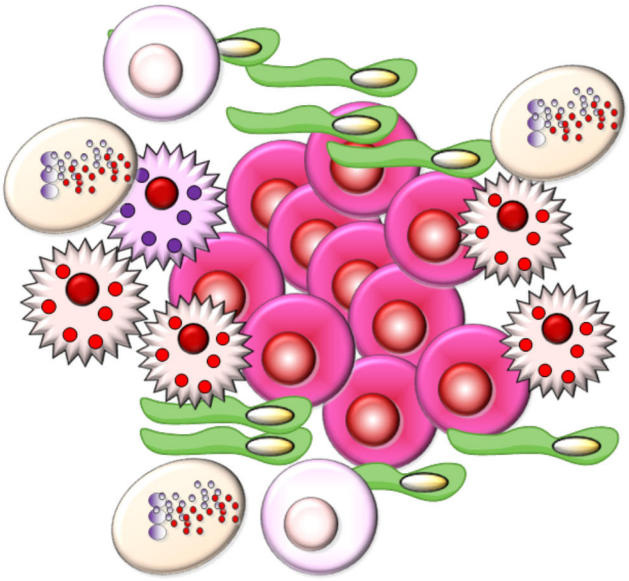

MACROPHAGES REDUX

September 2024

ABSTRACT

This Note examines macrophages and their impact on tumors. A great deal has been learned over the past two decades and control of macrophages may be essential for any effective therapeutic strategy, especially immunotherapeutics.

Terrence McGarty
TGL 209

NOTICE

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Furthermore, this document contains references to and quotes and modified charts and figures from papers and documents under the premise of “Fair Use” in order to present ideas and understandings in context. The Author has attempted to make any and all references to such material separate from those of the author per se and has referenced the source expressly in all cases. These documents are for the dissemination of ideas and have no commercial intent. 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 necessarily provide any new or fundamental results but merely attempt to assemble elements in a systematic and holistic manner.

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Contents

1 INTRODUCTION

Understanding cancer is an ever-moving target. If one reads Rosenberg's wonderful book from the early 1990s one sees how little we really knew. Over the next 30 years as Rosenberg continued his brilliant efforts to understand the immune system, we have seen progress but we also have been subjected to many new dimensions of what cancer is. From more detailed genomics, to epigenetic factors, through tumor micro environments. This Note is an attempt to update some of what is known about macrophages, perhaps just one new element, perhaps more.

We have previously examined various aspects of tumor associated cells[1]. Macrophages play critical roles in attacking or defending tumor cells. What these macrophages are and how they function is being more fully understood as time goes by. It is now understood that there are a multiplicity of phenotypical macrophages each excited in a specific manner and each eliciting signally in a similar fashion.

This Note examines some of the recent work on macrophages and cancer. The more that is understood the more complex it becomes. However the more one can see therapeutic targets. Two decades ago we saw the growth of such targets as CTLA-4 and PD-l. The deeper understanding of macrophages and the ability to control them may also present an additional set of therapeutic targets.

Macrophages have been found to have multiple phenotypes. Some helpful and some supportive of the malignancy. The macrophages are activated by various paths and in turn send out signals that attack or support the malignancy. One of the things that can become clear is that what we may know today could change dramatically as research on macrophages continues. However with what is currently understood, however, it is clear that macrophages and their affiliated cells and pathways present alternative options for new therapeutics.

We know that immunotherapy functions well in only 20% to 40% of the patients. If we try to target PD-1 for example, perhaps the tumor micro environment, TME, surrounded any tumor associated macrophages, TAM, shield the efficacy of the targeted therapeutic. Thus, conceptually, perhaps if we can target the TAM, break it loose from protecting the malignant cells then we can have a two shot therapeutic approach with increased efficacy.

We examine this issue herein.

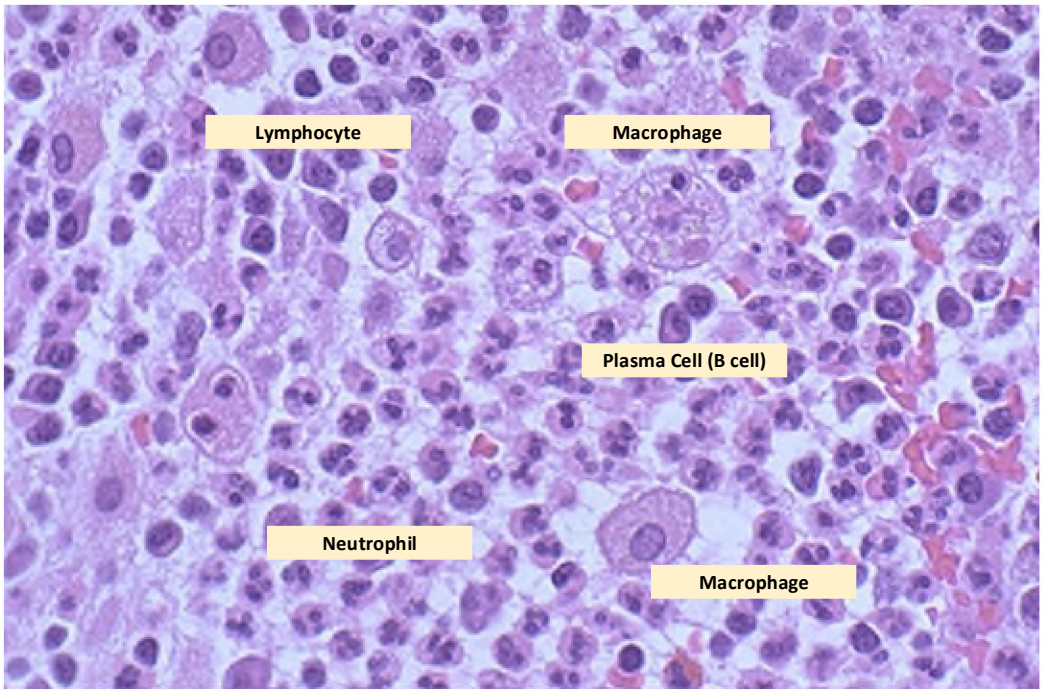
1.1 WHAT IS A MACROPHAGE

Macrophages are a complex set of cells that occupy the tissues of the body and often are considered part of the immune system attacking invaders after associating with T cells. From Abbas et al:

[1] See https://www.researchgate.net/publication/336116071_Tumor_Associated_Immune_Cells_On_the_one_hand_and_on_the_other_hand (2019),

After birth, cells of the monocyte-macrophage lineage arise from committed precursor cells in the bone marrow, driven by a cytokine called monocyte (or macrophage) colony-stimulating factor (M-CSF). These precursors mature into monocytes, which enter and circulate in the blood, where they have a short life span of approximately 1 to 7 days. Blood monocytes are efficiently recruited into tissue sites of infection or injury, and therefore most macrophages at sites of inflammation are monocyte-derived. Most long-lived tissue-resident macrophages are derived not from the bone marrow but from yolk sac or fetal liver precursors during fetal development. These cells have self-renewal capacity, so they can maintain stable numbers. They often assume specialized phenotypes depending on the organ. Examples are Kupffer cells lining the sinusoids in the liver, alveolar macrophages in the lung, and microglial cells in the brain. In the steady state, blood monocytes are recruited at a low rate into healthy tissues, where they differentiate into tissue-resident macrophages. This pathway of monocyte differentiation into tissue macrophages supplements the self-renewal of the fetally derived cells, and accounts for varying fractions of resident macrophages in different tissues.

The image below is an example of macrophages and associated cells.



https://webpath.med.utah.edu/INFLHTML/INFL073.html

1.2 Dr Jekyll and Mr Hyde?

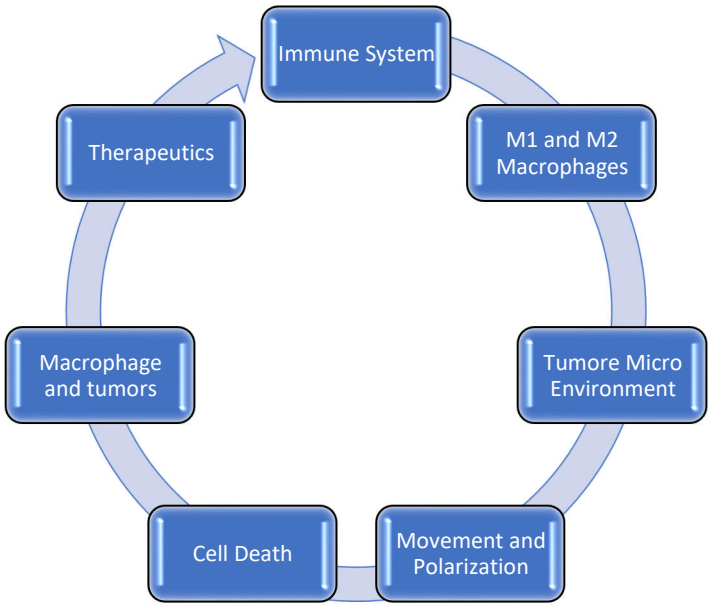
Macrophage can be "good" cells in attacking cancers or "bad" cells protecting and supporting malignant masses. We attempt in this note to examine both and the implications of therapeutics thereto. Macrophages can alter their phenotype almost at will. Understanding this fact, and understanding what facilitates this phenotypic alteration is essential in understanding the impact on malignancies.

However, as more is understood about cancers, more complexities are introduced. Macrophages are but one element in the development of cancers. Yet it may be the element that can facilitate other immunotherapeutic approaches.

1.3 Overview

We examine the following in this Note:

1. An overview of the immune system with a focus on macrophages
2. A detailed discussion of M1 and M2 macrophages with an analysis of the sub types of M2 macrophages
3. A review of the tumor micro-environment, TME, and currently perceived.
4. The elements of macrophage movement and polarization. Namely what attracts what type of macrophage and where.
5. Macrophages and Tumor specificity
6. The types of cell death and the relationship of the various macrophage phenotypes
7. Macrophages and therapeutic options



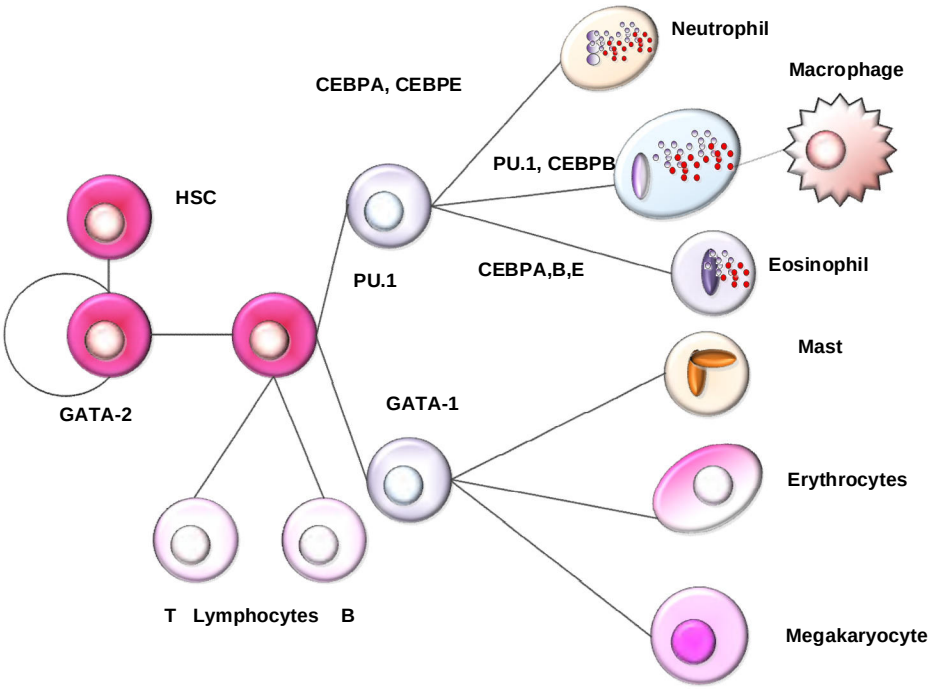
We also provide some observations regarding areas of current investigation regarding TAMs. Understanding TAMs and the therapeutic options could be a significant area of opportunity in cancer management. It is a challenge however since performing pathological studies could be costly and time consuming.

2 T CELL OVERVIEW

T cells are interactive partners with macrophages and cells which can attack and eliminate cancer cells. We provide an overview here so as to position T cells in the functioning of macrophages.

2.1 ORIGINS

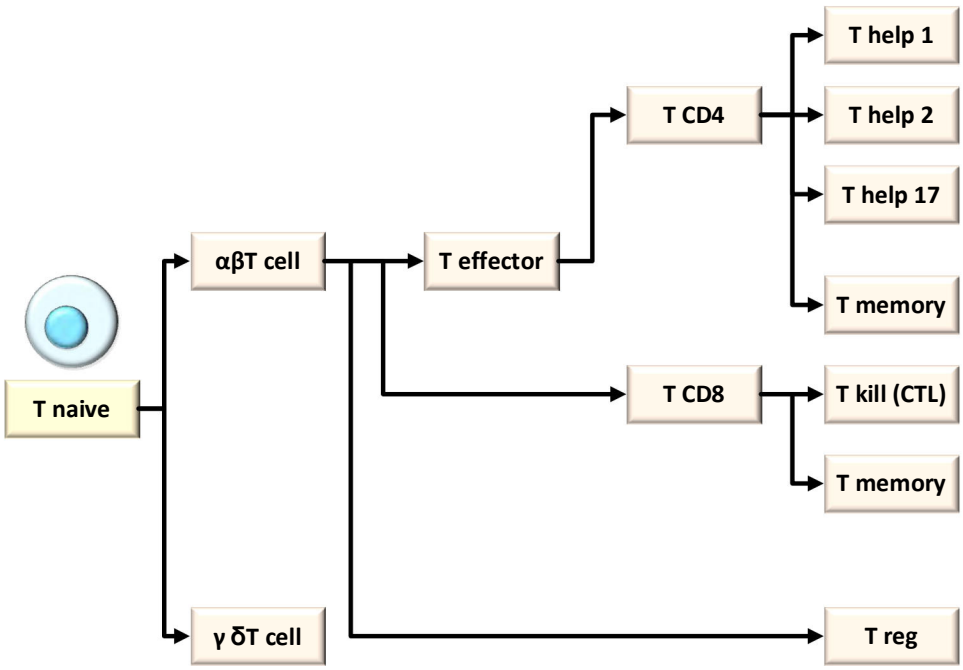
The Figure below is the classic paradigm of the multiplicity of blood cells. It is not complete and it also fails in many ways to portray the complexity of the various cells. Our interest is in macrophages and the simplicity of this paradigm fails to portray that. However it is a basis for moving forward.



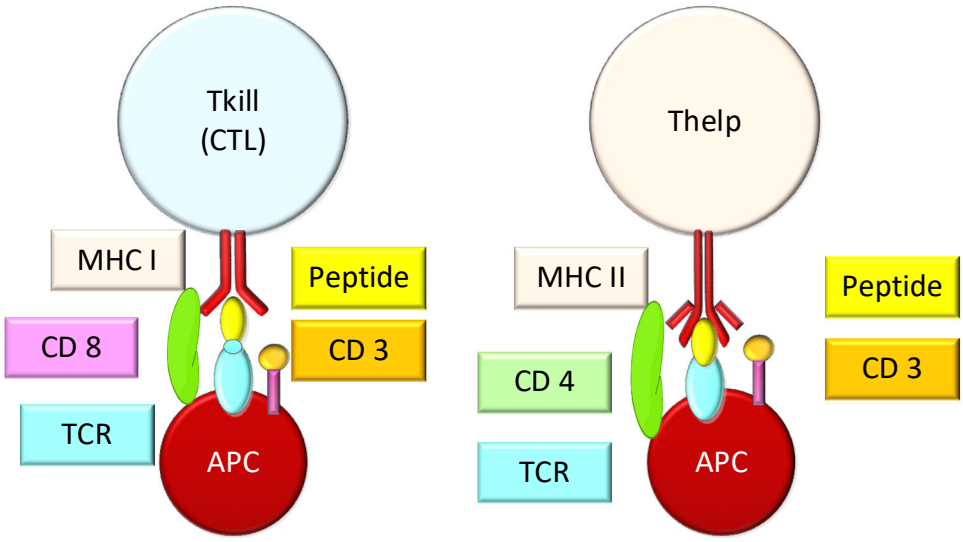
Now there are a large set of T cell variants each having separate functions. We demonstrate the development of some of the key ones below. The classic phrase, “Ontogeny recapitulates Phylogeny”, can be applied to the systematics of T cells below. It must be understood that over the past thirty years we have just begun to understand the complexity of T cell. T cells are key players in destroying infections, cancers, and a wide variety of invasive elements. They do so in conjunction with other cells and alone. T cells can be turned on, turned off, and just left in a dormant state.

A key observation below it that the T cell receptor, TCR, a means for identifying both antigens as well as self, has at this time two know variants, the αβ and the γδ. For the most part we focus on the first although the latter has substantial merit to be discussed later.

The next breakdown is between CD4 effector cells and CD8 killer cells. The “effector” cells do just that, effect other cells to kill the invader. The CD8 cells attack and kill the invader directly.

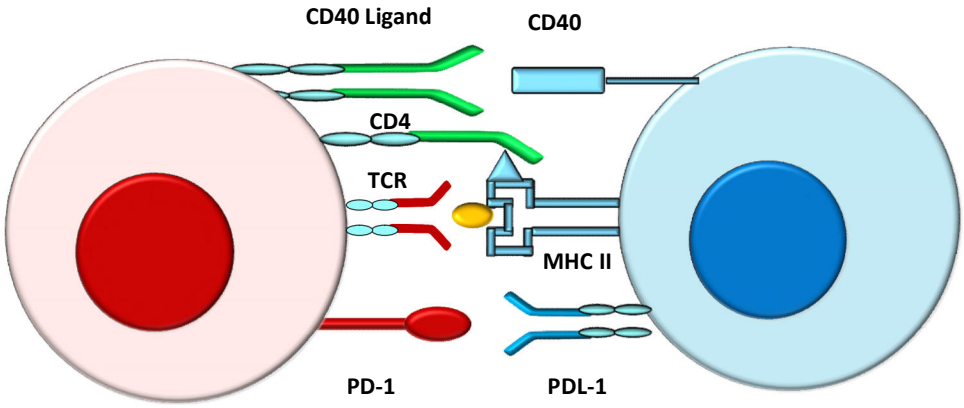


The graphic below depicts the difference between CD4 and CD8 cells.

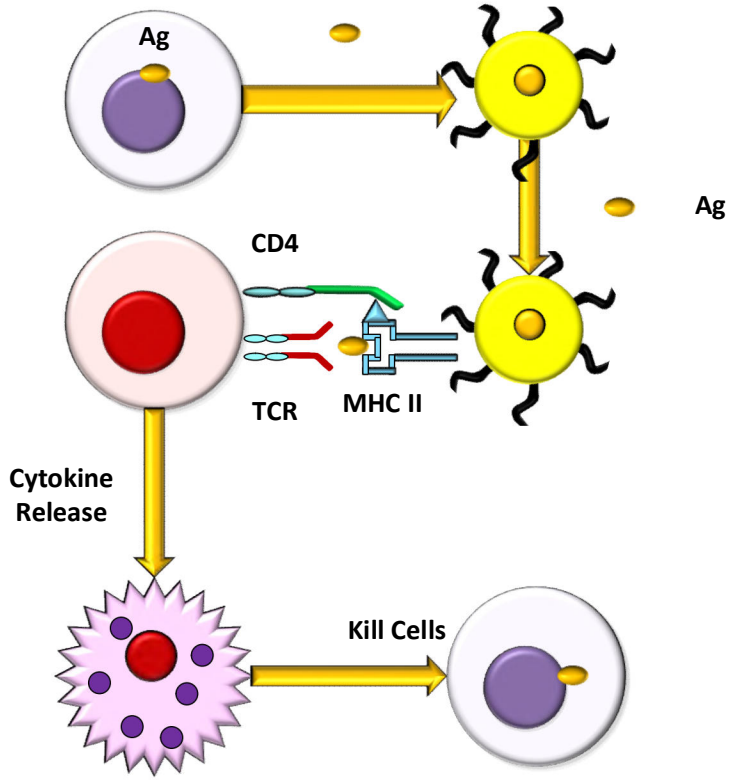


The CD8 cells get primed and then directly attach when seeing an Ag into the TCR. In contrast the CD4 cells assist (or "help") in the process, they effect the attack.

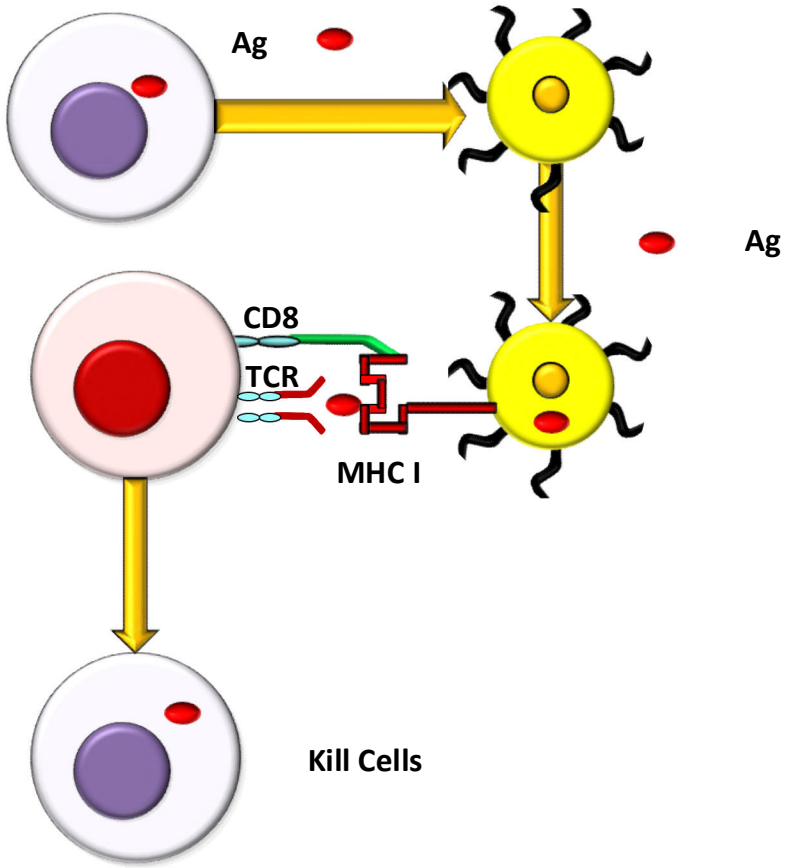
We demonstrate these two processes below. Note, the CD4 cells get primed via a dendritic cell and in turn the CD4 cell primes the macrophage.



The macrophage below does the killing of the Ag cells after it is primed by a CD4 cell. This macrophage is an M1 macrophage, an attacking macrophage. It is what one typically expects when Ags from foreign factors are present.



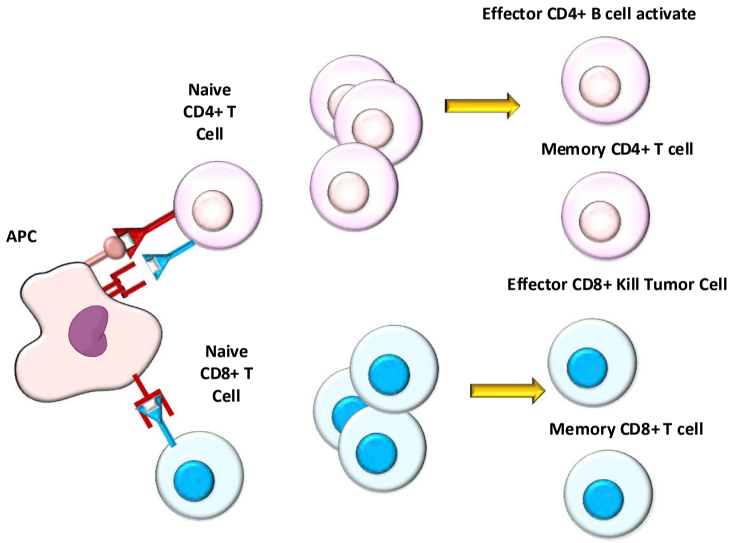
The CD8 system of attack is detailed below. It uses the CD8 cell directly and does not require a macrophage.



This is a highly simplistic approach. It is provided to reassert the role of a macrophage.

2.2 T Cell Receptors

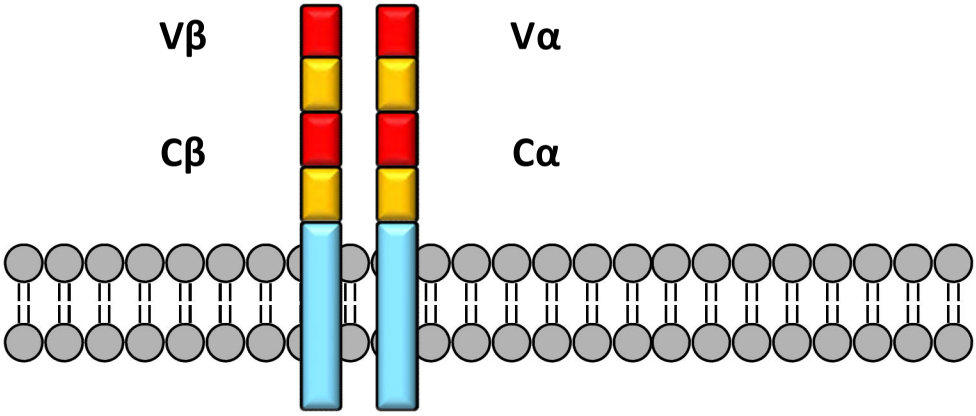
Let us now briefly discuss the T cell receptors. As shown below the TCR attaches to an MHC I or MHC II. It is matched to these proteins and MHC I is common on all nucleated cells (Note it is also known as HLA in humans),



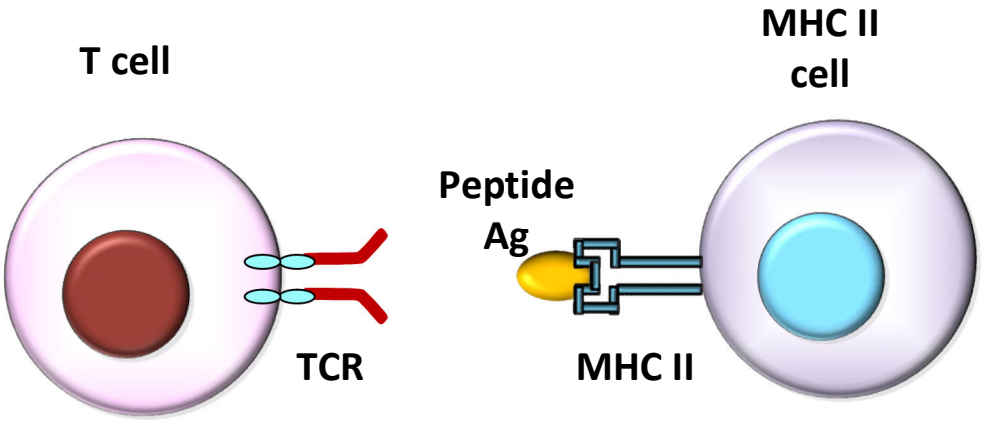
We now examine some details on these receptors, specifically the two well know types.

2.2.1 αβ Receptor

They first type is shown below with a variable and a constant domains. They are somewhat similar to MHC proteins for which the seek a bonding.



The bonding of a TCR and MHC II is demonstrated below with the MHC II presenting the AG to the CD4 T cell.



2.2.2 γδ Receptor

The second type of TCR is a γδ Receptor and Arias-Badia et al note:

γδ cells can possess both innate and adaptive pleiotropic functions that either enhance tumor progression or help to mediate tumor rejection, summarized in Fig. 1 and previously reviewed by others11,49–51 (Fig. 1). Tumor-infiltrating γδ populations have been known to contribute to anti-tumor immunity52–56 and can mediate strong anti-tumor responses via IFNγ and tumor necrosis factor (TNF) by cytolytic mechanisms. On the other hand, they can also possess immunosuppressive function.

For example, interleukin-17 (IL-17)-expressing γδ T cells, enacting the main γδ-mediated immunosuppressive pathway identified to date, can lead to the accumulation of myeloid-derived suppressor cells among many other functions. Tumor-infiltrating γδ T cells have also been described to suppress αβ T cell and dendritic cell function in a fashion reversible by engaging Toll-like receptor (TLR) signaling. Conversely, they have also been shown to function as professional antigen-presenting cells to αβ T cells in lymph nodes26,61 as well as to enable antibody production by B cells62 or to contribute to anti-tumor neutrophil infiltration.

Beyond IL-17+ γδ subsets, recent work has uncovered other markers for γδ T cell immunosuppression in IL-17− cells, such as promyelocytic leukemia zinc finger (PLZF) or amphiregulin (AREG) in tumor-infiltrating Vδ1 T cells in colorectal cancer. Tumor-infiltrating γδ T cells possessing a regulatory T (Treg) cell-like phenotype have also been associated with impaired dendritic cell and CD8+ T cell responses60,65 as well as with poor survival in breast66, pancreatic67 and colorectal cancers68 and multiple myeloma.

Treg cell-like γδ T cells expressing high levels of IL-10 and IL-17 were found to hamper immune surveillance in pancreatic ductal adenocarcinoma. An immunosuppressive role for IL-4-secreting γδ T cells has also been described in vitro.

The contribution of different γδ populations in anti-tumor immunity varies greatly between subsets and even tumor types. Vδ2+ T cells have been most studied in the context of antigen reactivity and have been shown to kill tumor cells upon antigen encounter46,. In addition, other subsets expressing alternative Vδ genes (Vδ2−) such as those encoding Vδ1, Vδ3 or Vδ5 have

also emerged as mediators of cytotoxic function in tumors52–. However, others questioned a therapeutic role for γδ cells in renal cancer by showing reduced tumor-infiltrating γδ T cell frequencies and lack of correlation with any of the clinicopathological features included in the study.

In the case of liver cancer, both Vδ2+ and Vδ1+ cells showing a CD69+CD49a+CD103+ tissue-resident memory phenotype have been recently associated with anti-tumoral activity. Overall, protumoral or anti-tumoral roles in γδ T cells cannot solely be predicted by subset or discrete marker expression. Ligand specificity and immune functional status, reviewed in the following sections, are therefore pivotal determinants of tumor-infiltrating γδ T cell fate. γδ ligands in cancer

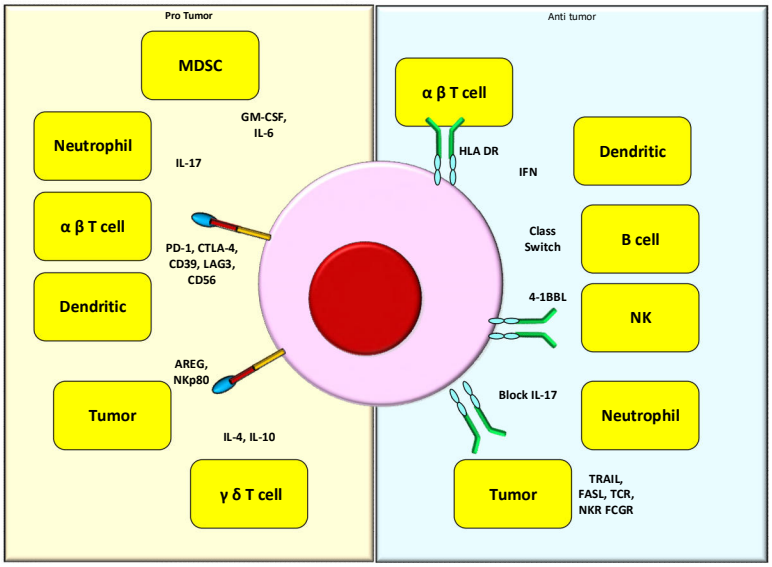
In contrast to αβ T cells, which rely on the recognition of processed peptides presented on MHCs, identified γδ ligands span a wide range of disparate molecules presented in different contexts, ranging from MHC class I-like proteins like CD1c or CD1d to co-stimulatory-like proteins like murine SKINT1 or the BTN protein family.

Vδ1 γδ T cells recognize multiple MHC-like proteins but also 'unexpected' self-ligands such as members of the heat shock protein family as well as other proteins such as MutS homolog 2 (MSH2) or other stress-related candidates. Vδ3 T cells can recognize CD1d-presented glycolipids and translocated annexin A2 in transformed cells as well as the MHC-like protein MR1 in an antibody-like recognition fashion, even though the latter has not been linked yet to tumoral tissue.

Vδ5 cells have been reported to recognize the endothelial protein C receptor (EPCR), also not yet linked to cancerous transformation. γδ T cells that are self-reactive toward specific MHC class I molecules have also been suggested.

