulomas

***Infectious**: Tropheryma whipplei, Listeria monocytogenes, non-tuberculous mycobacteria including M. leprae (lepromatous forms), H. capsulatum, Leishmania spp., Rhodococcus spp. (with malakoplakia)*

***Other**:*

Langerhans cell histiocytosis, granulomatous mycosis fungoides, juvenile xanthogranuloma, reticulohistiocytoma, Rosai Dorfman, pineal germinoma, seminoma/dysgerminoma, dendritic cell sarcoma, Erdheim-Chester disease, hemophagocytic lymphohistiocytosis, histiocytic sarcoma, interdigitating cell sarcoma, Langerhans cell sarcoma

Thus granulomas can be formed by a variety of drivers. The pathogens may be merely an irritant or it may be a complex microorganism.

3.3 External Components

The external elements of a granuloma are akin to those of TME. There are macrophages, fibroblasts, lymphocytes (T cells) and the like. As Williams and Williams had noted earlier:

Before considering the pathogenesis of granulomatous inflammation it is essential to review our knowledge of the three fundamental cells involved, namely the macrophage, the epithelioid cell and the multinucleated giant cell.

The name "mononuclear phagocyte system" was proposed in 1969 to describe the group of highly phagocytic mononuclear cells and their precursors which are widely distributed in the body, related by morphology and function, and which originate from the bone marrow.

Macrophages, monocytes, promonocytes and their precursor monoblasts are included, as are Kupffer cells and microglia. Labelling studies with tritiated thymidine have shown that granuloma cells, including both epithelioid cells and multinucleated giant cells, are also of the same lineage2-5 and it is claimed that monocytes in tissue culture may develop into epithelioid cells and giant cells.

The origin of tissue macrophages (histiocytes) from bone marrow precursors via circulating monocytes is now well established, the maturation process being accompanied by progressive morphological and functional changes which continue even when macrophages enter the tissues. The production of monocytes is under positive and negative feedback control, with peripheral macrophages and lymphocytes secreting factors that are both stimulatory and inhibitory to stem cell proliferation in the marrow.

Recruitment and localization of monocytes into inflammatory lesions is aided by two groups of substances.

The emigration of monocytes from the circulation is promoted by chemotactic agents including microbial products, complement components, fibrin degradation products and lymphokines while the immobilization of macrophages within a lesion is aided by other lymphokines including migration inhibition and macrophage adhesion factors.

Epithelioid cells are mononuclear cells with finely granular eosinophilic cytoplasm, vesicular nuclei, and indistinct cell boundaries which are usually found aggregated into clusters within certain granulomas. Their mononuclear phagocyte origin is not in doubt, but there remains controversy over the mechanisms by which epithelioid cells are formed, and in particular the role of cell-mediated immunity.

Epithelioid cells have been considered to be a hallmark of delayed hypersensitivity granulomas, a fact well illustrated in the pathology of leprosy, where epithelioid cells only occur with the appearance of cell-mediated immunity to the causative organism.

However there are now reports of epithelioid granuloma formation in genitally athymic "nude" animals suggesting that T cell function is not essential. Furthermore, although lymphokines induce dramatic changes in macrophages in vitro, the changes are not quite those of epithelioid transformation

Multinucleated giant cells are a regular feature of granulomatous inflammation.

There is now overwhelming evidence that they are macrophage polykaryons, produced by the fusion of macrophages, rather than by nuclear mitosis without cytoplasmic inflammatory giant cells have been divided into the Langhans (tuberculous) type, in which up to 20 nuclei are distributed centrally or around the periphery of the cell, and the foreign-body type with often very numerous haphazardly arranged nuclei throughout the cytoplasm.

However it is now clear that there is no fundamental difference between these two cell types, and there is no diagnostic.

Electron microscopy suggests that epithelioid cells division significance. Both types are commonly found to coexist in the same lesion, transitional forms have been described, and studies in tissue culture have shown that foreign body type giant cells "mature" into Langhans type cells, probably by movements of the intracellular cytoskeleton ...

Fibrosis is a common and important complication of granulomatous inflammation because it is often responsible for permanent tissue damage even after the causative agent has been eliminated. Thus hepatic and pulmonary fibrosis are important long-term complications of schistosomiasis and sarcoidosis respectively. However, until very recently, knowledge of how granulomas lead to fibrosis was very scanty.

It is clear that permanent fibrosis is not inevitable —most pathologists have seen lung biopsies with florid granulomatous inflammation being followed by apparent complete resolution and fibroblasts and collagen degradation, chiefly by neutral proteases. Macrophages have the potential to affect both sides of this balance. Their presence is highly desirable for successful wound healing and, when cultured under appropriate conditions they secrete substances which increase hydroxyproline production or stimulate proliferation in fibroblasts. Interleukin-1, a macrophage product which is closely related to endogenous pyrogen, is probably one such substance, while fibronectin, a glycoprotein secreted by macrophages with important roles in cellular adhesion, is a chemotactic agent for fibroblasts. On the other hand, macrophage supernatants which inhibit collagen synthesis have been described, and collagenase secretion by activated macrophages is well established.

Lymphocytes also have the potential for affecting fibrosis by their secretion of lymphokines which can induce fibroblast migration, proliferation and collagen synthesis. Studies on whole granulomas have also found them to contain substances that induce fibroblast proliferation. They probably originate from macrophages or lymphoid cells.

Moreover, a study of explanted granulomas of different types has found that collagen synthesis is most active in immunological granulomas and lowest in foreign body lesions, corresponding with findings in vivo described above. This suggests that cell-mediated immunity is of considerable importance in controlling fibrogenesis but whether this is achieved by a direct action of lymphoid cell products, or by an indirect effect of macrophage activation is uncertain. Epithelioid cells have been suggested as having a role in fibrosis,45 and while to date there is no

direct evidence for this, the degree of fibrosis in mycobacterial granulomas does correlate with their content of epithelioid cells.

Granulomatous inflammation represents a distinctive tissue reaction to an irritant in which the central cell is the mononuclear phagocyte cell, but which can be modified by other phenomena, especially hypersensitivity. The last 25 years have seen tremendous improvement in our knowledge of cell biology, immunology and macrophage function but in spite of this many mysteries continue to surround the pathogenesis of organised granulomas and the function and significance of their two distinctive cell types, epithelioid cells and giant cells.

Continued research into granulomatous inflammation is essential, not only for its theoretical value, but also for its important potential clinical implications. Better knowledge of the granulomatous process will both some granulomas, such as those of lepromatous leprosy or carrageenan are associated with little fibrosis.

3.3.1 Macrophages

As Duque and Descoteaux have noted

When macrophages are exposed to inflammatory stimuli, they secrete cytokines such as tumor necrosis factor (TNF), IL-1, IL-6, IL-8, and IL-12. Although monocytes and macrophages are the main sources of these cytokines, they are also produced by activated lymphocytes, endothelial cells, and fibroblasts. Additionally, macrophages release chemokines, leukotrienes, prostaglandins, and complement. All of these molecules, in concert, may induce increased vascular permeability and recruitment of inflammatory cells. Aside from local effects, these mediators also produce systemic effects such as fever and the production of acute inflammatory response proteins. The inflammatory response is beneficial for the host when the aforementioned cytokines are produced in appropriate amounts, but toxic when produced in a deregulated fashion. For example, excessive production of IL-1b and TNF triggers an acute generalized inflammatory response characteristic of septic shock and multi-organ failure (12).

3.3.2 Fibroblasts

Fibroblasts are common cells which often act as an effective shell about a granuloma. As Dick and Limaiem note[4]:

The fibroblast is one of the most abundant cell types present in the stroma. It has a variety of functions and composes the basic framework for tissues and organs. Under homeostasis, this cell is responsible for maintaining the extracellular matrix (ECM). During stress, fibroblasts adapt to their environment and have the ability to respond and send local signals. In times of injury, the fibroblast can transform phenotypes and synthesize the building blocks necessary to replace wounded tissue. During pathologic states, the extracellular matrix gets generated in excessive quantities, and collagen is deposited in a dysregulated manner often causing irreversible organ dysfunction or disfiguring appearance.

[4] https://www.ncbi.nlm.nih.gov/books/NBK541065/

Fibroblasts are a diverse group of cells. Within one organ system, there can be a great variety of functions. Within the integument, dermal fibroblasts in different locations have separate roles. The superficially located lineage involves the formation of the hair follicle and is responsible for reepithelization during wound healing; the deeper lineage is responsible for ECM generation. Fibroblasts are known for their plasticity; adipocytes, pericytes, endothelial and epithelial cells, otherwise known as terminally differentiated cells, can de-differentiate into fibroblasts. Stimulation of fibroblasts further increases susceptibility to epigenetic modifications. The ability of fibroblasts to transform is partly due to the variety of cell-surface adhesion receptors (integrins, syndecans, cadherins) that facilitate the communication of fibroblasts with their surroundings. One of these well-described fibroblast transformations is the transformation of fibroblast into the myofibroblast. Myofibroblasts are present in both healthy and pathologic tissues and contain features of fibroblasts and smooth muscle cells. These cells work in conjunction with vascular endothelial cells to form granulation tissue during times of wound healing.

3.4 Internal Pathway Components

A second major factor in understanding granulomas is the impact on extracellular and intracellular signalling whether through cytokines or internal pathways. We consider two key players here; VEGF and mTOR.

3.4.1 VEGF and VEGFR

From Nilson and Heymach:

VEGF is now known to be the prototypic member of a family of structurally related dimeric proteins including VEGF-B, VEGF-C, VEGF-D, and VEGF-E, as well as placental-growth factor (PlGF) -1 and -2. VEGF is essential for development because homozygous or heterozygous deletion of the VEGF gene is embryonically lethal.

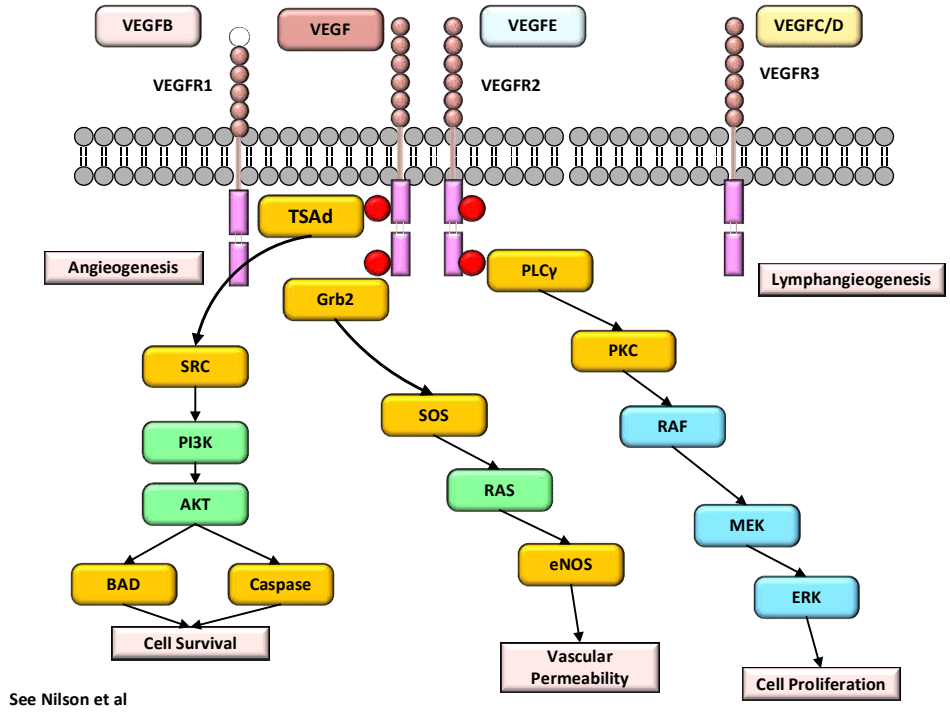
Indeed, VEGF family members are important in physiological angiogenic processes in the adult including wound healing, ovulation, and pregnancy, as well as pathological conditions such as cancer.

VEGF ligands activate angiogenic programs through binding of several receptors.

VEGFR-1 (Flt-1) binds VEGF, VEGF-B, and PlGF -1,2 and promotes recruitment of endothelial progenitors and monocyte migration. VEGFR-2 (Flk-1/KDR) is expressed on nearly all endothelial cells and binds VEGF, VEGF-C, VEGF-D, and VEGF-E.

Signal transduction through VEGFR2 has been shown to regulate endothelial cell proliferation, migration, and survival. In healthy adults, expression of VEGFR-3 is limited to lymphatic endothelium, although VEGFR-3 may also be expressed on tumor-associated blood vessels.

Through binding to VEGF-C and VEGF-D, VEGFR-3 is thought to facilitate the outgrowth of lymphatic vessels. Neuropilin (NRP)-1,2 have been demonstrated to be coreceptors for VEGF. NRP-1 binds VEGF165 and PlGF, and NRP-2 binds VEGF165 and VEGF-C.4 Unlike other VEGFRs, NRP-1,2 lack intracellular signaling domains. Although the specific role of NRP-1,2 in angiogenesis is not fully known, NRP-1,2 bind VEGF ligands and enhance their affinity to other VEGFRs.



From Nilson and Keymach:

Agent	Target within VEGF pathway	Type	Company
Bevacizumab	VEGF	mAB	Genentech
VEGF trap	VEGF	EC receptor domain	Regeneron
IMC-1121B	VEGFR-2	mAb	Imclone
Sunitinib	VEGFR-1,2,3	RTKI	Pfizer
Sorafenib	VEGFR-2,3	RTKI	Bayer
ZD6474,	VEGFR-2,3	RTKI	AstraZeneca
AZD2171	VEGFR-1,2,3	RTKI	AstraZeneca
Vatalinib	VEGFR-2	RTKI	Novartis
AMG706	VEGFR-1,2,3	RTKI	Amgen
Ag-013736	VEGFR-1,2,3	RTKI	Pfizer

As Duffy et al note a focus on CNS tissues but which apply more globally as well :

Vascular Endothelial Growth Factor (VEGF) is a major contributor to the growth of malignant tumors of the central nervous system. It stimulates tumor angiogenesis and vascular proliferation characteristic of high grade gliomas.

Elevated expression of VEGF is one the factors responsible for the virulent nature of these tumors. The production of VEGF by malignant glial cells in response to ionizing radiation contributes to treatment failure. The rat C6 glioma is similar to human gliomas with respect to VEGF pathophysiology. Interruption of VEGF-Receptor signaling in preclinical models effectively suppresses tumor growth and demonstrates the potential for anti-angiogenic therapy….

Intact VEGF-Receptor signaling is required for maturation of the central nervous system (CNS). Mutant mice heterozygous for VEGF die in utero and develop multiple anomalies including failure of vascularization of the neuroepithelium, disorganization of neuroepithelial cells, and underdevelopment of the forebrain.1 A single mutant allele can bring this about.

In mature brain tissue VEGF is distributed in areas surrounding the microvasculature where it may assist in maintaining the differentiated state.2 VEGF is also produced in response to CNS trauma. In response to cold thermal injury, VEGF isoform A is upregulated in astrocytes, inflammatory cells, and neovascular endothelium in the rat brain. Increased production of VEGF mRNA was demonstrated as early as six hours after injury by in situ hybridization …

High grade gliomas are incurable by current methods of treatment. They possess the ability to regenerate by mounting a vigorous angiogenic response. VEGF is central to the process. It is the main "accelerant" which fuels tumor growth before and after conventional treatment. In preclinical models, blockade of VEGF-receptor signaling disrupts angiogenesis which causes tumor shrinkage and growth delay. The magnitude of the effect is impressive. However, secondary growth factors (FGF's, PDGF, TGF etc) capable of stimulating angiogenesis are operative in high grade glioma. They can drive the angiogenic engine in the face of VEGF blockade.

Now Melincovici et al note:

Angiogenesis is an extremely complex process, influenced by multiple factors, some of them acting as proangiogenic agents, others as inhibitors of angiogenesis.

An extremely potent pro-angiogenic factor is vascular endothelial growth factor (VEGF) and, for this reason, there are numerous studies that demonstrated its implication in angiogenesis. During the embryonic period, the formation of new vessels occurs by the differentiation of endothelial cells from hemangioblasts (vasculogenesis).

Later, after birth, in certain physiological processes (menstrual cycle, pregnancy, wound healing and repair, etc.), new vascular networks are formed by angiogenesis, based on preexisting vessels (neoangiogenesis).

At the same time, data suggests that VEGF plays an important role in pathological angiogenesis, inducing the development and progression of certain pathological conditions in the postnatal period, such as: tumor growth and metastasis, macular degeneration, diabetic retinopathy,

inflammatory processes (e.g., rheumatoid arthritis), ischemic processes (myocardial ischemia), preeclampsia, etc..

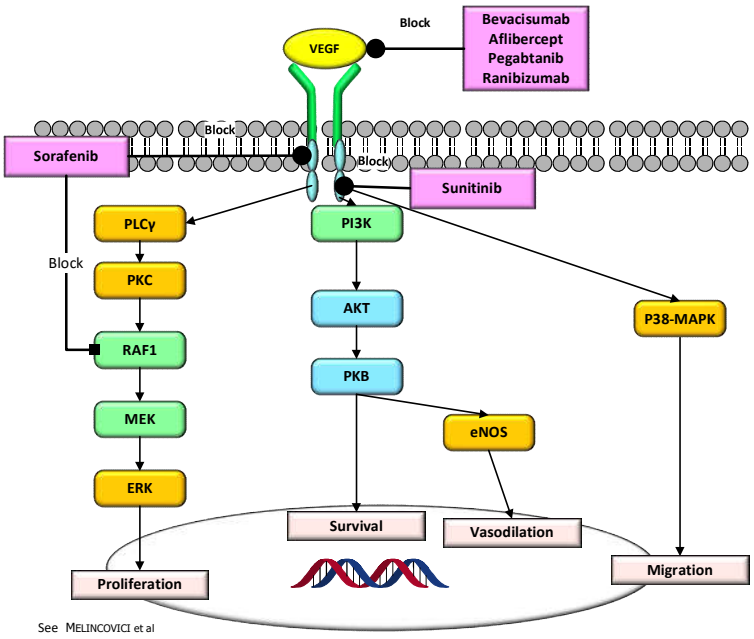
At present, increased attention is focused on the process of formation and development of certain new lymphatic vessels (lymphangiogenesis) The human VEGF gene, located on the 6p21.3 chromosome, is part of the VEGF/platelet-derived growth factor (PDGF) gene family, also called the cystine-knot superfamily of growth factors. From a structural point of view, VEGF is a 40-kDa heterodimeric glycoprotein, which contains the cystine-knot motif, characterized by the disposition of certain bisulfidic bridges in the protein structure.

Alongside VEGF, there are additional growth factors from the cystineknot motif family: PDGF, nerve growth factor (NGF) and transforming growth factor-beta (TGF-β). In humans, the VEGF family includes several members that perform various functions:

VEGF-A (which presents several isoforms), VEGF-B, VEGF-C, VEGF-D, VEGF-E (viral VEGF, in parapoxvirus 1), VEGF-F (snake venom VEGF) and the placenta growth factor (PlGF).

More recently, a new member has been added to this family, named the endocrine gland-derived vascular endothelial growth factor (EG-VEGF)

The authors then present putative therapeutics and targets:



Key Points

1. *Three vascular endothelial growth-factor receptors (VEGFRs) regulate vascular-endothelial, haematopoietic and lymphatic-endothelial cell function during development and in the adult.*

Many of these processes require balanced VEGFR signalling, which involves more than one of the VEGFRs.

2. *VEGFR1 signalling seems to be dispensable for endothelial-cell function, but it is essential for the migration of haematopoietic cells. A soluble splice variant of VEGFR1, which lacks the intracellular domain, might function as a VEGF 'trap', and is implicated in preeclampsia during pregnancy. VEGFR1 signal transduction might positively or negatively regulate VEGFR2 activity.*

3. *VEGFR2 is absolutely required for endothelial-cell development and survival of blood vessels. Tyrosine phosphorylation sites in VEGFR2 regulate kinase activity and binding of phospholipase C-γ, and the adaptor molecules TSAd, Shb and Sck. VEGFR2-blocking therapies are in use or are being tested for the treatment of human malignancies.*

4. *VEGFR3 is required for cardiovascular development and lymphangiogenesis. Certain VEGF family members might induce formation of heterodimers, which involves VEGFR2 and VEGFR3, thereby regulating the phosphorylation of VEGFR3 and consequent signal transduction.*

5. *Co-receptors (VEGF-binding molecules that might lack intrinsic catalytic function) such as heparan-sulphate proteoglycans and neuropilins are engaged in the VEGFR signalling complex in a manner that is guided by the VEGF isoform. Co-receptors modulate the duration and quality of VEGFR signalling by the formation of VEGF gradients and by stabilizing the signalling complex. Cell-cell and cell-matrix adhesion molecules that are regulated, for example, by blood flow, affect VEGFR signalling by allowing receptor activation in the absence of VEGF.*

6. *The signal from an activated VEGFR is influenced by several factors (the particular VEGF isoform, the possibility of homodimerization or heterodimerization with other VEGFRs, co-receptors or adhesion molecules) in the local milieu.*

Vascular endothelial growth-factor receptors (VEGFRs) regulate the cardiovascular system. VEGFR1 is required for the recruitment of haematopoietic precursors and migration of monocytes and macrophages, whereas VEGFR2 and VEGFR3 are essential for the functions of vascular endothelial and lymph endothelial cells, respectively. Recent insights have shed light onto VEGFR signal transduction and the interplay between different VEGFRs and VEGF co-receptors in development, adult physiology and disease.

From NCBI[5]:

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.

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

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.

The levels of VEGF are increased during infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), thus promoting inflammation by facilitating recruitment of inflammatory cells, and by increasing the level of angiopoietin II (Ang II), one of two products of the SARS-CoV-2 binding target, angiotensin-converting enzyme 2 (ACE2). In turn, Ang II facilitates the elevation of VEGF, thus forming a vicious cycle in the release of inflammatory cytokines.

As Harding et al (2019) note:

Many autoimmune and infectious diseases are characterized by the formation of granulomas which are inflammatory lesions that consist of spatially organized immune cells. These sites protect the host and control pathogens like Mycobacterium tuberculosis (Mtb), but are highly inflammatory and cause pathology. Using bacille Calmette-Guerin (BCG) and Mtb infection in mice that induce sarcoid or caseating granulomas, we show that a subpopulation of granuloma macrophages produces vascular endothelial growth factor (VEGF-A), which recruits immune cells to the granuloma by a non-angiogenic pathway. Selective blockade of VEGF-A in myeloid cells, combined with granuloma transplantation, shows that granuloma VEGF-A regulates granulomatous inflammation.

The severity of granuloma-related inflammation can be ameliorated by pharmaceutical or genetic inhibition of VEGF-A, which improves survival of mice infected with virulent Mtb without altering host protection. These data show that VEGF-A inhibitors could be used as a host-directed therapy against granulomatous diseases like tuberculosis and sarcoidosis, thereby expanding the value of already existing and approved anti-VEGF-A drugs.

1. *Blockade of VEGF-A Reduces Granulomatous Inflammation without Compromising Bacterial Containment*

2. *VEGF-A Regulates Granulomatous Inflammation through Monocyte Recruitment and Not through Vasculogenic Effects*

3. *Inhibition of VEGF-A Decreases Granulomatous Lung Inflammation and Improves Survival after Aerosol Infection*

4. *VEGF-A Expression Is Associated with P2RX7 in Mycobacterial Granulomas*

In summary, we show that after reaching a size where cell death or hypoxia becomes a feeder for cells, a subpopulation of macrophages in the lesions produce VEGF-A, which recruits more cells into the lesions.

The appearance of larger lesions likely reflects the underlying tissue pathology observed in granulomatous disease.

Our data also show the importance of VEGF-A as an immunomodulatory target. Given that many VEGF-A inhibitors are already safe and in clinical use, they hold unique promise as a host-directed therapy in the growing number of patients with drug-resistant Mtb.

Additionally, there are more than 100 human pathological conditions associated with granuloma formation. The discovery that VEGF-A from granuloma macrophages regulates granulomatous inflammation, and can be influenced with existing anti-VEGF-A therapeutics, may have broad implications for this class of diseases

3.4.2 mTOR

We start with a brief overview of mTOR. As NCBI states[6]:

The protein encoded by this gene belongs to a family of phosphatidylinositol kinase-related kinases. These kinases mediate cellular responses to stresses such as DNA damage and nutrient deprivation. This protein acts as the target for the cell-cycle arrest and immunosuppressive effects of the FKBP12-rapamycin complex.

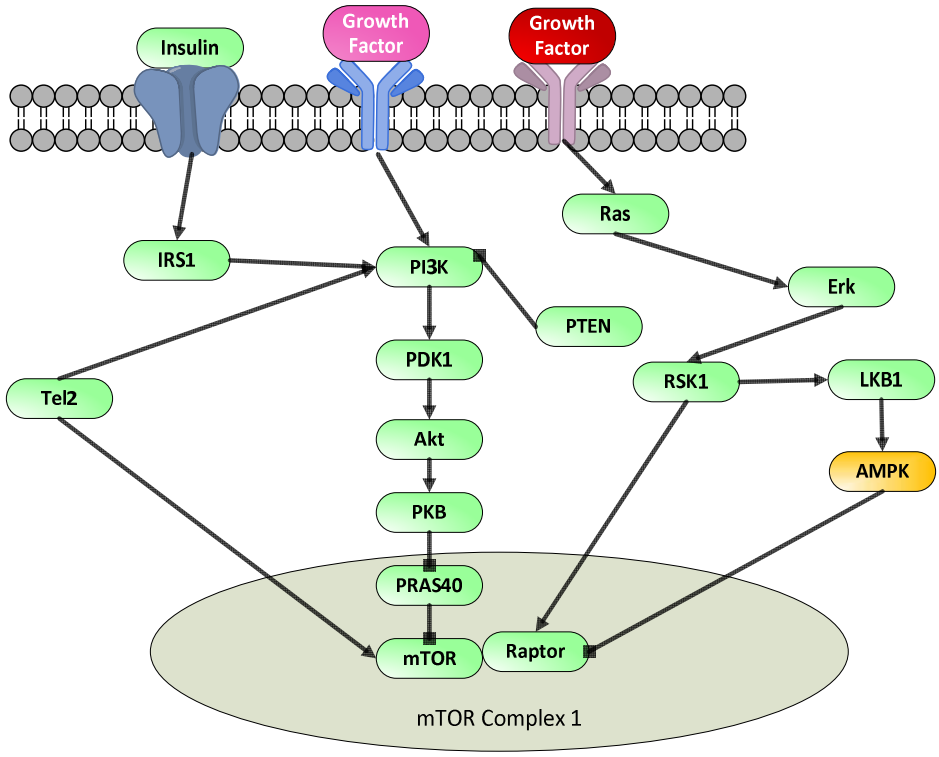
Now mTOR by itself plays a role only when conjugated with other products, namely those generating mTORC1 and mTORC2. We now briefly explain the structure of each of these two.

mTOR is a control protein that in involved in metabolic related pathways. mTOR, the mammalian target of rapamycin, is a gene product (1p36.2) is a protein which acts in a critical manner in interconnecting the genetic circuits in mammals, and especially man. It fundamentally controls glucose transport and protein synthesis. The pathway depicted below is a modification of the graphic from Weinberg (p 785) which shows mTOR in its two modes, one with Raptor

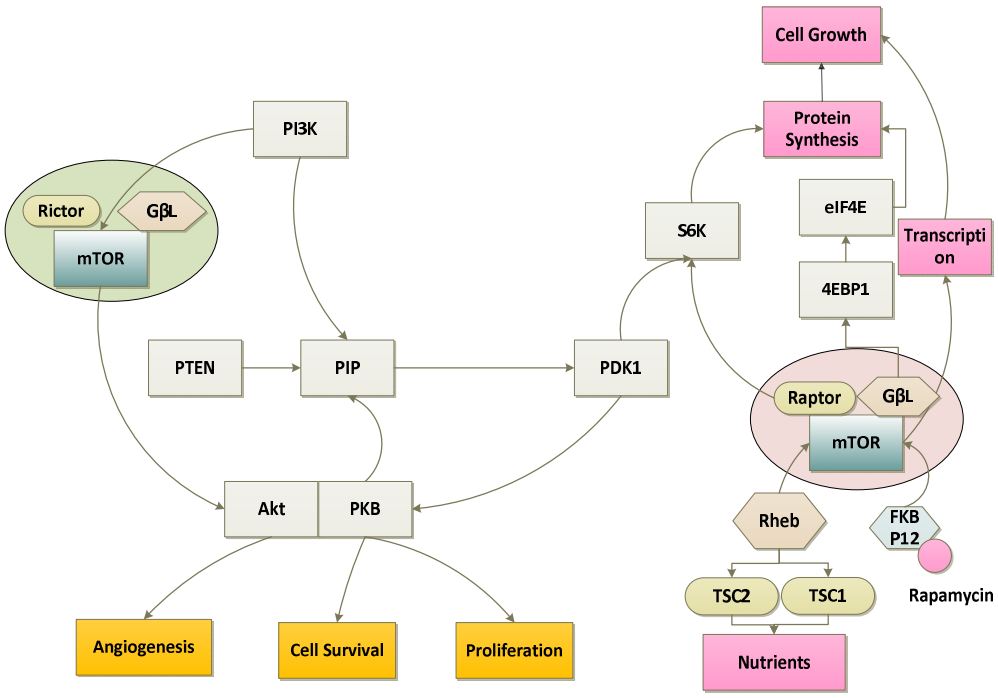
[6] https://www.ncbi.nlm.nih.gov/gene/2475

assisting and one with Rictor. The Rictor/mTOR mode activates the Akt pathway via the placement of a phosphate and this manages the protein synthesis portion. The inclusion of rapamycin will block the Raptor/mTOR path and reduce the protein synthesis and cell growth portion. The inhibitory effect on Akt/PKB by rapamycin is assumed to be the main factor in its anti-cancer effects.

We depict the mTOR C1 pathway below:



The following chart presents a more complex version of the mTOR C1 pathway (Raptor). This allows us to best understand the complex interactions. The mTOR C1 and C2 pathways are depicted in the combined chart below:



Looking at the complexity of the mTOR pathway it presents an interesting one for addressing PCa. Kinkaide et al (2008) indicate:

Among the major signaling networks that have been implicated in advanced prostate cancer are the AKT/mammalian target of rapamycin (AKT/mTOR) and MAPK pathways. Indeed, deregulated expression and/or mutations of the phosphate and tensin homolog tumor suppressor gene (PTEN) occur with high frequency in prostate cancer, leading to aberrant activation of AKT kinase activity as well as its downstream effectors, including the mTOR signaling pathway. In addition, many prostate tumors display deregulated growth factor signaling, which may result in activation of MAPK kinase 1 (MEK) kinase and ultimately ERK MAP.

Notably, previous studies have demonstrated that the AKT/mTOR and MAPK signaling pathways are alternatively and/ or coordinately expressed in advanced prostate cancer and function cooperatively to promote tumor growth and the emergence of hormone- refractory disease. These observations formed the basis for our hypothesis that targeting these signaling pathways combinatorially may be effective for inhibiting tumorigenicity and androgen independence in prostate cancer.

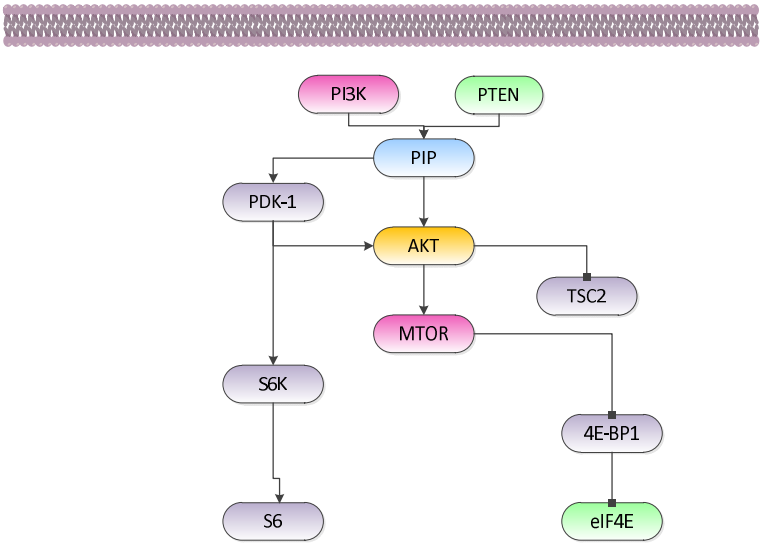
Kinkaide et al also demonstrate the creation of HGPIN via their work. This represents another pathway of HGPIN to PCa. LoPiccolo et al state:

The PI3K/Akt/mTOR pathway is a prototypic survival pathway that is constitutively activated in many types of cancer. Mechanisms for pathway activation include loss of tumor suppressor PTEN function, amplification or mutation of PI3K, amplification or mutation of Akt, activation of growth factor receptors, and exposure to carcinogens. Once activated, signaling through Akt

can be propagated to a diverse array of substrates, including mTOR, a key regulator of protein translation. This pathway is an attractive therapeutic target in cancer because it serves as a convergence point for many growth stimuli, and through its downstream substrates, controls cellular processes that contribute to the initiation and maintenance of cancer.

Moreover, activation of the Akt/mTOR pathway confers resistance to many types of cancer therapy, and is a poor prognostic factor for many types of cancers. This review will provide an update on the clinical progress of various agents that target the pathway, such as the Akt inhibitors perifosine and PX-866 and mTOR inhibitors (rapamycin, CCI-779, RAD-001) and discuss strategies to combine these pathway inhibitors with conventional chemotherapy, radiotherapy, as well as newer targeted agents. We (show) how the complex regulation of the PI3K/Akt/mTOR pathway poses practical issues concerning the design of clinical trials, potential toxicities and criteria for patient selection.

LoPiccolo et al show the more simplified pathway as follows:

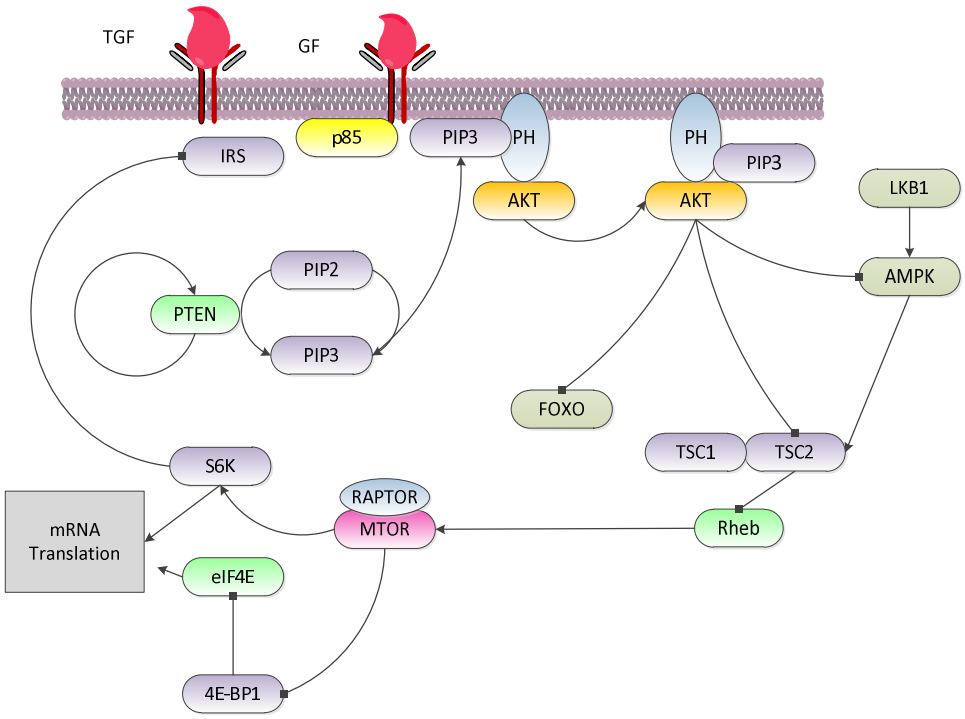


As we have shown with the more complex Weinberg model, here mTOR and PTEN play a strong role in the overall control. The authors show the points of possible control. The complexity of the pathways will be a challenge. It is less an issue of size complexity than a feedback and instability complexity. Nelson et al (2007) have demonstrated similar results as well.

Other researchers have also posited other simple models. We demonstrated the one by Hay as has been stated:

The downstream effector of PI3K, Akt, is frequently hyperactivated in human cancers. A critical downstream effector of Akt, which contributes to tumorigenesis, is mTOR. In the PI3K/Akt/mTOR pathway, Akt is flanked by two tumor suppressors: PTEN, acting as a brake upstream of Akt, and TSC1/TSC2 heterodimer, acting as a brake downstream of Akt and upstream of mTOR.

In the absence of the TSC1/TSC2 brake, mTOR activity is unleashed to inhibit Akt via an inhibitory feedback mechanism. Two recent studies used mouse genetics to assess the roles of PTEN and TSC2 in cancer, underscoring the importance of Akt mTOR interplay for cancer progression and therapy.