2.3 PRO AND ANTI-TUMOR

The interfaces between pro and antitumor and the communications of these is shown below:



2.4 CD8 CELLS

CD8 cells are fundamentally killer cells. They attach to MHC I which is on all cells. Failure of CD8 cells present a significant failure to manage cancer development. As van der Leun et al note:

The T cell infiltrates that are formed in human cancers are a modifier of natural disease progression and also determine the probability of clinical response to cancer immunotherapies. Recent technological advances that allow the single-cell analysis of phenotypic and transcriptional states have revealed a vast heterogeneity of intratumoural T cell states, both within and between patients, and the observation of this heterogeneity makes it critical to understand the relationship between individual T cell states and therapy response.

This Review covers our current knowledge of the T cell states that are present in human tumours and the role that different T cell populations have been hypothesized to play within the tumour microenvironment, with a particular focus on CD8+ T cells.

The three key models that are discussed herein are as follows:

(ii) *the dysfunction of T cells in human cancer is associated with a change in T cell functionality rather than inactivity;*

(2) antigen recognition in the tumour microenvironment is an important driver of T cell dysfunctionality and the presence of dysfunctional T cells can hence be used as a proxy for the presence of a tumour-reactive T cell compartment;

(3) a less dysfunctional population of tumour-reactive T cells may be required to drive a durable response to T cell immune checkpoint blockade…

The simplest distinction between T cells is that of the CD4+ and CD8+ T cell subsets. The evidence for a role of the CD8+ T cell subset in tumour control is compelling, as for instance reflected by a series of prognostic analyses (listed in refs4,14), the association between pretreatment intratumoural CD8+ T cell numbers and response to PD1 blockade15, and the clinical activity of CD8+ T cell-enriched cell products in melanoma. These observations explain the focus of most of the recent single-cell analyses, and also this Review, on the CD8+ T cell compartment.

However, we feel that it is also important to briefly describe the cell states that are assumed by CD4+ T cells in the tumour microenvironment (TME), as CD4+ T cells have been shown to play a substantial role in tumour control in both preclinical models and patient case studies.

Furthermore, prior data already revealed that distinct CD4+ T cell subsets are associated with either good or poor clinical prognosis4, suggesting that a more granular analysis of CD4+ T cell states is likely to yield further information on the role of different intratumoural CD4+ T cell pools. A brief overview of the CD4+ T cell states that have been identified in human …

Circulating and lymph node-resident CD8+ T cells are classically subdivided according to their state of differentiation into I T cells, effector T cells and subsets of memory T cells. The development of high dimensional profiling techniques such as cytometry by time of flight (CyTOF) and single-cell rNA sequencing has allowed the field to go substantially beyond this relatively coarse profiling of CD8+ T cells based on the expression of just a few protein markers and has over the past few years been used to profile T cell infiltrates in human tumours.

In three independent melanoma cohorts, the major intratumoural T cell populations that were identified on the basis of transcriptional profiling using different single-cell RNA-sequencing platforms displayed strong resemblance across the studies.

In one study, 'I' CD8+ T cells, marked by expression of the genes CC chemokine receptor 7 (CCR7), transcription factor 7 (TCF7), lymphoid enhancer-binding factor 1 (LEF1) and SELL (encoding L-selectin), and 'cytotoxic' cells, expressing, among other genes, perforin 1 (PRF1), granzyme A (GZMA), GZMB and natural killer cell granule 7 (NKG7), were identified20 le S1).

Likewise, 'I-like' (marked by expression of, among other genes, CCR7, LEF1, interleukin-7 receptor (IL7R) and TCF7) and 'cytotoxic effector' (for instance characterized by expression of the genes CX 3C chemokine receptor 1 (CX3CR1), PRF1, killer cell lectin-like receptor subfamily G member 1 (KLRG1) and fibroblast growth factor-binding protein 2 (FGFBP2)) cell states were defined in a second study.

In a third cohort, CD8+ T cell states with similar characteristics were observed, but were named differently, identifying a 'memory' state with expression of CCR7, IL7R, LEF1 and TCF7 (matching the I(-like) cells observed in the other two studies) and a 'cytotoxic' state defined by expression of Fcγ receptor IIIA (FCGR3A), KLRG1, PRF1 and GZMB.

Combined protein and gene expression analyses may be required to clarify whether the first of these two populations is composed of true I CD8+ T cells, memory CD8+ T cells, a stem cell-like subset of memory cells or a mixture of these, as the transcriptional profiles of these subsets display many similarities.

In the absence of data that conclusively settle this issue, we will here refer to this population as 'I-like'. The presence of these I-like CD8+ T cells at tumour sites represents somewhat of a conundrum: while cytotoxic effector cells are known for their capacity to home to peripheral tissues, I and (stem cell-like) memory T cells typically circulate through blood and lymphoid organs. One hypothesis may be that intratumoural I-like cells reside in the intratumoural lymph node-like aggregates that are referred to as tertiary lymphoid structures (TLS), but more work to substantiate this is clearly required. …

While CD8+ T cells are considered major drivers of antitumour immunity, CD4+ T cells also play a prominent role in tumour control, either by promoting or inhibiting antitumour responses. For instance, conventional CD4+ T cells (Tconv cells) can promote tumour control through stimulation of, among other cells, CD8+ T cells, natural killer (NK) cells and a broad range of other innate immune cell types (reviewed in ref.75).

In addition to this function of facilitating antitumour immune responses, Tconv cells can exert cytotoxic functions that result in killing of human leukocyte antigen (HLA) class II expressing tumour cells or inhibit tumour growth through secretion of interferon-γ (IFNγ) and tumour necrosis factor. In addition to the Tconv cell pool, a T follicular helper (TFH) cell-like population of CD4+ T cells that is characterized by expression of B cell lymphoma 6 (BCL-6) and the capacity to produce high levels of CXC chemokine ligand 13 (CXCL13) has been identified in multiple human tumour types. Although the exact role of T FH cells in tumour immunity is unclear, these cells may contribute "o the generation of tertiary lymphoid structures (TLS) at the tumour site and thereby shape intratumoural CD8+ T cell and B cell responses,

By contrast, tumour-resident regulatory T (Treg) cells have been shown to counteract tumour-specific immune responses by suppressing the infiltration and antitumour activity of, among other cells, CD8+ T cells and macrophages.

In point of fact, Treg cells stop the anti-tumor processes. In some cases it modulates the aggressive CD8 and CD4 processes, however it may actually inhibit them.

Single-cell RNA-sequencing studies have described a variety of CD4+ T cell states, including dysfunctional CD4+ T cells, I-like or memory CD4+ T cells, cytotoxic effector CD4+ T cells, Treg cells and TFH cells,

Notably, unlike the major CD8+ T cell states, these CD4+ T cell states do not appear to be ubiquitously present in all tumour types.

Another interesting observation of single-cell sequencing as well as cytometry by time of flight (CyTOF) studies has been that Treg cells in the tumour express higher levels of tumour necrosis factor receptor superfamily member 9 (TNFRSF9; encoding 4-1BB), inducible T cell co-

stimulatory (ICOS) and cytotoxic T lymphocyte-associated antigen 4 (CTLA4) than Treg cells in blood or adjacent normal tissue, possibly reflective of an activated state. In addition, the intratumoural Treg cell pool displays substantial diversity, as shown, for example, by the variable expression levels of TNFRSF9.

Furthermore, in melanoma, both T reg cells and TFH cells displayed levels of proliferation that were comparable to those observed in dysfunctional CD8+ T cells.

By analogy with the dysfunctional CD8+ T cell pool, it may be hypothesized that this proliferative signature reflects a response of these cell pools to a local (antigen) signal and suggests that both Treg cells and TFH cells may play pivotal roles in the intratumoural CD4+ T cell response.

As Gebhardt et al note:

Memory CD8+ T cells are broadly categorized into phenotypically distinct subsets that display various functional traits, such as developmental plasticity, longevity, self-renewal, recirculation, or residency.

The main subsets commonly considered in studies of tumour-associated T cells are outlined below.

Central memory T cells Central memory T cells (TCM cells) are a long-lived, constantly recirculating and self-renewing population with heightened developmental potential and enhanced capacity for recall expansion upon restimulation.

The expression of lymph tissue homing molecules such as CD62L and C–C chemokine receptor type 7 (CCR7) directs their continuous recirculation through lymphoid tissues via the blood. Upon reactivation, inhibitor of DNA binding 3 (ID3)-positive C–X3–C motif chemokine receptor 1 (CX3CR1)-negative TCM cells can generate all major subsets of effector and memory T cells2.

Stem cell-like memory T cells (TSCM cells) are a closely related and overlapping subset thought to excel in typical TCM cell traits, but phenotypically (CD44loCD45RA+Sca-1hi) seem to be less differentiated than TCM cells and are overall less frequent*.*

Effector memory T cells Effector memory T cells (TEM cells) are a population of cells with the capacity to rapidly secrete pro-inflammatory cytokines, but only intermediate longevity and limited developmental plasticity compared with TCM cells. *TEM cells largely lack expression of CD62L and CCR7 but express chemokine and adhesion molecules that allow for rapid infiltration of inflamed tissues.*

CX3CR1hi TEM cells share recirculatory surveillance of peripheral tissues with some of the more recently identified CX3CR1int peripheral memory T cells (TPM cells).

In humans, TEM cells also encompass more terminally differentiated cells with heightened effector activity but limited proliferative potential, called TEMRA …

Tissue-resident memory T cells Tissue-resident memory T cells (TRM cells) are a long-lived, non-recirculating population with developmental plasticity that take up residence in a broad range of tissues.

All TRM cells lack tissue exit receptors and commonly express CD69, whereas those in epithelial tissues additionally express the integrin subunit CD103 (ITGAE). TRM cells are characterized by a unique transcriptional and epigenetic signature distinct from TEM cells or TCM cells. Instead, they have elevated expression of tissue-resident regulatory transcription factors such as ZNF683 (homologue of Blimp1 in T cell (HOBIT)), B lymphocyte-induced maturation protein 1 (BLIMP1) and runt-related transcription factor 3 (RUNX3), and concomitant repression of factors that promote tissue egress such as Krueppel-like factor 2 (KLF2). TRM cells are key mediators of tissue immunity and afford frontline defence in barrier tissues such as skin and mucosa.

They can also control localized reservoirs of persisting pathogens or cancer cells when the antigen burden is low or disease is either subclinical or relapse-remitting in nature. Notably, TRM cells can also express a range of molecules commonly associated with terminally exhausted T cells (TTEX cells) such as thymocyte selection-associated high mobility group box protein (TOX), PD1 and CD39 in an antigen-independent manner.

In situations where the antigen burden is both high and unwavering, the extensive overlap between exhausted T cell and TRM cell phenotypes may conflate classification of either subset. …

Chronic antigen stimulation is the major driver of the dysfunctional trajectory of tumour-reactive CD8+ T cells in the tumour microenvironment (TME).

Conversely, cessation of antigen stimulation or localization of tumour-reactive CD8+ T cells to anatomical niches outside the TME, such as remote lymph nodes, where little to no antigen is present, may prevent a fraction of cells from acquiring features of exhaustion. Supporting this notion, ostensibly canonical memory T cells are commonly found in lymphoid or non-lymphoid peripheral tissues in preclinical studies following tumour eradication by therapeutic interventions, including surgical tumour excision, depletion of regulatory CD4+ T cells, immunotherapies, and tumour vaccination…

Indeed, a recent study employing a range of transplantable and genetic tumour models has demonstrated that tumour-reactive CD8+ T cells that transcriptionally and functionally resemble memory T cells generated after acute infection form in tumour-draining lymph nodes, where these cells serve as upstream precursors for TPEX cells and more terminally exhausted cells in the TME. Importantly, a similar population of tumour-reactive memory T cells have also been detected in hepatic lymph nodes from patients with liver cancer. Moreover, putative melanoma-reactive memory T cells have been shown to persist in patients for up to a decade following successful checkpoint blockade therapy.

Similarly, cancer-specific CD8+ T cells were detected in the blood of patients with lung cancer at least 3 years after therapy. Such examples illustrate that a proportion of CD8+ T cells responding to tumour-derived antigens can become long-lived memory T cells.

3 M1 AND M2 FUNCTIONS

Macrophages differentiate into several classes and subclasses. M1 macrophages can attack cancer cells whereas M2 types can protect and enhance them. M0 cells are those yet to be differentiated. This is a current reflection of the immune system. We must always be reminded that as one learns more and has more sophisticated tools to ascertain cells types that this systematics of macrophages may be altered significantly. But we start here and progress.

As Quaranta and Schmid note:

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

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

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

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

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

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

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

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

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

***Patrolling monocytes monitor the microvasculature under steady-state** conditions and rarely extravasate into tissue. However, they can rapidly accumulate in lung metastatic tissue and*

inhibit cancer cell seeding and growth by exerting anti-tumour functions through recruitment of NKs. Macrophages are a population of heterogeneous and plastic cells.

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

The phenotypic macrophage is a result of what it sees in the region in which it resides. It is a pleiomorphic cell. From Ruffell and Coussens:

Macrophages produce an array of cytokines, chemokines, polypeptide growth factors, hormones, matrix-remodeling proteases, and metabolites, many of which possess tumor-promoting activities.

Of course this will depend on how it is activated. They continue:

A caveat to some of these reported activities is that many findings originate from cell culture studies utilizing neoplastic myeloid cell lines or bone marrow-derived macrophages and, therefore, cannot account for the complex milieu of polarization signals to which macrophages would be exposed in vivo.

This includes the aforementioned CSF-1 and CCL2, prostaglandin E2 (PGE2), and damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 protein (HMGB1), extracellular ATP, and degraded extracellular matrix components …

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

VEGF is a powerful agonist for vascular expansion. This expansion is a critical factor for tumor growth.

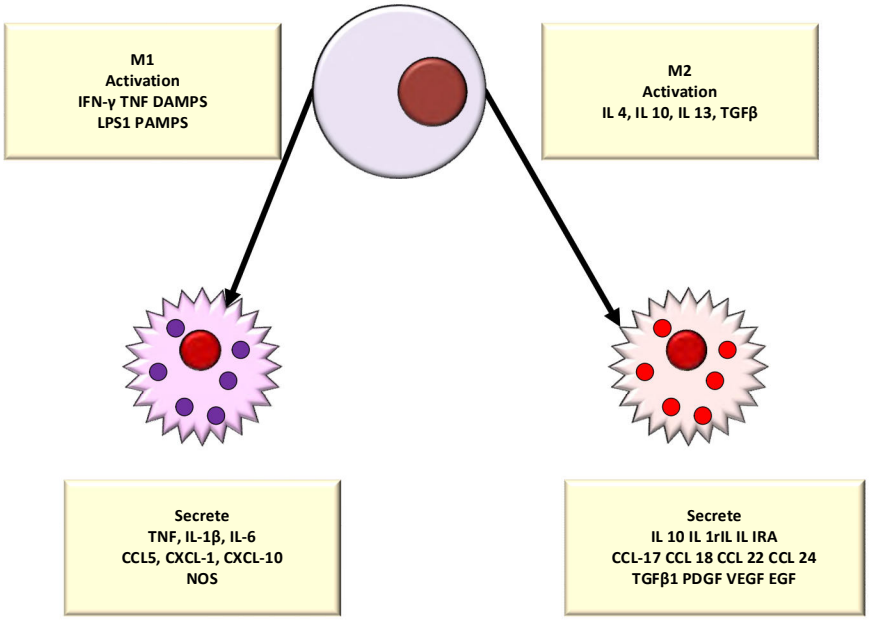
VEGF antagonists induce vascular normalization, and several studies have reported increased uptake of chemotherapeutics associated with this process, likely because of reduced vessel leakiness and interstitial fluid pressure.

Although macrophages are not necessarily a dominant source of VEGF-A in all tumor tissues, specific deletion of VEGF-A in macrophages via lysozyme Mpromoter-driven Cre recombinase revealed their role in driving abnormal vascular phenotypes in tumors.

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

3.1 MACROPHAGE INTERACTIONS

Macrophages can sustain multiple phenotypes. These phenotypes can be switched back and forth as we show in the graphic below. This Figure depicts the activation and the response, or secretion, states for macrophages. M1 respond to what one would suspect are infection states whereas M2 are often driven as adjuncts in support of tumors. However, in some sense the states of macrophages may be a balancing act in non-malignant states.

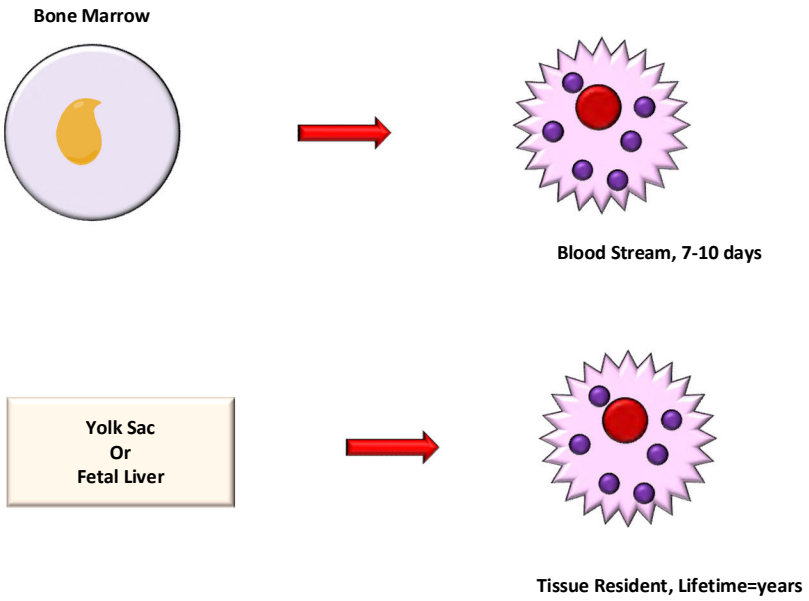


The above shows what each of the phenotypes produces, pro and ant tumors factors. I t also shows what activates an M0 phenotype transition to one of the other phenotypes.

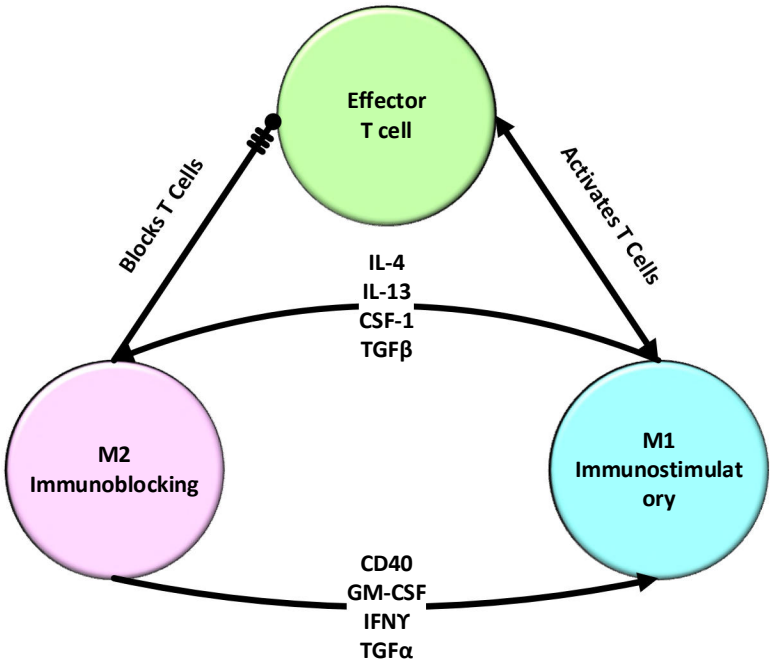
3.2 MACROPHAGE DEVELOPMENT

Macrophages arise from two sources; fetal organs and bone marrow. The fetal organ macrophages become resident in organs and manage to reproduce and proliferate there. The bone marrow cells start are monocytes and enter the blood stream, then they leave the blood stream where needed and become macrophages.

We demonstrate the concept below;



As we moted previously, macrophages get activated by T cells which are in turn activated by dendritic cells. The net result is that the macrophages have been brought into proximity of the Ag detected and then attack the Ag possessing entity. The graphic below also shows the ability to move between phenotypes which may be a useful therapeutic targeting.



3.3 MACROPHAGE PHENOTYPES

We now examine the macrophage phenotypes. The complexity is drive by what specific interactions polarize an M0 to one of the other states and in turn what that state functions as. As Basak et al note:

Multiple macrophage phenotypes have been identified so far based on diverse surface receptor expressions, secretory patterns, and activities.

The multiple unique markers that macrophages express on their membranes result in a range of phenotypes dependent on TME signals, leading to a high degree of plasticity.

While TAMs are closely associated with the tumor, cancer associated macrophage-like (CAML) cells are disseminated tumor cells found in peripheral blood.

While differentiated macrophages are a rare phenomenon as a circulating population of cells, CAML is defined as macrophages with phagocytosed tumor fraction, has been studied in breast, pancreatic, and prostate cancer, and may serve as a biomarker for these cancers. CAMLs are more commonly found in people with advanced malignancies than in those with benign tumors. Surprisingly, the number of CAMLs increases following chemotherapy, which may be related to the efficiency of the treatment because increased phagocytosing macrophages are proportionate to dying cancer cells.

… on the other hand, used data from a phase 2 clinical trial (NCT02525757) to show that the existence of giant CAMLs was associated with metastases and poor survival despite immunotherapy and chemoradiation.

Furthermore, patients with programmed cell death ligand 1 (PDL1) expressing CAML when treated with immunological checkpoint inhibitors (ICIs) had significantly greater overall survival than those who were not treated with ICIs in metastatic lung cancer.

The above observation is significant. As we noted earlier, immunotherapy works on a fraction of patients. The specific details of what inhibits such therapy is still be determined but one suspects that macrophages can and do play a major role.

In general, TAMs are classified as either traditionally activated anti-tumor M1 or alternatively activated pro-tumor M2 phenotypes.

According to in vivo wound healing studies, the early stages of wound healing are characterized by the activation of proinflammatory M1-like macrophages, which gradually give way to an anti-inflammatory M2-like macrophage phenotype. The macrophage colony-stimulating factor-1 receptor (CSF1R) is the primary lineage regulator of virtually all macrophages, regardless of origin. This class III transmembrane tyrosine kinase receptor is expressed by the majority of mononuclear phagocytic cells. IFNg and IL1b, secreted by Th1 cells and bacterial lipopolysaccharide (LPS), drive macrophages to polarize towards the M1 phenotype, whereas IL4 and IL13, secreted by Th2 cells, cause the M2 phenotype to be dichotomized.

Macrophages in the M1 end of the continuum have a proinflammatory phenotype and express the surface markers CD86 and CD64 *; MHC-II and macrophage receptor with collagenous structure (MARCO) ; nitric oxide synthase-2 (NOS2) and suppressor of cytokine signaling-1 (SOCS1); and pro-inflammatory cytokines (IL6, IL12, IL1b, and TNFa) and chemokine ligands (CCL2, CCL5, CXCL9, CXCL10, and CXCL11).*

All these indicators show their strong phagocytic and cytotoxic power, ability to draw T and B cells to the infection site, and prodigious ability to deliver antigens.

In contrast, pro-tumor M2 macrophages with surface markers CD36, CD206, and CD163 are immunosuppressive and anti-inflammatory, helping with tissue repair, angiogenesis, and phagocytosis to reduce and "clean up" after inflammation.

They are also Th2 activators and Th1 inhibitors (25, 35–37). M2-like macrophages are typically characterized by their poor ability to present antigens, having low IL12 and high IL10, IL4, and IL13 secretory profiles. M2 macrophages also express/secrete transforming growth factor-beta (TGFb), peroxisome proliferatoractivated receptor-gamma (PPARg), CCL14, CCL22, and arginase-1 (ARG-1).

M2-like macrophages are more functionally diverse than M1- like macrophages because they have many subtypes (M2a, M2b, M2c, and M2d), each with a distinct combination of cytokine and chemokine profiles.

1. ***M2a macrophages express higher levels of IL10, TGFb, and the chemokines CCL17, CCL18, CCL22, and CCL24, all of which are linked to Th2-polarized allergic inflammation. IL4 and/or IL13 stimulate the production of M2a macrophages.***

It is interesting to see the IL-4 and IL-13 as drivers. This pair is also frequent in such things as asthma and pruritis.

2. ***Immune complexes (ICs), LPS, Toll-like receptors (TLRs), or the IL1 receptor antagonist (IL1ra), on the other hand, sustains M2b macrophages, which are characterized by the production of TNFa, IL1b, IL6, IL10, and CCL1.***

3. ***A TGFb-, glucocorticoid (GC)-, prostaglandin E2-, and IL10-rich environment induces M2c macrophages, which continue to express IL10 and TGFb; thus, they are crucial regulators for inflammation resolution and tissue healing.***

4. ***Finally, M2d macrophages have been shown to contribute to <u>angiogenesis</u> by expressing vascular endothelial growth factor (VEGF) and IL10 when stimulated by TLR, adenosine A2A receptor ligands, and IL6.***

Angiogenesis via VEGF expression is an essential target for a therapeutic. However local VEGF production may or may not be the limiting factor. The authors note below that M2a also releases VEGF thus questioning a specific target.

In the context of cancer, these pro-tumor M2 macrophage subsets share the function of tumor development and immune response suppression via multiple pathways.

For example, VEGF and CCL18, which are released by M2a macrophages, promote breast cancer cell motility and angiogenesis. Furthermore, M2a macrophages contribute to the development of lung cancer cells via the IL4/STAT6 signaling pathway. STAT6- expressing M2a macrophages are required for tumor cell growth. IL4, which is released by both tumor cells and M2a macrophages, promotes more macrophages to polarize to the M2a phenotype, resulting in a positive feedback loop.

M2b macrophages proliferate and replace M1 macrophages as hepatocellular carcinoma (HCC) advances.

These cells release CCL1 in order to attract Th2 and Treg cells that express CCR8, thereby promoting a pro-tumorigenic environment. The CCL1/CCR8 signaling mechanism also enhances tumor cell motility, proliferation, and metastasis. ***Furthermore, M2b macrophages express higher levels of indoleamine 2,3-dioxygenase (IDO), IL10, and IL6, all of which are immunosuppressive factors.***

M2b macrophage-secreted IL10 promotes Treg cell differentiation from naive T cells , whereas secreted IL6 activates Th2 cells, which promote tumor progression*.*

In breast cancer patients, the percentage of circulating M2c macrophages is associated with a poor prognosis. *Yuan et al. demonstrated that M2c macrophages promote lung tumor growth. Kim et al. revealed evidence that IL10-induced M2c macrophages promote tumor development in mouse melanoma models. Furthermore, both in vitro and in vivo,* ***M2c macrophages enhanced endothelial cell mobility and tube formation, implying that M2c may boost tumor growth through increased angiogenesis****.*

M2d macrophages in gastric cancer release a number of pro-tumorigenic molecules, including IL10 and TGFb, to promote cancer cell proliferation and migration. M2d macrophages release VEGF and matrix metalloproteinase 9 (MMP9), which promote ECM breakdown, angiogenesis, and metastasis. *They also produce IL6 in various cancer. The canonical IL6/JAK/ STAT3 pathway is related to survival, angiogenesis, metastasis, proliferation, and drug resistance. M2d macrophages also express immunosuppressive factors such as IDO, IL10, and PDL1.*

The above provide some recent details on drivers and functions. The collection of M2 phenotypes create a tumor supportive environment. As Wang et al note:

The M1-like TAMs and M2-like TAMs are located at the two ends of the continuous dynamic TAM polarization axis, each possessing unique cell surface markers and functional factors, and play different roles in the TME (Fig. 2). The M1-like TAMs have pro-inflammatory properties. M1-like macrophages are stimulated by cytokines like interferon (IFN), colony stimulating factor (CSF), tumor necrosis factor (TNF).

Furthermore, lipopolysaccharides(LPS) have the capacity to interact with and engage the toll like receptor (TLR)4 located on the macrophage surface and promote M1- like macrophage polarization by acting on nuclear factor kappa-B (NF-κB) and interferon regulatory factor 3 (IRF3). Strongly presenting antigens and secreting many pro-inflammatory cytokines are two traits of M1-like macrophages.

M1-like macrophages secrete an extensive number of costimulatory molecules like cluster of differentiation (CD)86, CD60, and CD80, along with pro-inflammatory biomarkers including TNF-α, IL-1β, IL-6, IL-12, IL-23. They also highly express major histocompatibility complex (MHC) II molecules.

Notably, they do express IL-10, although at a lower level. M1-like macrophages release matrix metalloproteinases (MMPs) including MMP1, MMP2, MMP7, MMP9, and MMP. These enzymes are specialized in breaking down extracellular matrix (ECM) constituents.

M1-like macrophages generate chemokines like CCL2, CCL3 CCL5, C-X-C motif chemokine ligand (CXCL) 8, CXCL9, CXCL10, CXCL11, CXCL. Also, they release IFN-γ, inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS).

M1-like macrophages activate a strong T-helper1(Th1)-type immune response by releasing these inflammatory mediators to promote an inflammatory response that inhibits cell proliferation and kills pathogens and tumor cells in the human body, thereby exerting antitumor effects.

The M2-like TAMs, which present antigens poorly, are anti-inflammatory, unlike the M1 phenotype. There is no specific procedure that must be used to initiate the activation of M2-like macrophages.

The primary activation of M2-like macrophages is caused by triggering cytokines, which include transforming growth factor (TGF)-β, IL-4, IL-13, IL-10, and macrophage colony-stimulating factor (M-CSF)22,.

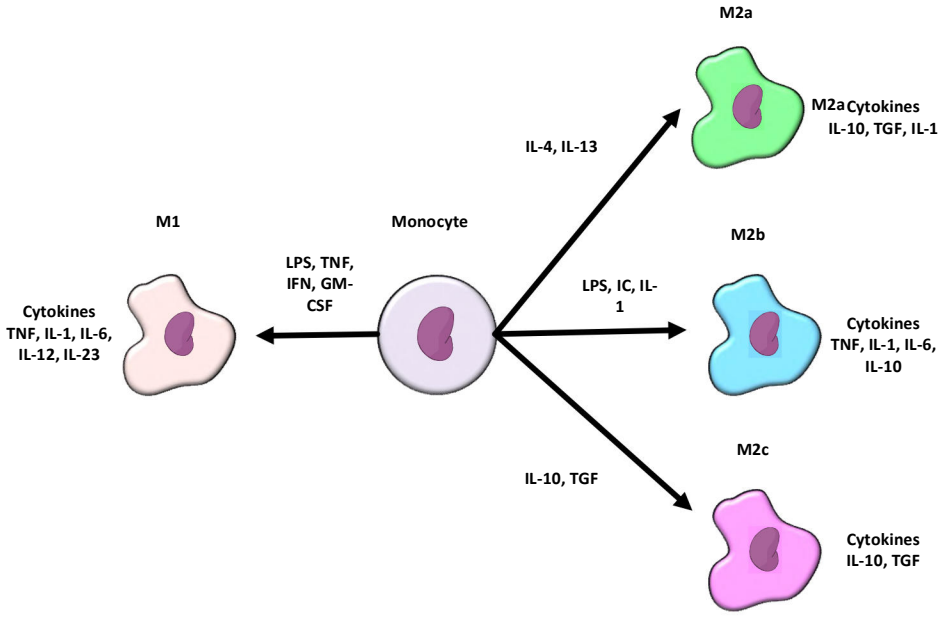
The input and output characteristics can be summarized as follows:

M2-like macrophages highly express CD163, CD206, CD200R, CD209, CD301 and chemokines like CCL1, CCL17, CCL18, CCL22, and CCL.

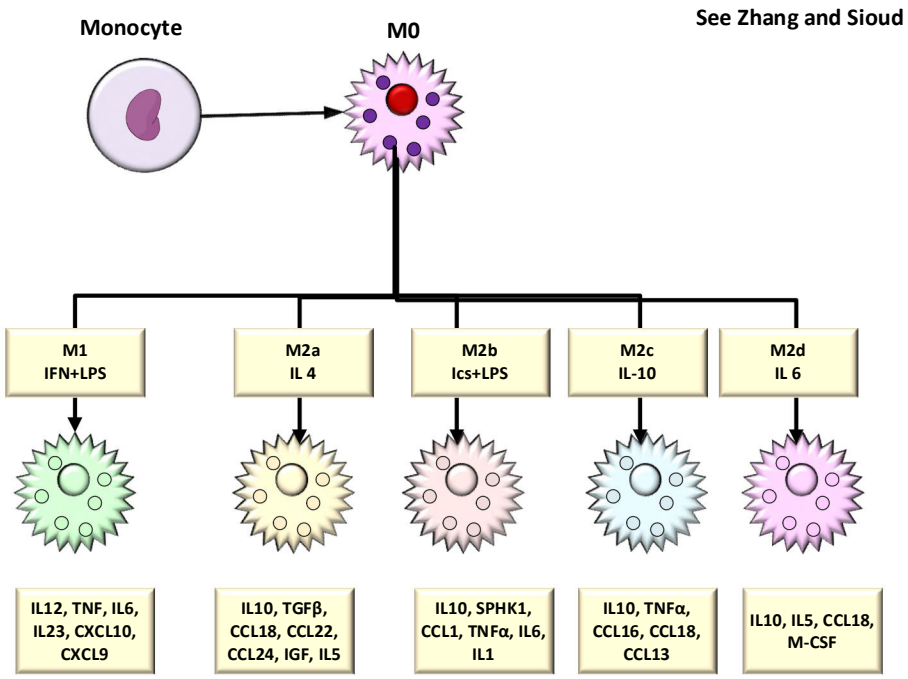
They release numerous anti-inflammatory factors, including TGF-β, IL-4, IL-13, IL-10, and IL- 1RA.