The Baldo et al model is quite similar to the Weinberg model shown initially. It clearly demonstrates the overall controlling influence of mTOR. As Baldo et al state:

There is a great body of evidence supporting consideration of the mTOR signaling system as an important network in cell regulation, differentiation and survival. mTOR is a sensor of mitogen, energy and nutritional levels, acting as a "switch" for cell-cycle progression from phase G1 to phase S.

The antibiotic Rapamycin, a potent mTOR inhibitor, has been known to the National Cancer Institute and recognized for its potential anticancer properties since the 1970s. The observation that cell lines from different cancer types exposed to low doses of Rapamycin underwent cell-cycle arrest in phase G1, provided the basis for considering mTOR as a target for cancer therapy.

Development of mTOR inhibitor compounds has proceeded empirically due to the lack of understanding of the precise molecular targets and the required dose of the new compounds . The development of Rapamycin analogs ("Rapalogs"), but also of other, structurally different, mTOR inhibitors, was directed at the selection of specific cancer type sensitivity and an optimization of pharmaceutical forms.

To give an example, Temsirolimus revealed clinical responses in patients with renal cell carcinoma in advanced stage. Temsirolimus was approved by the FDA on May 2007 for this therapeutic use and is being investigated in clinical trials for other cancer types (breast cancer, lymphoma, renal cancer, glioblastoma); significantly there are a considerable number of clinical studies involving mTOR inhibitors currently active worldwide…

The mTOR pathway controls cell size and cellular proliferation*.…nutrient metabolism, mRNA translation and cell survival control. Disruption of TOR leads to early embryonic death in flies and mammalian cells, indicating mTOR plays an important role in regulating cell survival. … deregulation of several mTOR components leads to modified cell proliferation patterns and, on the other, that many mTOR components are deregulated in several human cancers.*

*… **Therefore, inhibition of mTOR leads to slowing or arrest of cells in the G1 phase**. Translational control may have an important role in the balance of cell survival and death, and hence for apoptosis. Importantly, components of mTOR are deregulated in some human cancers, for example, breast and colon. Alteration of PI3-K/Akt is frequently observed in head and neck cancer .*

PTEN, a phosphatase that acts on PIP3 to convert it to PIP2, normally regulates the mTOR pathway negatively, and shows decreased activity in some tumors. A strong relation seems to exist between the sensitivity to the effect of Rapamycin and PTEN loss or deregulation. PTEN is frequently mutated in several cancers and in cancer-like syndromes like Cowden and Proteus syndromes…

Loss of PTEN function can occur in 26-80% of endometrial carcinomas*, …recent studies of human prostate cancer have shown that loss of PTEN is strongly associated with more aggressive cancers. The relationship between PTEN status and sensitivity to rapalogs has been questioned by several investigators. Some attention has recently been dedicated to the role of the mTORC2 complex in the mTOR pathway.*

In fact this complex, believed until recently to be completely insensitive to the effect of Rapamycin, after long-term exposure to Rapamycin is able to prevent mTOR-mediated Akt phosphorylation and the activation of the mTOR pathway. Another component, the TSC1/TSC2 complex located upstream of mTOR, is predicted to integrate signals derived from nutrients, cellular energy status and hypoxia into a common growth regulatory signal to the mTORC1 complex.

As Easton and Houghton state:

Proteins regulating the mammalian target of rapamycin (mTOR), as well as some of the targets of the mTOR kinase, are overexpressed or mutated in cancer. Rapamycin, the naturally occurring inhibitor of mTOR, along with a number of recently developed rapamycin analogs (rapalogs) consisting of synthetically derived compounds containing minor chemical modifications to the parent structure, inhibit the growth of cell lines derived from multiple tumor types in vitro, and tumor models in vivo.

Results from clinical trials indicate that the rapalogs may be useful for the treatment of subsets of certain types of cancer. The sporadic responses from the initial clinical trials, based on the hypothesis of general translation inhibition of cancer cells are now beginning to be understood owing to a more complete understanding of the dynamics of mTOR regulation and the function of mTOR in the tumor microenvironment. This review will summarize the preclinical and clinical data and recent discoveries of the function of mTOR in cancer and growth regulation.

The other observation here is that we often find multiple characterizations of the pathways. Namely there is no canonical form, and often a pathway is depicted to demonstrate a specific protein function. Thus we may see an emphasis on one set of proteins while others are neglected. As much as we currently attempt to unify this process we are left somewhat adrift in model development at this stage. This can be exemplified by now looking at the next section on LKB1. There we show its control over PTEN whereas in an earlier model we have it controlling AMPK. In reality there are multiple links as we have discussed. The literature can be even more confusing on this issue as well.

As Mendelsohn et al state:

It is now widely accepted that mTORC1 positively controls an array of cellular processes critical for growth, including protein synthesis, ribosome biogenesis, and metabolism, and negatively influences catabolic processes such as autophagy—all of which have roles in cancer pathogenesis. Elucidating the key downstream targets of mTORC1 driving these events is an intense area of research.

Originally, much of the study of mTOR relied on experiments in which rapamycin was used acutely to inhibit mTOR (which we now know was mTORC1) in cultured cells. This led to extensive characterization of the best known mTORC1 substrates eiF-4E-binding protein 1(4E-BP1) and S6 kinase 1 (S6K1), both of which regulate protein synthesis.3 In the unphosphorylated state, 4E-BP1 binds and inhibits the cap-binding protein and translational regulator eIF4E. When phosphorylated by mTOR, 4E-BP1 is relieved of its inhibitory duty, promoting eIF4E interaction with the eIF4F complex and the translation of capped nuclear transcribed mRNA.

Following co-regulatory phosphorylation by mTORC1 and another kinase called phosphatidylinositol 3-dependent kinase 1 (PDK1), S6K1 positively affects mRNA synthesis at multiple steps including initiation and elongation by phosphorylating several translational regulators. Although the preponderance of evidence indicates that S6K1 and 4E-BP1 are directly phosphorylated by mTOR, an unidentified phosphatase activity may also be involved in their regulation. For example, the rapamycin-sensitive phosphorylation site on S6K1 is rapidly dephosphorylated (i.e., within minutes) of exposure to the drug.

They continue:

Conditions that inhibit growth, such as decreased energy, low oxygen, and insufficient nutrients, are associated with the harsh microenvironment of poorly vascularized tumor. The ability of cancer cells to overcome these adverse conditions would promote tumor growth, putting the

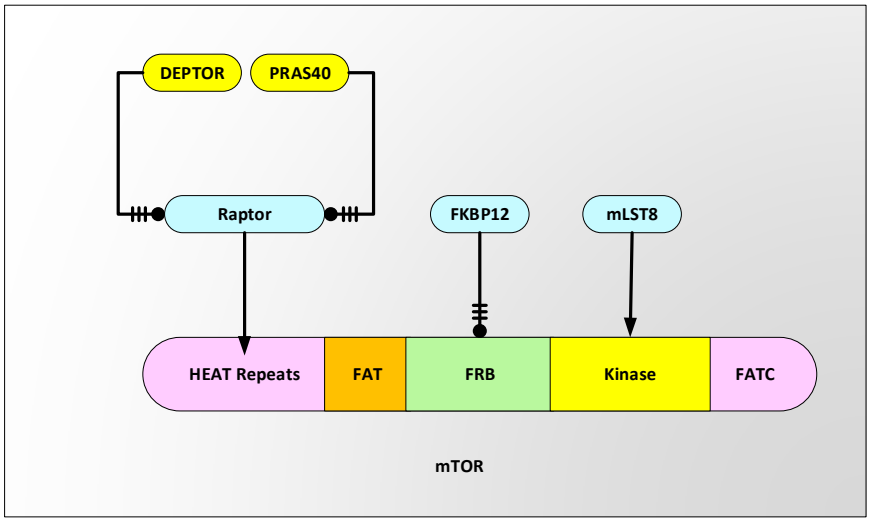
desensitization of mTORC1 signaling in the spotlight as a potential mechanism cancer cells could exploit to enhance their viability. Whether mutations in the amino acid– and glucose-sensing pathway that activates mTORC1 exist in cancer is not known. Mutations in the growth factor inputs to mTORC1 are prominent in cancer.

For example, mutations causing loss of PTEN function or oncogenic activation of PI3K or AKT are associated with many aggressive human cancers (Table 12-1).17-20 The findings that AKT promotes mTORC1 activity through TSC and PRAS40 suggest that cancers with elevated PI3K-AKT signaling may in part thrive because of an mTORC1-driven growth advantage. Activation of PI3K-AKT signaling also facilitates nutrient uptake by cells, which indirectly contributes to mTORC1 activity by localizing mTORC1 to lysosomes.

Therefore, understanding the contribution and relevance of mTORC1 signaling in the progression of cancers with aberrant PI3K-AKT signaling is an important area of research.

3.4.2.1 mTORC1

As we noted earlier mTORC1 has the most significant set of impacts on cell stability. Also as we noted there are upstream and downstream influences generated by this complex. We start with the structure of the mTORC1 complex as noted below:



The mTOR protein is composed of five sections, including the kinase element. The HEAT Repeats, as noted by Neuwald and Hirano are:

HEAT repeats correspond to tandemly arranged curlicue-like structures that appear to serve as flexible scaffolding on which other components can assemble. Using sensitive sequence analysis techniques we detected HEAT repeats in various chromosome-associated proteins, including four families of proteins associated with condensins and cohesins, which are nuclear complexes that contain structural maintenance of chromosome (SMC) proteins.

RAPTOR is the regulatory associated protein of mTOR[7]. RAPTOR is an mTOR binding protein.

As Saxton and Sabatini have noted:

In order to grow and divide, cells must increase production of proteins, lipids, and nucleotides while also suppressing catabolic pathways such as autophagy. mTORC1 plays a central role in regulating all of these processes and therefore controls the balance between anabolism and catabolism in response to environmental conditions... the critical substrates and cellular processes downstream of mTORC1 and how they contribute to cell growth.

Most of the functions discussed here were identified and characterized in the context of mammalian cell lines, while the physiological context in which these processes are important will be discussed in greater detail below.

Protein Synthesis mTORC1 promotes protein synthesis largely through the phosphorylation of two key effectors, p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP). mTORC1 directly phosphorylates S6K1 on its hydrophobic motif site, Thr389, enabling its subsequent phosphorylation and activation by PDK1.

S6K1 phosphorylates and activates several substrates that promote mRNA translation initiation, including eIF4B, a positive regulator of the 50cap binding eIF4F complex. S6K1 also phosphorylates and promotes the degradation of PDCD4, an inhibitor of eIF4B, and enhances the translation efficiency of spliced mRNAs via its interaction with SKAR, a component of exon-junction complexes.

The mTORC1 substrate 4EBP is unrelated to S6K1 and inhibits translation by binding and sequestering eIF4E to prevent assembly of the eIF4F complex. mTORC1 phosphorylates 4EBP at multiple sites to trigger its dissociation from eIF4E, allowing 50cap-dependent mRNA translation to occur.

Although it has long been appreciated that mTORC1 signaling regulates mRNA translation, whether and how it affects specific classes of mRNA transcripts has been debated. Global ribosome footprinting analyses, however, revealed that, while acute mTOR inhibition moderately suppresses general mRNA translation, it most profoundly affects mRNAs containing pyrimidine-rich 50 TOP or ''TOP-like'' motifs, which includes most genes involved in protein synthesis

Now the upstream influencers or drivers are detailed below from Seeboeck et al:

mTORC1 Upstream	
Rapamycin	rapamycin
FKBP12	FK506-binding protein 12 kDa
TSC	tuberous sclerosis complex
Rheb	Ras homolog enriched in brain
IGF-1 pathway	insulin/insulin like growth factor

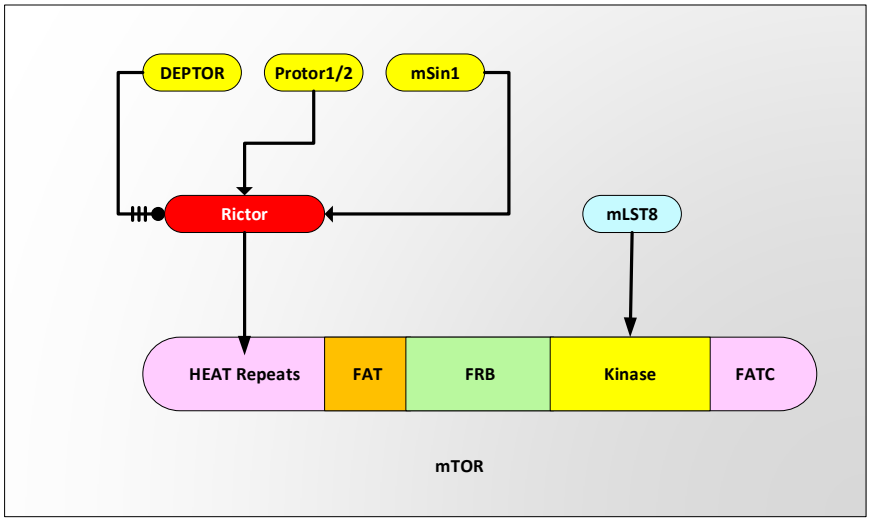
[7] https://www.ncbi.nlm.nih.gov/gene/57521

AKT	AKT serine/threonine kinase
mTORC2	promotes dissociation of PRAS40 from mTORC1.
Wnt	Wnt
TNFα 1	tumor necrosis factor α
AMPK	5'-AMP-activated protein kinase
REDD1	regulated in development and DNA damage responses 1

The above each in their own manner effects the actions of mTORC1. Rapamycin is a major driver when present. Some of these are exogenous to the cell itself such as the growth factors and others are part of the cell normal pathway. Note that mTORC2 has a driving factor as well. We shall briefly explore that next.

3.4.2.2 mTORC2

Now we consider mTORC2. From Seeboeck et al the structure appears as below:



Rictor is akin to Raptor. We see the same underlying mTOR base elements and then the complex binding to create the multiprotein complex. Now the drivers or upstream elements are shown below. Like mTORC1, it also is a driver here.

mTORC2 Upstream	
Rapamycin	rapamycin
FKBP12	FK506-binding protein 12 kDa
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
AKT	AKT serine/threonine kinase
mTORC1	Negative feedback loop between mTORC1 and insulin/PI3K signaling

Saxton and Sabatini have noted the downstream effects of mTORC2:

While mTORC1 regulates cell growth and metabolism, mTORC2 instead controls proliferation and survival primarily by phosphorylating several members of the AGC (PKA/PKG/PKC) family of protein kinases.

The first mTORC2 substrate to be identified was PKCa, a regulator of the actin cytoskeleton. More recently, mTORC2 has also been shown to phosphorylate several other members of the PKC family, including PKCd, PKCz, as well as PKCg and PKCε, all of which regulate various aspects of cytoskeletal remodeling and cell migration.

The most important role of mTORC2, however, is likely the phosphorylation and activation of Akt, a key effector of insulin/ PI3K signaling.

Once active, Akt promotes cell survival, proliferation, and growth through the phosphorylation and inhibition of several key substrates, including the FoxO1/3a transcription factors, the metabolic regulator GSK3b, and the mTORC1 inhibitor TSC2.

However, while mTORC2- dependent phosphorylation is required for Akt to phosphorylate some substrates in vivo, such as FoxO1/3a, it is dispensable for the phosphorylation of others, including TSC2. Finally, mTORC2 also phosphorylates and activates SGK1, another AGC-kinase that regulates ion transport as well as cell survival.

The mTORC1-dependent shift toward increased anabolism should only occur in the presence of pro-growth endocrine signals as well as sufficient energy and chemical building blocks for macromolecular synthesis. In mammals, these inputs are largely dependent on diet, such that mTORC1 is activated following feeding to promote growth and energy storage in tissues such as the liver and muscle but inhibited during fasting conserve limited resources. Here, we discuss the cellular pathways upstream of mTORC1 and the mechanisms through which they control mTORC1 activation.

3.4.3 TNF

Again as Duque and Descoteaux have note

Tumor necrosis factor (formerly known as TNF-a) is a 185- amino acid glycoprotein that was initially described for its ability to induce necrosis in certain tumors. It stimulates the acute phase of the immune response. This potent pyrogenic cytokine is one of the first to be released in response to a pathogen, and is able to exert its effects in many organs. As such, TNF is one of the main cytokines responsible for septic shock. In the hypothalamus, TNF stimulates the release of corticotropic releasing hormone, suppresses appetite, and induces fever. In liver, it stimulates the acute inflammatory response by elevating the synthesis of C-reactive protein and other mediators.

TNF induces vasodilation and loss of vascular permeability, which is propitious for lymphocyte, neutrophil, and monocyte infiltration. It helps recruit these cells to the inflammation site by regulating chemokine release. TNF, in concert with IL-17, triggers the expression of neutrophil

attracting chemokines CXCL1, CXCL2, and CXCL5 and can also augment the expression of cell adhesion molecules that facilitate diapedesis.

This in turn increases CXCR2-dependent neutrophil migration to the inflammation site. Being an inducer of the inflammatory response, excess amounts of TNF have been found to play pathological roles in ailments such as inflammatory bowel disease, psoriasis, rheumatoid arthritis, asthma, cancer, infectious diseases, and other auto-immune pathologies.

Some of these conditions are currently co-treated with monoclonal antibodies that neutralize this cytokine. In macrophages, TNF is released to the extracellular milieu via the constitutive secretion pathway, and its trafficking is the best understood of all cytokines.

Details on TNF trafficking will be discussed in another article of this issue. After synthesis in the ER, the SNARE proteins Stx6, Stx7, Vtib mediate the fusion of TNF-containing vesicles from the Golgi complex with VAMP3Crecycling endosomes. Thence, the Stx4/SNAP23/VAMP3 complex facilitates the passage of TNF from recycling endosomes to the cell membrane. Rho1 and Cdc42, two proteins that govern cell shape via actin remodeling, also regulate the postrecycling endosome trafficking of TNF to the plasmalemma.

Moreover, LPS was found to increase the expression of vesicle trafficking proteins that regulate TNF trafficking.

Finally, release of mature TNF from the plasmalemma requires cleavage of the membrane-bound precursor by the TNF-a-converting enzyme (TACE)….

3.4.4 IL-6

Also as Duque and Descoteaux have note

IL-6 is a pleiotropic cytokine that has both proinflammatory and anti-inflammatory functions that affect processes ranging from immunity to tissue repair and metabolism. It promotes differentiation of B cells into plasma cells, activates cytotoxic T cells, and regulates bone homeostasis. As with other proinflammatory cytokines, IL-6 is has been implicated in Crohn's disease and rheumatoid arthritis.

Similar to TNF and IL-1b, IL-6 is an endogenous pyrogen that promotes fever and the production of acute phase proteins from liver.

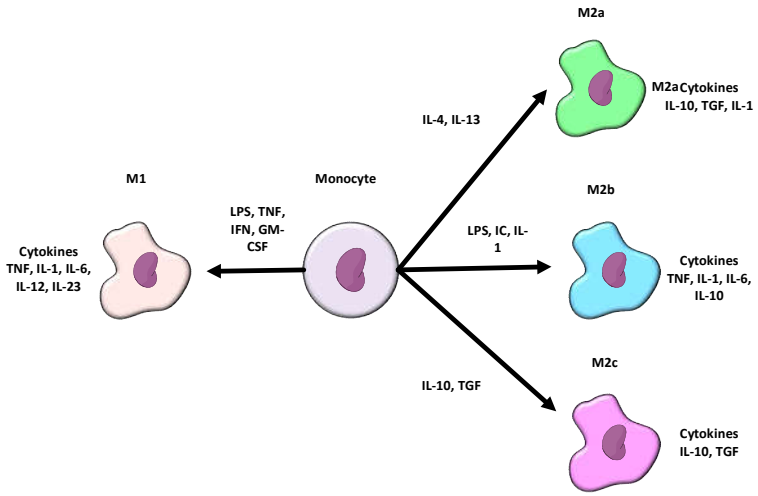
Proinflammatory properties are elicited when IL-6 signals in trans via soluble IL-6 receptors binding to gp130, which is ubiquitous in all cells. Inhibition of trans signaling via gp130 blockade in murine sepsis models rescues mice from widespread inflammation and death. IL-6 trans signaling also leads to recruitment of monocytes to the inflammation site, promotes the maintenance of Th17 cells, and inhibits T cell apoptosis and development of Tregs.

In contrast, antiinflammatory properties are elicited when IL-6 signals through the classical pathway, which occurs via the IL-6 receptor that only few cells express. The anti-inflammatory

properties of IL-6 are illustrated by IL-6−/− mice, which exhibit hepatosteatosis, insulin resistance, and liver inflammation.

IL-6 classic signaling also mediates apoptosis inhibition and the regeneration of intestinal epithelial cells

The authors then present the following:



Where they note:

Upon encountering different stimuli, monocytes turn into highly microbicidal (M1), or into immunosuppressive macrophages (M2). Stimuli can range from microbial substances to biochemical signals provided by the microenvironment of a given tissue. Many of the cytokines that bias macrophage phenotype are provided by surrounding lymphocytes or other non-immune cells.

Macrophage subtypes release a vastly different array of cytokines and chemokines that can either promote inflammation and sometimes tissue destruction, or wound healing and tissue repair. M1 macrophages are known to be tumor suppressive whereas M2 macrophages generally promote tumorigenesis. It is important to note that macrophage bias is a spectrum and is reversible. IC, immune complexes; ApC, apoptotic cells; Gluc, glucocorticoids

4 CLASSIC GRANULOMA DISORDERS

We now discuss some of the more common granulomatous diseases. Some of these have been well known as granuloma based and others are putatively posed as also being so.

4.1 TUBERCULOSIS (TB)

TB has been a long lasting disease which is both highly communicable as well as having high morbidity and mortality. Pandemics of TB had been common especially amongst crowded low income communities. It is also a prototypical granuloma disorder. As Ehlers and Schaible have noted:

A granuloma is defined as an inflammatory mononuclear cell infiltrate that, while capable of limiting growth of Mycobacterium tuberculosis, also provides a survival niche from which the bacteria may disseminate. The tuberculosis lesion is highly dynamic and shaped by both, immune response elements and the pathogen.

In the granuloma, M. tuberculosis switches to a non-replicating but energy-generating life style whose detailed molecular characterization can identify novel targets for chemotherapy.

To secure transmission to a new host, M. tuberculosis has evolved to drive T cell immunity to the point that necrotizing granulomas leak into bronchial cavities to facilitate expectoration of bacilli.

From an evolutionary perspective it is therefore questionable whether vaccination and immunity enhancing strategies that merely mimic the natural immune response directed against M. tuberculosis infection can overcome pulmonary tuberculosis in the adult population.

Juxtaposition of molecular pathology and immunology with microbial physiology and the use of novel imaging approaches afford an integrative view of the granuloma's contribution to the life cycle of M. tuberculosis. This review revisits the different input of innate and adaptive immunity in granuloma biogenesis, with a focus on the co-evolutionary forces that redirectimmune responses also to the benefit of the pathogen, i.e., its survival, propagation, and transmission.

As Rubin notes:

Granulomas are cellular aggregates that are the pathologic hallmarks of tuberculosis.

These chronic inflammatory lesions have long been considered to be necessary for containment of infection. A recent study … suggests that granulomas may help to promote infection, rather than simply contain it.

The vast majority of persons infected with Mycobacterium tuberculosis remain asymptomatic for life; at least 90% of infected adults never become ill. What is the basis for resistance to tuberculosis?

The high rates of clinical tuberculosis among persons infected with the human immunodeficiency virus and also among persons receiving cytotoxic therapy point to a critical role of the intact adaptive immune system.

However, the most important cellular player may be the macrophage, which has two mutually contradictory roles in tuberculosis.

On the one hand, activated macrophages are capable of killing or at least controlling the growth of M. tuberculosis. Granulomas are present in persons with intact immunity but absent or poorly formed in persons with poor immune responses; this observation supports the hypothesis that they are critical for limiting bacterial growth.

On the other hand, macrophages provide the primary growth niche for this intracellular organism; throughout infection, mycobacteria are largely intracellular. The hypothesis that granulomas limit bacterial growth is based largely on animal models that do not permit the observation of infection continuously over time. Granulomas are located deep in tissues; most models require that infected animals be killed to permit observation of the interaction between bacteria and host structures.

Thus, we have had to draw conclusions about a dynamic process from analyses at single time points. Although the zebrafish cannot be infected with M. tuberculosis, it is a natural host for the fish (and occasionally human) pathogen M. marinum. More importantly, the zebrafish embryo (which is also a host for M. marinum) is transparent, allowing easy visualization of living bacteria in a living host. Davis and Ramakrishnan infected zebrafish with M. marinum that expressed fluorescent proteins.

Phagocytic cells, the fish equivalent of human macrophages, then took up the labeled bacteria, allowing investigators to follow the fate of both infected and uninfected phagocytes over time by means of microscopy.

Infected cells appear to recruit uninfected phagocytes. As infected cells die, apparently via apoptosis, they are taken up by previously uninfected cells that themselves become infected. These cells provide a new growth niche for the pathogen and permit renewed bacterial growth.

… recently observed that growth of Mycobacterium marinum in the phagocytic cell of zebrafish (the zebrafish equivalent of the human macrophage) eventually leads to cell death. Infected cells recruit uninfected cells, some of which ingest dead infected cells; this provides a niche for further growth of the pathogen and permits egress of infected cells to produce new granulomas. Infection with bacteria that do not have the critical virulence region ESX1 results in less recruitment of uninfected cells and, consequently, fewer bacteria, smaller lesions, and fewer new granulomas.

4.2 SARCOIDOSIS

From Wing and Schiffman:

Sarcoidosis is a multisystem granulomatous disorder of unknown cause.

The lungs and thoracic lymph nodes are frequent sites of involvement.

Sarcoidosis is relatively common, with a prevalence of 1 to 40 cases per 100,000 people worldwide. A higher incidence of sarcoidosis is reported among Scandinavian, German, and Irish individuals residing in northern Europe. In the United States, the prevalence rates of sarcoidosis are 10.9 cases per 100,000 white individuals and 35.5 cases per 100,000 African Americans, with women in both groups being more frequently affected. Because sarcoidosis may be asymptomatic, the true prevalence may be higher. Sarcoidosis typically occurs in individuals between 10 and 40 years old.

Sarcoidosis is characterized by the formation in tissues of noncaseating granulomas that organize in an inner core of epithelioid histiocytes, CD4+ T lymphocytes, and giant cells, which are surrounded by a rim of lymphocytes, fibroblasts, and connective tissue (Fig. 17.9).

Granulomas are found in the airways or lung parenchyma in more than 90% of patients with sarcoidosis. Granulomatous angiitis may also be found in the lungs. The upper respiratory system, lymph nodes, skin, and eyes are commonly involved. Virtually any other organ may be affected, including the liver, bone marrow, spleen, musculoskeletal system, heart, salivary glands, and nervous system.

Subepithelial noncaseating granuloma, which is characteristic of sarcoidosis, from an endobronchial biopsy. The granulomas may be clinically silent or, if extensive, may disrupt normal organ structure and function. The cause of these lesions is unknown, but given the frequency of lung involvement, inhaled antigens ranging from bacteria (especially mycobacteria and Propionibacterium) to environmental substances have been hypothesized to trigger the onset of granulomatous inflammation.

This inflammation may be self-limited or may be propagated, possibly by repeated exposure to the unknown antigen or because of defective immune regulation. Familial susceptibility to sarcoidosis exists, and alleles of human leukocyte antigen (HLA) genes involved in antigen presentation and a mutation in the butyrophilin-like 2 gene (BTNL2), a possible immunoregulatory gene, have been associated with susceptibility to sarcoidosis.

A single causative antigen initiating granuloma formation may not exist, and sarcoidosis instead may represent a stereotypical inflammatory reaction to various antigens in a genetically susceptible host.

Sarcoidosis is associated with abnormal immune function as evidenced by cutaneous anergy and as exhibited in lung by an increased ratio of CD4+ to CD8+ T lymphocytes and increased concentrations of pro-inflammatory cytokines such as interferon-γ, interleukin-12, and tumor

necrosis factor-α (TNF-α). These derangements can be detected in the bronchoalveolar lavage (BAL) fluid and are consistent with an imbalance in the production of type 1 (TH1) and type 2 (TH2) helper T-cell cytokines, favoring the production of the former and promoting persistent inflammation.

Sarcoidosis may occur in the setting of immunomodulatory therapy, especially with interferon-α, or the immune reconstitution syndrome, occurring after initiation of antiretroviral therapy for human immunodeficiency virus (HIV) infection, highlighting the role of immune imbalances in the disorder.

As Iannuzzi et al note :

Infectious, organic, and inorganic agents are possible antigens in sarcoidosis.

Any causative microbe, if present, is probably cleared, leaving behind an undegradable product or initiating a cross-reacting immune response to self-antigen. Antigen-presenting cells (APC), in addition to producing high levels of tumor necrosis factor alpha (TNF-α), secrete interleukin-12, -15, and -18, macrophage inflammatory protein 1 (MIP-1), monocyte chemotactic protein 1 (MCP-1), and granulocyte macrophage colony-stimulating factor (GM-CSF).

A cardinal feature of sarcoidosis is the presence of CD4+ T cells that interact with APCs to initiate the formation and maintenance of granulomas. CD4+ T cells release interleukin-2 and interferon-γ. Activated CD4+ cells differentiate into type 1 helper (Th1)-like cells and secrete predominantly interleukin-2 and interferon-γ. The efficiency of antigen processing, antigen presentation, and cytokine release is probably under genetic control; evidence strongly supports a role for macrophage HLA and BTNL2 alleles in sarcoidosis susceptibility and phenotype.

However, T-cell genes that may confer a predisposition to sarcoidosis or affect the phenotype have not yet been identified. Sarcoidal granulomas are organized, structured masses composed of macrophages and their derivatives, epithelioid cells, giant cells, and T cells. Sarcoidal granulomas may persist, resolve, or lead to fibrosis. Alveolar macrophages activated in the context of a predominant type 2 helper (Th2) T-cell response appear to stimulate fibroblast proliferation and collagen production, leading to progressive fibrosis

Linke et al have noted:

Aggregation of hypertrophic macrophages constitutes the basis of all granulomatous diseases such as tuberculosis or sarcoidosis and is decisive for disease pathogenesis.

However, macrophageintrinsic pathways driving granuloma initiation and maintenance remain elusive.

Here we show that activation of the metabolic checkpoint kinase mTORC1 in macrophages by deletion of Tsc2 was sufficient to induce hypertrophy and proliferation resulting in excessive granuloma formation in vivo. TSC2-deficient macrophages formed mTORC1-dependent

granulomatous structures in vitro and showed constitutive proliferation mediated by the neo-expression of cyclin-dependent kinase 4 (CDK4).

Moreover, mTORC1 promoted metabolic reprogramming via CDK4 towards increased glycolysis, while simultaneously inhibiting NF-κB signaling and apoptosis.

Inhibition of mTORC1 induced apoptosis and completely resolved granulomas in myeloid TSC2-deficient mice. In human sarcoidosis patients mTORC1 activation, macrophage proliferation, and glycolysis were identified as hallmarks that correlated with clinical disease progression.

Collectively, TSC2 maintains macrophage quiescence and prevents mTORC1-dependent granulomatous disease with clinical implications for sarcoidosis. Granulomas are compact aggregates of mature macrophages with an increased cytoplasmic size whose membranes become interlaced leading them to be called epithelioid cells. They are usually formed and maintained in response to the continuous presence of either infectious stimuli such as bacteria, fungi, protozoa, trematodes and viruses, or in response to non-infectious foreign-body particles. Tuberculosis or schistosomiasis are prime examples of an infectious granulomatous disease, whereas non-infectious granuloma formation is observed in sarcoidosis, Crohn's disease, primary biliary cirrhosis but also in neoplasias.

Sarcoidosis is an enigmatic granulomatous disease of unknown etiology that most commonly affects the lung, lymph nodes, skin, and liver. The onset is gradual from an asymptomatic state to a progressive disease that persists in about one-third of patients and can become life threatening. Molecular signals or pathways that control disease progression are largely undefined. When treatment is required, corticosteroids are usually recommended, but they are associated with significant side effects. Due to the unknown etiology, there is currently no therapeutic approach targeting the pathogenetic mechanisms

As Shah et al observe:

Sarcoidosis is a granulomatous disease involving multiple organ systems including the lungs, kidneys, skin, joints, muscles, and eyes.

Data from A Case Control Etiologic Study of Sarcoidosis (ACCESS), which registered 736 patients, demonstrated pulmonary and skin involvement in 95% and 15.9% of cases, respectively.

Of note, there is an age, sex, and race predilection to end organ involvement, with African American females most commonly affected.

Majority of patients present with pulmonary involvement, which is pathologically characterized by the presence of non-necrotizing granulomas with exclusion of other causes. The suspected etiology is hypothesized to represent an autoimmune response to infections of Mycobacteria or Propionibacteria species or an unidentified environmental agent.

In the lung, the suspected inhaled exposure stimulates an antigenic response where granulomas are detected clinically by endobronchial biopsy (40–71% of patients). Histologically occur around bronchovascular bundles and follow a lymphangitic distribution (nearly 70%). Lymphocytic alveolitis composed of T-helper cells is commonly identified in open lung biopsies of sarcoidosis patients and is thought to be a precursor lesion to granuloma formation. .... A variety of non-specific inclusions have been histologically described in varying frequencies including Schaumann's bodies, asteroid bodies, birefringent crystals, and Hamazaki-Wesenberg bodies. During disease progression the granulomas can heal and undergo fibrosis. In up to a third of patients progressive fibrosis can occur and result in end-stage fibrosis. In nearly a third of patients, extrapulmonary sarcoidosis is the presenting symptom.

Renal sarcoidosis occurs either in association with other organ involvement, or as a renal limited form of sarcoidosis. The most common modes of presentation include hypercalcemia, hypercalciuria, nephrolithiasis, obstructive uropathy, and renal tubular defects. GIN, is a well described manifestation of renal sarcoidosis, and the frequency with which it occurs, as estimated by post-mortem studies ranges from 7% to 27% .

The granulomas are usually well-formed and non-necrotizing. The presence of Schaumann and asteroid bodies are not common. There are often interstitial and tubular deposits of calcium due to hypercalcemia.

As Drent et al note :

As mentioned previously, the histologic hallmark of sarcoidosis — granulomatous inflammation — is thought to be a dysregulated antigenic response to unknown environmental exposures in a genetically susceptible person. Loci that house genes involved in antigen presentation (e.g., loci in the HLA class II region and the butyrophilinlike 2 gene [BTNL2]) are linked to the development of sarcoidosis and to certain disease phenotypes (Table 1).

Subclinical inflammation begins with activation of membrane-bound patternrecognition receptors (e.g., toll-like receptors). When stimulated, macrophages and other innate immune cells promote transcription factors, resulting in the production of cytokines (Fig. 1).1,21-27 "Classically" activated macrophages are regarded as drivers of the inflammatory process (proinflammatory M1 type) associated with granuloma formation.

Cytokines can also promote CD4+ helper T cells and sometimes their differentiation into type 17 helper T (Th17) cells.1 Professional antigen-presenting cells (e.g., dendritic cells) are also activated to generate and display antigen peptides, which are recognized

4.3 CROHN'S DISEASE

From Wing and Schiffman:

The clinical presentation of Crohn's disease depends on the section of gastrointestinal tract involved and the type of inflammation.

Crohn's disease can involve any portion of the gastrointestinal tract; the most common site is ileocecal/ileocolonic (40% of patients), followed by isolated small bowel disease mostly affecting the terminal ileum (30%), and isolated colonic involvement (25%).

The remaining sites of Crohn's disease are rarely (5%) affected in isolation and include the esophagus, stomach, and duodenum. Symptoms in Crohn's disease often include right lower quadrant abdominal pain, fever, weight loss, diarrhea, and sometimes a palpable inflammatory mass on physical exam.

Hematochezia may be present with colonic involvement but is less common than in UC. The symptoms can often be present for months or years before a diagnosis is made, and in children, growth retardation may be the sole presenting sign. In contrast to UC, the inflammation in Crohn's disease is transmural and can result in deep ulcerations and the formation of fistulous tracts. Fistulas may form between different segments of bowel (e.g., enteroenteric, enterocolonic) or between bowel and skin (enterocutaneous), bowel and bladder (enterovesicular), or rectum and vagina (rectovaginal). Over time, as many as 30% to 40% of patients will develop perianal involvement with fissures, fistulas or abscesses.

Chronic inflammation can cause fibrosis and stricture formation, which in turn may result in partial or complete intestinal obstruction with the patient complaining of abdominal pain, distention, nausea, and vomiting. Strictures can also lead to stasis with subsequent small intestinal bacterial overgrowth.

Small bowel disease may lead to vitamin D deficiency. Extensive ileal mucosal disease may lead to malabsorption of vitamin B12 (resulting in a megaloblastic anemia and neurologic side effects if not corrected) and malabsorption of bile salts (resulting in diarrhea induced by unabsorbed bile salts and potential fat-soluble vitamin deficiency). Depletion of the bile salt pool can lead to the formation of gallstones. Weight loss may result from generalized malabsorption caused by loss of absorptive surfaces.

Chronic fat malabsorption leads to luminal binding of free fatty acids to calcium; this allows oxalate, which normally is poorly absorbed because it complexes to calcium in the gut lumen, to be absorbed in the colon. The increase in oxalate absorption increases the risk for urinary calcium oxalate stone formation. Patients with an ileostomy or chronic volume loss from diarrhea are also at increased risk for uric acid stones.