Additionally, M2-like TAMs express the inflammatory cytokines IL-6, IL-12, IL-23 and TNF-α at lower levels. M2-like macrophages highly express MMPs and autocrine ECM components such as fibronectin, betaigh3 (BIG-H3), ECM cross-linking enzymes, transglutaminase and bone bridging proteins25, thus participating in cell adhesion. M2-like macrophages promote Arginase (Arg)−1 and VEGF expression, which are involved in the biosynthesis of proline and polyamines. Proline promotes the construction of ECM, and polyamines are involved in cell proliferation.

Other factors secreted by M2-like macrophages that promote cell proliferation, such as platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF), are involved in angiogenesis. M2-like macrophages release immunosuppressive chemicals to block the Th1-type immune activity as well as boost the Th2-type immune activity. This activity reduces the control over inflammatory reactions while promoting tumor cell growth, drug resistance, angiogenesis, and tissue healing.



We follow Zhang and Sioud in the descriptions. Graphically they summarize the macrophage phenotypes as in the graphic follows:



3.3.1 M1

We first begin with further details on M1 macrophages. From Basak et al:

M1-like macrophages perform their anti-cancer function by first efficiently distinguishing cancer cells from surrounding healthy cells by recognizing altered or cancer-specific antigens.

Macrophages recognize altered carbohydrate structures (or glycosylation) that cancer cells occasionally present on their cell surfaces. Some tumor antigens, such as carcinoembryonic antigen and Tn antigen, are glycosylated molecules that lectin-like receptors on macrophage cell membranes recognize.

As they note, M1 are cancer cell killers. The notes below show how an effective infiltration by M1 macrophages can be highly anti-tumor. Yet as we noted, the changes could result in M1 becoming M2 phenotype and thus tumor supportive!

Secondly, M1 eventually kills cancer cells via directly inducing cytotoxicity, phagocytosis, and antibody dependent cell-mediated cytotoxicity (ADCC). The first step is somewhat slow, lasting 1 to 3 days and involving several stages such as the formation of ROS (reactive oxygen species) and RNS (reactive nitrogen species), as well as the production of IL1 and TNF to destroy cancer cells.

Activated anti-tumor M1 macrophages target tumor cells by generating ROS and nitric oxide (NO), causing DNA damage, cytotoxicity, and apoptosis.

M1 macrophages sustain themselves as well as induce NK cell and cytotoxic T-cell infiltration and activation in the tumor site by secreting substantial amounts of pro-inflammatory

cytokines IFNg and IL12 with anti-tumor activity, indicating an indirect mechanism of inhibiting cancer progression.

Recognizing cancer cells and effectively phagocytosing them, on the other hand, suggests their ability as innate immune effectors, which later cross-primes adaptive immune response by presenting antigens on their surface. M1 uses ADCC as an adaptive response, employing anti-tumor antibodies for opsonization, which is a quicker process. After adhering to the Fc region of the antibodies, macrophages can phagocytose cancer cells coated with antibodies. To avoid M1-mediated elimination, tumor cells downregulate recognition molecules and activate counter-signals, such as PD1 activation in TAMs

3.3.2 M2a

We now move on to some further details on the M2 class. From Zhang and Sioud:

The M2a macrophages historically represent the most widely studied M2 subset.

These macrophages were first described in 1992 by a study demonstrating that IL-4 stimulated murine peritoneal macrophages had enhanced surface expression of CD206 with increased functional activity.

Apart from IL-4, IL-13 is another cytokine that can polarize macrophages toward the M2a phenotype, which is characterized by the high expression of cell surface markers CD206, CD209, and Dectin-1.

The expression levels of CD14, CD163, and CD80/86 vary from low to medium. M2a cells produce IL-10, CCL17, CCL18, CCL22, and the amino-acid catabolizing enzyme Arg1. These phenotypic markers and intracellular factors define the functional activities of M2a. As above-mentioned, M2a macrophages express certain ***pattern recognition receptors (PRRs)*** *such as* ***CD206, CD209, and Dectin-1****, thus facilitating the sensing and elimination of invading bacteria, fungi, and parasites.*

The binding of pathogen-derived carbohydrates to PRRs triggers scavenging activities and activates downstream signaling cascades, leading to IL-10 production. Secreted IL-10 inhibits pro-inflammatory IL-12 production as well as the expression of co-stimulatory molecules CD80/86, which in turn renders M2a macrophages poor inducers of T cell activation and proliferation.

Tissue remodeling is another prominent feature of M2a.

In response to tissue damage, IL-4 is released and induces macrophages to differentiate toward M2a, resulting in the production of various cellular products that play major roles in matrix reorganization.

For instance, L-ornithine, one of the metabolites generated by Arg1, is a precursor of collagens and polyamines, which are major components of the extracellular matrix (ECM). Certain chitinase-like substances produced by M2a also function in matrix reorganization.

It has been reported that M2 but not M1-polarized macrophages expressed high levels of fibronectin, which plays an important role in tissue repair and cell motility.

Hence, fibronectin has been proposed as a potential biomarker for M2 macrophages. Fibronectin also induced M2 polarization. Indeed, Zhou et al. found that fibronectin-1 secreted from human head and neck squamous cell carcinoma, co-cultured with THP-1 macrophages, enhanced M2 polarization. Fibronectin also supports tumor progression by promoting tumor cell proliferation, invasion, and migration.

Fibroblasts are also elements of the TME and can be a highly protective barrier against therapeutics including immunotherapy. Again, targeting these may help. As we examine macrophages we see again and again multiple targets. In fact, those targets may be highly personalized to the specific patient. This presents a massive challenge in diagnosis and treatment.

Considering its detrimental roles in cancers, fibronectin is now recognized as a potential target for cancer therapy.

3.3.3 M2b

We now consider M2b from the above reference.

In 2002, Charles F. et al. found that the addition of TLR agonists (LPS) and immune complexes (ICs, antibody/antigen complexes) could convert macrophage phenotype from M1 to M2 by downregulating IL-12 production and upregulating IL-10.

This novel M2 subset was later termed M2b, and thus, LPS plus ICs are now considered as the classical inducers of M2b. This macrophage subset has several characteristics that make it distinct from the other M2 subsets.

First, the crosslinking of the Fcγ receptor (FcγR) on M2b macrophages induces high levels of anti-inflammatory IL-10 and low levels of IL-12 [17,21,53–56].

Second, M2b macrophages can bias Th1-cell responses toward Th2-cell responses, predominantly through IL-4 secretion [34,53,54]. Hence, M2b macrophages are also named regulatory macrophages.

Third, the polarization toward M2b macrophages require two stimuli, leading to the activation of several signaling events involving NFκB, PI3K/Akt, IRFs, and MAPKs [34,53,57,58] (Figure 2). Although the underlying mechanisms leading to cell polarization remain unclear, they resemble those induced by IC vaccines.

In vitro preformed IgG/antigen complexes as well as those formed following antibody therapy in vivo are multifaceted immune regulators. A landmark turning point for IC's immune regulatory functions was the discovery of FcγRs. Some of these receptors transmit activating signals via an immunoreceptor tyrosine-based activation motif (ITAM) on the associated common γ chain. Upon IC binding to these receptors, the ITAM/syk interaction is the primary signaling axis in phagocyte activation, leading to the activation of the PI3K, PLC, MAPK, and NF-κB signaling pathways. In the case of macrophage polarization, the binding of the ICs to one of the Fcγ receptors will not fully induce macrophage polarization on its own.

The cell needs to receive a second signal such as the binding of LPS and IL-1 to their respective receptors. It is only when these two stimuli coexist that the M1 phenotype can reprogram to M2b. Of note, M2b cells, but not other M2 subsets, produce high levels of CCL1.

The released CCL1 is essential in maintaining the M2b properties as its inhibition led to the conversion of M2b to M0 or M1 macrophages.

Other factors are proposed as good markers in discriminating M2b, but their use remains controversial. LIGHT (also known as CD258 or TNFSF14) is a secreted protein and can compete with the herpes simplex virus for cell-binding, thus inhibiting viral entry into the cells. LIGHT has been shown to be exclusively upregulated in M2b macrophages in comparison to other macrophage subsets such as M1 and M2a.

In contrast, Wang et al. showed that M2b derived from human blood monocytes primed by LPS plus IL-1β only expressed very low levels of LIGHT. Sphingosine kinase 1 (SPHK1) was also found to be solely upregulated in M2b , supporting its use as a marker. However the use of SPHK1 is controversial as the expression of SPHK1 is not restricted to M2b. For example, SPHK1 was found to be expressed at high levels in the M1 compared to M2 macrophages. In addition, its expression was observed in the M2c subset. CD86 as well as TNF-α were also claimed to be good markers to discriminate M2b from the other M2 subsets.

However, CD86 was also reported to be expressed at higher levels in human M2a in comparison to other M2 subsets. The L-arginine metabolism pathway has also been proposed as a way to discriminate between different M2 subsets. Murine M2b macrophages have been reported to produce high levels of NO, a metabolite of the iNOS pathway. In contrast, Ito et al. found neither iNOS nor Arg1 RNA expression in ICs and LPS primed murine macrophages.

In line with this finding, TAMs isolated from intermediate-stage hepatocellular carcinoma (HCC) patients were identified as M2b macrophages, but barely any iNOS mRNA expression was observed.

3.3.4 M2c

We now proceed to details on the third phenotype, M2c.

M2c are macrophages stimulated by IL-10, TGF-β, or glucocorticoids.

In comparison to other macrophage subsets, M2c macrophages express high levels of the cell surface markers CD163, Mer tyrosine kinase (MerTK), and Tie2 as well as low to medium levels of CD14, CD86, CD16, and CD206.

The expression levels of the surface markers described above depend on the culture conditions, especially the concentrations and type of stimuli (IL-10, TGF-β, or glucocorticoids), culture medium components (with or without serum), cell origins (murine or human), and cell types (primary cells or cell lines). Usually, M2c macrophages are characterized by the secretion of the pro-inflammatory cytokines IL-10 and TGF-β as well as the chemokines CCL16, CCL18, and CXCL13. In terms of L-arginine metabolism.

M2c macrophages share an identical metabolic state with M2a and produce Arg1. IL-10 is produced by virtually all types of leukocytes such as macrophages, T, and B cells. The IL-10 receptor (IL-10R) is a tetramer consisting of two IL-10R1 and two IL-10R2 subunits. As shown in Figure 3, IL-10R1 functions as a ligand binding site and associates with JAK1, whereas IL-10R2 acts as a signal transduction subunit and interacts with TYK2. Binding of IL-10 to IL-10R induces receptor auto-phosphorylation and subsequent recruitment of the downstream transcription factor STAT3.

Nuclear translocation of dimerized STAT3 activates the transcription of anti-inflammatory genes and downregulates the expression of pro-inflammatory cytokines [31,72–75]. IL-10 prevents monocytes from differentiating to dendritic cells (DCs) and skews macrophage polarization. Upon IL-10 exposure, macrophages upregulate the expression of an increasing number of genes associated with anti-inflammatory activities in a time-dependent manner. In the meantime, the ability to respond to M1 stimuli (LPS and IFN-U) is gradually lost through the inhibition of STAT1 and NF-κB, resulting in suppressed M1 activation.

As a consequence, M2c macrophages are also called <u>acquired deactivation macrophages</u> because they lose their ability for M1 polarization.

Of note, it is possible to revert M2c macrophages to M1 if STAT3-mediated signaling is blocked. Anderson et al. showed that human M2c macrophages were converted to M1 and exhibited an increased expression of pro-inflammatory cytokines upon delivering corosolic acid-loaded liposomes into the cells through CD163-mediated endocytosis.

Corosolic acid is a STAT3 inhibitor and the delivery of corosolic acid-loaded liposomes into macrophages prevents M2c polarization while favoring M1 polarization. Apart from STAT3-mediated signaling, lines of evidence suggest that IL-10 also signals through the PI3K/Akt and MAPK pathways. Signaling through these pathways results in the activation of a series of genes associated with anti-inflammation, matrix remodeling, angiogenesis, and phagocytosis. It should be noted that immunoregulation is a prominent feature of M2c macrophages. To regulate immune responses, M2c suppresses M1-favored inflammation though several mechanisms.

First, they deprive macrophages of their ability to polarize into M1 and produce pro-inflammatory cytokines, as described above.

Second, they capture and sequester inflammatory chemokines. The expression of certain inflammatory chemokine receptors such as CCR2 and CCR5 are upregulated in IL-10-primed M2c macrophages. Interestingly, these inflammatory chemokine receptors displayed on the cell surface function as decoy receptors as they cannot transmit signals. Instead, they capture inflammatory chemokines and initiate scavenging events.

Third, M2c cells are efficient in eliminating apoptotic cells via MerTK-mediated phagocytosis. Clearance of apoptotic cells not only leads to the release of anti-inflammatory cytokines IL-10 and TGF-β from macrophages, but substantial evidence suggests that the apoptotic cells themselves can also trigger antiinflammation responses. Moreover, M2c macrophages produce GAS6, which is the ligand for MerTK …

Emerging evidence implicates M2c macrophages in supporting tumor progression. For example, in patients with breast, it has been demonstrated that the percentage of circulating M2c macrophages is correlated with disease severity.

In line with this, Yuan et al. demonstrated that M2c macrophages promoted xenograft lung tumor growth in vivo and induced tumor cell invasion in vitro. Kim et al. provided indirect evidence showing that M2c promoted tumor growth in mice bearing melanoma or lymphoma. Pellino-1 protein was originally identified as a ubiquitin ligase that plays a role in regulating TLR signaling by acting as a scaffolding protein. In their study, they found that pellino-1 regulates STAT3 activation via enhancing STAT1 signaling, thereby damping IL-10-induced M2c macrophage polarization while favoring M1 polarization in mouse BMDMs. Consistent

3.3.5 M2d

Finally, the fourth variant M2d:

In relation to cancer, ***M2d macrophages promote tumor progression through two main mechanisms****.*

First, M2d cells can produce pro-tumoral factors.

For example, M2d macrophages were able ***to promote cancer cell proliferation and migration by secreting IL-10 and TGF-β in gastric cancer.***

Additionally, VEGF and MMP9, produced by M2d, are expected to induce angiogenesis and degradation of the extracellular matrix, facilitating tumor metastasis.

As described above, M2d cells are a source of IL-6 that is implicated in various tumor types such as ovarian cancer , breast cancer, gastric cancer , colorectal cancer as well as hepatocellular cancer. The IL-6/JAK/STAT3 canonical pathway regulates the expression of several genes linked to anti-apoptosis, angiogenesis, metastasis, proliferation, and drug resistance.

It is therefore not surprising that the IL- 6-STAT3 pathway is now considered as a major target for the treatment of cancer. M2d can also dampen normal immune responses, supporting the

tumor cell evasion from immune surveillance. M2d is known to express several molecules related to immune suppression such as the IDO, IL10, Siglec 15, and PD-1 ligands. All M2 subsets are poor in stimulating T cells. In conclusion, the different M2 macrophage subsets have in common the effects of tumor promotion and the suppression of effective adaptive immune responses.

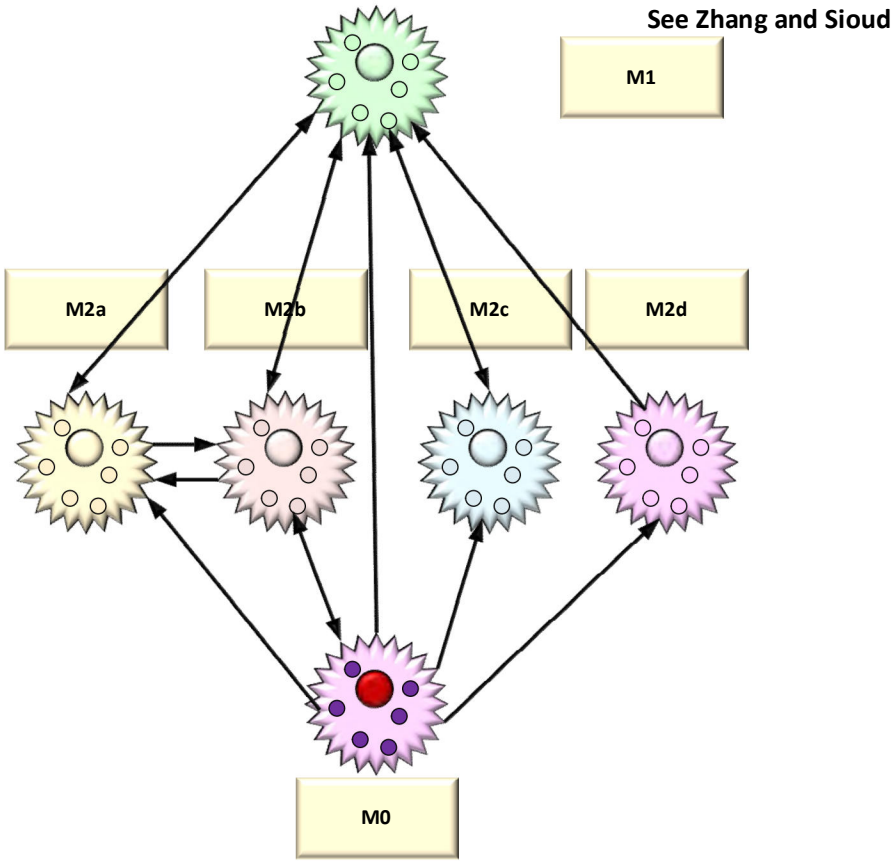
3.4 REPROGRAMMING

Macrophage phenotypes possess a level of plasticity. Namely they can be reprogrammed. From Zhang and Sioud:

Phenotypically and functionally distinct macrophage subsets co-exist in the TME.

Macrophages change their phenotypic and functional activities according to the stimuli they receive from the TME. This property can thus be exploited to reprogram tumor supportive M2 macrophages into anti-tumoral M1 macrophages. As indicated above, each M2 subset is polarized by certain stimuli that trigger particular cell signaling pathways.

Therefore, M2 may be reverted to M1 macrophages by interfering with the activities of these stimuli or by inhibiting particular cell signaling pathways. The known conversions between macrophage subsets are summarized in Figure 6 and Table 3. As depicted, macrophages can convert between different M2 subsets or revert to M1 macrophages under certain conditions. For instance, interfering STAT6-mediated signaling can be used to reprogram M2a back to M1.



3.5 SUMMARY

As Strizova et al note:

Macrophages are present at varying frequencies in all types of cancer and account for one of the most frequent immune cells in the tumor microenvironment (TME). The precursors of the macrophage pool in the TME are primarily tissue-resident macrophages and blood monocytes, with a smaller fraction deriving from MDSCs that are closely related to blood monocytes.

While the rapid differentiation of MDSCs into macrophages within the TME may occur, it is crucial to emphasize that this process is characterized by a high degree of complexity and remains context dependent. In this scenario, the differentiation of MDSCs into TAMs shows substantial heterogeneity, both across different cancer types and among individuals, and to date studies are still investigating the factors that induce changes in gene expression and cellular signalling pathways, leading to the transformation of MDSCs into TAMs.

Macrophages act as double-edged swords in cancer because of their ability to provide successful anti-tumour immunity and orchestrate the immunosuppressive TME.

Potent anti-tumour mechanisms include activation of innate and adaptive immunity, antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis and direct cytotoxicity. All these properties are attributed to properly functioning M1-like macrophages.

Nevertheless, under the influence of various factors, such as local hypoxia, high levels of lactic acid, inflammation and secretory molecules (cytokines, chemokines, etc.), macrophages gain impaired functions and protumoural properties. M2-like macrophages attempt to repair cancer tissue through immunosuppression, tissue remodeling and neovascularization. Tumor cells have learned to take advantage of these reparative mechanisms, which they find highly advantageous.

Consequently, they have adapted to exploit these mechanisms to the fullest extent which bolsters the growth and development of the tumor even further. M2-like macrophages express metalloproteinase, matrix remodelling enzymes and other growth factors, such as EGF, TGF-β and VEGF, which facilitate cancer cell proliferation, invasiveness, epithelial–mesenchymal transition and metastasis formation.

In addition to the production of immunosuppressive cytokines, various immune checkpoint molecules expressed by macrophages, such as B7-H4, PD-L1, PD-L2, TIM-3 and VISTA, drive immunosuppression within the TME. There is no unique nomenclature system for macrophages present in the TME. Most studies consider all macrophages in the TME as tumour-associated macrophages (TAMs), which are further divided into M1/M1-like and M2/M2-like macrophages. Some studies have described TAMs as a unique group that does not exist under normal physiological conditions and possesses a specific phenotype with both M1 and M2 characteristics.

Furthermore, few studies have described only M2/M2-like macrophages as TAMs. Additionally, many researchers have distinguished between M1/M2 macrophages and M1/M2-like macrophages.

M1 refers to macrophages induced in response to IFN-γ and bacterial products, whereas M1-like is a polarization state that promotes antitumour immunity and cytotoxicity.

The above is an important observation.

In contrast, M2 differentiation is driven by IL-4 and IL-13 while M2-like macrophages suppress effective adaptive immunity to promote protumoural conditions.

Likewise the above concerning M2 macrophages is a critical observation, IL-4 and IL-13 is a common driving duple. Namely this pair is seen in such issues as itch, asthma, and a multiplicity of chronic but not malignant disorders. Currently a Mab such as dupilumab is a antagonist for IL-4 and IL-13. It could be considered thus as a blocker of M2 phenotype differentiation. There do not seem to be any studies related to this however.

ScRNA-seq has emerged as a crucial technique for studying TAMs, enabling a deeper understanding of their plasticity and facilitating the identification of rare TAM populations. Remarkably, the exploration of these newly discovered macrophage subsets in tumors, such as CD74+ macrophages or macrophages with high expression of CCL8, has shed light on their potential roles in tumor progression. Tumour-promoting macrophages are highly variable.

Undoubtedly M2-like macrophages contribute to various hallmarks of cancer, however, one of the hallmarks is tumour-promoting inflammation, which in multiple ways may support the cancer cells.

M1-like macrophages largely contribute to the production of pro-inflammatory molecules such as IL-1β, IL-6, and TNF-α. The effects of TNF-α depend on its concentration, form (soluble/membrane-bound), receptor binding, and subsequent cell signalling. TNF-α may induce apoptosis in cancer cells; however, it can also stimulate tumour growth, survival, proliferation, invasion, and metastasis formation.

Similarly, IL-6 may facilitate tumour cell survival and migration, induce cancer stemness, and prime macrophages towards the M2-like phenotype. Additionally, IL-6 triggers the expression of key angiogenic key factors HIF-1α and VEGF. Similarly, IL-1β has been shown to increase angiogenesis, tumour growth, and invasiveness. Another factor that contributes to the development of tumour-promoting inflammation is ROS. Increased ROS production leads to genetic instability, another hallmark of cancer.

In conclusion, macrophages have a heterogeneous spectrum of phenotypes with M2-like macrophages being narrowly associated with tumour-promoting properties. It is worth mentioning that the M1-like macrophages can also exert these effects by facilitating the growth of cancer cells, promoting neovascularization, and contributing to the development of metastases. TME is extremely complicated, dynamic, and quickly evolving milieu. However,

differences exist among macrophage subpopulations within a tumour, reflecting TME heterogeneity. Besides TME, which is specific for a given tumour type and stage, the affected organ, macrophage origin, and microbiota also influence the final phenotype of macrophages. Thus, the phenotype of macrophages within the TME is complex, overlapping and plastic.

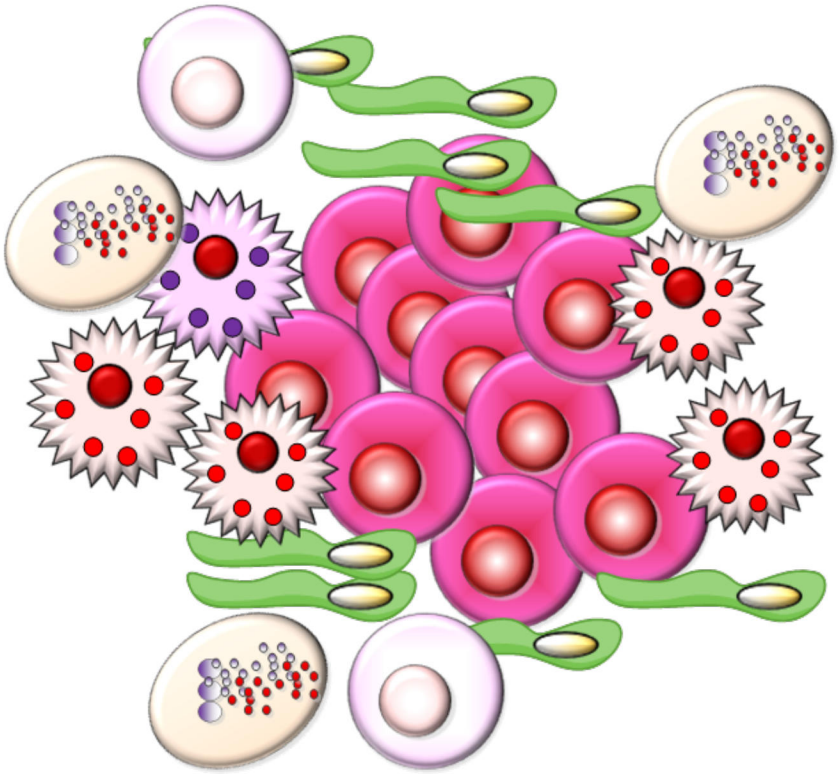
The classification of M1-like and M2-like macrophages is despite the complexities and numerous phenotypes of macrophages, useful for assessing the prognosis.

From Hesketh et al:

Product	M1	M2a	M2b	M2c	M2d
Marker Expression	CD14 CD16 CD68 CD86 CD80 MHC II high	CD14+ CD16++ CD68 CD163 CD206 CD3001, MHC-II low	CD14+ CD16++ CD68 CD86, MHC-II+	CD14+, CD16++, CD68, CD150, CD163 CD206	CD14+, CD16++, CD68
Cytokines	IL1β IL 6 IL12 IL 18 IL 23 TNF	IL-1ra, sIL-1R, IL-10	IL-1β, IL-6, IL-10, TNF	IL10	IL 10 IL 12 TNF
Chemokines	CXCL1 CXCl10 CXCL11 CXC:16 CCL2-5	CCL-17, 18, CCL22, CCL24	CCL1 CCL20 CXCL1, CXCL3	CXCL--13, CCL-16, CCL-18 CCR2	CXCL-10, CXCL-16, CCL-5
Signalling	STAT 1 STAT 4 SOCS 3	STAT 3	SOCS3	STA T 6	
Proteases	MMP 1, 2,3,7,9,12		MMP-1, 2, 9, 12,13, 14, TIMP-1		
Growth Factors	ROS RNS NO	EGF, TGFβ, IGF1 Arg1	COX 2	EGF, TGFβ, IGF1 Arg1	VEGF, TGFβ,

4 TUMOR MICRO-ENVIRONMENT (TME)

The tumor micro environment, TME, is the amalgam of cells that enhance and support the tumor cells. The Figure below is a graphic for this construct. The fibroblasts create a quasi-granuloma protecting the tumor cells they surround. There are neutrophils, leukocytes, and a multiplicity of macrophages. The cells communicate and interact and the tumor cells create a protective and supportive environment in which to thrive.



Tumors are not self-sufficient entities. They are not aberrant cells awash in a sea of normalcy. The create and in a sense are created by the microenvironment in which the grow and expand. We have examined some of the specific protein elements of the ECM and now we will examine the cellular components.

4.1 OVERVIEW

We begin with a brief discussion of the TME and then will proceed with some details. As Roma-Rodrigues et al have noted:

The development of effective anti-cancer therapies has been challenged by the overall complexity of tumors. The tumor heterogeneity is exacerbated during the progression of the cancer along with the maturation of the cellular and noncellular components of the tumor niche—the tumor microenvironment (TME).

The TME consists of extracellular matrix (ECM), stromal cells (such as fibroblasts, mesenchymal stromal cells, pericytes, occasionally adipocytes, blood and lymphatic vascular networks) and immune cells (including T and B lymphocytes, natural killer cells and Tumor-associated macrophages).

The TME is a protective crust surrounding many tumor clusters. They not only protect but also invigorate the tumor. Thus, as a therapeutic it is essential to both understand the TME as well as migrate around it. They continue:

The TME has progressively been shown to dictate aberrant tissue function and to play a critical role in the subsequent evolution of malignancies. Epithelial tumors display common features that allow for the setting of hallmarks that define cancer progression. Tumor initiation is based on a complex series of biological events occurring on a normal cell that will result in hyperplasia, uncontrolled growth and resistance to cell death. As tumor cells continue proliferation, the tumor increases in size with an associated remodeling of the TME. This is induced by hypoxia, oxidative stress and acidosis, due to an alteration of tumor cells metabolism, resulting in dysplasia, which is the appearance of a heterogeneous population of tumoral cells with different genetic and phenotypic traits.

These events are orchestrated by autocrine and paracrine communications with stromal cell and immune system adjacent to the tumor, coupled to an increased interstitial fluid pressure.

Once again, autocrine and paracrine communications between TME cells induce TME maturation and tumor progression, resulting in increased stiffness of the extracellular matrix, formation of blood and lymph vessels, possible appearance of necrotic regions and metastasis.

4.2 TME AND IMMUNE SYSTEM

With the introduction of immunotherapy, and even just simple monoclonal antibody targeting, getting around the TME is essential. As Whiteside had noted:

The tumor microenvironment is created by the tumor and dominated by tumor-induced interactions. Although various immune effector cells are recruited to the tumor site, their anti-tumor functions are downregulated, largely in response to tumor-derived signals. Infiltrates of inflammatory cells present in human tumors are chronic in nature and are enriched in regulatory T cells (Treg) as well as myeloid suppressor cells (MSC).

Recall that Treg suppress the tumor killing capacity of the other T cells.

Immune cells in the tumor microenvironment not only fail to exercise antitumor effector functions, but they are co-opted to promote tumor growth. Sustained activation of the NF-kB pathway in the tumor milieu represents one mechanism that appears to favor tumor survival and drive abortive activation of immune cells. The result is tumor escape from the host immune system.

Tumor escape is accomplished through the activation of one or several molecular mechanisms that lead to inhibition of immune cell functions or to apoptosis of anti-tumor effector cells. The ability to block tumor escape depends on a better understanding of cellular and molecular pathways operating in the tumor microenvironment. Novel therapeutic strategies that emerge are designed to change the protumor microenvironment to one favoring acute responses and potent anti-tumor activity

The author continues:

A tissue microenvironment of developing tumor is comprised of proliferating tumor cells, the tumor stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells.

It is a unique environment that emerges in the course of tumor progression as a result of its interactions with the host. It is created by and at all times shaped and dominated by the tumor, which orchestrates molecular and cellular events taking place in surrounding tissues. Immune cells present in the tumor include those mediating adaptive immunity, T lymphocytes, dendritic cells (DC) and occasional B cells, as well as effectors of innate immunity, macrophages, polymorphonuclear leukocytes and rare natural killer (NK) cells.

NK cells, which mediate innate immunity and are rich in perforin- or granzyme-containing granules, are conspicuously absent from most tumor infiltrates or even pre-cancerous lesions. Although NK cells represent 'the first line' of defense against pathogens and mediate potent antitumor cytotoxicity in vitro, in tumor milieu, they are infrequent, despite the fact that tumor cells frequently downregulate expression of HLA antigens and are enriched in MICA and MICB molecules. …

NK cells are powerful tumor killer cells. Understanding the reasons for their absence and means to enable them can be a powerful tool.

TIL clones with the specificity to a broad variety of the tumor-associated antigens can be outgrown from human tumors, confirming that immune responses directed not only at 'unique' antigens expressed by the tumor, but also at a range of differentiation or tissue-specific antigens, are generated by the host.

Although accumulations of these effector T cells in the tumor might be considered as evidence of immune surveillance by the host, they are largely ineffective in arresting tumor growth. Among CD4 + T cells present in the tumor, a subset of CD4+ CD25 high Foxp3 + cells is expanded (5–15% of CD3 +CD4+þ T cells in TIL) relative to their significantly lower frequency in the peripheral circulation of patients with cancer.

These cells are regulatory T cells (Treg) capable of suppressing proliferation of other T cells in the microenvironment through contact-dependent mechanisms or IL-10 and TGF-b secretion. They come in different flavors (for example, nTreg, Tr1) and are a characteristic feature of the microenvironment in human tumors.

Macrophages present in tumors are known as tumor associated macrophages or TAMs. They are re-programmed to inhibit lymphocyte functions through release of inhibitory cytokines such as IL-10, prostaglandins or reactive oxygen species (ROS).