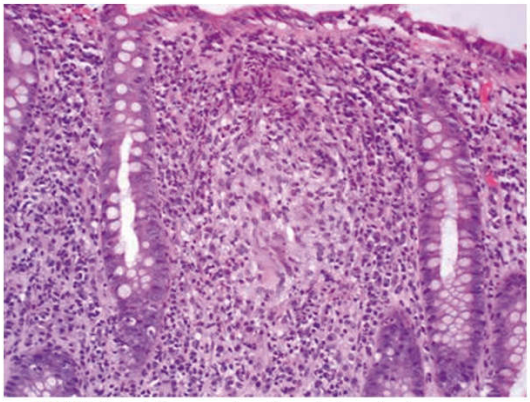


FIG. 38.8 Colonic biopsy specimen demonstrates a chronic inflammatory infiltrate with a granuloma in a patient with Crohn's colitis (hematoxylin and eosin stain).

4.4 ALZHEIMER'S

Alzheimer's is a neurologic degenerative disorder. It is interesting to see what role the granuloma structure plays in this disease. As Lemke and Huang note:

Dense-core plaques, whose centers contain highly polymerized and compacted aggregates of amyloid β peptides, are one of the two defining histopathological features of Alzheimer's disease.

Recent findings indicate that these plaques do not form spontaneously but are instead constructed by microglia, the tissue macrophages of the central nervous system. We discuss cellular, structural, functional, and gene expression criteria by which the microglial assembly of dense-core plaques in the Alzheimer's brain parallels the construction of granulomas by macrophages in other settings.

We compare the genesis of these plaques to the macrophage assembly of mycobacterial granulomas, the defining histopathological features of tuberculosis.

We suggest that if dense-core plaques are indeed granulomas, their simple disassembly may be contraindicated as an Alzheimer's therapy …

Granulomas are compact, organized collections of mononuclear phagocytes—primarily macrophages that develop in response to an unresolved infectious or foreign body stimulus. These structures are common, and are seen in schistosomiasis, after the inhalation of silica and metals in atherosclerosis, and following the deposition of foreign bodies or insoluble proteins or lipids. The most widely studied granulomas have been those that develop in tuberculosis (TB) and other persistent mycobacterial infections. TB is marked by the presence of pulmonary and extrapulmonary granulomas that are populated by macrophages, macrophage derivatives, and a

panoply of other immune cells. Although some TB granulomas are paucibacillary, these cells typically surround a community of Mycobacterium tuberculosis.

Traditionally, the TB granuloma has been viewed as a confinement structure that sequesters the bacillus.

This view has been challenged, however, since in addition to protecting the host from the bacterium, this granuloma also provides an environment that enables bacterial growth and dissemination.

In this Perspective, we suggest that the dense-core amyloid β (Aβ) plaques of Alzheimer's disease (AD), which were first described by Alois Alzheimer more than a century ago, are granulomas. We compare the construction of these plaques to the development of the TB granuloma.

Understanding dense-core plaque formation is important, since together with neurofibrillary tangles of the microtubule protein Tau, plaques are the defining histopathological feature of AD

The above presents an interesting interpretation of how this disease is reflected in terms of granuloma structures.

4.5 LEPROSY

Leprosy or Hansen's Disease has been the bane of many a society for millennia. It is in many ways a prototypical granulomatous disorder resulting on significant disfigurement. What makes this an interesting granuloma disorder is the continuing proliferation. As Shah et al note:

Mycobacterium leprae*, otherwise known as Hansen's disease, is primarily a tropical, mycobacterial infection which most often affects skin, nerves, and nasal mucosa. Mycobacterium leprae is an obligate intracellular gram positive, partially acid fast bacillus. Morphologically, it has a thick waxy coating which may prevent identification with traditional Gram stain. When it does stain,* ***the organism is Gram positive****.*

The mode of infection is suspected to be through aerosolized droplets, however direct infection from armadillos, rodents, or tattoos have been reported.

Leprosy exists as a spectrum of disease states, at each end are the paucibacillary (tuberculoid) and multibacillary (lepromatous) disease states. As the name suggests, paucibacillary is characterized by few lesions with a high resistance and paucity of organisms, whereas, multibacillary occurs with multisite involvement and a high bacterial load. Periods of disease evolution have been described and include tuberculoid, borderline-tuberculoid, borderline, borderline-lepromatous, and lepromatous leprosy.

Cellular response has been characterized with a similar spectrum, starting with an initial IL-2 and interferon gamma initiation (T-helper type 1) and eventually progressing to activation of T-

helper type II (IL-4, 5, 10) and cyclooxygenase II [19]. Diagnosis requires skin biopsy from the active edge of a lesion. Histologically, the tuberculoid and lepromatous forms have distinct appearances.

Lepromatous leprosy is rich with dermal parasitized macrophages, whereas the tuberculoid form is similar to the granulomatous inflammation by which its name is ascribed; epithelioid histiocytes with multinucleated giant cells and a lymphohistiocytic cuff. In addition, mild to severe disease states show increasing involvement with chronically inflamed blood vessels, dermal appendages, and hypertrophied nerves within the superficial and deep dermis.

In the mild form of tuberculoid leprosy, organisms are found in less than 50% of cases. Additionally, post treatment, granulomas can persist for 18 months. In the severe, lepromatous form, foamy histiocytes, eccrine structures, and endothelium are filled with bacilli. There is a relatively minor lymphocytic infiltrate seen in the lepromatous form. In rare cases, necrotizing vasculitis (Lucio's phenomenon) or a severe erythema nodosum-like histologic presentation can occur.

The characteristic diagnostic ***triad*** *of leprosy is* ***hypo-pigmented skin patches with thickened nerves, definite loss of sensation, and biopsy-proven organisms****.*

Given their partial acid-fast nature, the Fite stain rather than the Ziehl–Neelsen should be used to stain M. leprae. These bacilli also stain using GMS, which non-specifically stains most fungi and bacteria

Leprosy has been a complex granuloma type disease to treat mainly due to the protective nature of the granulomas. In many ways this represent the prototypical aggressive spectrum of granuloma disorders.

4.6 OTHERS

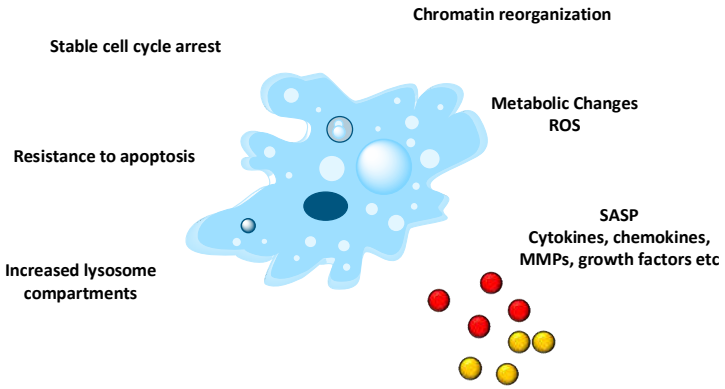
Shah et al present a Table for a multiplicity of granulomatous diseases on various organs. We highlight some in the modified Table below:

Organ	Infectious	Fungal	Viral	Autoimmune	Neoplastic
Lung	Mycobacterium	Aspergillus	Cytomegalovirus	Churg Strauss	Hodgkin
Skin	Actinomyces	Blastomyces	Cytomegalovirus	Granuloma annulare	Hodgkin
Kidney	Bartonella	Coccidiosis		Sarcoid	CLL
Liver	Brucella	Candida	Cytomegalovirus Hepatitis	Crohn's Cirrhosis	Hemophagocytic lymphohistocytosis
Lymph Node	Chlamydia	Histoplasma	Epstein Barr	Sarcoid	Dendritic Cell sarcoma

5 SENESCENCE

Senescence is the process where there is a stable cell cycle and the secretion of many potent cytokines and chemokines[8]. In contrast cell death has three variants[9]. Apoptosis or the falling apart of cells wherein the contents of the dead cells remain intact. Second is autophagy, is the self-eating of the cell, its self-digestion. Third is necrosis, or the death of the cell itself. Cell death and destruction is one way the body cleans up after itself. Senescence however is a process of lingering. We examine senescence here as a possible path in the overall process of granulomas. For in granulomas we have a stable but lingering accumulation of cells. In addition the cells do emit a set of cytokines and chemokines that enable it to survive. A particular characteristic of senescent cells is the process of senescence associated secretory phenotype, SASP. This is the process in senescent cells where there is an emission of chemokines, cytokines, growth factors and the like.

We depict some of the factors associated with senescence below.



5.1 FUNDAMENTALS

We can now begin with some further details on senescence. As Kumari and Jat note:

Cellular senescence is a stable cell cycle arrest that can be triggered in normal cells in response to various intrinsic and extrinsic stimuli, as well as developmental signals.

Senescence is considered to be a highly dynamic, multi-step process, during which the properties of senescent cells continuously evolve and diversify in a context dependent manner.

[8] See Serrano and Munoz-Espi

[9] See Green, also see Kumar and Asthana

It is associated with multiple cellular and molecular changes and distinct phenotypic alterations, including a stable proliferation arrest unresponsive to mitogenic stimuli.

Senescent cells remain viable, have alterations in metabolic activity and undergo dramatic changes in gene expression and develop a complex senescence associated secretory phenotype.

Cellular senescence can compromise tissue repair and regeneration, thereby contributing toward aging. Removal of senescent cells can attenuate age-related tissue dysfunction and extend health span. Senescence can also act as a potent anti-tumor mechanism, by preventing proliferation of potentially cancerous cells. It is a cellular program which acts as a double-edged sword, with both beneficial and detrimental effects on the health of the organism, and considered to be an example of evolutionary antagonistic pleiotropy.

Activation of the p53/p21WAF1=CIP1 and p16INK4A/pRB tumor suppressor pathways play a central role in regulating senescence. Several other pathways have recently been implicated in mediating senescence and the senescent phenotype.

…the molecular mechanisms that underlie cellular senescence and the senescence associated growth arrest with a particular focus on why cells stop dividing, the stability of the growth arrest, the hypersecretory phenotype and how the different pathways are all integrated …

Although senescence is associated with aging, cells can undergo senescence irrespective of organismal age due to different signals apart from telomere shortening. In accordance with this the use of transgenic mouse models have allowed the detection of senescent cells in different age related pathologies and enabled the development of genetic or pharmacological strategies to demonstrate that selective elimination of senescent cells can prevent or delay age-related tissue dysfunction to extend life span and improve health span.

Cellular senescence is a cellular program which acts as a double-edged sword with both beneficial and detrimental effects on the health of the organism, and thereby considered to be an example of evolutionary antagonistic pleiotropy.

Taken together, senescence is both a physiologically fundamental and pathologically relevant program, with its role depending on the context and the specific situation.

***Here, we review the different mechanisms controlling cellular senescence with a special focus on cell cycle arrest and SASP**. We detail the complexity of the mechanisms involved in SASP regulation, focus on the key mediators, characteristic hallmarks and the different pathways involved in manifesting cellular senescence as well as the cell cycle arrest and its key regulators along with the role of the DREAM complex and its associated components. The significance of cellular senescence in different contexts such as its role in vivo, in cancer and aging are also discussed.*

At the end we discuss the translational relevance and suitability for identifying and characterizing senescent cells in vivo to explore potential future avenues for exploiting the

benefits and preventing the detrimental aspects of senescent cells such as suppressing the SASP or selectively eliminating senescent cells to increase health span.

As Roger et al note :

Cellular metabolism changes are important for the function and fate of senescent cells. Although senescent cells do not divide, they display a very active but altered metabolism, with increased glycolysis and ***mTOR activity****.*

The increased metabolic demands are related to their increased size, elevated production of secreted proteins (SASP), and increased oxidative stress and endoplasmic reticulum (ER) stress after cell cycle exit.

This results in different metabolic needs compared with proliferating cells and requires changes to support these demands. ***Senescent cells exhibit a shift toward elevated glycolysis with an imbalance activity of glycolytic enzymes that results in a reduced energetic state when cell enter replicative senescence.*** *Increased aerobic glycolysis compensates for the reduced adenosine triphosphate (ATP) production caused by mitochondrial respiration decline during senescence. In the early stage of senescence, mitochondria do not function properly and display impaired oxidative phosphorylation capacity and reduced inner membrane potential, resulting in ROS overproduction.*

Due to their functional defects, the mass and number of mitochondria are increased in senescent fibroblasts.

Increased mitochondrial biogenesis is dependent on ATM-mediated activation of the Akt/mTORC1 phosphorylation cascade, leading to stimulation of the mitochondrial biogenesis regulator peroxisome proliferator-activated receptor gamma coactivator α (PGC1-α). Moreover, damaged mitochondria are insensitive to mitophagy (i.e., selective autophagy of mitochondria) and consequently, mitochondrial number and size are not properly regulated in senescent cells.

Removal of mitochondria in senescent cells disrupts the feedforward cycle that involves ROS production and persistent DDR activation, while preserving their cell cycle arrest. In these cells, SASP gene expression alteration is not caused by insufficient energy levels because ATP levels are high due to increased glycolysis.

Therefore, it seems that at least in some contexts, the execution of the senescence program is compromised not by insufficient energy levels but rather by mitochondrial oxidative metabolism status. Accordingly, a metabolic shift from glycolysis towards mitochondrial oxidative respiration through activation of mitochondrial pyruvate dehydrogenase is required to establish and stabilize the OIS-associated cell growth arrest.

Moreover, during OIS, fatty acid metabolism is altered, glucose consumption is enhanced, and the utilization of pyruvate in the tricarboxylic acid cycle and nucleotide deficiency are increased. Senescence induced by nucleotide deficiency causes aberrant DNA replication but can be

overcome by ATM inactivation through restoration of glucose and glutamine consumption. This supports the causative role of metabolic changes in senescence induction.

As Gorgoulis et al note :

Cellular senescence is a cell state triggered by stressful insults and certain physiological processes, characterized by a prolonged and generally irreversible cell-cycle arrest with secretory features, macromolecular damage, and altered metabolism. These features can be inter-dependent but for clarity are described here separately.

Cell-Cycle Arrest

One common feature of senescent cells is an essentially irreversible cell-cycle arrest that can be an alarm response instigated by deleterious stimuli or aberrant proliferation. This cell-cycle withdrawal differs from quiescence and terminal.

Quiescence is a temporary arrest state with proliferation re-instated by appropriate stimuli; terminal differentiation is the acquisition of specific cellular functions accompanied by a durable cell-cycle arrest mediated by pathways distinct from those of cellular senescence. In turn, senescent cells acquire a new phenotype.

Although the senescence cell-cycle arrest is generally irreversible, cell-cycle re-entry can occur under certain circumstances, particularly in tumor cells.

In mammalian cells, the retinoblastoma (RB) family and p53 proteins are important for establishing senescent cell-cycle arrest. RB1 and its family members p107 (RBL1) and p130 (RBL2) are phosphorylated by specific cyclin-dependent kinases (CDKs; CDK4, CDK6, CDK2).

This phosphorylation reduces the ability of the RB family members to repress E2F family transcription factor activity, which is required for cell-cycle progression. In senescent cells, however, the CDK2 inhibitor p21WAF1/Cip1 (CDKN1A) and CDK4/6 inhibitor p16INK4A (CDKN2A) accumulate. This accumulation results in persistent activation of RB family proteins, inhibition of E2F transactivation, and consequent cell-cycle arrest, which, in time, cannot be reversed by subsequent inactivation of RB family proteins or p53. This persistence is enforced by heterochromatinization of E2F target genes, the effects of cytokines secreted by senescent cells and enduring reactive oxygen species (ROS) production.

Notably, in senescent murine cells, ARF—an alternate reading frame protein of the p16INK4a gene locus that activates p53—also has an important role in regulating cell-cycle arrest.

Additional features of the senescent cell-cycle arrest include ribosome biogenesis defects and derepression of retrotransposons. However, currently no specific marker of the senescent cell-cycle arrest has been identified. For example, RB and p53 activation also occurs in other forms of cell-cycle arrest. Even p16INK4A, which is considered more specific to senescence, is expressed in certain non-senescent cells and is not expressed by all senescent cells. Thus,

detecting a senescence-associated cell-cycle arrest requires quantification of multiple factors and features.

5.2 AUTOPHAGY

We note that there may be a balance between senescence and autophagy. Thus it is useful to briefly examine it as well. As Glick et al note:

Autophagy is a self-degradative process that is important for balancing sources of energy at critical times in development and in response to nutrient stress.

Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins, clearing damaged organelles, such as mitochondria, endoplasmic reticulum and peroxisomes, as well as eliminating intracellular pathogens. Thus, autophagy is generally thought of as a survival mechanism, although its deregulation has been linked to non-apoptotic cell death. Autophagy can be either non-selective or selective in the removal of specific organelles, ribosomes and protein aggregates, although the mechanisms regulating aspects of selective autophagy are not fully worked out.

In addition to elimination of intracellular aggregates and damaged organelles, autophagy promotes cellular senescence and cell surface antigen presentation, protects against genome instability and prevents necrosis, giving it a key role in preventing diseases such as cancer, neurodegeneration, cardiomyopathy, diabetes, liver disease, autoimmune diseases and infections. This review summarizes the most up-to-date findings on how autophagy is executed and regulated at the molecular level and how its disruption can lead to disease.

What is autophagy? The term 'autophagy', derived from the Greek meaning 'eating of self', was first coined by Christian de Duve over 40 years ago, and was largely based on the observed degradation of mitochondria and other intra-cellular structures within lysosomes of rat liver perfused with the pancreatic hormone, glucagon. The mechanism of glucagon-induced autophagy in the liver is still not fully understood at the molecular level, other than that it requires cyclic AMP induced activation of protein kinase-A and is highly tissue-specific. In recent years the scientific world has 'rediscovered' autophagy, with major contributions to our molecular understanding and appreciation of the physiological significance of this process coming from numerous laboratories. Although the importance of autophagy is well recognized in mammalian systems, many of the mechanistic breakthroughs in delineating how autophagy is regulated and executed at the molecular level have been made in yeast (Saccharomyces cerevisiae)…

Now Drent et al also note:

Furthermore, sarcoidosis appears to be associated with activation of the metabolic checkpoint kinase mammalian target of rapamycin complex 1 (mTORC1), which can be involved in the granulomatous process by activating macrophages and their differentiation into epithelioid cells (which tend to aggregate) and multinuclear giant cells. A disturbed autophagy process that normally eliminates antigens will further stimulate granuloma formation. Thus, growing

knowledge about newer pathophysiological pathways, such as mTORC1 and dysfunctional autophagy and activation of the nucleotide-binding oligomerization domain–like receptor protein 3 (NLRP3) inflammasome, may guide the development of new therapeutic targets.

5.3 AUTOPHAGY VS SENESCENCE

Now we examine the connection between the above two processes. As Kwon et al have noted:

When mammalian cells and animals face a variety of internal or external stresses, they need to make homeostatic changes so as to cope with various stresses. To this end, mammalian cells are equipped with two critical stress responses, autophagy and cellular senescence.

Autophagy and cellular senescence share a number of stimuli including telomere shortening, DNA damage, oncogenic stress and oxidative stress, suggesting their intimate relationship.

Autophagy is originally thought to suppress cellular senescence by removing damaged macromolecules or organelles, yet recent studies also indicated that autophagy promotes cellular senescence by facilitating the synthesis of senescence-associated secretory proteins. These seemingly opposite roles of autophagy may reflect a complex picture of autophagic regulation on cellular senescence, including different types of autophagy or a unique spatiotemporal activation of autophagy.

Thus, a better understanding of autophagy process will lead us to not only elucidate the conundrum how autophagy plays dual roles in the regulation of cellular senescence but also helps the development of new therapeutic strategies for many human diseases associated with cellular senescence. We address the pro-senescence and anti-senescence roles of autophagy while focusing on the potential mechanistic aspects of this complex relationship between autophagy and cellular senescence …

Cellular senescence is a stress-activated genetic program that permanently prevents damaged cells from further proliferation, acting as a potent tumor suppressive mechanism. It is originally recognized as a response to telomere shortening caused by replicative exhaustion, yet since then many stresses including DNA damage, oxidative stress, and oncogenic stress have been also shown to activate cellular senescence.

In addition to cell cycle arrest, senescent cells have additional effector programs such as an enlarged flattened morphology, an altered chromatin structure called ***senescence-associated heterochromatin formation (SAHF)****, and a massive secretion of several factors called* ***senescence-associated secretory phenotype (SASP).***

Critical among these is the SASP that includes many cytokines, chemokines, proteases, growth factors, and extra cellular matrix (ECM), as it affects not only senescent cells themselves in an autocrine manner but also nearby cells and the tissue microenvironment in a non-cellautonomous manner.

For example, SASP factors can reinforce cell cycle arrest of senescent cells and mobilize the immune system to suppress tumorigenesis and promote an optimal tissue repair. When dysregulated, however, SASP factors can stimulate adjacent premalignant and malignant cells to promote tumorigenesis and may cause chronic inflammation, contributing to many age-associated diseases including aging itself.

Macroautophagy (hereinafter referred to as autophagy) is another critical effector program of cellular senescence. Autophagy, a major lysosomal degradation pathway, was originally recognized as a response to starvation, recycling cellular components to maintain energy homeostasis.

As in the case of cellular senescence, however, it has been shown that autophagy can be activated by a variety of stresses including those that cause cellular senescence. In fact, autophagy is found to increase in senescent cells but its roles in the regulation of cellular senescence are still under debate.

On the one hand, this phenotype can be viewed as 'cellular senescence by autophagy' (pro-senescence). Given the well-known homeostatic function of autophagy, on the other hand, it can be viewed as 'cellular senescence with autophagy' - a failed attempt to prevent cellular senescence by autophagy (anti-senescence).

In this review, we focus on the complex relationship between autophagy and cellular senescence.

We address each mode of action of autophagy on cellular senescence and provide an intriguing hypothesis of how autophagy may differentially modulate cellular senescence depending on its types or its unique spatiotemporal activation. ...

A type of autophagy involved: general autophagy seems to act as an anti-senescence mechanism by maintaining homeostasis under either normal or stress-induced conditions. However, its timing of activation might be a differential factor that we will discuss below ... ***When autophagy acts****'.*

When autophagy degrades *only a certain type of substrates, we collectively call it 'selective autophagy'. However, in fact, it is all different types of autophagy, as each has its unique substrate, very similarly to an E3 ubiquitin ligase in the ubiquitin proteasome system. So, when considered for the role of selective autophagy in the regulation of cellular senescence, one should consider more its substrates and autophagic receptors that allow for autophagy to have a specificity. For example, p62-dependent selective autophagy of GATA4 acts as an anti-senescence mechanism, yet LC3B-lamin B1-dependent selective autophagy of nuclear lamina acts as a pro-senescence mechanism. It will be of a particular interest to find additional autophagy receptor-substrate pairs that act in the senescence regulatory network. 2)*

When autophagy acts*: under normal conditions, general autophagy acts as an anti-senescence mechanism as mentioned above. Earlier action of general autophagy under stress-induced conditions also represents a homeostatic response, so it is mostly anti-senescent. Once cells over*

a certain time point during cellular senescence, however, general autophagy may become pro-senescent, as it may sustain a viability of senescent cells by decreasing the burden of stresses that senescent cells must cope with: non-senescence addiction. For example, senescent cells do not divide, so they cannot dilute toxic substances as in the case of fully differentiated cells. Senescent cells also secrete many factors, which elicit ER stress and an unfolded protein response.

All of them can disrupt proteostasis in the cell, which in turn eventually cause cell death unless general autophagy delays or dampens it. It will be critical to determine when will be a clear cut to differentiate the role of autophagy in controlling cellular senescence. This will be very likely context dependent, influenced by a type of senescence inducing stressors

6 PUTATIVE LONG HAUL DISORDERS

We now examine two cases which may involve a granuloma like disorder. These two are purely speculative but they have features seen in granuloma disorders, namely persistence and latency. As noted, these two may or may not involve a granuloma like element but raising the question may lead to a better understanding of the disease and granulomas.

6.1 UTI

UTIs are most common amongst women and most often it is an E coli infection. Men usually have a low incidence and low grade UTI. However a recent set of studies have shown infection from food poisoning. In the UC Berkeley study the authors note[10]:

A new research collaboration between UC Berkeley and the Centers for Disease Control and Prevention will study whether food is a significant source of the antibiotic-resistant bacteria that cause urinary tract infections, the most common bacterial infections in the developed world, which disproportionately affect women.

The CDC today awarded $560,000 to the research project, which is led by Lee Riley, a professor of infectious diseases at the UC Berkeley School of Public Health. The study is one of 34 projects the CDC is funding with $14 million through its Broad Agency Announcement to support activities related to the CDC Antibiotic Resistance Solutions Initiative and implement the tracking, prevention and antibiotic stewardship activities outlined in the National Action Plan for Combating Antibiotic-Resistant Bacteria.

The goal of these projects is to find new approaches to combat antibiotic resistance, including research on how the microorganisms that are naturally found in the human body, called the microbiome, can be used to predict and prevent infections caused by drug-resistant organisms. "Understanding the role the microbiome plays in antibiotic-resistant infections is necessary to protect the public's health," said CDC director Tom Frieden. "We think it is key to innovative approaches to combat antibiotic resistance, protect patients and improve antibiotic use." Treatment of urinary tract infections has become more difficult in recent years because E. coli, the most common bacteria that cause urinary tract infections, have become increasingly resistant to commonly used antimicrobial agents.

Researchers do not know how big a role food plays in spreading the antibiotic-resistant forms of the bacteria, which is the key question Riley's team aims to answer. "Understanding what proportion of multidrug-resistant urinary tract infections are attributable to food sources will change the way we calculate the burden of foodborne disease and the impact of antimicrobial use in food animal husbandry," Riley said. An estimated 11 percent of women in the United States report at least one physician- diagnosed urinary tract infection per year.

[10] https://news.berkeley.edu/2016/10/06/uc-berkeley-cdc-team-up-to-investigate-link-between-uti-food-poisoning/

Some urinary tract infections are acquired by the introduction of particular strains of E. coli into the bladder during sex, but these infections can also occur when E. coli are ingested, colonize in the intestine and then spread to the bladder.

The above mechanism has significant import. They continue:

In 1999, when Riley's lab began investigating how urinary tract infections were acquired, they found that in three geographically diverse communities, a single E. coli strain, called clonal group A, accounted for nearly half of the drug-resistant urinary tract infections in women. The strain was also found in 30 percent of males in the study.

The widespread prevalence of a single strain, which is known to have resistance to common antibiotics, suggested the E. coli was spread by an outbreak and not by sex.

Now a detailed study by Nordstron et al noted:

Urinary tract infections (UTIs) are among the most common bacterial infections worldwide. Disproportionately affecting women, UTIs exact a substantial public burden each year in terms of direct medical expenses, decreased quality of life, and lost productivity. Increasing antimicrobial resistance among strains of extraintestinal pathogenic Escherichia coli challenges successful treatment of UTIs.

Community-acquired UTIs were long considered sporadic infections, typically caused by the patients' native gastrointestinal microbiota; however, the recent recognition of UTI outbreaks with probable foodborne origins has shifted our understanding of UTI epidemiology. Along with this paradigm shift come new opportunities to disrupt the infection process and possibly quell increasing resistance, including the elimination of non-therapeutic antimicrobial use in food-animal production. ...

Taken together, the studies reviewed above provide compelling evidence that retail meat, particularly poultry, serves as an important reservoir for human exposure to antibiotic-resistant E. coli that is causing UTIs.

Thus, ***the term foodborne UTIs or FUTIs has been adopted to describe these infections.***

The traditional mode of foodborne diseases necessarily involves an infection or toxification of the gastrointestinal tract; however, in FUTIs, the etiologic agent causes no gastrointestinal pathologies. Likewise, with classic foodborne infections, ingestion is the rate-limiting step: if a susceptible host consumes a sufficient dose of a pathogenic microbe, disease will ensue.

The FUTI model requires at least two steps:

(1) a susceptible host ingests a uropathogen and

(2) an infectious dose of the uropathogen is transferred from the host's gastrointestinal tract to his or her urinary tract.

As shown above, the first step appears to occur regularly in the community; therefore, the ratelimiting step is expected to be the transfer of the uropathogen to the urinary tract. Given these important distinctions, FUTIs represent a significant shift from the classic foodborne illness paradigm and broadens the implications of antibiotic-resistant E. coli in the food supply.

We have recently seen this in a patient which resulted in significant infection and distress. To summarize:

1. The patient, Pt, had significant lab work in anticipation of an annual physical. All readings on blood and urine were well within normal range. The Pt had no UTI symptoms and no symptoms of any infection. The Pt had no sexual activity and was generally in excellent health.

2. Three days later the Pt consumed meat that had been in their refrigerator for several weeks at about 2 PM. By 10 PM the Pt has severe diarrhea and emesis thought to be food poisoning. However by 4 AM the following day the Pt was febrile with a measured temperature of 104.5. By 9 AM the Pt temperature was down to 101.5.

3. On the fourth day after lab work the Pt remained slightly febrile but no emesis or diarrhea. The Pt remained in bed.

4. On the 5th day the Pt was still febrile and admitted to the ED of a local hospital.

5. Tests showed significant septicemia and as well as significant UTI with a significantly enlarged prostate (>200 cc). The Pt was admitted and placed on a variety of antibiotics. The infection was identified as E coli.

6. The Pt was released on the 9th day from the initial tests. Neutrophils had peaked, platelets had been depressed, and urine had shown infection, blood and protein.

7. Three weeks after release the Pt had a bout of hematuria. Cystoscopy showed a sessile inflammation of the bladder and reduction in prostate size. After a 14 day use of ciprofloxacin a second cystoscopy revealed a total resolution of any infection.

After discussion amongst the author and the Urology team the suggestion was this was this rare form of food induced UTI, especially having the prior lab work as a baseline. The concern was a recurrent UTI with a possible latent storage of E coli in the inflamed prostate. However after 6 months the tests remained normal except for a slightly elevated neutrophilia. We believe that this was a food activated septicemia followed by a UTI with a quasi-granulomatous reservoir in the prostate. The Pt will continue to be followed.

As Roos et al note:

Urinary tract infections (UTIs) are among the most frequent bacterial infections in human beings, affecting millions of people each year. It is estimated that there are more than 10 million cases in Western Europe alone per year.

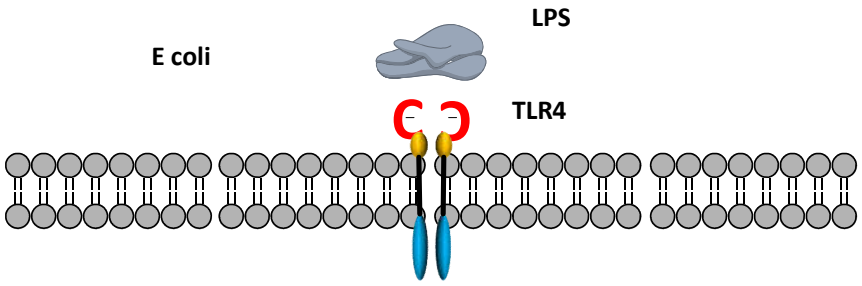
The recurrence rate is high and often the infections tend to become chronic with many episodes. UTI usually starts as a bladder infection but often evolves to encompass the kidneys and ultimately can result in renal failure or dissemination to the blood. UTI is classified into disease categories by the site of infection: cystitis (the bladder), pyelonephritis (the kidney) and bacteriuria (the urine).

Paradoxically, the most frequent form of UTI is asymptomatic bacteriuria (ABU), i.e. colonization of urine in the absence of clinical symptoms. ABU patients may carry large number of bacteria, more than 105 CFUmL1, for months or years without developing symptoms. ABU occurs in up to 6% of healthy individuals and 20% of elderly individuals. Most patients with ABU do not need treatment and in many cases the colonizing organism actually helps to prevent infection by other more virulent bacteria. Escherichia coli are responsible for more than 80% of all UTIs and cause both ABU and symptomatic UTI. The ability of uropathogenic E. coli (UPEC) to cause symptomatic UTI is associated with the expression of a variety of virulence factors such as adhesins (e.g. type 1 and P fimbriae) and toxins (e.g. haemolysin).

Bacterial adherence is generally considered to be a pivotal step in the infection of host tissue surfaces submitted to hydrodynamic flow forces. The human urinary tract is submitted to significant hydrodynamic shear forces, and fimbriae-mediated adherence to the urinary tract epithelium is generally believed to be important for the bacteria to resist removal by urine flow and to establish in this niche. The three primary fimbrial adhesins associated with UPEC strains are type 1, P and F1C fimbriae. Type 1 fimbriae are mainly associated with cystitis, and confer binding to a-D-mannosylated proteins such as uroplakins, which are abundant in the bladder. Expression of P fimbriae is primarily linked to pyelonephritic strains. P fimbriae recognize the a-D-galactopyranosyl-(1–4)-b-D-galactopyranoside moiety present in the globoseries of glycolipids located in

6.1.1 The Immune System and Bacteria

As a brief reminder the immune system responds to bacteria lipopolysaccharides in E coli. The TLR 4 Toll Like Receptor on the macrophage gets activated which starts the process of as shown below.



Macrophage activation of the immune system. M0 to M1

We now examine further details about E coli that are important in understanding common UTI and the possible granuloma opportunities.

6.1.2 E. Coli

As Raeispour and Ranjbar have recently noted:

E. coli is considered as the cause of 80–90% of UTIs that today is one of the most common bacterial infections.

Because of unreasonable use of antibiotics, the bacterial resistance has been raised. In this study, we reported a high value of multidrug resistance among the uropathogenic E. coli strains. Resistance to cefepime was very high (100%) and after that the strains were resistant to cefalothin (74%) and cefpodoxime (67%). Also high sensitivity to imipenem, vancomycin and doxycycline (100%), amikacin (92%) and nitrofurantoin (90%) have been observed. High levels of susceptibility to imipenem, amikacin, nitrofurantoin and also high levels of resistance to tetracycline and ampicillin have been reported in other studies in Iran [6, 13]. Our results in some cases are consistent with those reported by Niranjan and Malini. They evaluated antibiotic resistance of 119 E. coli isolated from UTI patients.

The isolates were resistant to ampicillin (88.4%), amoxicillin (74.4%), norfloxacin (74.2%), ceftriaxone (71.4%) and sensitive to amikacin (82.6%), nitrofurantoin (82.1%) and imipenem (98.9%). No resistance to vancomycin, imipenem and doxycycline was observed among the studied isolates. In previous studies a high sensitivity to imipenem has been also reported. These antibiotics seems to be a good choice for the treatment of UTI caused by E. coli but it should be considered that unlimited use of these antibiotics can gradually lead to increasing antibiotic resistant. Shakya, did a research on antibiotic resistance of E. coli strains isolated from Indian children. The results showed that the strains have been resistant to nalidixic acid (45%), tetracycline (37%), ampicillin (37%), trimethoprim/sulfamethoxazole (29%), amoxicillin/clavulanic acid (29%), imipenem (0.0%)

As Servin has noted:

For extracellular colonization and internalization, microbial pathogens develop molecular interactions with the host cell surfaces.

Bacterial pathogens, including pathogenic E. coli, have developed on their surfaces adhesins and invasins responsible for the recognition and binding of specific membrane bound host molecules acting as receptors. In some cases, activation of complex signal transduction cascades associated with these host cell molecules follows the binding of adhesins and invasins within the active sites on these molecules.

In many instances adhesins and invasins are located on the bacterial surface in extended hair-like appendages named pili or fimbriae or in amorphous outer membrane-associated structures termed a-fimbrial sheaths.

The Afa/Dr family of adhesins contains representatives having fimbrial, afimbrial, and nonfimbrial architectures. The structural assembly genes coding for Afa/Dr adhesins have a similar organization, consisting of operons of at least five genes. Genes A to D, which encode accessory proteins, are highly conserved in the different family members, whereas gene E, which encodes the adhesin molecule itself, is more divergent. On the basis of a similar genetic organization of the gene clusters involved in the biogenesis of adhesins and/or binding to the common epithelial cell receptor decay-accelerating factor (DAF, CD55), Nowicki et al. have proposed that the Afa/Dr family of adhesins currently includes 13 human adhesins, i.e., AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr, Dr-II, F1845, Nfa-I, AAF-I, AAF-II, AAF-III, the bovine adhesin AfaE-VII, and the AfaE-VIII adhesin found in humans and animals.

Only the human adhesins AfaE-I, AfaE-III, Dr, Dr-II, and F1845 have been fully explored with regard to their genetic organization, receptor recognition, and involvement in Afa/Dr DAEC pathogenicity. In addition, it was noted that the EAEC adhesins AAF-I, AAF-II, and AAF-III are probably more distantly related members of the Afa/Dr family of adhesins. In particular, despite similar genetic organizations of the gene clusters involved in the biogenesis of these three adhesins and Afa/Dr adhesins, it remains important to explore whether or not EAEC adhesins recognized the Afa/Dr receptors, type IV collagen, DAF (CD55), and/or carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs), which play a pivotal role in Afa/Dr DAEC pathogenesis.