Myeloid suppressor cells (MSC) accumulating in human tumors are CD34+ CD33 + CD13+ CD15- bone marrow-derived immature dendritic cells, an equivalent to CD11b þ/ Gr1 þ cells in mice. They promote tumor growth and suppress immune cell functions through copious production of an enzyme involved in L-arginine metabolism, arginase 1, which synergizes with iNOS to increase superoxide and NO production, blunting lymphocyte responses and by induction of iNOS in surrounding cells. …

Polymorphonuclear leukocytes are infrequently seen in infiltrates of human tumors, with the exception of nests of eosinophils that may be present in association with tumor cells in various squamous cell tumors, for example. In contrast, granulocytes tend to be a major cellular component of many murine tumor models. This disparity may be because of a different nature of infiltrates, which in man are chronic rather than acute. Acute cellular responses may be long gone by the time human tumors are diagnosed, biopsied and examined.

Inflammatory cells present in the tumor microenvironment either contribute to tumor progression or actively interfere with its development. It is clear today that the former takes precedence, largely because the tumor generally proceeds to establish mechanisms responsible for its 'immune evasion' or escape from the immune intervention.

The tumor not only manages to escape from the host immune system, but it effectively contrives to benefit from infiltrating cells by modifying their functions to create the microenvironment favorable to tumor progression. To this end, immune cells infiltrating the tumor together with fibroblasts and extracellular matrix forming a scaffold supporting its expansion, contribute to establish an inflammatory milieu that nourishes the tumor and promotes its growth. Tumor escape from the host is facilitated by the ability of human tumors to actively subvert antitumor immunity by downregulating or completely suppressing local and systemic innate as well as adaptive antitumor immunity by a variety of mechanisms as discussed below.

Recently Arneth noted:

The TME refers to the cellular environment in which tumors or cancer stem cells exist. Cancer stem cells are cells in a tumor with the abilities to self-renew and drive tumorigenesis.

Previous studies have isolated unique cancer stem cells in samples from patients with breast, hematopoietic, colon, lung, and brain cancers.

These cells help improve the understanding of the TME, but pose significant challenges in the diagnosis and management of cancer.

The TME encompasses the surrounding immune cells, blood vessels, extracellular matrix (ECM), fibroblasts, lymphocytes, bone marrow-derived inflammatory cells, and signaling molecules.

Interactions between malignant and nonmalignant cells create a TME that affects cancer development and progression. The nonmalignant cells in the TME often play a protumorigenic function at all phases of carcinogenesis by stimulating uncontrolled cell proliferation. In contrast, malignant cells invade healthy tissues and spread to other body parts through the lymphatic or circulatory system.

The TME comprises different cellular components.

The first is endothelial cells, which play a key role in tumor development and tumor cell protection from the immune system.

Tumor angiogenic vessels usually branch outwards from preexisting vessels or are derived from endothelial progenitor cells. In this way, these cells offer nutritional support for tumor growth and development.

The second major component is immune cells, such as granulocytes, lymphocytes, and macrophages.

These cells are involved in various immune responses and activities, such as inflammatory reactions orchestrated by the tumor to promote survival. The most prominent immune cell type in the TME is the macrophage.

Macrophages have diverse functions that are linked to cancer development and progression; they promote the escape of tumor cells into the circulatory system and can suppress antitumor immune mechanisms and responses.

Evidence from previous studies has revealed that macrophages can help circulating cancer cells extravasate at distant sites, such as the lungs, which can lead to the persistent growth of metastatic colonies. An increasing number of studies have revealed that tumor-associated macrophages (TAMs) can augment, mediate, or antagonize the antitumor activity of irradiation, cytotoxic agents, and checkpoint inhibitors. The final cell type in the TME is the fibroblast.

Fibroblasts allow cancer cells to migrate from the primary tumor location into the bloodstream for systemic metastasis. Furthermore, fibroblasts provide a reliable passage for endothelial cells undergoing angiogenesis in the tumor.

The role of the ECM in cancer development and progression has been examined in previous studies. The ECM consists of a network of macromolecules, including glycoproteins, collagens, and enzymes, that support biomechanical activities and functions in the body. Importantly, the ECM is composed of active tissue components that influence cell adhesion, proliferation, and communication.

The cellular growth factors found in the matrix near other cell membranes, such as integrins, are implicated in the ability of cells to communicate with the TME. The ECM further influences the migration of cancer cells by altering its physical properties, composition, and topography. The

adhesion gradient and the ECM concentration determine the speed at which cancer cells migrate from one region to another.

4.3 Detailed Elements of TME

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

4.3.1 Cancer-Associated Fibroblasts.

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.

4.3.2 Adipocytes.

Other cells also contribute to the TME. Frequently we see adipocytes in tumor clusters. As the previous author notes:

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.

This may be the issue of chicken vs egg. Namely does obesity, namely excess adipocytes enable cancer or are they added to protect a malignancy. We know that obesity, thus excess adipocytes, results in inflammation. Inflammation, namely chronic inflammation, is clearly a driver of malignancies. The authors continue:

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.

4.3.3 Perivascular Cells.

The next set of cells are perivascular.

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.

4.3.4 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. 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.

4.3.5 Tumor-Associated Macrophages.

We have already discussed TAMs separately. Now in the context of the TME we observe a few remarks. 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.

4.3.6 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.

4.3.7 T-Regulatory Cells.

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.

4.3.8 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. The author notes:

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.

4.3.9 Other Stromal Cells.

Finally, there are several other cells:

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. …

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

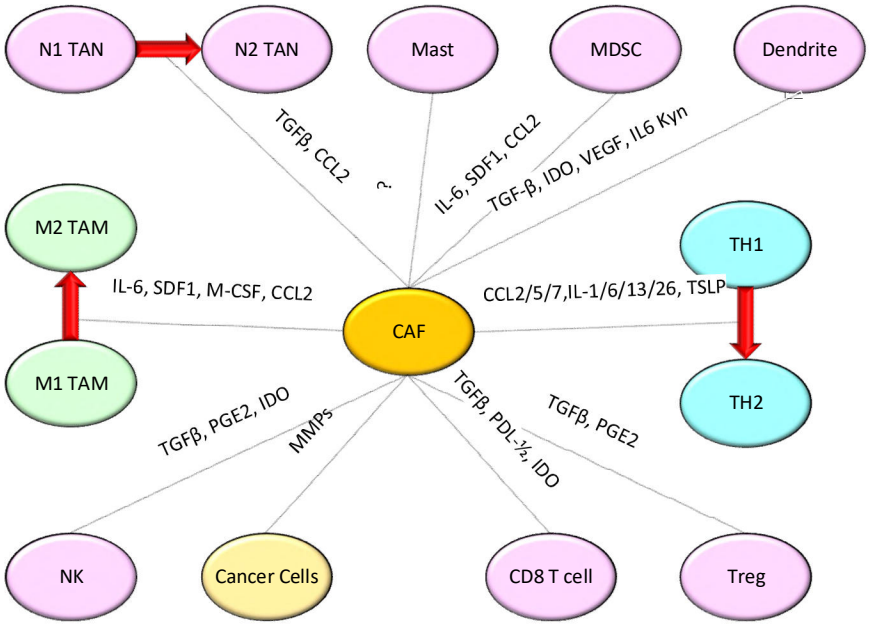
correlated with the EMT and cell migration in CD133+ pancreatic CSCs. In addition to cancer cell invasion, hypoxia contributes to drug resistance by maintaining CSCs in a quiescent state to confer resistance to chemotherapeutics that commonly target actively dividing cancer cells.

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.

4.4 LINKAGES

The Figure below is an example of the interactions of fibroblasts. The signalling also allows for phenotypic changes as with macrophages which we have discussed above.



Before considering the attack on cancer cells with immunotherapeutic methods, one must understand that they establish themselves in a protective environment, the tumor microenvironment, TME. The tumor micro-environment (TME) is the complex of interacting aggregate tumor cells. The immune system generally acts to attack and eliminate invaders but the TME has the capability to modify many of these functions. The presence and structure of the TME must be understood for each malignant form so as best to attack with immune cells. If not done so, then the attacking agents may just "bounce off" this protective shell.

As Tredan et al have noted:

Resistance of human tumors to anticancer drugs is most often ascribed to gene mutations, gene amplification, or epigenetic changes that influence the uptake, metabolism, or export of drugs from single cells. Another important yet little-appreciated cause of anticancer drug resistance is the limited ability of drugs to penetrate tumor tissue and to reach all of the tumor cells in a potentially lethal concentration.

To reach all viable cells in the tumor, anticancer drugs must be delivered efficiently through the tumor vasculature, cross the vessel wall, and traverse the tumor tissue. In addition, heterogeneity within the tumor microenvironment leads to marked gradients in the rate of cell proliferation and to regions of hypoxia and acidity, all of which can influence the sensitivity of the tumor cells to drug treatment. In this review, we describe how the tumor microenvironment may be involved in the resistance of solid tumors to chemotherapy and discuss potential strategies to improve the effectiveness of drug treatment by modifying factors relating to the tumor microenvironment. …

Solid tumors are organ-like structures that are heterogeneous and structurally complex. They comprise cancer cells and stromal cells (i.e., fibroblasts and inflammatory cells) that are embedded in an extracellular matrix and nourished by a vascular network; each of these components may vary from one location to another in the same tumor.

The TME is thus the complex assembly of the cells, the ECM, the vasculature and its arrangement and cohesiveness. The TME is a complex infrastructure which tends to protect the mass of cancer cells and this protection allows them to proliferate while being protected against any of the attempt of the immune system to battle them. The authors continue:

The effectiveness of drug therapy is impaired by limited delivery of drugs to some regions of tumors and by effects of the tumor microenvironment on drug activity and on the metabolism and proliferation of tumor cells. Agents that improve drug delivery or activity by targeting the tumor microenvironment, especially in hypoxic regions of tumors, represent an important future direction for cancer therapy.

Adding vascular-disrupting agents that increase the extent of the hypoxic/acidic region might enhance the anticancer activity of various drugs that show increased efficacy against acidic cells, hypoxia-activated prodrugs, or bacteriolytic therapies. The development of methodologies to characterize causes of drug resistance related to the tumor microenvironment has considerable potential to improve the outcomes of patients following systemic treatment of solid tumors.

The same effects to drug therapy will apply to immunotherapy and must be addressed accordingly. Nyberg et al further note:

The tumor microenvironment is a mixture of extracellular matrix molecules, tumor cells, endothelial cells, fibroblasts and immune cells. Tumor growth and metastasis formation are dependent on the growth of blood vessels into the tumor mass. The tumor microenvironment contributes to this pathological angiogenic process.

The extracellular matrix and basement membranes are a source for endogenous angiogenesis inhibitors, such as endostatin. On the other hand, many extracellular matrix molecules can promote angiogenesis by stabilizing blood vessels and sequestering pro-angiogenic growth factors. The majority of stromal cells in carcinomas are fibroblasts. Carcinoma- associated fibroblasts show a distinct phenotype from normal fibroblasts.

The mechanisms how the tumor- associated fibroblasts regulate angiogenesis are not fully known, but they are suggested to be an important source for growth factors and cytokines recruiting endothelial cells. The immune cells, particularly macrophages and neutrophils are another source for angiogenesis-regulating chemokines, growth factors and proteases. Taken together, the tumor microenvironment is a complex unorganized tissue of various cell types and extracellular matrix that can regulate the pathological angiogenic switch.

Zhang et al have recently described various technique as how to modify the TME so as to be more accepting of immunotherapy. As Bassani et al note:

Strong evidences suggest that the presence of inflammatory cells within the TME plays a crucial role in the development and/or progression of tumors. Among the host-dependent biological features of the tumor hallmarks defined by Hanahan and Weinberg, there are "evading immune destruction" and "tumor-promoting inflammation", which together with the immune cell-mediated orchestration of angiogenesis, point out the key role of the immune system in neoplastic disease.

As a consequence of their functional plasticity, several immune cells, can modify upon stimuli delivered by the components of TME their phenotypic and functional features; this leads to a reduced killing of tumor cells, the expression of a tolerogenic/immunosuppressive behavior and the acquisition of pro-angiogenic activities, thus promoting tumor expansion. NK cells are innate lymphocytes that can potentially control tumor growth by their cytotoxic activity.

Classical NK cells are distinct from innate lymphoid cells (ILCs) although they share with ILC1 several phenotypic features; indeed, NK cells are key cytolytic effectors of innate immunity while ILC1 are generally non-cytotoxic or weakly cytotoxic but they show a central role in response to certain infections and are also involved in tissue remodeling homeostasis, morphogenesis, metabolism, repair, and regeneration. ….

ILC and NK cells originate from a common lymphoid progenitor (CLP). GATA3 or TOX/NFIL3/ID2/ETS1 drive the distinction between common innate lymphoid progenitor (CLIP) and the NK cell progenitor (NKP), respectively. Finally, T-bet/EOMES expression in NKPs govern NK cell differentiation. Natural killer cell subsets can differ according to tissue distribution that is related to distinct homing properties and/or local maturation

5 MACROPHAGE MOVEMENT AND CHEMOKINES

The attraction of a macrophage to a specific cell can be accomplished via the use of a chemokine and its related receptor. If a cell presents a specific chemokine on its surface then the complement receptor on let us say a macrophage can draw that macrophage to that cell expressing the chemokine. We examine this characteristic in some detail. It should be noted that this can be another therapeutic target approach.

As Quaranta and Schmid note:

Macrophages originate from three different developmental pathways. All tissue embryonic macrophages derive from macrophage precursors in the yolk sac and fetal liver. During adulthood, fetal macrophages are replaced gradually by macrophages derived from bone marrow hematopoietic stem cell (HSCs). Some types of tissue resident macrophages, including bone osteoclasts, epidermal Langerhans cells, lung alveolar macrophages, microglia and liver Kupffer cells develop from embryonic macrophages and persist in adult tissues independently of replenishment by Ly6Chigh monocytes originated from HSCs during adulthood. Instead, other types of tissue macrophages such as intestine, dermis, heart and pancreas macrophages undergo a continuous turnover in adulthood by recruitment of circulating monocytes which differentiate into macrophages upon tissue infiltration.

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

There are different markers to identify monocyte-macrophages diversity in human and mouse. In human, circulating monocytes, which originate from the bone marrow, can be classified in two subsets: CD14+ CD16neg 'inflammatory' or 'classical' and CD14+ CD16+ 'patrolling' or 'non-classical' monocytes.

In the same way, mouse 'inflammatory' monocytes are classified as CD11b+ Ly6Chigh CCR2high CX3CR1low, in contrast 'patrolling' monocytes are CD11b+ Ly6Glow CCR2low CX3CR1high. Patrolling monocytes monitor the microvasculature under steady-state conditions and rarely extravasate into tissue. However they can rapidly accumulate in lung metastatic tissue and inhibit cancer cell seeding and growth by exerting anti-tumour functions through recruitment of NKs. Macrophages are a population of heterogeneous and plastic cells.

Once resident in tissues, macrophages acquire a distinctive phenotype in response to different signals present in the immediate microenvironment. Environmental stimuli, like IFNγ or microbial products, like the lipopolysaccharide molecule (LPS), induce a classical activation of macrophages and skew them toward an M1-like or 'classically activated' macrophage phenotype. M1-like macrophages mediate anti-microbial and tumoricidal response by secreting inflammatory cytokines, such as TNFα, IL-12, reactive oxygen species and nitric oxide (NO), by up-regulating the expression of major histocompatibility complex (MHC II) and by promoting a TH1-type of response.

Alternatively, if the microenvironment becomes populated by different types of cytokines and growth factors, like the TH type-2 cytokines IL-4 and IL-13, macrophages are stimulated to acquire an alternative activation state, resulting in an M2-like subtype.

M2-like polarized macrophages are characterized by expression of anti-inflammatory cytokines, such as IL-10, lower expression of pro-inflammatory cytokines, up-regulation of scavenger receptors, such as mannose receptors (MRC1/CD206 and CD163) and reduced ability to activate adaptive immune response. As such, M2-like macrophages may facilitate resolution of inflammation and promote tissue repair after the acute inflammation phase. …

Monocytes are recruited into the tumour site by chemokines secreted by tumour and stroma cells including vascular endothelial growth factor (VEGF), 55mmune55ring 3A (SEMA3A), CCL2 and C-X-C motif ligand (CXCL)12. In tumours, the signals that orchestrate macrophage functions can vary between different tumour types, or even between different parts of the same tumour resulting in diverse TAM phenotypes.

TAMs with a relatively M1-like skewed phenotype are found to be associated with the early phases of tumour development or with regressing tumours. Classically activated M1-like macrophages can kill tumour cells and they mediate tissue-destructive reactions by taking part in the early elimination phase of 55mmune-editing orchestrated by CD8+ cytotoxic T lymphocytes and interferons.

Often early-stage tumors have M1 macrophages, and even some NK cells. However once these disappear, we see the development and infiltration of M2 cells.

Generally, TAM polarization toward an M2 phenotype seems to be a common feature for many cancers although their relative abundance depends on the tumour type. TAMs have properties correlated with angiogenesis, immunosuppression and promotion of cancer growth and metastasis. For example, M2-like TAM are found in perivascular and hypoxic regions of mouse and human tumours ; angiopoietin receptor TIE-2+ monocyte/macrophages are important for angiogenesis and they are associated with M2 skewed phenotype and tissue remodelling activity.

5.1 CHEMOKINES

We have referred to chemokines in the previous discussions. It is worth a summary of them separately. Simply chemokines are a subclass of cytokines. They are proteins with a general structure which we shall discuss. From Hughes and Nibbs

The chemokines (or chemotactic cytokines) are a large family of small, secreted proteins that signal through cell surface G protein-coupled heptahelical chemokine receptors.

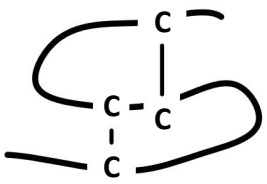
They are best known for their ability to stimulate the migration of cells, most notably white blood cells (leukocytes). Consequently, chemokines play a central role in the development and

homeostasis of the immune system, and are involved in all protective or destructive immune and inflammatory responses.

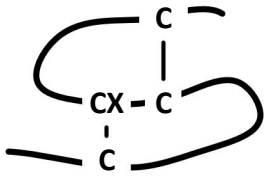
Classically viewed as inducers of directed chemotactic migration, it is now clear that chemokines can stimulate a variety of other types of directed and undirected migratory behavior, such as haptotaxis, chemokinesis, and haptokinesis, in addition to inducing cell arrest or adhesion.

However, chemokine receptors on leukocytes can do more than just direct migration, and these molecules can also be expressed on, and regulate the biology of, many nonleukocytic cell types. Chemokines are profoundly affected by post-translational modification, by interaction with the extracellular matrix (ECM), and by binding to heptahelical 'atypical' chemokine receptors that regulate chemokine localization and abundance. This guide gives a broad overview of the chemokine and chemokine receptor families; summarizes the complex physical interactions that occur in the chemokine network; and, using specific examples, discusses general principles of chemokine function, focusing particularly on their ability to direct leukocyte migration.

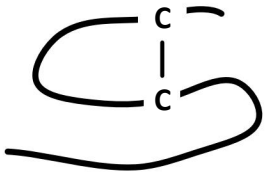
We show the classes below:



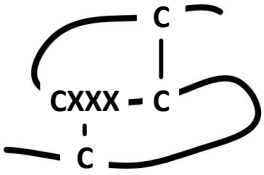
CC



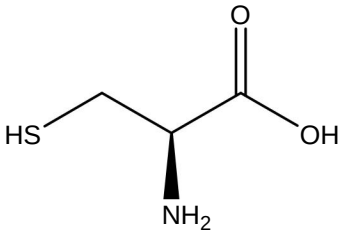
CXC



C

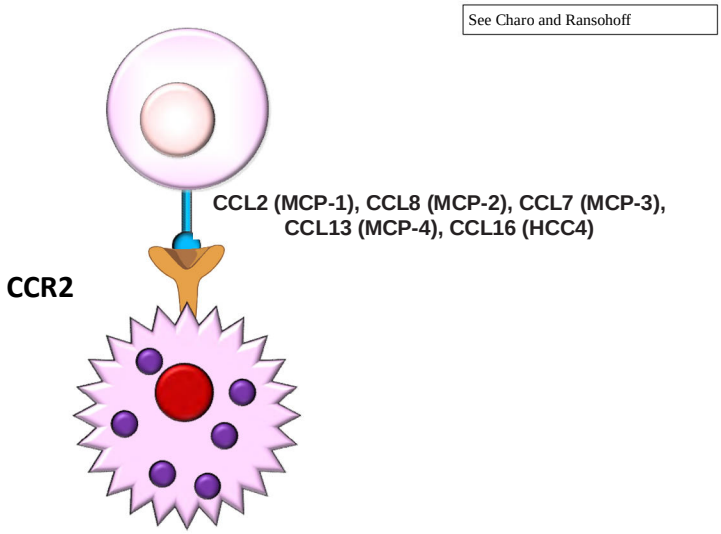


CXXXC



cysteine

A typical interaction of chemokines is shown below. There are chemokine ligands and in turn matching receptors. There are related pathways and activation mechanisms.



5.2 Chemokines and Macrophages

Now we have referred to chemokines in our discussion of macrophages in the TAM. We now examine this in some detail. As Bule et al note:

Aberrant expression of chemokine ligands or chemokine receptors has long been associated with dysfunctional lymphoid organ development and a defective or exacerbated immune response.

Evidence now shows that chemokines are also key molecules for development and disease progression in the context of cancer.

The infiltration of immune cells into the tumor microenvironment (TME) is a determinant factor in cancer prognosis.

Although chemokine signalling is crucial in recruiting immune cells with antitumor effects, such as CD8+ T cells, Thelper 1 (TH1) cells and natural killer (NK) cells, chemokine ligand secretion and chemokine receptor expression is often altered in the TME.

This often leads to the recruitment of pro-tumorigenic immune cells such as myeloid derived suppressor cells (MDSCs), tumor-associated neutrophils (TAN), tumor-associated macrophages (TAM) and regulatory T cells (Treg cells). Proliferation of these cells as the disease progresses leads to the suppression of effector lymphocytes, and is associated with worse prognosis in patients with various types of cancer.

Additionally, chemokines can directly target non-immune cells in the TME, including tumor cells and vascular endothelial cells, which often display pathological chemokine receptor expression.

Therefore, they can promote tumor cell proliferation, angiogenesis, cancer stemness, cancer invasiveness and metastasis. By acting directly and indirectly on the tumoral immune response, the chemokine system modulates tumor-immune and biological phenotypes and regulates cancer progression, which ultimately impacts therapy responses and patient clinical outcomes …

Macrophages are mainly recruited into the TME through the CCL2–CCR2 signalling pathway. Tumoral expression of CCL2 correlates with the number of TAM in many tumors and is often associated with poor patient prognosis.

Like Treg cells, TAMs can also inhibit tumor-associated antigens (TAA)-specific CD8+ T cell activation, which are capable of engaging tumor cells in an antigen-specific manner and drive antitumor immunity by secreting effector cytokines …

Although the CCR2–CCL2 axis appears to be the main driver of TAM and MDSC recruitment, other chemokines have also been shown to contribute to the process. Increased CCL5 expression correlates with increased TAM infiltration and disease progression in breast cancer, while CCR5-expressing MDSCs have been shown to be more immunosuppressive than their CCR5− counterparts.

Elevated CCL5 expression is also associated with disease progression in pancreatic, gastric, and ovarian cancer. Other chemokines reported to induce monocyte recruitment to tumors are CCL7, CCL15, CXCL8 and CXCL12. Interestingly, a cascade involving CCL2 and CCL3 has been described, in which TAMs, derived from CCL2-recruited MDSCs, secrete CCL3, which further promotes macrophage retention in the tumor and tumor metastatic sites.

As Mempel et al note:

CCR2 and CCR5 as well as their MCP/MIP cluster ligands (Table 1) are key elements of the inflammatory monocyte recruitment programme discussed subsequently but are also expressed by activated TH1 cells and CTLs and their ligands can be induced by IFNγ (in addition to IL-1 and other cytokines).

CCR2 is also expressed by TH2 cells, but CCR2-deficient mice fail to mount appropriate type 1 responses in favour of exaggerated type 2 responses in pulmonary infections. Therefore, both CCR2 and CCR5 are often considered part of the type 1 response programme. These chemokines are assumed to facilitate T cell interactions with monocytes or macrophages in inflamed tissue environments, for instance, to regulate antimicrobial activities in the latter.

CCR5 can also optimize CD8+ T cell priming in lymph nodes by guiding them to DCs that have been induced to express CCL3 and CCL4 and licensed through previous cognate interactions with CD4+ TH cells.

Expression of CCR5 ligands can also be elicited by direct viral infection of DCs to optimize their interactions with antiviral CD8+ T cells. CCR5 likely plays similar roles in immune responses to cancer, as its expression on both CD4+ and CD8+ T cells is, for example, required for maximal antitumour immunity in mouse models of fibrosarcoma and breast cancer. Finally, activated

CTLs can themselves become a rich source of the CCR5 ligands CCL3 and CCL4. As a result, antigen recognition by T cells, for instance, on cancer cells, may cause them to produce a chemokine trail for other CCR5-expressing T cells in the TME and thereby facilitate swarming behaviour, as demonstrated in a tumour spheroid model67 (Fig. 2a).

Such T cell swarming may be important when cooperativity between multiple CTLs is required for the killing of cancer cells with high resistance to cytotoxicity … The second of the two transmembrane chemokines, CX3CL1, is constitutively expressed on epithelial cells, endothelial cells, smooth muscle cells, neurons and DCs. As with CXCL16, it is further induced by the inflammatory cytokines IFNγ, TNF and IL-1β, as well as by transforming growth factor β (TGFβ), for example, on fibroblasts and astrocytes. Its sole receptor CX3CR1 is expressed on tissue-resident macrophages as well as monocyte subsets and their progeny, where it is important for the removal of dead cells, neural development and plasticity as well as the regulation of fibrotic processes95–97, although the precise mechanisms of its involvement are not in all cases fully understood. …

Monocytes represent a lineage of the mononuclear phagocyte system. They continuously egress from the bone marrow in a strictly CCR2-dependent fashion and circulate in the bloodstream initially as so-called classical or 'inflammatory' CCR2Hi monocytes with a half-life of about 1 day1. As such, they can be recruited at a low rate to tissues at steady state to produce macrophages that complement tissue-resident macrophages seeded during embryogenesis to maintain tissue homeostasis*.*

Monocytes are recruited at a much higher rate, along with neutrophils, to injured or infected tissues where they contribute to the inflammatory response, but can also give rise to more long-lived macrophages and monocyte-derived DCs. The remaining circulating CCR2Hi monocytes either undergo apoptosis or differentiate first into intermediate and then into non-classical, 'patrolling' monocytes that downregulate CCR2 and instead upregulate CX3CR1, and crawl along and patrol the luminal aspect of the endothelium of blood vessels, with an extended half-life of several days to a week in mice and humans.

Monocytes are found in the TME during all stages of tumour development, in which they can give rise to TAMs with a broad range of differentiation states that variably exhibit characteristics of IFNγ-induced classical M1 or IL-4-induced alternative M2 polarization1. Despite the aforementioned potential of inflammatory monocytes to support type 1 immune responses, and despite reports of TAM tumoricidal activities under some conditions144, reducing their accumulation in tumours by interfering with CCR2 or its ligand CCL2 generally enhances T cell immunity and reduces growth of a wide range of tumours145–149, indicating that their pro-tumoural activities most often outweigh their antitumoural ones.

Although it is now acknowledged that CCR2 can facilitate both monocyte bone marrow egress and tumour tissue entry148, recruitment to inflammatory sites is not critically dependent on CCR2 (refs. 150,151). This suggests that other chemokine receptors expressed by human inflammatory monocytes, including CCR1, CXCR1 and CXCR2 (ref. 152), may represent alternative mechanisms for tumour entry. Targeting these receptors could therefore complement CCR2-directed treatments to reduce monocyte accumulation in tumours.

5.3 CHEMOKINES AS TARGETS

We are always looking for therapeutic targets. Chemokines may present another such opportunity. As Mantovani et al noted:

Distinct chemokine repertoires associate with M1 and the various forms of M2 macrophage activation. LPS activation of monocytes or macrophages results in the NF-kB dependent transcription of inflammatory chemokines, such as CXCL1, 2, 3, 5, 8, 9 and 10 and CCL2, 3, 4, 5, 11, 17 and 22. In addition, LPS and IFN-g induce the expression of CXCL10, CXCL9 and CCL5. LPS mediates induction of the CXCL10, CXCL9 and CCL5 genes through the activation of the transcription factor IFN regulatory factor-3 (IRF-3), which results in IFN-b expression and subsequent STAT1 (signal transducer and activator of transcription 1) activation.

The spectrum of chemokines produced during classical activation (M1) amplifies delayed-type hypersensitivity (DTH) reactions and resistance to intracellular pathogens and tumors. CXCL16 and CX3CL1 share the property of being transmembrane chemokines. CXCL16 is expressed in macrophages and DCs and is induced by IFN-g and TNF. *It acts on CXCR6-expressing cells (T cells, NKT cells) in membrane-bound and shed forms. Hence, it provides a loop of amplification of cell–cell interaction and recruitment in polarized type I responses.*

M2-inducing signals generally inhibit the expression of M1 chemokines. The TLR- and IFN-g-dependent induction of CXCL10, CCL5 and CXCL9 is inhibited by IL-4 and IL-10. The inhibitory effects of IL-10 on LPS-activated macrophages rely on both STAT3-dependent mechanisms and the inhibition of NF-kB*.*

Furthermore, IL-10 directly inhibits CXCL10 and CXCL9 gene expression through the inhibition of STAT1 phosphorylation. Glucocorticoids also suppress CXCL10 production by LPS-treated macrophages through the inhibition of STAT1. IL-10 also alters KC mRNA stability by preventing the usually stabilizing effect of LPS.

The proto-oncogene c-Maf might also have a role in IL-10 activity. This transcription factor represents one of the physiological mediators of IL-10 activity, and when overexpressed it inhibits transcriptional activation of the IL-12p35 and p40 genes and it also upregulates IL-10 and IL-4. Suppression of the transcriptional activation of IFN-gand LPS-responsive genes by IL-4 requires STAT6, which acts by sequestering coactivator molecules required for the action of STAT1 and NF-kB. M2 macrophages obtained from mice implanted intraperitoneally with the filarial nematode Brugia malayi display an IL-4-dependent inhibition of the proinflammatory chemokines CCL3 and CCL4.

Mouse M2 macrophages have a crucial role in the correct cytokine balance during parasite infection. Overall, evidence suggests that signals inducing M2 polarization (e.g. IL-10, IL-4, IL-13) downregulate NF-kB and STAT1 activities, and thus act as a common mechanism to limit the induction of inflammatory chemokines associated with the development of type I immunity and inflammation.

M2-inducing signals are not simple inhibitors of proinflammatory chemokines, in that they induce expression of a specific subset of chemokines generally associated with a type II response. IL-4 and IL-13 selectively induce CCL24 , CCL17, and CCL22 in M2a macrophages, with inhibition by IFN-g. IL-4 also induces the production of CCL2, a chemokine associated with Th2 polarization by analysis of gene targeted mice.

The CCR4 agonist CCL17, produced by M2a cells, along with IL-10, inhibits the CpG-mediated M1 activation. The M2-associated agonist CCL22 is processed and inactivated by dipeptydilpeptidase IV (CD26), expressed on a variety of cell types and, among lymphocytes, preferentially on Th1 cells. CCL22 is also recognized and inactivated by the promiscuous receptor D6, a decoy and scavenger for inflammatory CC chemokines expressed on lymphatic endothelium.

In spite of its promiscuous recognition of CC chemokines, D6 binds and scavenges CCL22 but not the processed forms [CCL22, CCL22 and CCL22]. Thus, CCL22, produced by polarized M2a cells, is tuned at the level of induction, processing and scavenging. CCL18 expression is induced by Th2-associated cytokines, such as IL-4, IL-13, and IL-10, whereas it is inhibited by IFN-g. IL-10 induces production of both CCL18 and CCL16 in M2c human monocytes and macrophages.

Perhaps unexpectedly, CCL16 activates macrophages for tumor cytotoxicity. However, consistent with a role in type II inflammation, CCL16 attracts eosinophils through the histamine receptor type 4. The receptor for CCL18 has not been identified. CCL18 attracts naive T cells and is produced by tumor-associated macrophages. Recruitment of naive T cells in a microenvironment dominated by IL-10, which inhibits DC maturation, might result in tolerance and immunoregulation. As discussed earlier, IL-10, together with LPS, stimulates CXCL13 production in human monocyte-derived DCs and, less prominently, in monocytes.

The CXCL13 receptor CXCR5 is expressed on B cells and on a subset of CD4C T cells, which home to lymphoid follicles and provide help for antibody production. Therefore, CXCL13 facilitates a three-party interaction among monocytes or DCs, CXCR5C follicular type Th cells and B cells. In addition to CXCL13, IL-10 induces IL-7 under the same conditions. CXCR5, and its cognate ligand CXCL13, and IL-7 are all part of a cytokine cascade that sustains the organization of secondary lymphoid tissues. Therefore, IL-10-induced CXCL13 in M2 cells and DCs might promote the organization of extranodal lymphoid follicles in chronic inflammatory conditions

Mempel et al note regarding cancers and chemokines:

In principle, cancer cells of all tissue origins seem to be capable of activating or repressing expression of any chemokine gene.

However, in line with their complex interactions with the immune system, successful cancer cell clones are likely selected for their expression of chemokines that attract immune-suppressive and tissue repair-promoting cells such as most neutrophils and TAMs and selected against expression of chemokines that attract antitumour effector cells. In addition to the tissue of origin,

oncogene activation as well as epigenetic reprogramming can shape their chemokine expression profile in ways that support a favourable immune contexture.

For instance, gain-of-function mutations in the gene encoding β-catenin or loss-of-function mutations in genes encoding its negative regulators are frequent and strongly correlated with poor T cell infiltration in human melanoma2. In a mouse melanoma model, β-catenin signalling was found to repress cancer cell expression of the CCR5 ligand CCL4, which resulted in poor accumulation of cDC1s and intratumoural CD8+ T cell activation, suggesting a potential general mechanism by which melanoma cells restrict T cell inflammation2. An alternative, epigenetic mechanism to limit type I immunity has been observed in human ovarian cancer cells, in which both enhancer of zeste 2 (EZH2)-mediated histone H3K27 trimethylation and DNA methyltransferase 1 (DNMT1)-mediated DNA methylation directly repress CXCL9 and CXCL10 genes.

Cancer cells can also rid themselves of immunogenic chemokines by genomic deletion, as has been shown for loss of CXCL13 in chromosomally unstable colorectal carcinoma, which was correlated with reduced T and B cell infiltration and shortened survival of patients2. Different mechanisms also enable cancer cells to increase expression of chemokines that support their malignant behaviour.

For instance, reduced expression of the RAF kinase inhibitory protein (RKIP) is characteristic of human invasive and metastatic triple-negative breast cancers. This is reportedly explained by the ability of RKIP to inhibit expression of the transcriptional regulator high mobility group AT-hook 2 (HMGA2), which in turn drives expression of the TAM recruiting CCR5 ligand CCL5. Reduced RKIP, therefore, leads to increased CCL5 release by cancer cells and thereby increased recruitment of pro-metastatic TAMs1.

Also, in the context of experimental breast cancer in mice, a CRISPR loss-of-function screen for tumour suppressor genes identified the RHO-signalling regulator guanine nucleotide-binding protein subunit α-13 (GNA13). Loss of GNA13 was found to enhance CCL2 secretion (a ligand of CCR2 on monocytes), which promoted TAM accumulation and a pro-tumour microenvironment.

A different study found that the oncogene MYC drives expression of the translational repressor fragile X messenger ribonucleoprotein (FMRP). As CCL7 mRNA is a target for repression by FMRP, MYC activation restricts CCL7 expression2. Surprisingly, although CCL7 is another ligand for CCR2 expressed on monocytes and its increase will likely enhance monocyte recruitment to the TME, the authors of this study found that rescuing expression of CCL7 by deleting FMRP in cancer cells also enhances T cell infiltration and restricts the growth of experimental PDAC tumours, further highlighting the fact that enhancing monocyte recruitment can have opposing effects in different contexts, perhaps depending on their differentiation into tumour-supportive or tumoricidal TAM states.

These few examples probably only represent a small fraction of the mechanisms by which cancer cells can modulate the expression of chemokines to produce favourable growth and immune-

suppressive environments for themselves, some of which can conceivable be targeted therapeutically.

6 MACROPHAGES AND TUMOR INTERACTIONS

Macrophages have multiple tumor interactions. M1 macrophages can be deployed to destroy a malignant cell. However the class of M2 macrophages can become supports of the tumor. 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.

This is a brief summary of the four current types of M2 macrophages.

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…*

***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.

The detailed functions will be examined.

6.1 SPECIFIC INTERACTIONS

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.

6.2 VARIOUS TUMORS AND TAMS

As Ammendola et al note:

Breast cancer (BC) is the most common malignant tumor in women, and distal metastasis of highly invasive breast cancer cells is the major cause of death in these women. BC could be divided into three groups: BC expressing hormone receptor, estrogen receptor (ER+) or progesterone receptor (PR+), BC expressing human epidermal receptor 2 (HER2+) and triple-negative breast cancer (TNBC) (ER−, PR−, HER2−).

Regarding BC, a strong correlation with M2 type macrophages has been proven in murine models, while in vitro studies have shown that TAMs co-cultivated with breast cancer cells upregulate the production of matrix metalloproteases, stimulating tumor growth and angiogenesis.

The preliminary data suggests, intuitively, that an abundance of TAMs would have a certain negative prognostic role in BC; however, this correlation remains controversial. … concluding that TAMs infiltration was associated with an aggressive behaviour, in the form of reduced overall survival (OS), disease free survival (DFS) and relapse free survival (RFS). However, inconsistencies emerge from the use of different biomarkers to identify TAMs population: CD68 was deemed more accurate than CD206 or CD163 in this regard. Moreover, … high density of M2 type in TNBC is associated with poor prognosis and increased risk of metastasis.

Immunohistochemical studies have shown that specific markers like CD 136 and CD 204, which can be used like target during chemotherapy, characterize M2 population. Moreover, Chen et al. have found that M2 phenotype promotes metastasis both in breast cancer and in gastric cancer in murine models via an increase in chinase 3 like 1 protein (CHI3L1).

So, CHI3L1 interacts with interleukin-13 receptor α2 (IL13Rα2) on the membrane of cancer cells, promoting the production of matrix metalloproteases via the activation of mitogen activated protein kinase (MAPK) pathway.

TAMs and Gastric Cancer Although GC also originates from chronic inflammation or Helicobacter pylori infection M2 type macrophages play a crucial role in GC development,

because their presence and density modify the prognosis of tumour and the resistance to treatment.

Different studies have already described the relationship between GC and macrophage infiltration; for example, Sammarco et al. have demonstrated how TAM infiltration changes the prognostic factor in surgically resectable GCs. Furthermore, the treatment of GC, angiogenesis has become the cornerstone of chemotherapy. Novel therapeutic agents are prepared to reduce neo-angiogenesis, such as ramucirumab, or others that target CSF-1R such as emactuzumab. In addition, Eum et al. have shown that the macrophages found in the malignant ascites of advanced gastric cancer patients express an M2 phenotype, and have associated this finding with a worsened prognosis.

Macrophages play also a prognostic role, according to a study conducted by Svensson MC et al. In 148 patients with resectable Esophageal and Gastric (EG) adenocarcinoma, an Immunohistochemical analysis was conducted, highlighting that M2 type CD68+/CD163+ determinate a poor prognosis, instead of the presence of CD68+/CD163-, despite the use of neoadjuvant chemotherapy (NAC).

For locally advanced EG Adenocarcinoma, it has been shown that FLOT scheme in NAC in CD68+/CD163- cluster promotes the overall survival, the regression in size of the primary tumor and the reduction of distant metastases.

…high M2 type and total TAMs density were correlated to low overall survival (OS), whereas, a high M1 type density with increased OS. The progression of EG adenocarcinoma depends on specific cluster, not only on Macrophage's type.

Another interesting aspect regards the relationship between exosomes and TAMs and their correlation in GC progression. GC related exosomes, recruiting PD1+ TAM and inhibiting CD8+ T cells, are capable to increase tumoral progression. Furthermore, GC exosomes transfer ApoE into GC cells, by PI3K/Akt pathway, and can model the cyto-skeleton, promoting tumoral cell migration to distant sites.

TAMs and Colo-rectal Cancer Similar controversies emerge regarding CRC, as some studies highlight a positive prognostic role of TAMs, while others associate an abundance of TAMs with a worsened prognosis.

For instance, … several studies have underlined the role of TAMs in locally advanced colorectal cancer, describing an unfavorable prognostic role despite early surgery. …in 1,008 CRC biopsies that the number of TAMs does not differ between CRCs treated with chemotherapy and CRCs that have not been treated. On the other hand, it is pivotal to expand upon this field of research, particularly about the impact of TAMs in hepatic metastasis due to their involvement in the promotion of angiogenesis.

Takasu et al. studied the effect of TAMs in hepatic secondary lesions in 71 patients, who underwent curative surgery (R0) for CRC and were diagnosed with liver metastasis. According to this study, TAM density is high in small tumors and is correlated with less aggressive features.

Several studies have shown the different impact of M1 and M2 type on CRC. M1 macrophages demonstrate to have a poor correlation with tumoral progression; mean-while M2 macrophages are strictly correlated with the presence of liver metastases and dedifferentiated tumors.

Besides, it was hypothesized that the M1/M2 ratio could be used to predict liver metastases in CRC. For example, in a cohort of 360 patients a simple blood test was performed, analysing peripheral blood mononuclear cells. The results show a rise of these cells in CRC.

Hence, the ratio M1/M2 may be used like a novel biomarker for the treatment and its prognostic value in CRCs.

TAMs, Angiogenesis and Lymphoagenesis Angiogenesis and lymphangiogenesis are phenomena that occur mainly during embryogenesis, because their presence is reduced during growth when they start limited their presence to sites of wound healing and inflammation. The role of angiogenesis is significant in cancer, as it is known to drive tumor growth. Various stimuli deriving from innate immune cells can drive the angiogenetic process during tumor growth, primarily the production of pro-angiogenic factors within the TME.

Tumor lymphan-giogenesis plays a fundamental role in the development of metastasis and may occur both within the primary tumor and/or in the tumor periphery.

Angiogenesis and lymphan-giogenesis are driven by both stimulatory and inhibitory signals. Vascular endothelial growth factor (VEGF)-A is a known agonist of VEGFR2 found on blood endothelial cells (BECs).

VEGF-C and VEGF-D play a key role in the survival of lymphatic endothelial cells (LECs), along with their proliferation and migration, through the engagement of VEGFR3. VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) bind to three endothelial receptors: VEGFR1, VEGFR2 and VEFGR3. VEGF-A promotes the survival, proliferation, sprouting and migration of BECs, increases endothelial permeability and has a proinflammatory role.

It is also involved in lymphangiogenesis: both directly, by binding to VEGFR2/VEGFR3 heterodimer receptor, and indirectly by stimulating the production of VEGF-C and VEGF-D by immune cells (e.g., macrophages, mast cells). PlGF and VEGF-B bind to VEGFR1 on BECs, along with various immune cells and pericytes. Angiopoietins (ANGPT1 and ANGPT2) bind with Immunoglobulin-like and EGF-like domains-1 (TIE1) and TIE2 receptors and modulate angiogenesis and lymphangiogenesis through the engagement of Tyrosine Kinase. ANGPT1, expressed by pericytes, encourages BEC survival, whereas ANGPT2, secreted by BECs, acts as an autocrine and paracrine TIE2 ligand.

Numerous studies show that the pro- or an-ti-tumorigenic function of immune stromal cells is cancer specific regarding different solid tumors (breast, prostate, pancreas, gastric and colorectal), and depends on the stage of the tumor and on their localization within the microenvironment. Evidence shows that certain subsets of these cells may play a protective role

whereas other types may have a pro-tumorigenic function. Single-cell mapping of peri-tumoral and intra-tumoral immune cells might aid in defining the roles of different subtypes of immune stromal cells in the onset and progression of various solid tumors. Angiogenesis is a key component of cancer as it plays a crucial role in tumor growth.

Furthermore, lymphangiogenesis, defined as the development of new lymphatic vessels, is involved in the metastatic process of many kinds of tumor. Many innate immune cells can stimulate tumor growth by encouraging angio-genesis, mainly by producing angiogenic molecules within the TME.

For example, macrophages are involved in the production and secretion of metal-loproteinase-9 (MMP-9) which degrades the extracellular matrix, releasing the VEGF stored within.

The angiogenetic process is characterized by two different biological pathways: the first one is the MyD88-dependent pathway leads to the activation of nuclear factor kappa (NF-KB) and mitogen-activated protein kinase (MAPK).

The second one is the TIR-domain-containing adapter-inducing interferon-β dependent pathway causes the activation of serine/threonine-protein kinase-1 and receptor-interacting serine/threonine-protein kinase.

These intracellular cascade signals, in the end, stimulate TAMs to secrete various pro-angiogenic factors, such as VEGF, TP, FGF-2, TNF-α, IL-1, -6, -8. In fact, an increased expression of TLRs can be found in tumor cells, cell lines, and tissues. Additionally, angiogenesis is controlled by both stimulatory and inhibitory signals.

7 MACROPHAGE THERAPEUTICS

As we have demonstrated macrophages in the TAM are targets for therapeutics. By targeting them it would break down the wall of the TMA and potentially all effective use of existing therapeutics. We examine some of the efforts in this area.

7.1 OVERVIEW

We use the recent work by Mantovani et al to examine the multiple targets in TAM as a means to address TAM therapeutics. This is a recent set of therapeutic targets. As the authors note:

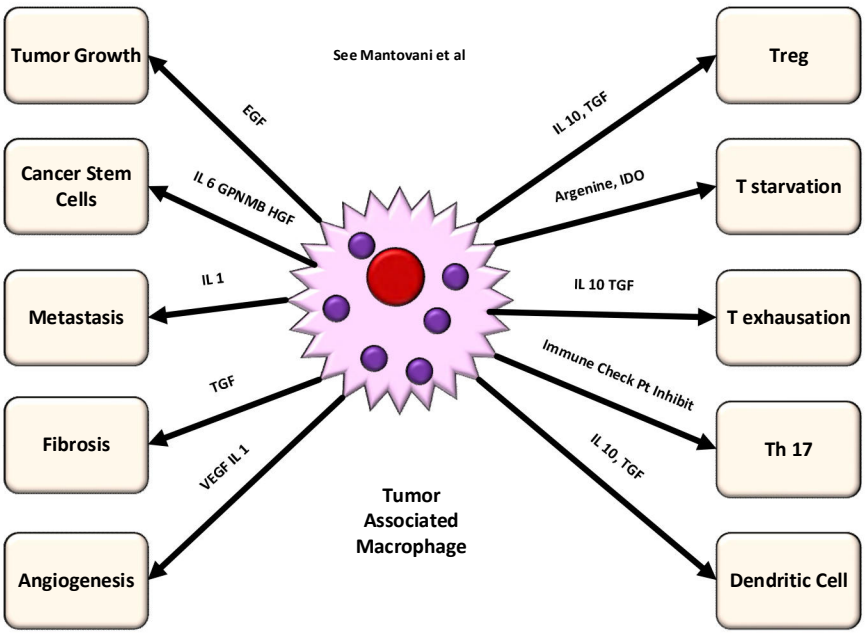
Tumour-associated macrophages are an essential component of the tumour microenvironment and have a role in the orchestration of angiogenesis, extracellular matrix remodelling, cancer cell proliferation, metastasis and immunosuppression, as well as in resistance to chemotherapeutic agents and checkpoint blockade immunotherapy.

Conversely, when appropriately activated, macrophages can mediate phagocytosis of cancer cells and cytotoxic tumour killing, and engage in effective bidirectional interactions with components of the innate and adaptive immune system.

Therefore, they have emerged as therapeutic targets in cancer therapy. Macrophage-targeting strategies include inhibitors of cytokines and chemokines involved in the recruitment and polarization of tumour-promoting myeloid cells as well as activators of their antitumorigenic and immunostimulating functions. Early clinical trials suggest that targeting negative regulators (checkpoints) of myeloid cell function indeed has antitumor potential.

Finally, given the continuous recruitment of myelomonocytic cells into tumour tissues, macrophages are candidates for cell therapy with the development of chimeric antigen receptor effector cells. Macrophage-centred therapeutic strategies have the potential to complement, and synergize with, currently available tools in the oncology armamentarium.

As Mantovani et al note:



The authors provide the following as explanation:

The pro-tumour functions of tumour-associated macrophages (TAMs) are diverse and act at different phases of tumour development. TAMs release nitric oxide (NO) and reactive oxygen intermediates (ROI), which cause DNA damage and genetic instability during the initiation phase.

TAMs produce epidermal growth factor (EGF) and several mediators such as IL-6, hepatocyte growth factor (HGF) and GPNMB, which support cancer stem cell expansion. At later stages, TAMs contribute to metastatic spread by releasing IL-1 and transforming growth factor-β (TGFβ), which are also involved — together with several proteases — in extracellular matrix (ECM) remodelling and pathological fibrosis.

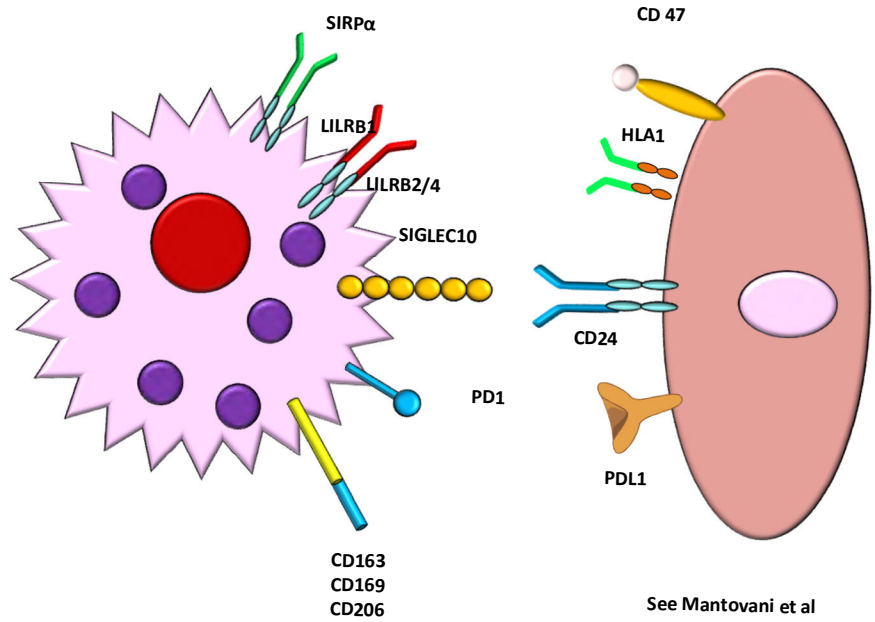
TAMs are a critical source of angiogenic factors: vascular endothelial growth factor (VEGF) and pro-angiogenic chemokines. TAMs are drivers of immunosuppression in the tumour microenvironment.

Secretion of IL-10, TGFβ, prostaglandins and indoleamine 2,3-dioxygenase (IDO) promote the expansion of regulatory T (T reg) cells, inappropriate skewing of dendritic cells towards an immature and tolerogenic state, and T cell metabolic starvation.

Immunosuppressive TAMs are characterized by a high expression of immune-checkpoint molecules (PDL1, PDL2, B7-H4) causing T cell exhaustion. EMT, epithelial– mesenchymal transition; ILC3, type 3 innate lymphoid cell; TH17, T helper.

Thus TAMs can provide extensive suppression factors preventing immunotherapeutic efficacy. However understanding these targets yield putative therapeutic options.

The authors continue with specific targets as shown below:



The authors details the above as follows:

Overview of myeloid checkpoints and inhibitory receptors expressed by tumour-associated macrophages (TAMs) and their ligands expressed on tumour cells or cell debris. These include the receptor/ligand pairs signal regulatory protein-α(SIRPα)–CD47, LILRB1–HLA1, sialic acid-binding immunoglobulin-like lectin 10 (SIGLEC10)–CD24, and PD1–PDL1, which inhibit phagocytosis, and macrophage receptor with collagenous structure (MARCO), CD169 and mannose receptor scavenger receptors.

Clever 1, triggering receptor expressed on myeloid cells 2 (TREM2) and P-selectin glycoprotein ligand 1 (PSGL1) are also depicted. Targeting of Clever 1 and TREM2 does not specifically interfere with phagocytosis but with immunosuppressive activation.

Now specific checkpoints and inhibitors detailed include:

SIRPα and CD47. *CD47 is expressed on normal cells and serves as a 'don't eat me' signal, instructing mononuclear phagocytes and neutrophils expressing SIRPα to spare host cells from removal181–1. Loss of CD47 is associated with ageing of red blood cells and allows their disposal, an illustration of the importance of this pathway.*

Tumour cells overexpress CD47 in many cancer types, disguising as healthy cells and avoiding phagocytosis. CD47-targeting approaches include both, antibodies anti-CD47 and anti-SIRPα….

Emerging evidence points to combination as the key to success of immune-based strategies. The strength of therapeutics disrupting the CD47–SIRPα axis resides in the possibility to

concomitantly render macrophages more phagocytic and increase their antigen load, thus enhancing antigen presentation to T cells185 (Fig. 3b). Therefore, in a stepwise manner, CD47 approaches may be synergistically effective in combination with T cell checkpoint inhibitors, first improving phagocytosis and antigen presentation and second unleashing a response of activated T cells

The SIGLEC family. *SIGLEC molecules are membrane* ***proteins that bind sialic acid and engage in cell–cell interactions****.*

These proteins contain tyrosine-based inhibitory receptor motifs (ITIMs) in their cytoplasmic tail, which are typically components of those immune receptors that inhibit and suppress activation signals, thus regulating the functions of several immune cells.

The LILRB family*. Downregulation of MHC class I molecules is probably one of the best-known mechanisms of evasion used by cancer cells to circumvent recognition by T cells1. However, tumour cells can exploit MHC class I as a mechanism of evasion from phagocytosis by interacting with LILRB family members. LILRB1 is an MHC-binding protein widely expressed on immune cells and enriched on TAMs197; it contains an ITIM motif and transduces an inhibitory signal. LILRB expression was associated with the inhibition of phagocytosis of cancer cells.*

In fact, its role as a myeloid checkpoint was discovered by analysing cancer cell lines resistant to the anti-CD47 antibody1. The expression of MHC class I by tumour cells correlated with the degree of their resistance to anti-CD47, and phagocytosis induced by the anti-CD47 antibody was restored by LILRB1-blocking antibody

PD1. *PD1 expression by TAMs inhibits phagocytosis and tumour immunity94, twisting the traditional view of the PD1–PDL1 axis as a specific T cell checkpoint. PDL1 expression on cancer cells may thus concomitantly enable evasion from T cell cytotoxicity and macrophagemediated phagocytosis, suggesting that blockade of this axis might unleash antitumour immunity by both adaptive and innate mechanisms.*

The mechanism of phagocytosis inhibition triggered by the engagement of PD1 on macrophages has not yet been elucidated nor have the signals inducing PD1 upregulation. SIRPα, LILRB1 and PD1 all contain an ITIM domain, which could be instrumental for the downstream signals inhibiting phagocytosis, but a formal demonstration has not been provided.

On this basis, studies aimed at monitoring response in patients with cancer undergoing checkpoint inhibitor treatment should consider the myeloid compartment as a potential target and as a predictive biomarker

As Vanmeerbeek et al note:

As described above, TAMs are greatly influenced by the dying cancer cells, which is intensified by anti-cancer therapies, and in turn, can also contribute to immunotherapy resistance For this

reason, combining traditional therapies together with TAM-targeting therapies has been emphasised over the last decade. So far, ***there are three main strategies for targeting the TAMs to overcome their pro-tumour properties:***

(I) Limiting their recruitment;

(II) repolarisation toward M1-like TAMs; and

(III) total TAM depletion.

Considering a large amount of TAM targeting strategies, we focus our further discussion on only those approaches that have been, or are currently being, tested in a human context. Of note, it is important to keep in mind that these therapies are always used together with cell death inducers. Therefore, the results from these agents are always intertwined.

7.2 Limiting the Recruitment of TAMs

In order to reduce TAM accumulation at the tumour site, antagonists against chemokine receptors, such as CCR2, have been developed. CCR2 functions as a receptor for CCL2 and is mainly expressed by TAMs, monocytic cells, dendritic cells (DCs) and endothelial cells but also neutrophils and lymphocytes.

CCL2 binding to its receptor has an anti-apoptotic effect and promotes angiogenesis and cell migration [135,136]. It has been suggested that blocking the CCL2-CCR2 axis can inhibit the recruitment of TAMs to the tumour site. Initial studies inhibiting CCR2 by the use of antibodies have shown reduced tumour growth and improved efficacy of chemotherapies in multiple murine tumour models. Unfortunately, clinical trials using CCR2 targeting agents, such as carlumab, a human anti-CCR2 antibody, alone or in combination with chemotherapy, resulted in only a short-term CCR2 suppression in the serum and no anti-tumoural response.

Additionally, it has been shown that the destabilisation of CCR2 after therapy can cause increased cancer progression.

Additionally, CCR2/CCR5 dual inhibitors have been put forward as a potential manner of decreasing TAM recruitment. Presently, a phase I/II clinical trial is testing the potential of BMS-813160, anti-CCR2/CCR5, in colorectal and pancreatic cancer (NCT03184870).

**Another pathway targeted to limit TAM accumulation is the CXCL12/CXCR4 axis. The binding of CXCL12 to CXCR4 is involved in multiple biological processes such as proliferation, angiogenesis and metastasis of cancer.*

Inhibiting CXCR4 with AMD3100/plerixafor has been tested in clinical trials for patients with colorectal or pancreatic cancer. Indeed, AMD3100/plerixafor did increase the amount of intra-tumoural T cells, although the limited treatment period in these particular studies did not result in any treatment response. Furthermore, multiple similar studies using different CXCR4 antagonists have been started and are currently ongoing.

6.2. Repolarisation of TAMs

The second mode of action for TAM-targeted immunotherapy is reprogramming the TAM compartment to be more immunogenic and anti-tumoural.

Presently, a lot of efforts have been directed at inhibiting the CSF1R-CSF1 axis. This is either achieved by the use of monoclonal antibodies against CSF1R or CSF1 or by the inhibition of tyrosine kinases downstream of CSF1R. Current CSF1R targeting therapies... As of yet, there has not been a consensus about the mode of action of CSF1R inhibition.

Some studies have shown evidence that suppressing the CSF1R-CSF1 axis will repolarise the TAM compartment while others demonstrate partial depletion of the M2 TAMs. Many clinical trials are targeting the CSF1R axis as monotherapy but also in combination with treatment regimens such as chemotherapy are currently ongoing. So far, there have only been some anecdotal reports of clinical responses.

For example, 3 out of 146 patients with solid tumours receiving BLZ945 showed partial response. Furthermore, additional analysis of peripheral blood monocytes did not show any changes after treatment. Another strategy to block the immune suppressive properties of M2 TAMs is by controlling the TAM polarisation. Toll-like receptor activation is considered to be an attractive strategy for achieving more M1-like polarisation.

Multiple TLR agonists are currently being used in clinical trials as an anti-cancer treatment, although they are not specifically targeting TAMs. Currently, efforts have been made to develop strategies more targeted towards TAMs, such as R848, TLR agonists, or nanoparticles containing mRNA or TLR agonists showing promising results. Additionally, increasing phagocytosis by blocking CD47, a well-known 'do notx eat me' signal (i.e., efferocytosis inhibitor), has been shown to cause TAM repolarisation. This surface ligand found on all cells modulates efferocytosis by interacting with the signal regulatory protein alpha (SIRPα) on TAMs.

When a target for phagocytosis expresses CD47, the internalisation step of phagocytosis gets inhibited, blocking phagocytosis altogether.

Using CD47 targeting antibodies such as magrolimab, one can increase phagocytosis and therefore enhance the M1 phenotype.

However, the use of anti-CD47 was not effective as a monotherapy. However, the combination of magrolimab with an anti-CD20 antibody caused a 36% complete response rate in non-Hodgkin's lymphoma (NHL), albeit in a small cohort. So far, multiple phase I clinical trials focusing on CD47 suppression have been initiated and have shown limited results, largely due to toxicity concerns due to CD47's ubiquitous expression in many non-tumour organ systems. Finally, anti-SIRPα clinical trials have also started to address the concerns about the side effects of anti-CD47 immunotherapy.

6.3. Depletion of TAMs

As mentioned above, CSF1R targeting studies have reported a partial depletion effect in the TAM compartment. For this reason, a large portion of the TAM-depleting studies are using CSF1R targeting therapies. Higher concentrations of CSF1R blockers have been found to be depleting the TAMs as well as the resident macrophages. In general, more off-target effects can be expected from high-dose therapies, something that is not yet clear for anti-CSF1R. Apart from anti-CSF1R, one of the chemicals for TAM depletion used in pre-clinical models is clodronate liposomes.

Liposomes encapsulating clodronate are endocytosed by phagocytes. Since the clodronate cannot cross the phospholipid bilayer, it will accumulate, ultimately causing apoptosis. However, this approach is non-specific and highly cytotoxic because of its capacity to target not only pro-tumoural macrophages but also the entire phagocyte compartment.

6.4. CAR-Macrophages

The use of chimeric antigen receptor (CAR) macrophages as an anti-cancer therapy is one of the most recent approaches being explored. CARs are synthetic receptors bioengineered to recognise specific target antigen.

Generally, T cells are used as a host for the CAR to generate a robust T-cell response. Similar to T-cell CARs, Macrophage CARs consist of an extracellular domain for specific antigen recognition, a hinge domain, a transmembrane domain of CD8α and an intracellular domain of FCER1G, MEGF10, MERTK or CD3ζ molecules for downstream signaling. Binding to the CAR induces phagocytosis of the cancer cells with the specific antigen, which is subsequently presented to T cells to initiate anti-cancer immunity.

Momentarily, the second generation of CAR macrophages is being developed that focuses on the improvement of T-cell activation and antigen presentation. Additionally, special attention is being paid to potentiating the anti-cancer phenotype of the CAR macrophages [168,170]. Presently, a first in human clinical trial is investigating the therapeutic potential of CT-0508, autologous second-generation macrophages expressing an anti-HER2 CAR, in metastatic solid cancers (NCT04660929).

So far, mouse studies have revealed that CT-0508 causes phagocytosis of cancer cells specifically, thereby decreasing tumour growth and increasing survival.

However, its efficiency in the human context remains to be determined. Of note, although the United States (US) Food and Drug Administration (FDA) has granted a fast development track for the CT-0508 modality, there are still some concerns. At this point, upscaling CAR macrophages is difficult since there is still an expansion problem. For this reason, novel strategies are being created that utilise pluripotent stem cells (iPSCs) that can be differentiated towards myeloid/macrophage lineages.

We believe if these obstacles are overcome soon…

7.3 TARGETS

As cancer therapeutics have evolved, they rely upon the ability to have selected tumor cell targets to use as a focus. The targeting of the tumor cell as well as the protecting TAM allow for a mor comprehensive approach. As Chen et al note:

Cancer promotion and advancement: Chronic inflammation may be linked to tumor beginning since it was shown that there were many inflammatory cells in tumor biopsy samples. This is true for gastric and colon cancer. This is because oncogene activation or chronic inflammation (from infection or exposure to irritants) may trigger the production of pro-inflammatory transcription factors, including NF-κB, STAT3, and HIF-1α.

To attract macrophages, cancer cells may produce cytokines and chemokines (TNF-α and IL-6), which may activate these factors.309 The production of a mutagenic microenvironment aids cancer development by macrophages, which may release inflammatory mediators like IL-6, TNF, and IFN-γ, growth factors like epidermal growth factor (EGF) and Wnt, proteases, ROS, and nitrogen compounds.

… found that TAM-derived IL-17 and IL-23 were associated with colon cancer development and progression…. found that IL-6 produced by TAMs promoted HCC growth by activating the STAT3 signaling pathway, suggesting that IL-6 was involved in HCC formation. To sum up, TAMs may play a wide variety of roles in the onset and progression of cancer. Invasion, metastasis, and angiogenesis.

The spread of cancer via invasive cells and distant organs is the leading cause of mortality. Because of their enhanced motility and the degradative enzymes they produce, cancer cells can break away from the initial tumor and invade other places, where they may develop new tumors.

EMT refers to the process through which epithelial cells acquire mesenchymal characteristics and acquire malignant biological traits such as invasion and metastasis. All through the EMT process, tumor cells give up cell-to-cell intersections and apical-basal polarity because of E-cadherin suppression and secure an adaptable phenotype of mesenchymal cells. Naturally, macrophages partake in the EMT procedure by discharging different dissolvable factors, for example, TGF-β, TNF- α, IL-1β, and IL-8.315

Recent investigations have shown that TAMs enhance metastasis and help regulate the EMT process. … TAMs boost the invasion and metastatic potential of CRC cells by inducing an EMT. Furthermore, CCL2 production upon activating this axis may aid in macrophage recruitment. High TCF4 expression was also linked to macrophage recruitment and polarization in metastatic locations. In addition, it was shown that the CCL2/CCR2 signaling pathway promoted metastasis. …

It was shown that TAMs might release CCL2, which induced MCF10A to develop an EMT and an invasive phenotype by increasing endoplasmic reticulum oxidoreductase-1 (ERO-1) and matrix metalloproteinase-9 (MMP9). Similarly, TAM-secreted CCL5 may significantly

increase prostate cancer cell invasion, metastasis, and EMT through activation of the β-catenin/STAT3 signaling pathway. With the help of CCL5 binding to CCR5 in macrophages, malignant phyllodes tumor could attract and repolarize TAMs, activating the AKT signaling pathway.