Finally, it has been established that Afa/Dr adhesins are assembled via the chaperone-usher pathway and that the Afa/Dr family of adhesins are members of the FGL group of the chaperoneusher class of E. coli adhesins

E coli can be an aggressive infection with a characteristic of adherence. As Johnson had noted:

Adherence to solid substrates is a property common to many pathogenic microorganisms, including viruses, grampositive and gram-negative bacteria, yeasts, and protozoa.

By attaching to host structures, microbial pathogens avoid being swept along by the normal flow of body fluids (blood, urine, intestinal contents) and eliminated, although host cells with adherent bacteria can be shed, thereby eliminating the organisms despite attachment.

Attachment is considered a necessary first step in the colonization of host mucosal surfaces and a precedent to invasive infection in many situations. Uroepithelial-Cell Adherence and Hemagglutination In the late 1970s it was recognized for the first time that strains of E. coli causing UTI typically agglutinate human erythrocytes despite the presence of mannose (mannoseresistant hemagglutination [MRHA]) and adhere to human uroepithelial cells. Also, adherence to uroepithelial cells is usually unaffected by mannose (mannose-resistant adherence) and is more common among strains exhibiting MRHA than among those exhibiting only mannose-sensitive hemagglutination.

The close association observed in individual strains between epithelial-cell adherence and MRHA was explained by the discovery that among most urinary isolates, both properties are mediated by fimbriae. Fimbriae as Mediators of Uroepithelial-Cell Adherence and MRHA The observation that both MRHA and epithelial-cell adherence are mediated by fimbriae is consistent with the results of studies by Duguid et al.. They established that the agglutination of erythrocytes by clinical isolates of E. coli is due to bacterial attachment to and cross-linking of erythrocytes via thin fiberlike appendages, which these investigators termed fimbriae (from the Latin word for threads or fringe).

Brinton later named these structures pili (from the Latin word for hairs) and showed that they retained their hemagglutinating capacity when sheared from bacteria and purified. Fimbriated strains also bind to leukocytes, platelets, spermatozoa, yeast cells, pollen, latex beads, and spores, demonstrating that hemagglutination is one example of the general phenomenon of bacterial attachment rather than a unique interaction of bacteria with erythrocytes. Fimbriae are morphologically and functionally distinct both from flagella, which are thicker, longer, more flexible appearing, and responsible for motility but not for attachment, and from sex pili, which are thicker and function in conjugation but not in attachment to other surfaces.

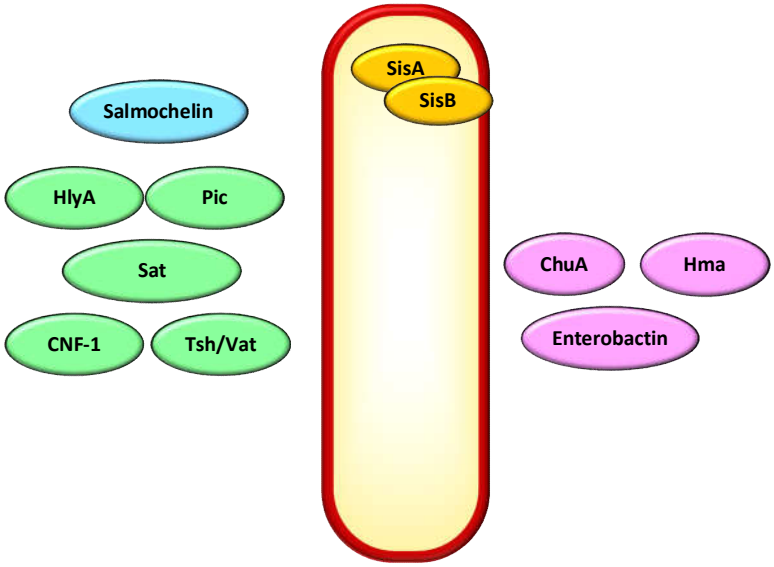
According to Brinton's structural analysis, type 1 fimbriae (which can also serve as a model for other fimbrial types) have a diameter of 7 nm, a length of 0.5 to 2 ,um, and a 0.2- to 0.25-nm-diameter central axial hole. They are composed of repeating subunits polymerized in a helix, with 3 and 1/8 subunits per turn. Whether the term fimbriae or pili is preferable for these adherence organelles is controversial. Favoring the term fimbriae are its priority, the simple adjectival form (fimbrial), and the distinction it emphasizes between adhesive appendages and sex pili. On the other hand, pili is simpler, there is a readily understood singular form (pilus), and there is a simple term for structural subunits (pilin). The dispute over which term should take precedence is moot, however, since both are in common use and are generally understood to be synonymous.

From Nielubowicz and Mobley we have the following:

The urinary tract is a common site of bacterial infections; nearly half of all women experience at least one urinary tract infection (UTI) during their lifetime. These infections are classified based

on the condition of the host. Uncomplicated infections affect otherwise healthy individuals and are most commonly caused by uropathogenic Escherichia coli, whereas complicated infections affect patients with underlying difficulties, such as a urinary tract abnormality or catheterization, and are commonly caused by species such as Proteus mirabilis. Virulence and fitness factors produced by both pathogens include fimbriae, toxins, flagella, iron acquisition systems, and proteins that function in immune evasion. Additional factors that contribute to infection include the formation of intracellular bacterial communities by E. coli and the production of urease by P. mirabilis, which can result in urinary stone formation. Innate immune responses are induced or mediated by pattern recognition receptors, antimicrobial peptides, and neutrophils. The adaptive immune response to UTI is less well understood. Host factors TLR4 and CXCR1 are implicated in disease outcome and susceptibility, respectively. Low levels of TLR4 are associated with asymptomatic bacteriuria while low levels of CXCR1 are associated with increased incidence of acute pyelonephritis. Current research is focused on the identification of additional virulence factors and therapeutic or prophylactic targets that might be used in the generation of vaccines against both uropathogens.

Some of the E coli factors are shown below:



6.1.3 Chronic UTI

As Schaeffer and Nicolle have noted:

Bacteria that are established in the prostate may be impossible to eradicate owing to limited diffusion of antibiotic agents into the gland or to the presence of colonized prostate stones….

Now perhaps those “stones” are in fact granulomas protecting the underlying pathologic organism. We shall examine this possibility. The authors continue:

As men age, they acquire structural and functional abnormalities of the urinary tract that impair normal voiding; the most common is benign prostatic hyperplasia, which can cause urinary tract infection owing to obstruction and turbulent urine flow. Acute bacterial prostatitis (prostate infection) is a severe, potentially life-threatening systemic infection.

Chronic bacterial prostatitis may manifest as recurring urinary tract infections, usually with the same bacterial strain iso lated with each episode.

Bacteria that are established in the prostate may be impossible to eradicate owing to limited diffusion of antibiotic agents into the gland or to the presence of colonized prostate stones.

Older populations often have coexisting conditions, such as diabetes mellitus, that are associated with an increased susceptibility to infection.

Urologic coexisting conditions, such as incontinence or urinary retention, facilitate the acquisition of bacteriuria owing to an increased exposure to interventions such as catheterization. However, prospective studies have not shown associations between postvoiding residual urine volume and bacteriuria or symptomatic urinary tract infection in men. The most consistent predictors of asymptomatic bacteriuria are markers of functional disability, including incontinence, immobility, and dementia. A gram-negative organism is isolated from 60 to 80% of samples from older men living in the community who have urinary tract infections.

E. coli is the most common organism; other Enterobacteriaceae such as Klebsiella pneumoniae and Proteus mirabilis are isolated less frequently. Enterococcus species are the most common gram-positive organisms. Specific E. coli strains and virulence traits correlate with clinical presentation. Strains that are isolated from men with pyelonephritis or febrile urinary infection are the most virulent, followed by strains isolated from men with cystitis; colonizing fecal strains tend to be the least virulent.

In men without indwelling urinary catheters who live in an institution and who have bacteriuria, E. coli is also the most common pathogen isolated, but P. mirabilis, Pseudomonas aeruginosa, and multidrugresistant strains are increasingly frequent.1 In a study conducted in Spain, men were more likely than women to have extended-spectrum betalactamase strains isolated from the urine; older age and nursing home residence were also associated with increased risk of these strains.

As Dikshit et al have noted:

***Recurrent urinary tract infections (UTIs)** caused by **uropathogenic E. coli (UPEC)** are common and morbid infections with limited therapeutic options.*

*Previous studies have demonstrated that persistent intracellular infection of **bladder epithelial cells (BEC) by UPEC** contributes to recurrent UTI in mouse models of infection.*

However, the mechanisms employed by UPEC to survive within BEC are incompletely understood.

In this study we aimed to understand the role of host vesicular trafficking proteins in the intracellular survival of UPEC. Using a cell culture model of intracellular UPEC infection, we found that the small GTPase Rab35 facilitates UPEC survival in UPEC-containing vacuoles (UCV) within BEC. Rab35 plays a role in endosomal recycling of transferrin receptor (TfR), the key protein responsible for transferrin–mediated cellular iron uptake. UPEC enhance the expression of both Rab35 and TfR and recruit these proteins to the UCV, thereby supplying UPEC with the essential nutrient iron. Accordingly, Rab35 or TfR depleted cells showed significantly lower intracellular iron levels and reduced ability to support UPEC survival. In the absence of Rab35, UPEC are preferentially trafficked to degradative lysosomes and killed. Furthermore, in an in vivo murine model of persistent intracellular infection, Rab35 also colocalizes with intracellular UPEC.

We propose a model in which UPEC subverts two different vesicular trafficking pathways (endosomal recycling and degradative lysosomal fusion) ***by modulating Rab35****, thereby simultaneously* ***enhancing iron acquisition and avoiding lysosomal degradation of the UCV within bladder epithelial cells.*** *Our findings reveal a novel survival mechanism of intracellular UPEC and suggest a potential avenue for therapeutic intervention against recurrent UTI.*

The bladder infection as a source of recurrence is one proposal. However one should also consider the prostate especially in the case of BPH.

As Chen et al note :

The gut and urinary tract are very distinct habitats from the perspective of their metabolic, immunologic, and microbial features.

The gut is home to our largest population of microbes, whereas the bladder is considered a normally sterile environment, guarded by physical and biological barriers to microbial invasion. Studies of the molecular pathogenesis of UTI in a mouse model have identified numerous virulence factors, including adhesins, toxins, iron acquisition systems, capsular structures, flagellae, pathogenicity islands (PAIs), and components important for biofilm formation (13).

Among adhesins, UPEC strains typically encode a multitude of chaperone/usher pathway (CUP) pilus gene clusters. CUP pili contain adhesins at their tips that play critical roles in host-pathogen interactions, recognizing specific receptors with stereochemical specificity. For example, FimH, the type 1 pilus tip adhesin, binds mannosylated glycoproteins, as well as N-linked oligosaccharides of b1 and a3 integrins that are expressed on the luminal surface of the bladder epithelium (urothelium) in humans and mice. Type 1 pilus–mediated binding can lead to invasion of UPEC into mouse and human bladder epithelial cells.

Invading UPEC can be expelled from the host cell, or they can "escape" into the cell's cytoplasm where they replicate rapidly and form a biofilm-like structure, composed of 104 to

105 organisms, known as an intracellular bacterial community (IBC). Bacteria in the IBC are protected from antibiotics and from immune responses. IBCs are transient; after maturation, UPEC can disperse from the IBC, exit their host cells, enter the lumen of the bladder, and subsequently invade other urothelial cells. One primary host defense that eliminates IBCs is exfoliation, where urothelial cells undergo an apoptotic-like cell death, detach from the underlying transitional epithelium, and are eliminated in the urine.

Exfoliated bladder epithelial cells containing IBCs have been observed in urine collected from women with recurrent UTI but not in healthy controls or in cases of UTI caused by Gram-positive pathogens. However, exfoliation exposes underlying cell layers of the urothelium. Subsequent UPEC invasion of these underlying cells in mice results in formation of additional intracellular structures termed quiescent intracellular reservoirs (QIRs). Bacteria in the QIR are dormant, are resistant to antibiotic treatment, and elude recognition by host immune defenses. Mouse models have been used to demonstrate that bacteria in QIRs can contribute to recurrent infection after antibiotic treatment has rendered the urine sterile.

6.1.4 Other Infections

As Martinez-Medina et al have noted:

A large number of mucosa-associated E. coli isolates per patient were analyzed in the present study to allow for a better comparison of mucosa-adhering bacteria between controls and IBD patients. By determining whether they belonged to the AIEC group, it was possible to compare the pathogenic features of a large collection of AIEC and nonAIEC subtypes. New information about the ecological parameters of the whole E. coli population and AIEC pathovar is provided for healthy subjects and ***CD (Crohn's Disease)*** *patients.*

Virulence genotyping of the 22 different AIEC subtypes collected contributes to a better description of the AIEC pathovar. Quantitative PCR revealed higher E. coli counts in I-CD patients in comparison to C-CD, IC-CD, and controls, which is in agreement with previous studies; these results were irrespective of the zone sampled along the bowel.

However, no difference in E. coli diversity was found between CD patients and controls. In fact, E. coli subtypes were found to be host-specific, and the same clones were associated with the ulcerated and nonulcerated mucosa of CD patients.

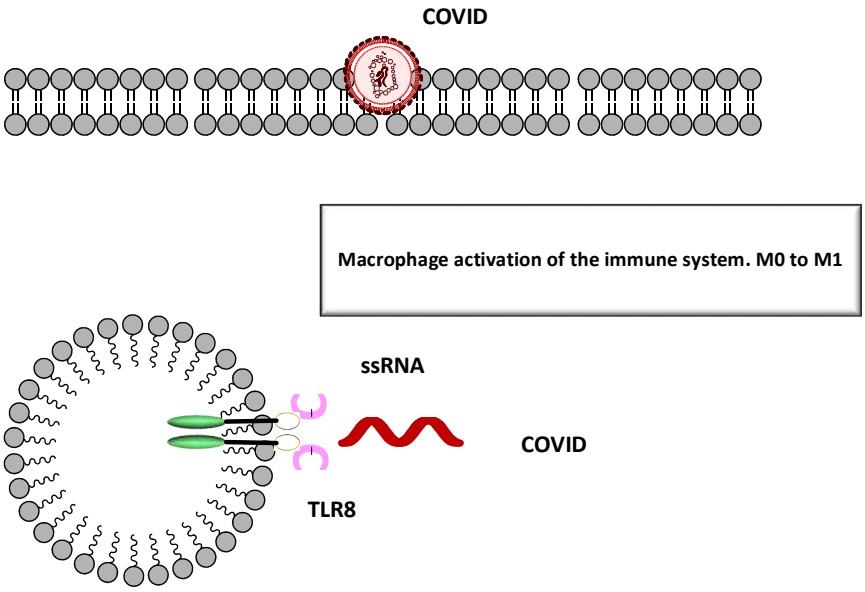
Although a genetic relationship among E. coli isolated from CD patients has been described by ribotyping, no genetic relatedness was observed by PFGE in this study… Moreover, phylogenetic groups A, B1, B2, and D were equally distributed in CD patients and healthy subjects, with B2 being the most abundant. The majority of mucosa-associated E. coli from both CD patients and controls showed "uropathogenic" features, which are characteristic of B2 and D phylogroups. These features have already been described for the resident colonic microbiota in normal mucosa and are thought to possess a fitness or colonization function

6.2 COVID

The recent corona virus pandemic was driven by a specific virus that has been called COVID. This virus isa structured as a common corona virus with the surface coated with a spike protein.

6.2.1 The Immune System and Viruses

COVID is a single stranded mRNA virus. Thus it must penetrate the cells, which it accomplished by attaching to the ACE 2 receptor. Once inside it disassembles and TLR8 responds which in turn drives the macrophage response (see Howley and Knipe).



6.2.2 Long Haul Infections

As Venkataramani and Winkler have recently noted:

Some patients who have recovered from an infection have reported transient or even lasting cognitive dysfunction. This includes patients who have been infected with SARS-CoV-2, many of whom, including those with mild disease, have reported deficits in attention, executive functioning, language, processing speed, and memory — symptoms collectively referred to as "brain fog." Together with increased incidence of anxiety, depression, sleep disorder, and fatigue, this syndrome of cognitive impairment contributes substantially to the morbidity of post–Covid-19 conditions (also called "long Covid").

Nevertheless, Covid-related brain fog is difficult to diagnose and to separate from other reasons for the symptoms in an individual patient, because neurocognitive longitudinal data for patients are rarely available. (On a population level, however, cognitive decline after Covid has been documented.1) Physicians are generally reluctant to accept a condition as an organic disease

without a pathobiologic concept or the ability to measure the disease in a given patient, as is the case with post-Covid brain fog.

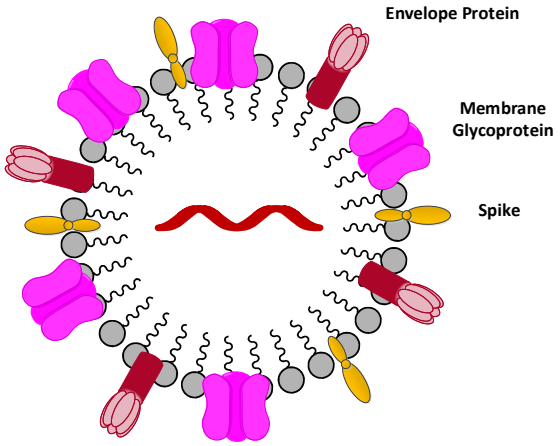
Results of a study recently reported by Fernández-Casta- ñeda and colleagues may represent a pivot in our understanding of this sequela.2 Using a mouse model, the investigators explored how mild respiratory infections of SARSCoV-2 could lead to neuroinflammation and subsequent brain damage through multilineage neural cell dysregulation (Fig. 1). The investigators modeled mild respiratory Covid in a mouse expressing the viral-entry receptor for SARSCoV-2 (angiotensin-converting enzyme 2 in humans) in the trachea and lung by delivering SARS-CoV-2 intranasally. They detected no SARSCoV-2 in the brain but found signs of neuroinflammation in elevated levels of chemokines in cerebrospinal fluid and serum, each with a distinct time course. These changes led to activation of microglia in subcortical and hippocampal white-matter regions (but not in gray matter), with distinct effects on specific neural cell populations. Of note, these findings were supported by similar results in a small group of patients who were found to have SARS-CoV-2 infection and no severe lung damage at the time of death.

Microglia are resident macrophage cells in the central nervous system. Although they contribute to the homeostasis of the central nervous system and refinement of neuronal networks by removing dendritic spines and synapses during the development of neurons, microglia can transition to an activated, neurotoxic state, as seen in this mouse model. In the subcortical white matter, microglial activation was associated with loss of both oligodendrocyte precursors and mature oligodendrocytes; consistent with this loss, there was also loss of myelin and myelinated axons for at least 7 weeks after the infection began.

Myelin insulates axons and is critical to the speed of electrical conduction along neurons and to axonal metabolism. The loss of myelinated axons impairs the structure and function of neuronal networks.