Myofibroblast differentiation and invasion were further aided by TAM-generated CCL18 binding to the myofibroblast receptor PIPTNM3. It was observed by Lan et al. that CCL26, when combined with CCR3, might cause TAM invasion. CCL26 upregulation by phosphatase of regenerating liver-3 (PRL-3) promoted TAM infiltration, invasion, and metastasis in CRC. TAMs co-cultured with NSCLC cells produced conditioned media that promoted tumor cell invasion through EMT and B-Crystallin (CRYAB) overexpression, which induced lung cancer metastasis in vivo.

According to Han's results, TAMs promote osteosarcoma metastasis and invasion by increasing the production of COX-2, MMP9, and phosphorylated STAT3, which induces EMT. Some TAMs express EMT-inducing substances, such as TGF-β and IL-6. In addition, it has been shown that TAMs release EGF, which may induce EMT by activating the EGFR/ERK1/2 signal pathway in cancer cells. The M2 macrophage expresses chitinase 3-like protein 1 (CHI3L1), advancing breast cancer and gastric cells. A system upsetting macrophage activities by genetic strategies lessens the tumor cell's endurance in pulmonary vessels and annuls tumor penetration into the lung.

Selected macrophages trigger the PI3K/Akt survival signaling pathway in recently scattered breast cancer cells by drawing in vascular cell adhesion molecule-1 (VCAM-1) employing α4 integrins. It is accepted that metastasis isn't essential to be an advanced late activity in tumor progression. Auxiliary body organs are sufficiently primed by the primary tumors and direct organ-explicit dispersal before entering tumor cells.

Moreover, those "prepared" destinations are inclined to metastasis and are presented as the idea of pre-metastatic niches (PMNs). PMNs are efficiently organized and determined by essential macrophages. They were prepared for the circulation system and then bunched in the premetastatic destinations by an assortment of tumor-derived factors, such as exosomes, CSF-1, CCL2, TNF-α, VEGF, TGF-β, PLGF, and tissue inhibitor of metallopeptidase (TIMP)… Moreover, the tissue-resident macrophages, for example, osteoclasts, pulmonary alveolar macrophages, and liver KCs, were likewise associated with organizing PMN development upon incitement.

Besides, macrophages similarly build up associated metabolic cross-talk with immune cells like dendritic cells and Th1 cells and suppress their related tumoricidal and additional tumor antigen-exhibiting features, advancing the flourishing of those recently held-up tumor cells in a strategy for immunosuppression. Angiogenesis. TAMs may indirectly impact tumor development by increasing angiogenesis and their potential to promote inflammatory processes connected to cancer.

The increased oxygen and food requirements of cancer cells need the initiation of angiogenesis. Neovascularization included a wide range of factors like hypoxia, hyperosmotic pressure, and

angiogenic factors like VEGF, TGF-β, COX-2, placenta growth factor (PGF), fibroblast growth factor (FGF), angiotensin (Ang), and chemokines, is essential for tumor invasion and metastasis. Tumor cells expressed HIF in hypoxic regions, which produced proangiogenic molecules (including VEGF-A and FGF-2). Consistent with these observations is the discovery that HIF-1 may stimulate VEGF expression in hypoxic glioma.

… EGF released by TAMs could activate the EGFR on the surface of tumor cells, thereby increasing VEGF/ VEGFR signaling and helping ovarian cancer cells proliferate and invade. TAMs were shown to stimulate tumor angiogenesis by Cui's group through increased TGF-1 and IL-10 production, stimulating endothelial cell proliferation.

Indirectly aiding angiogenic invasion, TAMs produce proteases such as MMP9, MMP2, and MMP3, which allow them to destroy ECM.

Since an abnormal Wnt/β-catenin signaling cascade promoted cancer formation, it is clear that this route plays a role in cell proliferation, apoptosis, invasion, and metastasis. TAMs were shown to increase the expression of Wnt7b (a member of the Wnt family of ligands), which may encourage tumor neovascularization.

Recurrence and CSC.

The ability to self-renew and give rise to a diverse population of tumor cells distinguishes CSCs from other tumor cells. discovered that TAMs, via STAT3 signaling, may produce IL-6, promoting HCC stem cell growth. TAMs generate chemokines, including CXCL8 and CXCL12, which may instruct cancer cells to acquire a CSC-like character and sustain stemness in oral squamous, HCC, and renal cell carcinoma. The association between hyaluronic acid (HA) (the ligand of CD44) and CD44 was enhanced by HAS2 in TAMs obtained from patients with head and neck squamous cell carcinoma, as demonstrated by Gomez's group.

The PI3K-4EBP1-SOX2 signaling pathway was activated when HA coupled to CD44, which enhanced stemness.339,340 TAMs secrete milk-fat globule-epidermal growth factor-VIII (MFG-E8), which activates STAT3 and the Shh signaling pathway in CSCs, resulting in CSCs exhibiting treatment resistance and enhanced tumorigenicity.341 The S100 calcium-binding protein A9, a secreted protein associated with inflammation and poor survival in HCC patients, was considerably upregulated by TAMs, reinforcing stem cell-like features through the activation of NF-kB signaling.

Furthermore, it has been demonstrated that TAMs may promote cancer stem cell maintenance by stimulating the TGF-β1/Smad2/3 pathway and the ERK1/2 pathway in glioblastoma. TAM-induced increase of CSC stemness in HCC and lymphoma may be attenuated by inhibiting the WNT/β-catenin pathway, as shown by a large body of in vivo and in vitro investigations.

These findings prove that TAMs promote the formation, survival, and proliferation of CSCs and other stem cell subtypes (including mesenchymal stem cells) in TME.

The authors continue:

Macrophages-targeted therapy

Over the past few decades, substantial preclinical and clinical progress has been made in understanding macrophage biology and its clinical relevance in human diseases. Therefore, macrophage-targeted therapy is emerging, and some have been translated into clinical trials.

Several immunotherapeutic approaches may benefit from macrophage depletion, such as CCL2 vaccination and ICIs such as PD-1 and CTLA4. Anti-CSF1R antibodies and other treatment methods focused on TAMs are now being tested in many ongoing clinical studies…

Moreover, over the last several years, macrophages have gained more and more attention as a potential immunotherapy component for treating cancer. Due to their usefulness in existing therapeutic approaches, they have emerged as a prime candidate for future advances in cancer therapy. Immunotherapy has emerged as the gold standard, given the shortcomings and shortages of conventional cancer therapies.

Several FDA-approved cancer immunotherapy therapies use direct and indirect macrophage targeting ME to counter their negative impact. Bisphosphonates can be taken up by phagocytes to deplete TAMs by inducing cell apoptosis. Currently, bisphosphonates are used clinically with decreased disease recurrence, metastasis, and overall mortality for breast cancer. Among them, clodronate, one of the non-nitrogen bisphosphonates, is artificially loaded by liposomes.

It can induce apoptosis of macrophages and inhibit tumor growth. ***Zoledronate****, a third-generation nitrogen-containing bisphosphonate, has been shown to exhibit selective cytotoxicity towards MMP9-expressing TAMs and reduce the infiltration of TAMs, decrease tumor angiogenesis, and inhibit tumor progression.*

Similarly, BLZ-945 (a CSF-1R inhibitor) and chemotherapy drugs (such as doxorubicin and epirubicin) can specifically target and deplete TAMs. In addition, inhibiting macrophage recruitment is the second strategy for TAM-targeting strategy treatment.

Many inhibitors, such as inhibitors of ANG2 (Trebananib), CCL2/CCR2 (Carlumab and PF-04136309), CCL5/CCR5 (Leronlimab and Maraviroc), CSF- 1/CSF-1R (Emactuzumab and Pexidartinib), and VEGF have been shown to inhibit macrophage recruitment for tumor growth.404 Macrophage reprogramming is crucial to reshaping their potential immune-stimulatory role as the significant phagocytes and professional antigen-presenting cells (APCs) within the TME. Generally, normal cells can express anti-phagocytosis molecules called "phagocytosis checkpoints" to avoid self-elimination by phagocytes.

Signal regulatory protein alpha (SIRPα) is a vital immunoreceptor tyrosine-based inhibitory motifs (ITIM)-bearing inhibitory receptors expressed on macrophages.

Tumor cells can become active in a "don't eat me" signal and avoid macrophage phagocytosis by over-expression of CD47 to recognize SIRPα, thereby leading to patients' poor survival.

Studies showed that blocking the CD47-SIRPα interaction by CD47 antibodies, a phagocytosis checkpoint inhibitor promotes phagocytosis in TAMs and enhances cancer immunotherapy, chemotherapy, and other combined therapy. … the combination of anti-CD47 antibody and PD-L1 blockade improved innate and adaptive immune checkpoint response rates and potentiated the vaccinal effect of antitumor antibody therapy in a mouse B16F10 model.

Reprograming M2-like TAMs toward M1-like TAMs represents an attractive strategy for macrophage-targeting treatment. CSF1/ CSF1R signaling pathway has positive roles in macrophage biology, including survival, proliferation, differentiation, and phagocytosis. Stephen et al. reported that CSF-1R blockade with PLX3397 improved the efficacy of adoptive cell therapy (ACT) in the mouse melanoma model. CSF-1R blockade reduced the ability to unleash the immune-stimulatory capacity of TAMs with a skewing of MHC II low to MHC II hi macrophages.

In addition, macrophage treatment with CD40 agonists, such as Sotigalimab and Selicrelumab, can significantly upregulate the expression of MHC, promotes the secretion of inflammatory cytokines, actives DCs, and induce cell polarization of M1-like TAMs. Furthermore, in clinical trials, blocking PI3Kγ by ***Eganelisib or Umbralisib*** *has been developed to turn on an "immune-stimulatory program" in immunosuppressive macrophages.*

This dramatic shift of TAMs is benefit in modulating the TME and promoting ICIs treatment against cancers.

Many macrophage-targeting agents have been developed with different approaches for cancer therapy, including previously unmentioned CXCL12-CXCR4 inhibitors, TREM inhibitors, SIGLEC10-CD24 inhibitors, and TLR agonists.

7.4 CHEMOKINES

As Mempel et al note:

There are numerous ways in which our understanding of the roles of the chemokine system in the TME can support efforts to treat cancer. Most immediately, chemokines and their receptors can serve as powerful biomarkers, both to reveal the strategies a particular cancer type or the cancer of an individual patient employs to evade or distort the antitumour immune response and to characterize the magnitude and the quality of the response that does ensue. As already alluded to, the abundance in the TME especially of chemokines associated with type 1 immunity or of chemokine receptors associated with tissue residence, either on their own or as part of inflammation scores, can predict favourable patient outcomes, including in response to immunotherapy.

Exploiting the detailed information on all chemokines expressed by all cells of the TME obtained by single-cell RNA-sequencing of well-annotated clinical tumour samples and, in the near future, from spatial transcriptomics studies, may lead to a much more comprehensive collection of patterns that can even more accurately predict responses to different types of immunotherapy in individual patients. In addition to providing biomarkers, the chemokine system can be targeted

therapeutically in various ways, as briefly discussed subsequently. Increasing chemokines in the TME

In an effort to achieve TME accumulation of therapeutic chemokines thought to preferentially attract antitumour immune cells, those chemokines have been fused to antibody fragments that bind to cancer cell-expressed surface proteins. In one instance, in which CXCL10 was fused to an antibody single-chain variable fragment (scFv) binding epidermal growth factor receptor vIII (EGFRvIII) expressed on glioma cells, its intracranial injection enhanced the recruitment of adoptively transferred therapeutic T cells to the central nervous system and improved survival in a mouse model of glioblastoma.

In another study, a fusion of CXCL10 to an anti-human endoglin scFv improved therapy with cytokine-induced killer cells in a human xenograft model of HCC2. In an alternative approach to targeting chemokines to cancer cells, fusion of the CCR5 ligand CCL4 to the collagen-binding domain of the glycoprotein von Willebrand factor has been shown to enhance its tumour accumulation upon systemic administration compared with the native chemokine, presumably through binding to exposed collagen in the TME.

In line with a role for CCL4 in the recruitment of CCR5-expressing cDC1s or their precursors, this treatment increased cDC1 accumulation in the TME and rendered otherwise treatment-resistant B16 mouse melanoma tumours responsive to ICT2. A second strategy to enhance chemokine concentrations in the TME relies on oncolytic viruses as genetic vectors to drive their in situ expression, for instance, of IP-10 cluster chemokines in an effort to enhance the local accumulation of CXCR3-expressing effector lymphocytes of the type I immunity response programme.

However, surprisingly, in one study in which a CXCL9-encoding oncolytic virus was examined, it was not found to enhance T cell infiltration compared with the native virus, presumably because the virus infection in itself induced sufficient endogenous expression of CXCL9, and the ectopically expressed chemokine was therefore redundant. Finally, cell therapy products have been used as vehicles to deliver chemokines to the TME.

Chimeric antigen receptor (CAR) T cells engineered to express the CCR7 ligand CCL19 (along with the lymphoid-tissue cytokine IL-7) have been shown to enhance the accumulation of both the CAR T cells themselves and endogenous T cells and DCs in the TME and exhibited single-agent antitumour activity in preclinical solid tumour models.

Preliminary observations also suggest potential efficacy in patients with HCC or PDAC2. Moreover, CCL21 has been reported to be even more efficacious than CCL19 in this cell therapy setting in mouse models of PDAC, bladder cancer and HCC2. Targeting chemokines In contrast to antitumour T cells, monocytes and granulocytes are generally viewed as having predominantly pro-tumoural activities, leading to efforts to reduce levels of chemokines such as CCL2 and XCL8 that support their accumulation in the TME. However, although CCL2 neutralization indeed limits the accumulation of monocyte-derived TAMs and reduces tumour growth as well as metastasis in mouse models, the neutralizing human CCL2 antibody carlumab

did not provide therapeutic benefit in patients with cancer, despite reducing serum levels of CCL2.

Similarly, although the human CXCL8 antibody HuMax-IL-8 reduced human (cancer cell-derived) CXCL8 in the serum of mice and granulocytes in the TME, and attenuated the in vivo growth of breast cancer xenografts, it has so far not produced objective responses in patients with cancer.

However, in the light of disease stabilization in some patients, HuMax-IL-8 continues to be evaluated in combination therapies2. Targeting cells via chemokine receptors On the basis of observations that tumour-infiltrating Treg cells express particularly high levels of CCR4 and CCR8, these receptors have been examined as targets for the selective depletion of immune-suppressive T reg cells in the TME while sparing their counterparts in healthy tissues to maintain immune homeostasis.

The FDA-approved CCR4 antibody mogamulizumab has achieved Treg-cell depletion and disease stabilization in several patients with solid cancers in a phase I study and one partial response in a patient with an advanced oesophageal cancer in a phase Ib study, encouraging ongoing combination studies. However, these studies predictably also noted effects on the frequencies of TH2, TH17 as well as CD8+ memory T cells, the latter being considered as a potentially limiting factor in the antitumour efficacy of this treatment2.

However, mogamulizumab is an effective therapy for the CCR4-expressing cutaneous T cell lymphoma variants, mycosis fungoides and Sezary syndrome. With respect to CCR8, antibodies against this receptor have shown selective depletion of the most clonally expanded Treg cells in the TME and produced encouraging antitumour activity effects in syngeneic mouse cancer models.

The human CCR8 antibody BMS-986340 is now being explored in a range of solid cancers2. Beyond the use of antibody-dependent cellular cytotoxicity (ADCC)-competent antibodies to target chemokine receptors for cell depletion, another strategy is to exploit their endocytic properties to target antigenic cargo to the lysosomal pathway, for example, by using fusions of tumour antigens to CCL20 to target CCR6-expressing APCs2. This concept has been further developed into a DNA-based vaccine that encodes for a fusion of the CCR5 ligand human CC motif chemokine ligand 3-like 1 (CCL3L1) with human papillomavirus 16 (HPV16) antigens for the treatment of HPV-associated cancers.

This particular plasmid vaccine is designed for intramuscular injection and local expression to attract and target the HPV antigens to CCR5-expressing DCs intended to activate T cells in tumour-draining lymph nodes.

However, one can imagine developing similar designs to target not only antigens but also other biomolecules to cells of the TME on the basis of their chemokine receptor expression profile.

As Ozga et al note:

Role of chemokines in cancer treatment Immunotherapy harnesses the patient's immune system to destroy tumors by relieving effector cell dysfunction and inhibiting suppressive immune cell populations. The immune contexture predicts responsiveness to immunotherapy, with hot tumors being the most responsive and cold tumors being the least responsive. There is growing interest in combining different cancer treatment approaches to overcome tumor resistance and sensitize cold and altered tumors for more effective immunotherapy.

Modulation of chemokine expression during cancer treatment contributes to the efficacy of as well as the resistance to therapy and will be reviewed in this section. Checkpoint blockade therapy targets immunosuppressive molecules on the surface of T cells to restore their effector function, enabling better tumor control. The efficacy of checkpoint blockade therapy strongly correlates with the preexisting immune response. Patients with T-cell-inflamed tumors that are enriched for T-cell-recruiting chemokines, such as CCL5, CXCL9, CXCL10, and CXCL11, are most likely to benefit from checkpoint blockade therapy.

However, responses are not guaranteed in these patients, indicating that immune cell infiltration into the TME is necessary, but not sufficient, for a clinical response. Indeed, recent data correlate responsiveness to checkpoint blockade therapy with the presence of a specific subset of T cells that are characterized by expression of the transcription factor TCF. Moreover, our group has recently found that the chemokine receptor CXCR3 and its ligand, CXCL9, were important for the response to anti-PD-1 therapy in mouse tumor models.

Upregulation of CXCL9 expression by cDC1 following PD-1 blockade enabled cDC1 to specifically activate CXCR3-expressing CD8+ T cells that are not terminally exhausted, thereby facilitating the generation of an effective antitumor response to eliminate tumor cells. CXCL9 is not only essential for the clinical response to anti-PD-1 treatment but also has been demonstrated to be pivotal for the efficacy of TIM-3 blockade therapy in mouse models. Anti-TIM-3 promoted the expression of CXCL9 by cDC1s, which triggered the intratumoral response of CD8+ T cells, enhancing the responsiveness to paclitaxel chemotherapy. Therefore, lack of response in some patients with Tcell-inflamed tumors might be a result of low abundance of TCF-1+ cells and/or impaired cross-talk with cDC1s within the TME.

Adoptive cell transfer therapy using chimeric antigen receptor T cell therapy and in-vitro-expanded autologous T cells has also shown clinical promise. However, one of the significant hurdles that limits the efficacy of adoptive cell transfer therapy in the treatment of solid tumors is the restriction of adoptively transferred T cell infiltration into the tumor bed due to abnormal tumor vessels and an immunosuppressive TME.

Thus, adoptive cell transfer therapy alone might not be sufficient to treat patients with cold and altered tumor profiles. To endow T cells with a more exceptional ability to migrate into tumors, …

However, the translation of these preclinical studies is urgently needed to determine if manipulation of the chemokine system can enhance the efficacy of adoptive cell transfer therapy for cancer patients.

Radiotherapy and chemotherapy are designed to induce death in rapidly proliferating malignant cells. Although the efficacy of radiotherapy and chemotherapy was initially attributed to the direct cytotoxic effects on malignant cells, it is now well appreciated that it is mediated, at least in part, through the activation of an anti-tumor immune response. Radiotherapy and chemotherapy can trigger the induction of an immunogenic cell death of cancer cells, promoting the cross-presentation of tumor-derived antigens and subsequent activation of adaptive anti-tumor T cell responses.

Therefore, there is growing interest in developing novel optimal radiotherapy and chemotherapy protocols to efficiently trigger T cell responses.

Moreover, several trials testing the synergy of checkpoint inhibitors with radiotherapy or chemotherapy are ongoing. The efficacy of radiotherapy and chemotherapy likely rely on the infiltration of cDC1s into the TME. Indeed, recent findings suggest that CCR7-dependent migration of cDCs from the TME into

TDLNs is critical for efficacy of chemotherapy. Therefore, cold tumors that interfere with cDC1 recruitment might show limited benefit from these treatment regimens unless the treatment itself enhances APC accumulation in the TME, as demonstrated in a murine model of fibrosarcoma. In this cancer type, treatment with anthracycline induces the expression of CCL2 in the TME, which recruits functional APCs and stimulates the generation of functional anti-tumor T cell responses.

Nevertheless, chemotherapy and radiotherapy might be more beneficial in cold and altered tumors that do not interfere with cDC1 recruitment. In these tumor types, chemotherapy and radiotherapy might unleash the expression of T-cell-recruiting chemokines within the TME. For instance, in a murine model of breast cancer, irradiation-induced CXCL16 expression was critical for the homing of CXCR6-expressing CD8+ Teff cells into the tumor.

Furthermore, another chemokine axis was found to mediate a similar effect in a mouse model of melanoma in which irradiation triggers the production of type I and II IFNs, upregulation of CXCL9 or CXCL10, and subsequent recruitment of CXCR3-expressing Teff cells. Similar to radiotherapy, treatment of tumor-bearing mice with chemotherapy leads to intratumoral expression of chemokines, such as CXCL9, CXCL10, and CCL5, which drives recruitment of CD4+ and CD8+ T cells into the tumor bed.

These chemokines are also upregulated in patients with melanoma who responded to chemotherapy, and their expression correlated with CD4+ and CD8+ T cell infiltration, tumor control, and patient survival. In addition, tumor cells increase expression of CXCL10 in response to anthracycline-based chemotherapy, which is important for anti-tumor T cell responses. Another mechanism of action of chemotherapy might include interference with chemokine-mediated immunosuppressive pathways.

7.5 ALTERNATIVES

There are other alternative targets associated with the macrophage suppression. From Wellhausen et al we have the following:

CD47: Cell-surface protein that acts as a 'don't eat me' signal by interacting with signal regulatory protein-α (SIRPα) on macrophages, protecting cells from macrophage-mediated destruction …

A different approach to improve immunotherapy responses in solid tumours is combining engineered T cell therapies with additional immunotherapies that can recruit macrophages and NK cells such as blocking CD47 with antibodies.

However, blocking the cancer cell–macrophage crosstalk through the CD47–signal regulatory protein-α (SIRPα) axis not only augments the phagocytic activity of tumour-associated macrophages against tumour cells but also leads to the clearance of the therapeutic CAR T cell or TCR T cell populations.

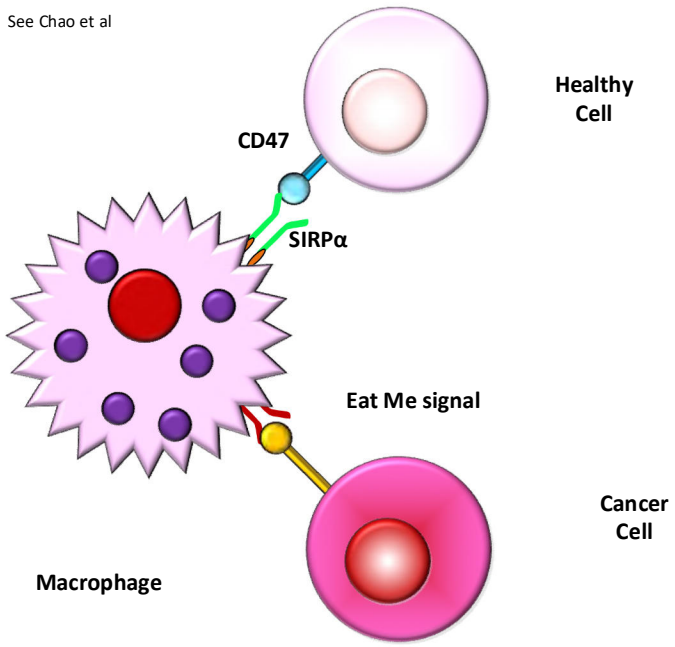
To overcome this challenge, Yamada-Hunter et al. engineered CAR T and TCR T cells to express a CD47 variant (47E) that was not blocked by anti-CD47 antibodies and thus engaged SIRPα to provide a 'don't-eat-me' signal to tumour-associated macrophages. This provided the T cells with selective resistance to macrophage clearance, enhancing their antitumour efficacy in combination with anti-CD47 blocking antibodies.

As Chao et al note:

In recent years, immunotherapies have been clinically investigated in AML and other myeloid malignancies. While most of these are focused on stimulating the adaptive immune system (including T cell checkpoint inhibitors), several key approaches targeting the innate immune system have been identified.

Macrophages are a key cell type in the innate immune response with CD47 being identified as a dominant macrophage checkpoint. CD47 is a "do not eat me" signal, overexpressed in myeloid malignancies that leads to tumor evasion of phagocytosis by macrophages. Blockade of CD47 leads to engulfment of leukemic cells and therapeutic elimination.

Pre-clinical data has demonstrated robust anti-cancer activity in multiple hematologic malignancies including AML and myelodysplastic syndrome (MDS). In addition, clinical studies have been underway with CD47 targeting agents in both AML and MDS as monotherapy and in combination. This review will describe the role of CD47 in myeloid malignancies and pre-clinical data supporting CD47 targeting. In addition, initial clinical data of CD47 targeting in AML/MDS will be reviewed, and including the first-in-class anti-CD47 antibody magrolimab.



Wang et al discuss various alternative targeting. They note as follows:

Antitumor nanodrugs targeting M2-like TAMs

Nanomaterials have significant potential for enhancing the effectiveness of tumor immunotherapy with the advancement of nanobiotechnology due to their benefits in specific targeted drug transport, accurate localization of drug release, ease of surface functionalization, and high bioavailability of pharmaceuticals.

Antitumor nanodrugs utilizing TAMs as a delivery medium. TAMs exhibit significant homing properties, able to migrate directionally and accumulate in tumor tissue through the detection of specific signals in the TME. Their integral role in promoting tumor progression, invasion, and metastasis is well-established.

Moreover, TAMs also have the capacity to function as vehicles for the delivery of antitumor therapeutics, including drugs, liposomes, and nanoparticles, directly to tumor sites and even into the tumor cells themselves.

The homing and delivery capabilities of TAMs determine their potential use as cellular vectors for therapeutic delivery.

Leveraging TAMs for the targeted delivery of antitumor nanomedicines is a prospective avenue within the evolving landscape of antitumor therapy. This TAM-mediated drug delivery strategy is also known as the "Trojan Horse" approach1. This technique entails ex vivo loading of nanodrugs into TAMs, which are subsequently reintroduced into the patient. These TAMs migrate to inflammatory sites and are then recruited into the tumor tissue to release the loaded nanomedicines.

This strategy can be applied to thermal ablation and radiotherapy of tumors. For instance, Au nanoshells are nanoparticles with a silica core and a thin Au shell. After coculturing with TAMs, Au nanoshells are phagocytized, internalized within TAMs, and these TAMs carry and accumulate them in the hypoxic core regions of tumors, improving the targeting of gold nanoparticles and enhancing the efficacy of tumor thermal ablation therapy. Recent years have seen a downtrend in the exploration of M2-like macrophages as vectors for nanomedicine delivery.

This paradigm shift can be attributed to M2-like TAMs' immunosuppressive role in the TME and the difficulties associated with selective targeting. Such constraints not only compound the sophistication of therapeutic conveyance but also risk augmenting tumor advancement. Despite these challenges, the advancement of new research methods and technologies still holds the potential for breakthroughs in future treatment strategies. Antitumor nanodrugs acting on M2-like TAMs.

Pro-inflammatory macrophages, known as M1-like TAMs, possess potent phagocytic capabilities and the ability to directly kill tumor cells.

Under the influence of relevant chemokines, pro-inflammatory macrophages can efficiently migrate to tumor lesions to exert these functions. Therefore, nanomaterials that target M2-like macrophages to reshape the TAMs phenotype, induce M2-like macrophages to polarize toward M1-like macrophages, and inhibit tumor growth also hold significant research value.

Currently, several nanotechnological strategies for acting on M2-like macrophages have been established, including termination of M2-like macrophages recruitment, specific targeting and eliminating M2-like macrophages, and converting M2-like macrophages into M1-like macrophages. Recent advances in explicitly enhancing antitumor immune responses by targeting M2-like macrophages with nanomaterials have demonstrated considerable promise.

Repolarize M2-like to M1-like macrophages *TAMs exhibit remarkable plasticity, capable of phenotype switching under various factors influences. Therefore, the targeted reprogramming of TAMs from M2 to M1 phenotype represents a critical area of oncological research. To attenuate tumor drug resistance, researchers have undertaken numerous experiments and created a variety of related drugs that induce the polarization from M2-like to M1-like macrophages including but not limited to CSF-1R antagonists, PI3Kγ inhibitors, bromodomain-containing protein 4 (BRD4) inhibitors, Signal-regulatory protein alpha (SIRPα) inhibitors and histone deacetylase (HDAC) inhibitors.*

Pexidartinib is a polygenic tyrosine kinase inhibitor that targets CSF-1R to significantly mitigate macrophage tumor infiltration1. Pexidartinib was shown … to upregulate BCL-2-associated X protein (BAX), CRISPR-associated protein 3 (Cas3), TNF-α, IFN-γ, and IL-6, and downregulate Ki-67, IL-13, IL-10, TGF-β, and Arg-1.

Additionally, Pexidartinib enhanced CD3 + CD8 + T cells infiltration in the TME by inhibiting the CSF-1/CSF-1R axis. Consequently, Pexidartinib could attenuate the polarization of M2-like TAMs, potentially overcoming the esophageal adenocarcinoma (EAC) model's resistance to PD-

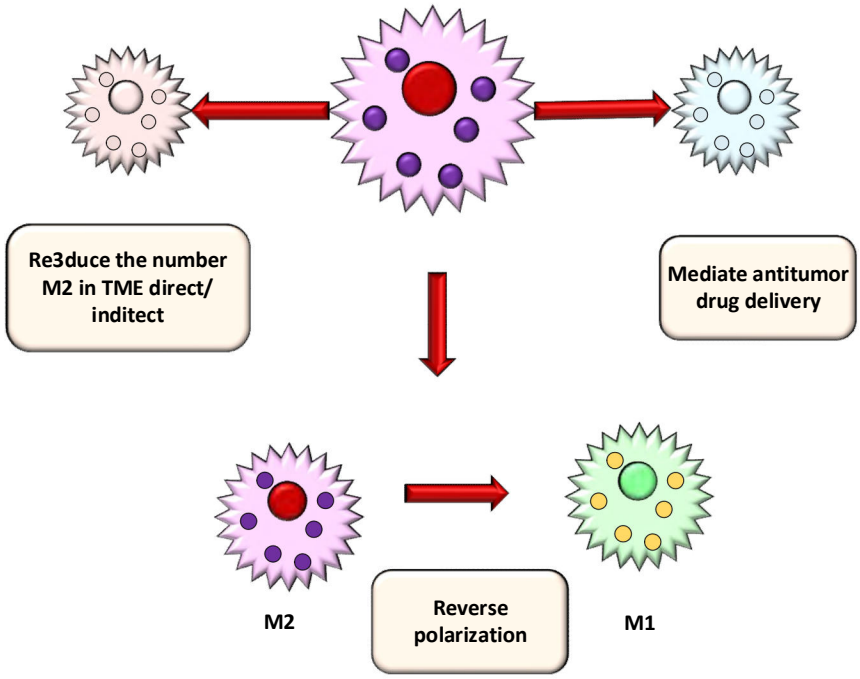
1/PD-L1 axis blockade. AZD5153, a specific BRD4 inhibitor, reprograms TAMs from M2 to M1 phenotype, which in turn promotes the secretion of pro-inflammatory cytokines. This secretion cascade activates CD8+ cytotoxic T lymphocytes (CTLs), thereby enhancing the responsiveness of high-grade serous ovarian cancer (HGSOC) to anti-PD-L1 therapy1.

Evorpacept, a CD47-SIRPα inhibitor, has been shown to enhance antitumor immune response in preclinical models by promoting phagocytosis of macrophages, driving the phenotype shift of M2-like to M1-like TAMs, and boosting cytotoxic T cell effector functions.

Evorpacept has also shown encouraging initial combination therapy activity in PhaseI clinical trial for solid tumors1.Preclinical data suggest that the combination of a CD40 agonist and anti-PD-1/anti-PD-L1 inhibitors improves survival in mouse tumor models compared with the use of either alone.

The co-administration of a CD40 agonist and anti-PD-1/antiPD-L1 inhibitors elevates PD-L1 expression in tumor-infiltrating monocytes and TAMs, biases the TAM populations toward the inflammatory M1 phenotype, thereby inhibiting tumor-induced immune resistance.

Wang et al argue that the three methods discussed can be demonstrated in the Figure below;



The authors remark

Current major antitumor approaches targeting M2-like TAMs to overcome tumor drug resistance.

These approaches mainly encompass three aspects: reducing the number of M2-like TAMs in the TME through direct or indirect methods, thereby diminishing their role in promoting tumor

growth; using M2-like TAMs as antitumor drug delivery mediums; and inducing the re-polarization of M2-like TAMs to the M1 phenotype, thus restoring their anti-tumor activity and enhancing the immune response in the TME.

These strategies collectively form a comprehensive therapeutic approach to overcoming tumor drug resistance by targeting M2 TAMs.

8 CELL DEATH

Cells die off by a variety of means. We examine some major ones here. An excellent reference is the work of Green. The intent here is to present cell death in the context of TAM functions and interaction.

As Vanmeerbeek et al note:

In a tumour, different cancer cell death modalities occur in a programmed or nonprogrammed manner, depending on the level and type of stress.

Cancer cell death is mainly caused by cellular stress due to DNA damage, endoplasmic reticulum stress, toxins, hypoxia, low levels of glucose and amino acids or other stress inducers triggering ***programmed cell death (PCD)*** *or* ***non-programmed cell death*** *pathways leading to the release of prominent factors, i.e., cytokines (such as type I IFNs), chemokines (e.g., CCL2, CXCL1) or other danger signals.*

A major non-PCD pathway is necrosis, which happens in an accidental manner due to stress induced by infection or physico-chemical injury.

This type of ***accidental cell death results in high inflammation caused by the sudden release of intracellular components*** *(including damage-associated molecular patterns, in short DAMPs), which will attract various phagocytes, including TAMs, to clear the dying/dead cells.*

Herein, the amount and composition of the release DAMPs can be a defining factor behind the M1-like or M2-like activities of TAMs.

The author begins with apoptosis, one of the better understood means of cell death. They continue:

On the contrary, PCD is, by definition, a more regulated manner of cell death, based on a mechanistically orchestrated signalling pathway. 'Physiological' apoptosis is a commonly known PCD, triggered by different pathways which release 'find me' and 'eat me' signals, e.g., phosphatidylserine (PS), opsonins, modified ***intercellular adhesion molecule 3 (ICAM-3)*** *and* ***complement system components*** *recognised by phagocytic cells including TAMs.*

Initially, apoptosis and the cell clearing process were considered as being immunologically 'silent' or even tolerogenic, but studies have shown that certain stimuli (which are discussed later in this review) can induce immunogenic apoptosis (also called 'immunogenic cell death' or ICD).

Two major routes can induce apoptosis*, i.e.,*

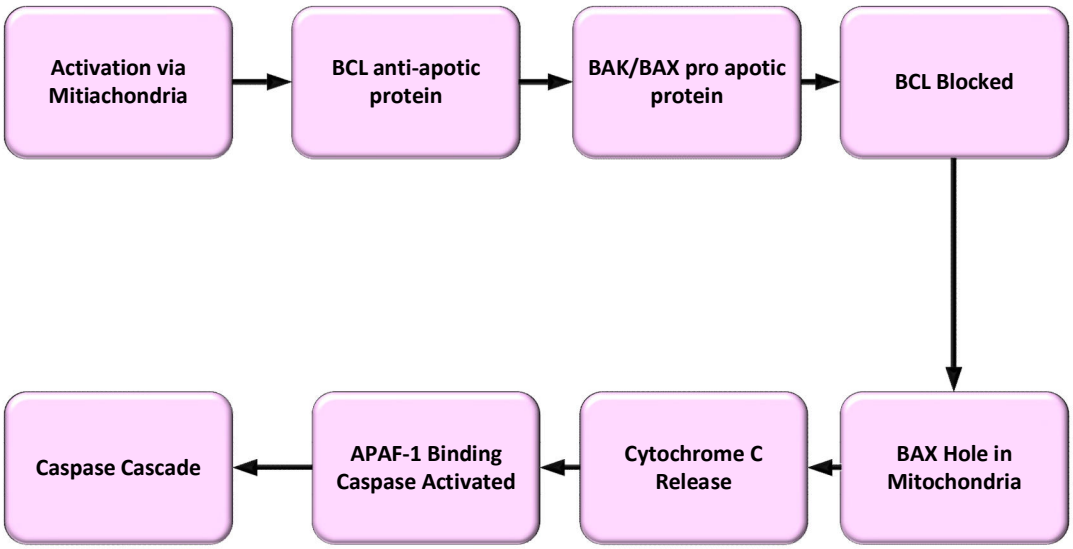
the extrinsic (death receptor-elicited) and

intrinsic (mitochondrial stress-driven) pathways,

both depending on the activity of caspases cleaving and activating downstream cellular substrates.

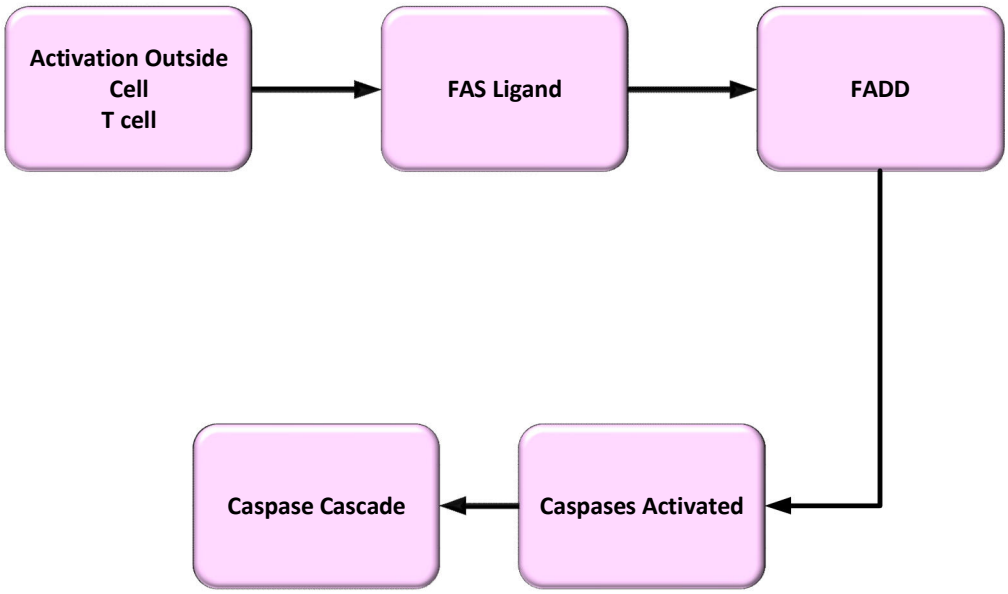
The intrinsic pathway is triggered by a diversity of intracellular stress signals, especially DNA damage or intracellular organellar stress, e.g., cytochrome c, released from the mitochondria. In the cytoplasm, cytochrome c binds and activates apoptosis protease activating factor-1 (Apaf-1) and induces the formation of the apoptosome, which will initiate the cell death pathway via caspases.

The intrinsic pathway is shown below"



The extrinsic pathway is activated by extracellular signals engaging their cognate death receptors, e.g., tumour-necrosis factor (TNF) or FAS receptors, which are located on the cellular surface. This interaction leads to the formation of the death-inducing signalling complex (DISC), which activates caspases responsible for the degradation of chromosomes and ultimately leads to apoptosis.

The extrinsic pathway is shown below:



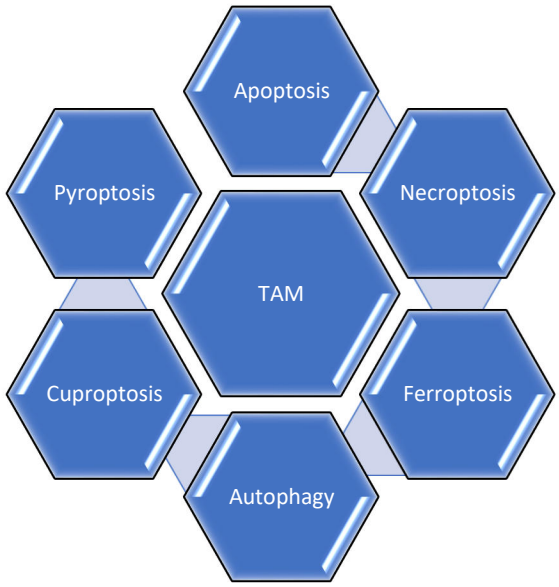
However, when caspases are inactivated or deficient due to, for example, somatic mutations, a programmed form of necrosis cell death, also called necroptosis, can be activated as a back-up mechanism. This cell death modality has morphological similarities with accidental necrosis but differs in molecular pathways.

Since most normal cells engage extrinsic apoptosis, which is less likely to be inflammatory, necroptosis has multiple elements in common with (extrinsic) apoptosis, such as the initiating receptor complexes. Whenever extrinsic apoptosis fails to be initiated, proximal initiator receptors of extrinsic cell death (e.g., TNF receptor-1, death receptor 4/5, FAS receptor, Toll like receptor 3 and 4, Z-DNA binding protein1) will provoke downstream activation of three major pro-necroptotic molecules, i.e., mixed lineage kinase domain such as pseudo kinase (MLKL) and receptor-interacting serine/threonine kinase 1 and 3 (RIPK1/RIPK3).

The activation of these proteins is induced by the engagement of death receptors such as TNF receptor or TNF-related apoptosis-inducing ligand (TRAIL) and leads to the formation of protein complexes that will eventually cause membrane pores resulting in necroptosis

8.1 ENDOGENOUS

Cell eventually die. They way they die is varied. Some death is “natural”, cells get old, some are the result of stress, cells lack food etc, some are from internal attack. Macrophages play a role in many of these processes. As Shao et al note:



The authors proceed to define:

Apoptosis

Apoptosis Efferocytosis *is the process by which phagocytes remove apoptotic cells that are programmed to death, a process by which apoptotic cells are "buried," hence the term efferocytosis.*

Moreover, macrophages will be involved in and may influence this process, mostly negatively, in all tissue development, maintenance of endostasis, and disease processes … activated antigen-specific FasCD8+ T cells interacted with FasLCD11b+F4/80+ monocyte-derived macrophages and underwent apoptosis to promote liver metastasis.

M2-type TAMs inhibit apoptosis and promote cancer metastasis. In hepatocellular carcinoma, M2-type TAMs increase the level of Cancer stem cells (CSC) and thus reduce sorafenib-induced apoptosis. In the triple negative breast cancer (TNBC) mouse model, surgical trauma accelerated primary tumors exhibiting an increase in TAMs, particularly M2-type macrophages, which accelerated tumor progression and lung metastasis.

Macrophages, immune cells that are essential in the tumor microenvironment, may be cultured by tumor cells into a pro-tumor phenotype to promote tumor progression and metastasis. The mechanisms mediating the interrelationship with tumor cells and macrophages in the TME ***remain challenging to describe.***

It was shown that α-ketoisovalerate (KIV) and α-keto-β-methylpentanoic acid promote macrophages to become pro-tumorigenic, while KIV promotes pro-inflammatory effects in macrophages, affecting inflammatory signaling pathways, phagocytosis, apoptosis and redox homeostasis.

M2-type TAMs also play an oncogenic role in lung adenocarcinoma (LUAD) promoting tumor progression. The macrophage atlas was constructed, and the infiltration rate of M2-type TAM subpopulations was found to be higher in the proficiency of mismatch repair (pMMR)-colorectal cancer (CRC) tumor tissues than in mismatch repair deficiency (dMMR)-CRC tumor tissues, and M2 polarization trajectories revealed apoptosis of M2-type TAM in dMMR. These findings suggested a potential function of apoptosis in tumor suppression and enhancement of immunotherapeutic effects.

Autophagy

Autophagy-dependent *cell death Macrophages to interact at TME with other immune cells through guiding intercellular contacts or secreting various effector molecules.*

TAM has an integral part to play in tumor progression, autophagy, and angiogenesis owing to its heterogeneity and intense plasticity.

Similarly, combining tumor cells with other immune cells can drive the recruitment and polarization of macrophages. LC3-associated phagocytosis (LAP) contributes to various cellular processes, especially in immunity. Stabilizing phagosomes through a macroautophagy mechanism in human macrophages can maintain antigen presentation on MHC class II molecules. Erk3 was found to bind to HSC70 (70 kDa) and lysosomal-associated membrane protein type 2 A (LAMP2A), which are two core components mediating chaperone molecular autophagy (CMA). EGLN3-catalyzed hydroxylation antagonizes CMA-dependent Erk3 destruction, and inactivated EGLN3 inhibits macrophage migration, efferent cell increases and M2-type polarization, and improves LLC cancer growth by reprogramming the TME.

Necroptosis

Cell death determines the response of the surrounding environment, while immune activation against cell death depends on the activation of the mortality pathway.

Apoptosis and necroptosis are the main cell death mechanisms and usually lead to an opposite immune response. Apoptotic death usually leads to an immunosilencing answer, while necroptosis death releases molecules that promote inflammation, a process known as necroinflammation.

Macrophages induce tumor necroptosis via derived TNFα in vivo, a process that also requires programmed cell death ligand 1 (PD-L1), GSDMC, and caspase-8, all of which are needed for the induction of tumor necrosis by macrophage-derived TNFα in vivo.

As a pan-cancerous feature associated with partial necroptosis, intra-tumor high potassium (K) has shown immunosuppressive potency against T cells , and some of the studies demonstrated that intra-tumor high K inhibits the anti-tumor capacity of TAMs. DNA hypomethylating drugs increase tumor infiltrating effector T cells, increase M2-type macrophages and lead to increased tumor necroptosis in a pancreatic cancer model

As Mieier et al note:

Most metastatic cancers remain incurable due to the emergence of apoptosis-resistant clones, fuelled by intratumour heterogeneity and tumour evolution.

To improve treatment, therapies should not only kill cancer cells but also activate the immune system against the tumour to eliminate any residual cancer cells that survive treatment. While current cancer therapies rely heavily on apoptosis — a largely immunologically silent form of cell death — there is growing interest in harnessing immunogenic forms of cell death such as necroptosis.

Unlike apoptosis, necroptosis generates second messengers that act on immune cells in the tumour microenvironment, alerting them of danger. This lytic form of cell death optimizes the provision of antigens and adjuvanticity for immune cells, potentially boosting anticancer treatment approaches by combining cellular suicide and immune response approaches.

In this Review, we discuss the mechanisms of necroptosis and how it activates antigen-presenting cells, drives cross-priming of CD8+ T cells and induces antitumour immune responses. We also examine the opportunities and potential drawbacks of such strategies for exposing cancer cells to immunological attacks.

Now back to the initial sources:

Pyroptosis

Immunogenic cell death (ICD) is a highly inflammatory form of death, pyroptosis provided to alleviate immunosuppression and facilitate a systemic immune response in solid tumors.

Gasdermin-E (GSDME) in tumors enhanced anti-tumor immunity by activating pyroptosis, and GSDME expression simultaneously enhanced phagocytosis of TAMs on tumor cells, *along with the number and function of tumor-infiltrating CD8+T lymphocytes and natural killer cells. Gasdermin-D (GSDMD) activated by inflammatory vesicles in macrophages is cleaved by caspase-1 to produce N-GSDMD fragments, which are then aggregated in the plasma membrane to form pores that increase membrane-permeability, resulting in IL-1β release and pyroptosis.*

Previous studies have shown that amino acid metabolism in colon cancer produces pivotal enzymes that are involved in regulating colorectal cancer through cell death and that these key metabolic enzymes may lead to immune escape from colorectal cancer through pyroptosis leading to macrophage death.

Pyroptosis-releasing factor activates GSDMD-cleaved caspase-1 in macrophages, leading to cytokine release and subsequent cytokine release syndrome (CRS), which inhibits chimeric antigen receptor (CAR) T cell therapy for patients.

Sorafenib induces macrophage pyroptosis and triggers natural killer cell-mediated cytotoxicity against hepatocellular carcinoma.

In vitro trials have shown that adipose-derived mesenchymal stem cells (ADSCs)- derived exosomes were absorbed by macrophages, and miR-17–5p in ADSC exosomes reduced Ang II-induced TXNIP and decreased the triggering of macrophage pyroptosis , thereby affecting abdominal aortic aneurysm (AAA) progression. Inhibition of macrophage pyroptosis activation and inflammation can inhibit the development of Xanthoma. DRD2 triggers programmed cell death in breast cancer by regulating the tumor microenvironment, promoting M1 polarization of macrophages, and triggering GSDME to perform pyroptosis

Ferroptosis

Ferroptosis is thought to be a novel form of discovery differing in that it is not a form of apoptosis and is iron-dependent lipid hydroperoxides, involving iron, lipid, and amino acid metabolism. Cytokines, some produced mainly by macrophages, have been reported to be able to induce or inhibit the procedure of ferroptosis in different manners.

Periodic studies related to the influence of macrophage function by amino acid metabolism found that there are eight critical enzymes involved in amino acid metabolism in colonic TAMs , namely ACADM, ACADS, GPX4, GSR, HADH, HMGCL, HMGCS1 and IDH1, are closely associated with GPX4, a critical protein of ferroptosis, which may regulate metabolism in colorectal cancer through ferroptosis. APOC1 is expressed highly in TAMs of HCC tissues , and inhibition of its expression can reverse the M2-type to M1-type through the ferroptosis in TAMs of HCC, thereby reshaping TIME and improving immunotherapy for HCC. Macrophages, in contrast to tumor cells, are inherently passive to iron-dependent cell death triggered by lipid peroxidation.

Single-cell RNA sequencing (scRNA-seq) provides a deeper understanding of cellular behavior in the complicated tumor microenvironment by analyzing single-cell populations. Recently, human primary macrophages were found to be able to respond to RSL3, an inhibitor of ferroptosis against GPX-4, by upregulating the expression of the iron transport protein ferritin, and the interaction of Nrf2 and BACH1 was able to induce ferritin expression and enhance the anti-ferroptosis effect of human macrophages.

Nuclear factor-erythroid 2-related factor 2 (NFE2L2) is specifically expressed in tumor macrophages and is associated with ferroptosis and occurrence in cervical squamous cell carcinoma (CESC). Studies on HBV-positive hepatocellular carcinoma cells found that their secreted exosome miR-142–3p promotes HCC progression by inducing ferroptosis in M1-type macrophages

Cuproptosis

Microelements, which are indispensable in living organisms, also exhibit cytotoxicity when their concentrations exceed the threshold for maintaining homeostatic mechanisms.

But what few people know is that there are actually many metal-induced deaths, besides ferroptosis , there is also zinc death (excess zinc can trigger non-apoptotic cell death by inhibiting adenosine triphosphate (ATP) synthesis*), and* ***cuproptosis.***

Researchers have named this mechanism of copper ion-induced cell death "Cuproptosis" , new and unique form of regulated cell death that is closely related to immunity and occurs in a variety of cancers. *In head and neck squamous cell carcinoma (HNSCC), a strong correlation between macrophages and cuproptosis was found by analysis of RNA-seq data and relevant clinical data.*

8.1.1 Apoptosis

We now consider some cell death mechanisms in detail. As Pfeffer and Singh note:

Apoptosis is the cell's natural mechanism for programed cell death.

It is particularly critical in long-lived mammals as it plays a critical role in development as well as homeostasis. It serves to eliminate any unnecessary or unwanted cells and is a highly regulated process. There are a wide variety of conditions that will result in the apoptotic pathway becoming activated including DNA damage or uncontrolled proliferation.

The apoptotic pathway is activated by both intracellular and extracellular signals.

There are two different pathways that lead to apoptosis: the intrinsic and extrinsic pathways that correlate with the signal type.

They are also referred to as the mitochondrial and death receptor pathways, respectively.

The intracellular signals include DNA damage, growth factor deprivation and cytokine deprivation , whereas the most common extracellular signals are death-inducing signals produced by cytotoxic T cells from the immune system in response to cells that are damaged or infected.

The pathways converge at the executioner caspases. As soon as apoptosis is signaled, changes start to occur within the cell. These changes include activation of caspases which cleave cellular components required for normal cellular function such cytoskeletal and nuclear proteins.

As a result of caspase activity, apoptotic cells begin to shrink and undergo plasma membrane changes that signal the macrophage response.

Apoptosis is carried out by caspases (cysteine aspartyl-specific proteases) which are a class of cysteine proteins that cleave target proteins. The caspase protease activity is essential to successful apoptosis as they cleave hundreds of various proteins. There are four initiator caspases (caspase-2, -8, -9, 10) and three executioner caspases (caspase-3, -6, -7).

The executioner caspases cleave the target proteins that eventually leads to the death of the cell. The pathways are highly regulated so that apoptosis will only occur if signaled. The intrinsic pathway, in particular, is regulated by the B-cell lymphoma-2 (BCL-2) protein family which include proapoptotic effector proteins, proapoptotic BH3-only proteins, and antiapoptotic BCL-2 proteins.

The antiapoptotic BCL-2 proteins inhibit apoptosis through the inhibition of the proapoptotic BCL-2 proteins, BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist killer (BAK). BH3-only proteins inhibit the antiapoptotic BCL-2 proteins. …

Intrinsic Pathway

The intrinsic mechanism of apoptosis uses the mitochondria and mitochondrial proteins. Cells with damaged DNA or upregulated oncogenes can stimulate this pathway. Additional stimuli for this pathway includes growth factor deprivation, surplus Ca2+, DNA-damaging molecules, oxidants and microtubule targeting drugs. The overall pathway is regulated by the BCL-2 family of proteins.

Various apoptotic stimuli result in the upregulation of BH3-only proteins, which then activate both BAX and BAK. BAX is regulated by p53 , a tumor suppressor gene.

Once activated, BAX and BAK oligomerize, which leads to mitochondrial outer membrane permeabilization (MOMP). MOMP is the defining event of intrinsic apoptosis and is considered the point of no return. The permeabilization allows the release of intermembrane proteins like cytochrome c, second mitochondria-derived activator of caspase (SMAC) and Omi. Upon the release of cytochrome c, the apoptosome is formed from cytochrome c, apoptotic protease-activating factor-1 (APAF-1), dATP and procaspase-9. Within the apoptosome, procaspase-9 is converted into caspase-9 which activates the executioner caspases-3 and -7. The executioner caspases quickly begin to break down proteins leading to cell death.

Extrinsic Pathway

The extrinsic pathway uses extracellular signals to induce apoptosis. Cell death signals, also known as death ligands, bind to tumor necrosis factor (TNF) family death receptors. Some death ligands include Fas ligand (Fas-L), TNF-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor (TNF). An adaptor protein is recruited to the death receptor; adaptor proteins include Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD). Initiator procaspases-8 and -10 bind to the adaptor protein, forming the death-inducing signaling complex (DISC) [4,14]. The procaspases have a death effector domain (DED) that binds to the adaptor protein at its DED. Procaspases-8 and -10 are activated by DISC. Executioner caspases-3, -6 and -7 are then activated and begin the cleavage of proteins and the cytoskeleton leading to cell death. DISC is regulated by the inhibitor, c-FLIP, which is homologous to caspase-8 yet lacks caspase activity.

As Morana et al note:

Apoptosis represents a tightly regulated and evolutionarily conserved cell death programme, performing key functions in normal physiological processes such as embryogenesis and adult tissue homeostasis, but also well renowned for its role as a tumour suppressor mechanism. Apoptosis is the normal physiological cell death response to many stimuli, infection or damage, including that which follows cytotoxic drug treatments or radiotherapy programmes, that generate irreparable DNA lesions.

The molecular machinery in apoptosis is well characterised and critically requires the activation of caspase proteases, either through an intrinsic pathway initiated through mitochondrial outer membrane permeabilization (MOMP) or triggered at the cell surface by the activation of death receptors, such as Fas and DR4/5, by their death-inducing ligands, for example, FasL and TRAIL, respectively.

The role of apoptosis in cancer has received much attention, with resistance to apoptosis being widely accepted as an acquired characteristic of cancer cells, endowing them with survival advantages that promote tumour evolution and outgrowth, as well as treatment failure.

Consequently, the efficacy of cancer treatments is strictly dependent not only on the cellular damage they cause, but also on the cells' ability to activate their apoptosis programme. Over recent decades, research in cancer therapy has largely focused on the development of improved drugs and radiation therapies aimed at inducing maximal tumour cell death with resultant regression of tumour volume and blockade of aggressiveness. Accordingly, significant efforts have been made to inhibit the underlying molecular mechanisms by which tumour cells may evade apoptosis.

In recent years, however, more attention has been given to observations that, in diverse cases, high-grade cancers with poor prognosis often contain relatively high levels of constitutively apoptotic cells; we and others have proposed that dying tumour cells may help determine net survival and expansion and evolution of the tumour population as a whole …

While causative relationships between the aggressiveness of malignant disease and the levels of tumour-cell apoptosis remain largely theoretical, additional correlative evidence can be drawn from observations that high expression of pro-apoptotic effectors may not correlate with low aggressiveness of disease and vice versa. This is illustrated, for example, by pro-apoptotic Bax expression or caspase activation being associated with aggressive disease and by anti-apoptotic Bcl-2 being linked to less aggressive states.

Furthermore, cell death may foster genomic instability and niche creation which, in the context of nascent and progressing tumours, could lead to repopulation by tumour cell clones with more aggressive properties. As we will discuss, accumulating evidence indicates that the apoptosis programme also has the capacity to provide significant pro-oncogenic signals—here from a cell population (rather than single-cell autonomy) perspective—which feed into the cell birth/cell death imbalance that leads to cancer.

We believe that innate regenerative responses to apoptosis are hijacked in cancer in order to promote and retain net tumour growth and progression. Of particular importance in this regard is the well-established knowledge that apoptotic cells attract and polarise macrophages to M2-like reparatory and regenerative activation states with the potential to promote cancer evolution and progression through diverse pathways …

Tumour-associated macrophages (TAMs) constitute a significant proportion of the cellular compartment of the TME in diverse malignancies where, in many cases, they are clearly active in clearance of apoptotic cells.

As we discuss later, TAM accumulation and pro-oncogenic activation, at least in certain cancers, is closely coupled to tumour growth and angiogenesis.

However, little is yet known of the relative contributions of the various find-me, eat-me and anti-inflammatory signaling mechanisms and molecules described above in the pro-oncogenic responses of TAMs to apoptotic tumour cells.

Notably, the protein tyrosine kinase MERTK (an indirect PS receptor) is functional in signaling not only phagocytosis of apoptotic cells but also anti-inflammatory/immunosuppressive responses; its inhibition can suppress cancer growth (reviewed in). It seems likely that phagocyte receptors will orchestrate context-dependent immune responses to apoptotic tumour cells whether dependent on, or independently of, PS; future work will define what receptors are important in specific tumours. An informative example is post-partum breast carcinoma, which tends to present as an aggressive, metastatic disease. In a mouse model that used the involuting mammary fat pad as a breast cancer transplant microenvironment, the critical importance of constitutive apoptosis, MERTK-dependent efferocytosis and TGFβ production were demonstrated. …

Macrophages are major cellular components of multiple classes of tumours and, although they have proven power to kill tumour cells in their classically activated state (often known as M1), their predominant function in cancer tends to be to support tumour growth via multiple mechanisms, including activation of angiogenesis, production of growth and survival factors, and support of invasion and metastasis, while also suppressing anti-tumour immunity.

This reparatory macrophage phenotype—often referred to as M2- like—is typical of macrophages responding to apoptotic cells.

Similar to apoptosis, TAM accumulation is correlated with poor prognosis in diverse cancer types.

It has been proposed that the TME conditions the activation state of TAMs, inducing a switch from anti- (M1) to pro-tumour (M2-like). Of note, apoptotic cells are not only key players in inducing M2-like activation of macrophages, but are also readily engulfed by M1 macrophages and dominantly suppress M1 anti-tumour activity.

That apoptosis profoundly and dominantly affects macrophages is further demonstrated by its ability to imprint a reparatory memory on macrophages and elicit a dominant migratory response signal in flies, observations which suggest that apoptosis may provide dominant signals to TAMs in the TME.

TAMs are the most commonly encountered cells that, identified through their obvious efferocytotic activity, clearly interact with apoptotic cells in the TME.

While it seems obvious that efferocytosis could provide TAMs with mechanisms for nutrient recycling and transmission from apoptotic to healthy tumour cells, little is yet known about the biology of this process or its relevance to tumour growth.

It is very clear, however, that efferocytosis functionally programmes macrophages to produce arrays of cytokines and other bioactive molecules with pleiotropic effects that can promote tumour growth through proliferation signalling, regeneration and repair responses and anti-tumour immune silencing.

Amongst the most renowned of these are the anti-inflammatory mediators TGF-β1, IL-10 and PGE2, all of which are known to have pleiotropic effects on their target cells. A case in point in relation to the last of these factors is the 'phoenix rising' repopulation pathway, which was first described as an apoptotic cell-driven regeneration pathway active in skin wound-healing and liver regeneration.

… the pathway based on the activation of effector caspase-3 and -7, which generate arachidonic acid through iPLA2 cleavage and activation. Subsequent conversion of arachidonic acid by cyclooxygenases 1 and 2 into PGH2 is followed by conversion into PGE2 by PGE2 synthase. PGE2 was found to be the critical effector of stem cell proliferation, repair and regeneration. While this pathway may generate PGE2 release from apoptotic cells themselves, it also has elements of an efferocytic response to apoptosis, since cyclooxygenases are activated in macrophages by apoptotic cells and PGE2 is a common resultant product. The caspase-3-dependent production of PGE2 has also been reported to support the stimulation of tumour re-growth in mice following therapy-induced apoptosis in models of breast cancer, melanoma, bladder carcinoma and glioblastoma. PGE2 has additional oncogenic roles in suppressing adaptive immunity and promoting angiogenesis.

Because of the inherent plasticity of macrophages, the polarisation of TAMs is finely tuned by their environmental cues, including apoptotic cells.

In order to understand the activation status of TAMs interacting with apoptotic tumour cells in situ, we previously investigated the transcriptomic profile of efferocytosing TAMs in starry-sky NHL (SSTAMs) isolated from their TME by laser capture microdissection.

SS-TAMs were found to display an M2-like status, characterised by the upregulation of gene clusters known to be associated with

(1) apoptotic cell clearance and anti-inflammatory responses (e.g., MSR1, LRP1, MERTK, AXL, GAS6, CD36, CD93, LGALS3, ABCA1 and TGFB1), and

(2) survival, proliferation, angiogenesis, repair and remodeling (e.g., MRC1, ANPEP, GPNMB, HMOX1, PLAU, CTSB, CTSD, CTSL, IGF1, PDGFC, FN1 and MMP12).

Although the relative importance of most of these genes remains unproven with respect to their requirements for NHL growth, we have shown, subsequently, that NHL TME deficiencies in MERTK or LGALS3 (the gene encoding galectin-3) severely impair starry-sky NHL growth. Together, these studies demonstrate in a tumour context that efferocytosis imparts oncogenic properties to the TME.

Much remains to be learned about how apoptosis drives the M2-like phenotype of TAMs in the ORN, though it is notable that TAM production of lactate appears to be an important pathway in metabolic programming of pro-tumour, M2-like macrophage activation.

Intriguingly, efferocytosis stimulates aerobic glycolysis and lactate production in phagocytes leading to the generation of an anti-inflammatory milieu via SLC family activation

As Su et al (2013) note:

Apoptosis is the best-understood mechanism of programmed cell death.

It is recognized by distinct morphological characteristics of cells, such as cellular shrinkage with nuclear chromatin condensation and nuclear fragmentation. It functions as a homeostasis mechanism to maintain cell populations, as well as a defense mechanism in the presence of toxic agents. Apoptosis can be triggered by diverse cellular signals.

These include intracellular signals produced in response to cellular stresses, such as increased intracellular Ca2+ concentration, oxidative damage caused by ***reactive oxygen species*** *(ROS) , and hypoxia. Extrinsic inducers of apoptosis include bacterial pathogens , toxins , nitric oxide , growth factors , and hormones.*

Depending on the apoptosis-inducing signal, two different apoptosis pathways have been identified: the intrinsic pathway characterized by mitochondrial outer membrane permeabilization (MMP) and the release of mitochondrial cytochrome c; and the extrinsic pathway, which is initiated by death-receptor stimulation. There is an overlap between these pathways as the extrinsic pathway usually also activates the intrinsic pathway, and both pathways result in the recruitment and activation of cysteine-aspartic acid proteases (caspases).

Intracellular apoptotic signals trigger the intrinsic pathway, starting with the activation of different BCL-2 homology 3 (BH3) domain-only proteins. Activated BH3-only proteins bind to antiapoptotic BCL-2 proteins, preventing them from binding to and inhibiting the multi-BH domain proapoptotic proteins, BAX and BAK. This allows homodimerization of BAX and/or BAK in the outer mitochondrial membrane, forming channels that increase MMP to permit the release of cytochrome c as well as other apoptosis effectors. Cytochrome c then associates with

apoptotic protease-activating factor 1 (APAF-1) and caspase- 9 to form a complex called the apoptosome.

The apoptosome activates effector caspases leading to cell death. Intracellular stress such as DNA damage results in the transcriptional upregulation of proapoptotic proteins like p53-induced protein with a death domain (PIDD). PIDD recruits receptor-interacting protein (RIP)-associated ICH- 1/CED-3 homologous protein with a death domain (RAIDD) and caspase 2 to form a 700 kDa complex called PIDDosome.

Caspase 2 activated inside the PIDDosome induces apoptosis via cleavage of BH3-only proteins like BID, and subsequent MMP. The extrinsic pathway starts with the stimulation of specific death receptors upon binding of their ligands, like tumor necrosis factor-related apoptosisinducing ligand (TRAIL) and tumor necrosis factor (TNF).