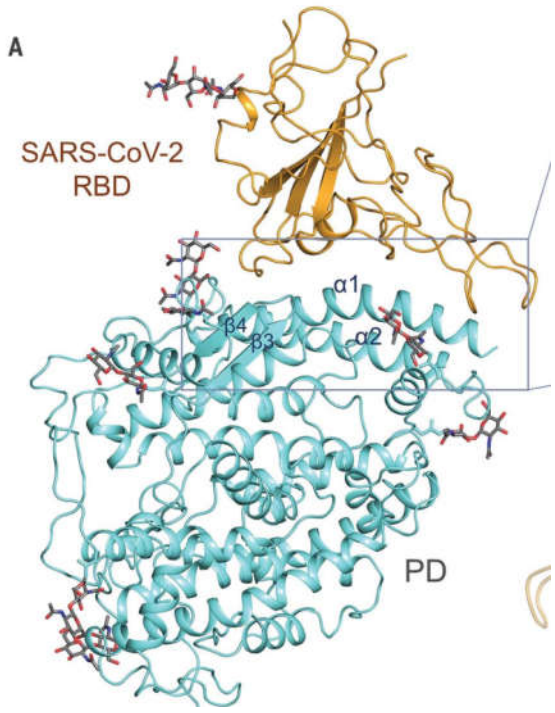
6.2.3 Virus Basics

The details of the COVID virus are depicted below.



COVID is a corona virus which is a single stranded RNA virus as shown above[11]. RNA is a sequence of nucleic acids on a sugar like backbone (ribose). There are about 30,000 nucleotides in this RNA. The virus has an outer shell and on top of that there are spike proteins that extend out. These spike proteins are used by the virus to find a cell to attach to.

The virus enters the human generally through the nasal passages. The virus surface spike protein needs to find a cells that has a surface protein that the spike can attach to. A protein is a collection of connected amino acids. The amino acids are connected in a complex manner and they have pockets of positive and negative charges. The COVID protein (SARS-Coc-2) seeks a cells with a surface protein that matches. This then allows it to attach. We show an example below.



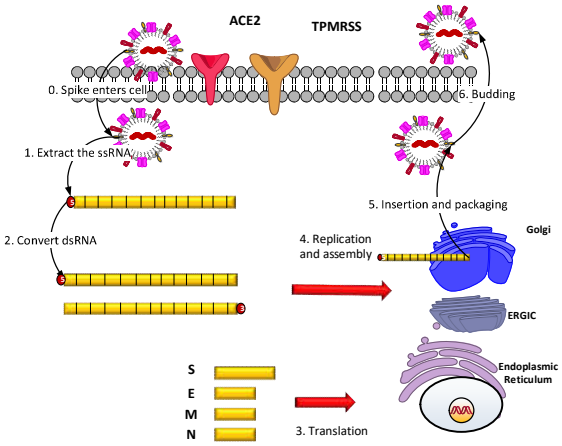
In humans the cell surface protein called ACE2 inhibitor is a good match for the COVID spike. Thus when the virus particle sees this protein on a cell it will attach, enter the cell, and commence its replication.

However, and this is very important, the shape of a protein is temperature dependent. The higher the temperature the more unwrapped the protein becomes and the more it can find a place to attach to a cell. For example, it has been noted that the spike protein and ACE2 binding is poor at 94F a temperature in the nasal cavities but very strong at 99F in the lung. This appears to be reflected in the fact that upper respiratory symptoms such as sore throat and runny nose do not occur. However, and this is critical, the virus can remain in the nasopharynx and can spread. The time that this quasi-dormant stage remains viable appears to be 24 to 48 hours.

[11] https://www.telmarc.com/Documents/White%20Papers/173Corona.pdf

The attack point in humans is the ACE2 receptor. This protein is almost the same in all humans but not quite. There are about 140 variants in ACE2. It appears that the variants do get reflected in how aggressive the viral attack is. For example in East Asians the ACE2 is a poorer match that Europeans and Africans have a stronger match and thus a more virulent form than any others. However, the variants may have a different set of relative affinities.

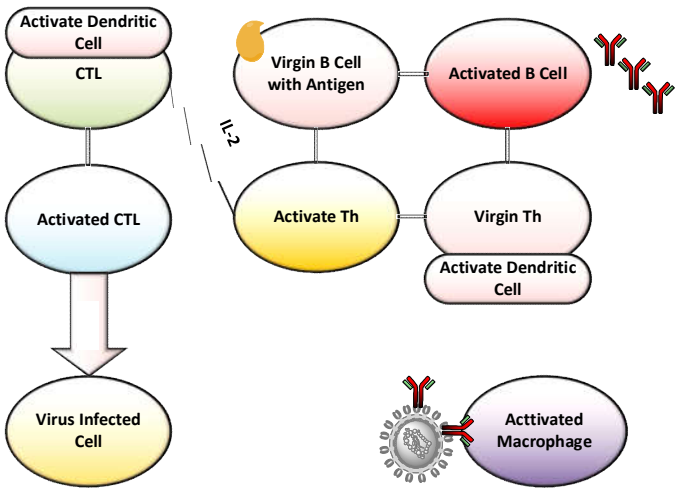
The picture below demonstrates how the virus uses the cells capabilities to replicate.



The virus attaches to human cells via the ACE2 receptor, a surface protein. It enters the cell where it sheds its surface proteins and uses the human cell to start reproducing itself. It can reproduce many times and create new virus elements while also damaging the cell. The cell is damaged as a result of both proliferation as well as the cells trying to rid itself of the virus invader by pumping out cytokines such as IL-6, which is a strong cytokine.

The virus invades cells and kills them as well as creating massive cytokine, attacking proteins, which try to consume the virus but manages to destroy remaining healthy cells as well. In the lung, the result of the massive response by the self's own immune system, the alveoli or lung's oxygen and carbon dioxide transfer system gets blocked resulting in death to the patient.

The immune system is a powerful set of cells and proteins that attack various invaders.



The immune system is comprised of two basic arms. The first is the innate which attacks any invader and is almost immediate. The second is the adaptive which attacks specific invaders and has a long term memory. The basic cells in the adaptive are B and T cells. B cells get activated by antigens such as a spike protein and create antibodies. T cells assist in the process and get activated and become killer cells, cytotoxic T lymphocytes, CTL, which then go after the virus cells.

A vaccine is a method that activates the persons immune system to recognize and attack a specific entity such as the virus.

Antibodies are proteins generated by the human in response to an antigen. An antigen is some other foreign substance such as a spike protein. The antibodies are generated by B cells in the body. The B cells are presented with the antigen in the lymph nodes by scavenger cells called dendritic cells. The B cells have the ability to have near infinite mutations of antibody generation and when one of these sees a matching antigen it multiplies at a great rate and produces massive amounts of antibodies.

The antibodies, they are proteins, then go through the human system and when they see an antigen such as a spike they attach and send out signals to T cells to come and attack this antigen. The T cells then attack using cytokine, attack proteins, which kill the virus before it can do much harm.

Memory T cells are T cells which remember the antigen and stay resident for very long periods in the cells which were attacked[12]. They can resume the work as killer cells when they see another attempt by the virus. It is difficult to measure memory T cells since they are tissue resident and not on the blood stream.

[12] https://www.telmarc.com/Documents/White%20Papers/189MTC.pdf

A vaccine works by enlisting the immune system to attack the virus before it can cause great harm. In the case of COVID we have the spike protein to identify the virus and we then train the immune system to attack when it sees the spike.

The vaccine sends into the human system just the spike protein[13]. This spike must get into our cells where it can reproduce like the total virus, but we just want the spike by itself. The spike mRNA is generally harmless and it gets released into the body where it gets recognized by our immune system, generally dendritic cells, which carry the mRNA as an antigen back to the lymph system where the B cells try to match it. If we get a good B cell match, the B cells start producing antibodies, Ab, which flood the human. These Ab are then ready to attack anything that has this spike protein. It may also produce memory T cells that reside in our system.

If I am vaccinated can I still get COVID? Yes. It does depend on what one means by "getting COVID". The virus can again try to invade the person but your immune system will attack. It may then be a mini infection with little or no symptoms. It depends on one's antibody load, immune response and memory T cell capacity. At present there is no significant data indicating how significant this is.

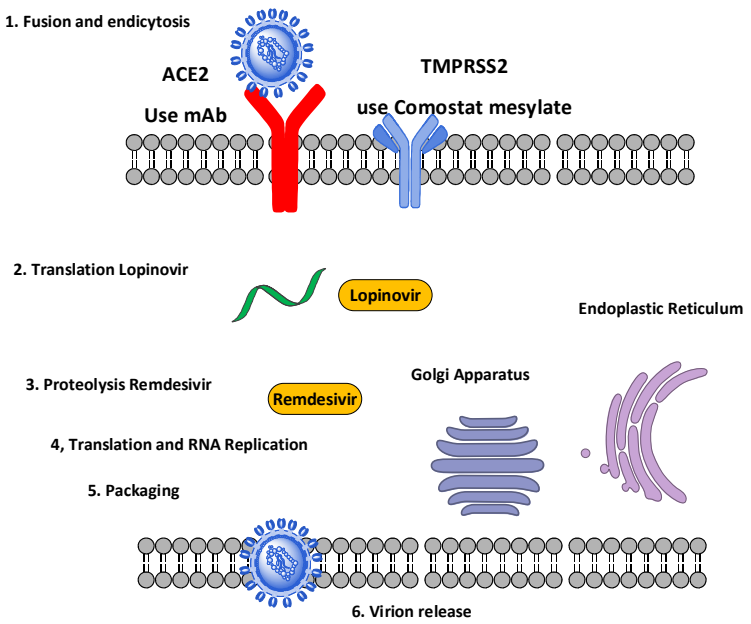
If I am vaccinated can I still infect others? Yes, and this is a real concern. Recall that the virus may enter the nasopharynx and not cause a disease but can be sent out in aerosols to others without even being aware. Furthermore, if vaccinated one may have a strong immune response when the virus attempts to enter the cells. Moreover, the immune response may depend on the conformation of the spike protein which we noted is temperature dependent as well. Thus nasopharynx viruses may cause no problem and just liger like the proverbial Typhoid Mary syndrome.

How Long Does a Vaccine Immunity Last? Good Question. No answer yet but the CDC should have been working on this but they appear at this time not to know. It is however a simple enough process to find out. Tracking say 8 age groups, 6 ethnic groups, two sexes (yes XX and XY if you must for those of the woke persuasion) give about 100 groups with 100 samples in each group. That is 10,000 tests once a week measuring Ab titers. The data should be made public. However it does not appear that the CDC is even aware of such tests. We really need better public health.

Sequellae to the infection can be significant. Such things as myocarditis or heart muscle inflammation has been noted as has many other sequellae. The sequellae may be the result of the immune system over-reacting to the virus, often via the pathway.

The following figure represents some of the dynamics of the virus and therapeutics that may be applied.

[13] https://www.telmarc.com/Documents/White%20Papers/180Update.pdf



Treatment must address several factors[14]. One is the virus itself. This can be dealt with by antiviral medications. The cytokine storm elicited by IL-6 can be blocked by IL-6 inhibitors[15] and infections with classic antibiotics. Recent use of antibody therapy also appears useful.

Briefly, the spike is a protein. It is about 1,000 amino acids long. It is produced from the mRNA of the virus in a segment approximately 3,000 nucleotides long. The coding of 3 nucleotides per amino acid is the translation metric. Now the spike protein is a combination of positively, negatively, and neutrally charged amino acids which become wrapped in a complex manner. The wrapping can be modified by temperature, pH, and many other factors. In a similar manner the ACE2 receptor is a protein and the two can attach to one another depending on charge and conformation.

Of course, it may be much more complex. We can get the above discussed mutation but at the same time many other mutations could occur which make that variant die out.

The main observation however is that we really do not want many variants. Thus if an immune compromised patient gets COVID they are subject to many more variants occurring and as such can become a source of many aggressive variant productions.

process. Frankly the reports referenced appear to justify masks as the starting point and then attempt to match limited data.

[14] https://www.telmarc.com/Documents/White%20Papers/175COVID.pdf

[15] https://jamanetwork.com/journals/jama/fullarticle/2781880

The problem with ascertaining the efficacy of masks is the complexity of the situation. Is it outside or inside, summer or winter, ventilated or not, homogeneous high quality masks or cheap off the shelf types, large congregations, high density, excess excitations, and the list goes on. The best one can say about masks is; it depends.

6.2.4 Reinfection: Real or just a Granuloma

As Skankey and Breeden note:

If the relapse episode was not due to an active infection, then the best explanation would be a post-infectious immune mediated response. Post-infectious immune mediated responses related to COVID-19 infections have been reported.

However, an immune mediated respiratory response, mimicking that of acute COVID-19 viral pneumonia, has not been reported. In our patient, There was acute development of fever, tachycardia, profound hypoxia, hypercapnia, bilateral infiltrates, marked increase in C-reactive protein, and marked increase of ferritin, which is what is encountered in acute COVID-19 infection. A minimal increase in the procalcitonin is a strike against a hospital acquired pneumonia. Additionally, blood in sputum cultures were no growth. The minimally elevated BNP makes pulmonary edema or congestive heart failure less likely. These findings provide strong evidence for acute infection.

As Abrokwa et al noted:

In this review, we found that recurrence of a positive SARS-CoV-2 test among previously recovered cases is a commonly-reported phenomenon within the first few weeks from recovery. While some of these cases follow exposure, confirmed SARS-CoV-2 re-infections are rare. Fifty percent of genetically confirmed cases of re-infection were observed within 90 days after initial disease. Evidence on onwards transmission and predictive markers is limited but existent. With this high rate of recurrence of SARS-CoV-2, and mixed evidence of the risk to public health, policy makers need to re-consider current policies of contact tracing and quarantine regulations.

7 OBSERVATIONS

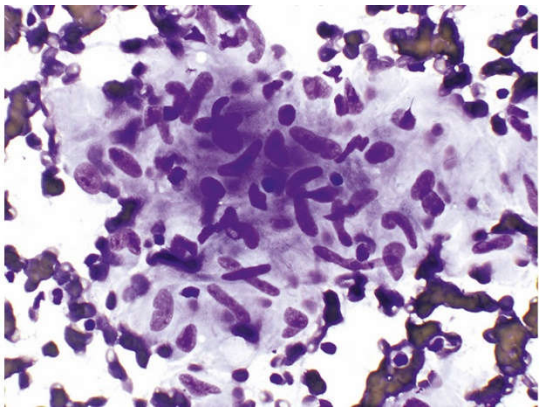
We now make several observations. Some are speculative but may be of merit. They are based upon the review we have performed herein.

7.1 COMMONALITY WITH TME AND GRANULOMAS

We believe that there is reasonable evidence to demonstrate a parallelism between TME and granulomas. Not that they are identical but that they has comparable functional elements.

7.2 DIAGNOSIS OF GRANULOMAS

Diagnosing granulomas have generally been accomplished by biopsy. An example is a sarcoid granuloma[16].



Not the collection of cells into a well-defined granuloma. One must then be able to identify most of the cells and this can involve a complex set of markers.

7.3 THERAPEUTIC APPROACHES

Excision may be useful but for many of the granulomatous diseases one has little success because of their extensive proliferation. Targeting the surrounding cells may help but to do so specific surface markers are essential.

7.4 IMAGING TECHNOLOGY FOR IDENTIFYING GRANULOMAS

Identifying granulomas ranges from the Leprosy identification on the skin to the imaging of the lung for TB. However identifying small granulomas such as may be the case with a UTI or a COVID demands two things. First where to look. Second resolution on a much higher level.

[16] See Cibas and Ducatman, Cytology, Elsevier, 2021

One may examine the MRI resolution. The resolution equation for MRI scanning is[17]:

$$S(m) = \frac{TBW(KHz)}{\gamma(MHz/T)G(mT/m)}$$

Thus the smaller the slice the smaller the bandwidth and the larger the gradient. The smaller the BW the longer the scan and the larger the gradient the bigger the magnet. Thus a 3T magnet allows for better gradient. It is not at all clear, however, that we can achieve the 210 micron or better in a timely manner.

We can use a simple example. Consider H and 1.5T magnet. We know:

$$\gamma = \frac{\omega}{B} = \frac{64MHz}{1.5T} = 40(MHz/T)$$

Thus if we use say a 1 KHz BW and a slice of 100 (mT/M) we obtain:

$$S(m) = \frac{1KHz}{40(MHz/T)100(mT/m)}$$

$$= \frac{1}{4000}(m) = 250\mu$$

Thus this is in the range of an epithelial layer. Now as we increase to say a 7.5T magnet we obtain a resolution of 50µ. If the granuloma is composed of the pathogen and then a layer of macrophages and then fibroblasts, we know that a cell nucleus is about 20µ and a cell about 50µ. Thus a mass of 1 fibroblast, 2 macrophages, the pathogen one can have a granuloma of 6+ cells, say 7, each at 50µ which is a diameter of 350µ. That is barely perceptible by a standard 1.5T MRI but with a 7.5T MRI we have reasonable resolution. However the search time is increased significantly. Perhaps an AI based algorithm may be useful.

7.5 Comparisons of TME and Granulomas

We provide a brief comparison between the two elements discusses:

Element	*TME*	*Granuloma*
Extracellular		
Macrophages (M1 and M2)	Yes	Yes
Fibroblasts	Yes	Yes
T-reg cells	Yes	Yes
NK Cells	Yes	Yes
Adipocytes	Yes	UNK
Perivascular Cells	Yes	UNK

[17] See Westbrook and Talbot, p 139

Element	*TME*	*Granuloma*
Intracellular		
VEGF	Yes	Yes
mTOR	Yes	Yes
CDKs	Yes	Yes
JAK/STAT	Yes	Yes
IL-6 etc	Yes	Yes

7.6 Metabolic Elements of Granuloma Persistence

We know that cancer cells have a strong metabolic element in their sustainability, proliferation, and metastasis. One may then ask of such a parallel exists also with granulomas.

7.7 What are the temporal dynamics of a granuloma?

When we examine granulomas we see a static process. There is little if any research demonstrating the dynamics of granulomas. Namely from the time of formation throughout a life cycle. In addition we do not appear to have any information regarding the drivers of any dynamics of a life cycle. This would logically be a significant information base to have because it may play upon possible therapeutics.

Also one should better understand the dynamics in the context of senescence and autophagy.

7.8 What are the spatial properties

Likewise to the temporal characteristics the spatial characteristics also are helpful to understand. Namely does the local cellular structure have a significant role to play on the microstructure of the granuloma? Is the granuloma growth wholly self-contained or does it reflect local cellular properties.

7.9 What limits the proliferation?

Granulomas are generally localized. The initiate growth about the pathogen and then growth stops at a certain pont. Leprosy is an exception. However new granulomas may arise as in sarcoid disease.

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