The binding of ligands causes trimerization of these death receptors, resulting in clustering of their death domains and recruitment of Fas-associated death domain (FADD) and caspase 8, to form the death-inducing signaling complex (DISC). Caspase 8 is activated inside the DISC and can then promote cell death, either by activating effector caspases or by cleaving the BH3-only protein BID to initiate mitochondria-dependent apoptosis.

8.1.2 Autophagy

As noted previously, autophagy is a "self-eating" in a cell. Often result from stress and as Green notes a less well understood process compared to apoptosis. Yet again a TAM association is present. As Chen et al note:

Regulation and evasion of macrophage autophagy.

Autophagy is an effective cellular strategy of enclosing materials or pathogens in the cytoplasm into a double-membrane cellular organelle called the autophagosome, which disintegrates substrates with the help of lysosomes.

The process of autophagy is coordinated into different capacities and procedures of the immune system.

It is a substantial obstruction component to ensure the body's organization against external pathogenic invaders and threat signals, assuming an essential function in the enlistment and guideline of inflammatory responses in innate immune cells.

This remarkably detailed process combines more than 30 autophagy-related genes (Atgs) as operating units and immune signaling pathways. Atgs, serine/threonine kinase ULK1, and Beclin-1, in contrast to Atg14 and type III phosphatidylinositol 3-kinase Vps34 Atgs, advance the structure of a cup-shaped separation membrane to inundate the load after autophagy has been initiated. Similarly, the cell-deathinhibiting action seems to be achieved by ULK1 phosphorylation of S357 inside the intermediary motif of RIPK1.

According to the study, ULK1 is a possible modulator of RIPK1-induced cell death. Macrophages perceive pathogens by surface-induced receptors, in this way, overwhelming and processing them. Macrophages with M1 increment and discharge huge measures of inflammatory factors, for example, TNF-α, IL-1, iNOS, IL-6, and several chemokines, C-C chemokine ligand 2/4 (CCL2/4) chemokine and (C-X-C theme) ligand 8/11 (CXCL8/11) chemokine, that can initiate the immunity intervened by Th1 cells, aberrant inflammation, endotoxic distress, and organ damage.

In macrophages, xenophagy has, for the most part, been described during bacterial disease. Immunity-related GTPase family M protein in human macrophages is interested in xenophagy by advancing ROS yield and selecting autophagy apparatus after PAMP introduction. Eventually, the autophagosome sends invading intracellular microorganisms to the lysosome for disintegration.

Autophagy modifies the surface expression of the phagocytic receptors apparatus and manages phagocytosis circuitously.

Macrophages deficient in Atg protein Atg7 increase the upregulation of MARCO and MSR1 binary class A scrounger receptors, encouraging the phagocytosis activity of M. bovis BCG and Mycobacterium tuberculosis (MTB). Furthermore, autophagy can down-regulate inflammasome actuation through numerous components. The upregulation of IL-1β and pyroptosis is brought about by the loss of Atg7 in alveolar macrophages. Features and levels of autophagy subordinate extraordinarily to the macrophage microenvironment.

The nearness of nutrient D in serum upgrades macrophage autophagy considerably by employing the induction of cathelicidin antimicrobial peptide. Moreover, human macrophages' 1,25(OH)2D3 enhances innate immune effectors and cathelicidin production with TLR2/1 stimulation. T cells can likewise trigger an autophagocytosis response in MTB-infested macrophages in humans. At long last, microbiota may impact autophagy activity as well. Currently, the upregulation of autophagy genes in macrophages is brought about by the probiotic Bacillus amyloliquefaciens, which outcomes in the enhanced killing of Escherichia coli.

A large cluster of strategies is built by intracellular bacterial pathogens to offset antibacterial resistances in macrophages, and autophagy response is no particular case. Cytosolic L. monocytogenes keep from engulfing via autophagy machinery by using two virulence factors, ActA and InlK.

To avoid being killed by macrophages, microbial pathogens have developed complex strategies. It has proven a vital model organism to decode the molecular processes of the interactions between pathogenic bacteria and macrophages using L. monocytogenes, S. aureus, or Yersinia spp. Following transmission, Francisella evades the phagosome and enters the host cell cytoplasm, replicating extensively.

As Su et al (2015) note:

Autophagy is an evolutionarily conserved catabolic process in which intracellular membrane structures package protein complexes and organelles to degrade and renew these cytoplasmic components.

It is thus critical for cell growth regulation and internal homeostasis.

Autophagy is physiologically a cellular strategy and mechanism for survival under stress conditions.

When over-activated under certain circumstances, excess autophagy results in cell death. To date, three types of autophagy have been identified: macroautophagy, microautophagy, and chaperone mediated autophagy.

We only discuss macroautophagy in this review; therefore, henceforth, "autophagy" specifically refers to macroautophagy. Autophagy is a multi-step process that includes nucleation, elongation, and autophagosome and autolysosome formation and that is executed by a series of highly conserved genes termed autophagyrelated genes (ATGs). Autophagy is often triggered by nutrient deprivation, ROS, hypoxia, drug stimuli, and endoplasmic reticulum (ER) stress via complex signal transduction pathways. Alterations in the autophagy machinery may lead to diverse pathological conditions, such as neurodegeneration, ageing, and cancer.

Mammalian target of rapamycin complex 1 (mTORC1), class I PI3K, AKT, class III PI3K, Beclin-1 and p53 are critical components of the autophagic pathway that have become major targets of autophagy-related drug design. Numerous small molecules have been found to target these components and to play a role in tumor treatment. For example, rapamycin and its derivatives (i.e., rottlerin, PP242 and AZD8055) target the PI3K/AKT/mTOR signaling pathway to induce autophagy; spautin-1 and tamoxifen regulate Beclin-1 activity to inhibit and promote autophagy, respectively; and oridonin and metformin trigger p53-mediated autophagy and cell death. The role of autophagy in cancer metastasis is complex, as reports have indicated both pro-metastatic and antimetastatic roles of autophagy.

Stage-specificity may affect the cellular response to autophagy during cancer metastasis. During the early stage of cancer metastasis, autophagy may act as a suppressor of metastasis by restricting tumor necrosis and inflammatory cell infiltration and by alleviating oncogene-induced senescence. These processes may help to reduce the invasion and dissemination of cancer cells from the primary site.

During the advanced stages of metastasis, autophagy tends to act as a promoter of metastasis by promoting ECM detached metastatic cell survival and colonization in a distant site and by inducing metastatic cells that fail to establish contact with the ECM in the new environment to enter dormancy.

In Su et al (2013) the authors note:

Autophagy is a cell-survival pathway conserved in all eukaryotes. It involves the selective degradation of cellular components, including long-lived proteins, protein aggregates, damaged cytoplasmic organelles, and intracellular pathogens, resulting in the recycling of nutrients and the generation of energy.

Basal levels of autophagy are required for cellular homeostasis. Autophagy is upregulated under stress conditions, including extracellular stress such as nutrition deprivation, hypoxia, and infection and intracellular stress such as that caused by accumulation of damaged proteins and organelles and high bioenergetic demands.

It allows lower eukaryotes to survive starvation, while in mammals, it is thought to be involved in many physiological and pathophysiological processes, including antiaging mechanisms, differentiation and development, immunity, and elimination of microorganisms. Autophagy is a highly regulated process executed by autophagy-related effectors, many of which are called ATG proteins. The first committed step of autophagy is vesicle nucleation in which macromolecular assemblies selected for degradation are surrounded by isolation membranes called phagophores.

The vesicle nucleation process is executed by a protein complex whose core comprises the class III phosphatidylinositol-3-kinase (PI3Kc3 or VPS34) which catalyzes phosphorylation of phosphatidylinositol to phosphatidylinositol 3-phosphate; the PI3Kc3 regulatory subunit (p150 or VPS15), a myristylated serine/threonine kinase that phosphorylates PI3Kc3 and recruits it to the membrane; and the BCL-2 interacting protein (Beclin 1 or ATG6), which appears to be a protein interaction hub. More recently, Ambra 1, identified as a positive regulator of autophagy that interacts with Beclin 1, was shown to be part of the core complex.

Further, the core complex variably associates with various other proteins such as ATG14, UV radiation resistance-associated gene (UVRAG), vacuole membrane protein 1 (Vmp1), endophilin B1 (Bif-1), and Beclin 1 associated RUN domain containing protein (Rubicon), forming complexes that have distinct functions in membrane trafficking processes.

8.1.3 Necroptosis

From Su et al:

Necrosis was originally considered to be an accidental and unregulated cell death.

Accumulating evidence has shown that necrosis can be induced and proceed in a regular manner like apoptosis, although in a caspaseindependent fashion.

Regulated necrosis is termed “programmed necrosis” or “necroptosis” to distinguish it from necrosis caused by physical trauma.

Necroptosis can be induced by the activation of the TNF receptor superfamily, T cell receptors, interferon receptors, Toll-like receptors (TLRs), cellular metabolic and genotoxic stresses, or

various anti-cancer agents. It can be pharmacologically inhibited by chemical compounds such as necrostatin-1 (Nec-1). The formation of the "necrosome" by receptor-interacting protein kinase 1 (RIP1) and RIP3 is one of the most critical characteristics of necroptosis. It is a multi-step process that contains three key checkpoints.

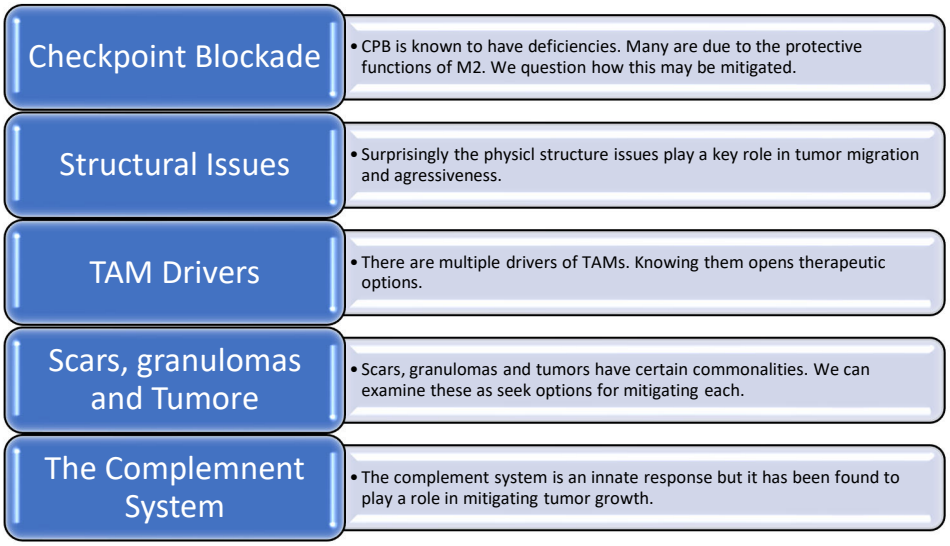
For example, in TNF-α mediated necroptosis , at the first checkpoint, the E3 ligases cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2 induce RIP1 ubiquitination , which blocks necroptosis via NF-κB-dependent or -independent mechanisms. The removal of ubiquitin chains from RIP1 by the deubiquitinase cylindromatosis (CYLD) is critical for the packaging of Complex IIa (including caspase-8, FADD, and RIP1) and Complex IIb [(including caspase-8, FADD, RIP1, RIP3, and mixed lineage kinase domain-like (MLKL)]. At the second checkpoint, activated caspase-8 cleaves and abolishes the activities of RIP1, RIP3, and CYLD

8.2 EXOGENEOUS

We have discussed the various forms of external cell death. Namely B and T cells, NK cells, cytokines and the like. We have considered some in this Note. As to exogeneous cancer cell death unfortunately cancer cells have a variety of means of avoiding them, with the TME complex being just one.

9 OBSERVATIONS

We discuss several extension on what we have presented herein. Namely the issues are as shown below:



These are added issue highlighted here and build upon what we have examined herein.

9.1 MACROPHAGES CAN EFFECT BLOCKADE ON IMMUNE CHECK-POINT BLOCKADE.

As Hofer noted:

Although promising against a wide range of cancers, there are many patients that are not responsive to ICB treatments.

In this issue of Nature Cancer, Scolaro et al.2 show that ICB-resistant pancreatic cancer cells overexpress cytidine deaminase resulting in the excessive production of uridine diphosphate (UDP), which — when excreted — creates an immunoprotected environment in the tumor with fewer cytotoxic (CD8+) T cells.

The authors propose that this function depends on the UDP-mediated recruitment of tumor-associated macrophages, which inactivate the T cells and prevent them from targeting the cancer cells Notably, the inhibition of cytidine deaminase or UDP signaling reverses ICB resistance in pancreatic tumor-engrafted mouse models, suggesting the potential use of such inhibitors in future ICB-treatment regimes. The present study was initiated by comparing publicly available transcription profiles of tumor tissues from responsive and resistant ICB-treated patients across various cancer types. Cytidine deaminase was then found to be commonly overexpressed in the resistant cancers.

Further investigation of pancreatic ductal adenocarcinoma cells and tumor tissues revealed that high levels of cytidine deaminase expression were correlated with increased intracellular and extracellular levels of UDP, enhanced recruitment of P2Y6-positive macrophages and fewer

cytotoxic T cells. … the proposed pathway begins with tumor cells taking up cytidine as a source for the production and excretion of UDP.

Extracellular UDP then activates macrophages via the P2Y6 receptor, which leads to macrophage recruitment into the tumor microenvironment and clearance of cytotoxic T cells. The effect of UDP signaling on macrophage recruitment was substantiated by an in vitro migration assay, whereas the clearance of T cells was assessed solely based on cell numbers with no mechanistic details. However, tumor-associated macrophages have been previously reported to induce cytotoxic T cell exhaustion.

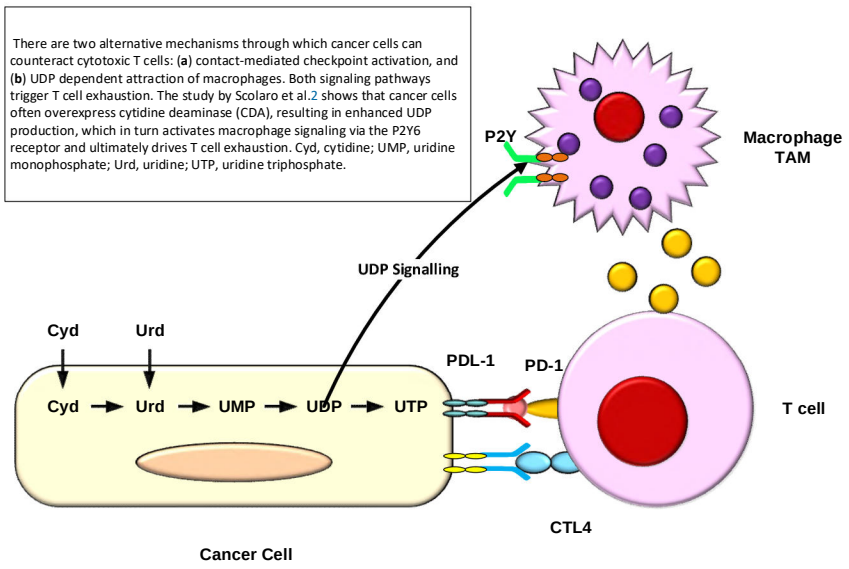
The signaling between macrophages and cytotoxic T cells is complex and involves direct cellular contacts as well as cytokine-mediated interactions.

Interestingly, Scolaro et al. show that it was necessary to block both cytidine deaminase and PD-1 receptor signaling for a full effect on T cell clearance, indicating that the UDP signaling alone has limited efficacy.

Based on the findings, the authors proposed that ICB treatment can be combined with inhibitors of cytidine deaminase or the P2Y6 receptor.

The immunomodulatory role of UDP and the P2Y6 receptor is well established but the major focus has previously been on innate immunity, inflammation and autoimmunity4. The limited literature available on the UDP–P2Y6 signaling axis in cancer includes reports showing that increased UDP levels or inhibition of the P2Y6 receptor is linked to enhanced migration of breast cancer cells, and that knocking out the P2Y6 receptor in mice leads to reduced lung metastases in a melanoma model.

Although the first study proposed that P2Y6 receptors are expressed by cancer cells5, the melanoma study aligns with the present findings that the P2Y6 receptor is highly expressed on tumor-associated macrophages and neutrophils. However, the results by Scolaro et al.2 indicate that macrophages are the most relevant cell population involved in P2Y6-mediated signaling, and that the cancer-related effects of this pathway are not limited to metastases



9.2 Mechanical or Structural Forces and key to the TME

The TME and related malignant cells create a structure. That structure is built upon the electrostatic forces of ligand and receptors as well as that of various other proteins. The strength of that structure is an added factor in the development of the tumor mass. As Yuan et al note:

Although many interactions between the stroma and transformed epithelial cells are influenced by secreted cytokines and growth factors, others can be triggered by acute, rapid changes in cell–cell and cell–ECM interactions, or by aberrant mechanical forces resulting from oncogenic mutations. Indeed CAFs secrete type I collagen and are responsible for most of the stromal ECM. The ability of tumour cells to invade depends upon the stiffness of their surroundings. In young, healthy tissues, stromal ECM is often stiff, impeding tumorigenesis and often requiring ECM-degrading enzymes to facilitate invasion.

As tissues age, stromal ECM becomes weakened, particularly in sun-exposed or mechanically stressed regions, probably contributing to the marked age-related rise in tumorigenesis. The stiffness of the basement membrane also contributes to tumour progression. In basal cell carcinomas, for instance, basement membrane proteins are secreted at a fast pace, inducing the formation of a softer, viscoelastic basement membrane that can contain lesions and keep tumours benign.

Of additional note, resistance to Hedgehog inhibitors is determined by basal cell carcinoma tumour architecture, with cells in close contact with the basement membrane constituting the resistant population. By contrast, skin epithelium harbouring HRASG12V mutations form cutaneous SCCs, which have thinner and stiffer basement membranes that rupture more easily in the face of the strong mechanical forces emanating from the stiff keratin pearls that typify these cancers.

These examples illustrate how fundamental changes in the biology established early by a particular oncogene can have profound non-genetic effects on later steps in tumour progression.

The importance of local mechanical forces as non-genetic factors in tumour progression has also been investigated in studies using a higher mutational burden. For example, in an array of mouse and human cancer cell lines grown in different patterns and hydrogel stiffness, geometric cues alone were shown to enhance CSC features in clonal tumouroids, including proliferation, migration, invasion and survival.

This principle has been documented in vivo in a model of pancreatic ductal adenocarcinoma driven by KrasG12D and loss of Trp53, in which it was demonstrated that the geometry of an incipient lesion can predict the aggressiveness of the tumour. Thus, the diameter of the duct where the pancreatic tumours arise determines whether the lesion will grow into the lumen (endophytically) or fold externally (exophytically).

Exophytic lesions interact closely with the TME78, so they also recruit pro-tumorigenic CAFs, facilitating tumour progression.

Another example of how the mechanical landscape of the extracellular space affects tumorigenicity and invasiveness comes from studies on human hepatocellular carcinoma lines, where stiffening of the ECM induces their secretion of exosomes, which in turn leads to NOTCH activation and growth enhancement upon xenografting.

Extracellular fluid viscosity can also reprogram breast cancer cells in vitro into disseminating phenotypes that favour metastasis, affecting the actin cytoskeleton, sodium and calcium transport and contractility.

Although not generally considered we believe these factors must be understood.

9.3 TAMs ARE KNOWN SIGNIFICANT DRIVERS IN MULTIPLE CANCERS

As Li et al note regarding colon cancer:

Many components of the immune microenvironment are implicated in proliferation, which is a primary hallmark of tumor. At present, the role of TAM in CRC proliferation has been widely reported. Through secreting transforming growth factor b1 (TGFB1), TAMs have abilities to upregulate vascular endothelial growth factor (VEGF) and interleukin-6 (IL6), and the latter binds with IL6 receptor on the tumor cell surface to promote CRC proliferation via activating STAT3. The extent of STAT3 activation is affected by diet, and dieting is considered a potential therapy that promotes anti-tumor immunity.

Under fasting conditions, M2 polarization was inhibited, resulting in limited proliferation and increased apoptosis of CT26 colon cancer cells. A current study provided an important observation that serum starvation caused differentiation markers of T-reg, TGFB1, and FOXP3 rising. This evidence leaves us some questions that if TAMs could play a role in T-reg by

modulation of TGFB1, fasting combined TGFB1 neutralizing antibody might show more potency against CRC proliferation. Based on the above research, eliminating M2 TAMs is regarded as an effective anti-cancer therapy. Yet, in eliminating macrophages of Ccr2−/− mice, TAMs can still proliferate and release cytokines to promote the growth of CRC.

So, in addition to the activation of the M0 alternative pathway, there are other ways to generate M2 macrophages. Meanwhile, CRC could escape immune supervision through this bypass, and figuring out associated specific mechanisms would bring new understanding to clinical therapy. CRC Metastasis During the malignant transformation, cancer cells undergo function acquisition and alteration due to genetic mutation. Among all the functions, the capacity of invasion is an essential criterion for judging malignant diseases.

Many studies have observed that TAMs accelerate tumor invasion mainly by regulating the epithelial–mesenchymal transition (EMT) process during which tumor cells gradually discard epithelial characteristics and obtain mesenchymal phenotypes, generating circulating tumor cells and tumor stem cells.

After a positive correlation between TAMs and the EMT marker snail was observed, TAMs were confirmed to release transforming growth factor b (TGFB) to activate the TGFB/ Smad2,3-4/Snail signaling pathway, and restraining the pathway with TGFB receptor inhibitor might reverse metastasis. Apart from TGFB, TAMs secret other cytokines, namely, IL4 and IL6, and the former indicates the formation of M2 phenotype, while the latter activates the STAT3 signaling pathway to trigger EMT in CRC.

EMT is an interregulating course, and there is an urgent need to lock the TAM/ EMT axis.

In recent years, intestinal microbiota has attracted much attention in immunomodulation. The microbiota dysfunction can induce TAMs secreting IL6 and TNF to launch EMT of HT29 colorectal adenocarcinoma cells. Maybe intestinal microbiota is an excellent target to attenuate the M2 phenotype. In-depth analysis of M2 macrophage-derived exosomes (MDE) shows high expression of miR-155-5p, while the microRNA also participates in metabolism-related gene transcription of intestinal microflora.

The findings provide the basis of intestinal microflora regulation served as one target of inhibiting CRC metastasis

9.4 SCARS, GRANULOMAS AND TUMORS

Scars, granulomas and tumors have certain morphological similarities. They take certain seed like cells and then surround them with protective cells. As Jeschke et al note:

The function and contribution of innate immune cells have become better understood in skin wound healing although the role of certain immune cells (adaptive) remains unclear.

Immune cells are crucial to help orchestrate the inflammatory process after injury. Immune cells migrate to the wound site, and the activation and recruitment of immune cells are initiated by

and depend on signals generated by damage- and pathogen-associated molecular patterns as well as several key chemokines (such as CXCL7, CXCL4 (PF4), CXCL1 (GROa), CXCL5, CCL5 (RANTES), CCL3 (MIP1a)) and cytokines (such as platelet-derived growth factor (PDGF) receptors, and transforming growth factor-β (TGFβ)) released by degranulating platelets.

Neutrophils are recruited early to the wound site and are responsible for clearing invasive microorganisms through phagocytosis.

Natural killer T cells (NK T cells) infiltrate soon after neutrophils, during the early inflammatory phase and produce chemokines that promote proliferation during healing such as by releasing macrophage inflammatory protein 2 (MIP2; also known as CXCL2).

Fibrocytes are a unique subpopulation of leukocytes that proliferate at wound site after injury and become fibroblast-like cells (they have been associated with hypertrophic and keloid scar development). Mast cells respond to any form of cutaneous injury by arriving early to degranulate and release their molecular cargo (histamine and chemokines) near nerve endings and blood vessels, which act as pro-inflammatory molecules and increase proliferation and vascular permeability …

Monocytes and macrophages arrive later and start to release cytokines and recruit fibroblasts into the wound site, initiate signals for angiogenesis and are responsible for removing dead leukocytes.

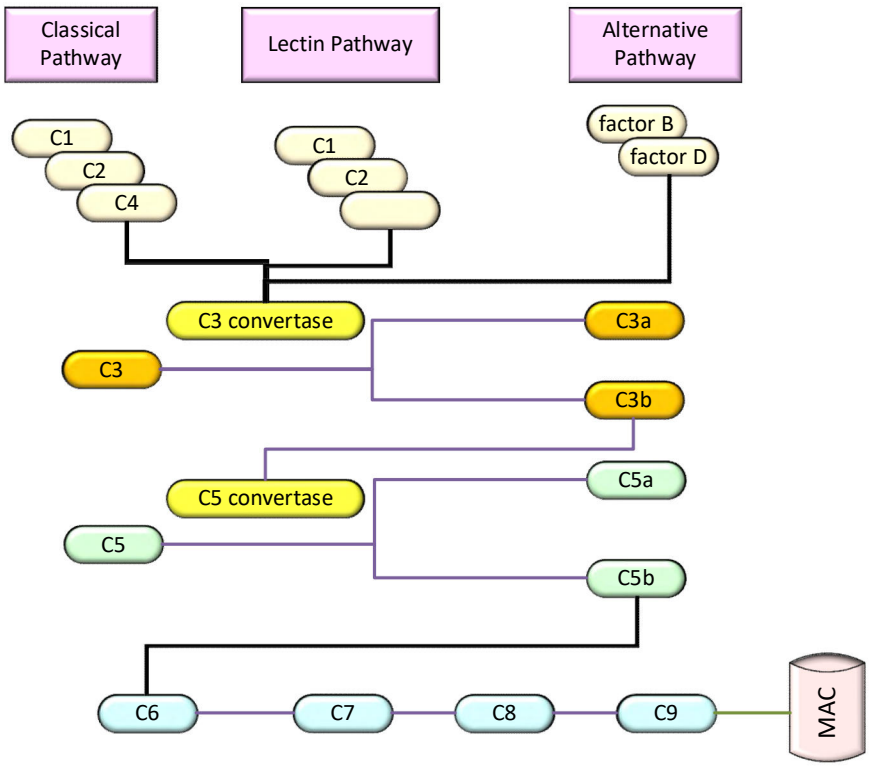
Both tissue-resident macrophages (arise from different lineages, dependent and independent of haematopoietic stem cells) and circulating bone marrow-derived monocytes have an important diverse function (anti-fibrotic (M1) or pro-fibrotic function (M2)) in generating and resolving the inflammatory response.

In addition, fibrocytes are considered to be hybrid mesenchymal–haematopoietic cells, having been identified in various fibrotic disorders such as keloid scars as the precursors of abnormal fibroblasts. γδT cells are thought to release certain growth factors responsible for epidermal migration.

Naive T helper (TH) cells (CD4+) can present as TH1 (exposed to IL-12) or TH2 (exposed to IL-4) phenotype in response to certain antigens and/or cytokines. In hypertrophic scars, it has been shown that there is a shift towards TH2 cell phenotype with increased IL-4 production. The role of innervation in cutaneous healing and scar formation needs further exploration even though developmental studies suggest a reciprocal positive association

9.5 THE COMPLEMENT AND TAMS

The complement system is an element of the innate immune system. Complement proteins are generally produced in the liver and work their way through the system looking for invaders. The three pathways leading to the cell destroying MAC are depicted below.



Now as Roumenina et al have noted:

The interactions of malignant cells with supporting and reactive non-transformed host cells are orchestrated by the density, location and functional activity of the latter and by soluble mediators released into the tumour microenvironment (TME).

Frequently neglected elements of the TME are the components of the complement system, produced by the tumour and infiltrating cells or originating from the circulation.

Complement is a key player in the innate immune defence against pathogens and in the maintenance of host homeostasis. It is composed of more than 50 plasma components produced mainly by the liver and released into the circulation as well as receptors expressed on the membranes of different cell types. The individual components interact with each other in the extracellular space.

Recent discoveries have made clear that complement effectors can also be generated intracellularly, leading to locally occurring complement activation, and that complement proteins have non-canonical functions, which are independent of the plasmatic cascade.

Cumulative evidence over the past 10 years has proved that complement proteins are present in the TME and that malignant and infiltrating cells have the capacity to produce in situ a large spectrum of these components.

Complement and cancer is an emerging field and most of the phenomena have been described in a single study or for a single type of cancer. Nevertheless, a solid body of evidence has

accumulated to demonstrate that the functionality and level of expression of complement proteins by malignant cells or in the TME can modulate the fate of the tumour. In cancer, the impact of complement is diverse, ranging from antitumour defence to potent tumour promotion….

Complement is a central part of immunity that serves as a first line of defence against pathogens and stressed host cells.

The complement system is composed of plasma proteins that react with one another to opsonize pathogens, inducing a series of inflammatory responses that concomitantly help immune cells to fight against infections and to maintain homeostasis2. The initiation of the complement cascade is dependent both on the context (for example, the nature of the trigger or the type of antigen) and on the tissue location ….

Direct impact of complement effectors on tumour cells. In addition to promoting inflammation, C3a and C5a could directly affect fundamental processes of tumour cells, such as survival, proliferation, migration and stemness (Fig. 3b,c). Anaphylatoxin receptors are expressed on certain cancer cells6. Multiple reports show that these tumour cells also express C3 and/or C5 and generate C3a and C5a, acting in an autocrine manner. The impact of this signalling on tumour cells ranges from stimulation of proliferation40,63 to maintenance of a multipotent state of glioblastoma stem-like cells64, induction of the epithelial-to-mesenchymal transition (EMT)39,65, changes in invasiveness and morphology66, and promoting stemness. Specifically, C3a enhances tumour cell proliferation, migration and stemness in mouse cutaneous squamous cell carcinoma and this activity was correlated with activation of the WNT–β-catenin pathway.

If the cascade proceeds to terminal MAC formation, the sublytic levels of C5b-9 mediate signalling, promoting cancer cell cycle progression.

Briefly, in tumour cells, C5a triggers expression of matrix metalloproteinases (MMPs), increases tumour cell migration and invasiveness, enhances the release of pro-angiogenic factors and induces EMT. Anaphylatoxins also facilitate tumour dissemination by stimulating a hypercoagulable state (an increased predisposition to form blood clots) and NETs, and adapt specific organ environments to metastatic spread*.*

In addition, C5a induces CXC-chemokine ligand 16 (CXCL16)- mediated osteoclastogenesis and the generation of an immunosuppressive microenvironment in a mouse model of lung cancer bone metastasis. Pharmacological blockade or genetic deficiency of C5aR1 was sufficient to reduce lung metastases in a breast cancer mouse model.

Specifically, C5aR1 signalling promoted regulatory T (Treg) cell generation and suppressed T cell responses in the lungs in this context. In addition, C5aR1 expression in patients with gastric cancer is associated with cancer progression, liver metastasis and poor prognosis.

Cancer cell-derived C3a also promotes leptomeningeal metastasis by activation of C3aR on the choroid plexus epithelium, thus disrupting the blood–cerebrospinal fluid barrier in vivo. In theory, the complement cascade may lead to tumour cell killing within primary tumours or metastases, if sufficiently strongly activated by host antitumoural immunoglobulin M (IgM) or

IgG, or by therapeutic antibodies, and if abundant MACs are inserted into the cell membrane. Yet, in the context of cancer, current evidence suggests that complement can only proceed to cell-killing MAC assembly following treatment with targeted therapeutics (such as monoclonal antibodies that target tumour cells74). A large body of evidence has demonstrated that this escape from complement killing is in part linked to a high expression of complement regulators at the tumour cell surface6

Proposed mechanism of classical complement pathway activation and its consequences on tumour progression in tumours with 'aggressive complement'.

1. *C1q is produced by tumour-associated macrophages (TAMs) (**step 1**)*
2. *and contributes to a tumour-promoting phenotype of these cells (**step 2**)*
3. *and T cell exhaustion (**step 3**).*
4. *Secreted C1q promotes adherence of tumour cells to the extracellular matrix (**step 4**)*
5. *and neoangiogenesis (**step 5**).*
6. *A particular feature of clear cell renal cell carcinoma (also known as kidney renal clear cell carcinoma (KIRC)) is that the tumour cells produce C1r and C1s **(step 6)***
7. *and allow formation of a functionally active C1 complex **(step 7)**, capable of activating the classical pathway.*
8. *Moreover, immunoglobulin G (IgG) deposits on tumour cells serve as C1 ligands (**step 8**) to initiate the cascade.*
9. *The tumour cells also produce the subsequent components of the complement cascade, which enable C4 and C3 activation fragment deposition (C4b, C4d and C3b, iC3b, C3d; note that not all of these are shown on the schematic for simplicity) (**step 9**).*

Anaphylatoxins C3a and C5a are released, exerting their action on the tumour cells and on the microenvironment. The ensemble of these processes contributes to tumour progression and poor prognosis for patients with cancer. This model is based on the data for KIRC and is potentially applicable to other tumour types within the 'aggressive complement' group. C3aR, C3a receptor; C5aR1, C5a receptor 1; MDSC, myeloid-derived suppressor cell; PDL2, programmed cell death 1 ligand 2.

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