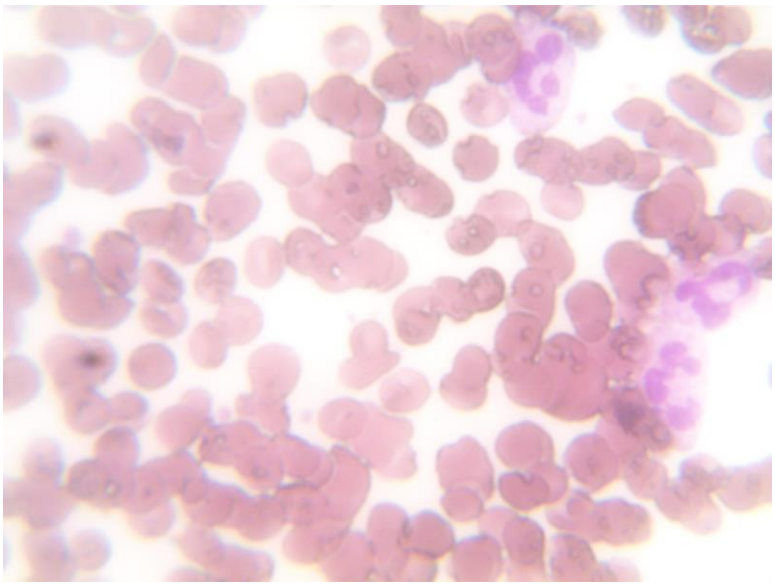

Neutrophils and Inflammation

January 2023

ABSTRACT

This note focuses on the dynamics of neutrophil control and proliferation driven by an inflammatory state. It examines each element in some detail and attempts to elucidate possible therapeutic options.

Terrence McGarty
TGL 195

Notice

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

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1 INTRODUCTION

Neutrophils are the most numerous of the granulocytes. They have a generally short life time and are the fastest responders of the innate immune system. Dead neutrophils are the pus one finds in infected areas. Many physicians look at neutrophils are measure of possible infections. Yet the complexity of neutrophil generation and response is yet to be fully understood. We consider the case of a chronic inflammatory condition and its impact on neutrophils in this note. Unlike acute infections which we generally assume to be somewhat fluent in, chronic inflammation, in our study osteoarthritis, presents a more complex scenario.

1.1 A CLINICAL EXAMPLE

Let us consider the following clinical example[1].

An 80 year old male patient presents with a stiffness in the right knee. The knee and the foot appear somewhat inflamed. An X ray presents a simple case of osteoarthritis on the right knee on the lateral side. On palpation there also is a popliteal cyst (Baker's cyst) which was drained and an injection of methylprednisolone was provided. The patient was advised to use a meloxicam as a NSAID and return if complications were to arise. The PCP some sixty days later did a CBC and it was unremarkable except for a slightly elevated neutrophil count, 7.3, but the knee and foot appeared unremarkable. Prior neutrophil count was 5.2 three months earlier just after the steroidal injection. The patient still complained of mild discomfort and a tightness in the knee.

Thus is this increase may be attributed to the recurrence of the osteoarthritic inflammation. The question we examine herein is; what are the elements associated with this increase in neutrophils and is this a common mechanism or a unique?

1.2 THE PARADIGM

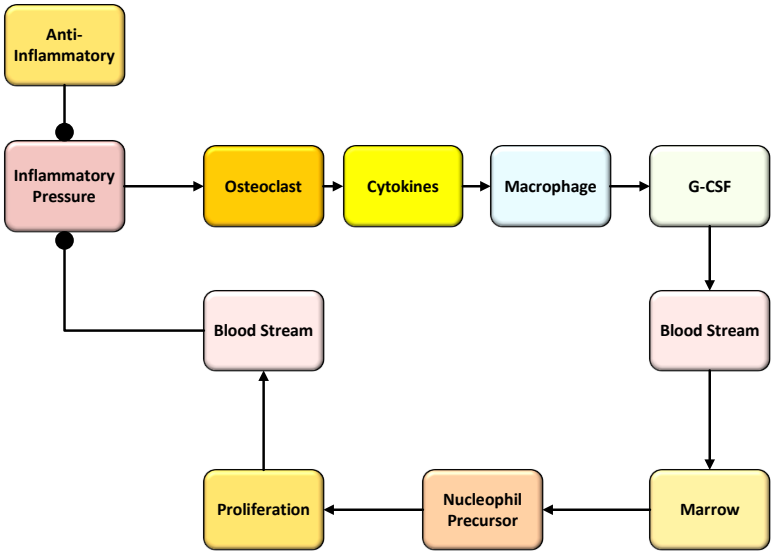
We seek to examine the above clinical example from a systems approach. Namely what is the driver or initiator of the process, specifically given an osteoarthritic inflammatory state of a chronic nature, what is being produced and how does that ultimately drive the generation of neutrophils? Then with the increased neutrophils, what actions are they put to that may be beneficial to the inflamed site?

Thus the question we address herein is:

How do neutrophils respond to chronic inflammation, such as osteoarthritis and is this chronic inflammation response prototypical of many other such chronic inflammations? If not, then what are the significant differences? Finally, can these neutrophil enriched chronic inflammations become sources for the generation of cancers and if so which ones?

[1] See Sharma for another clinical example.

We use a paradigmatic model of this process as shown below. We will go through this in detail below.



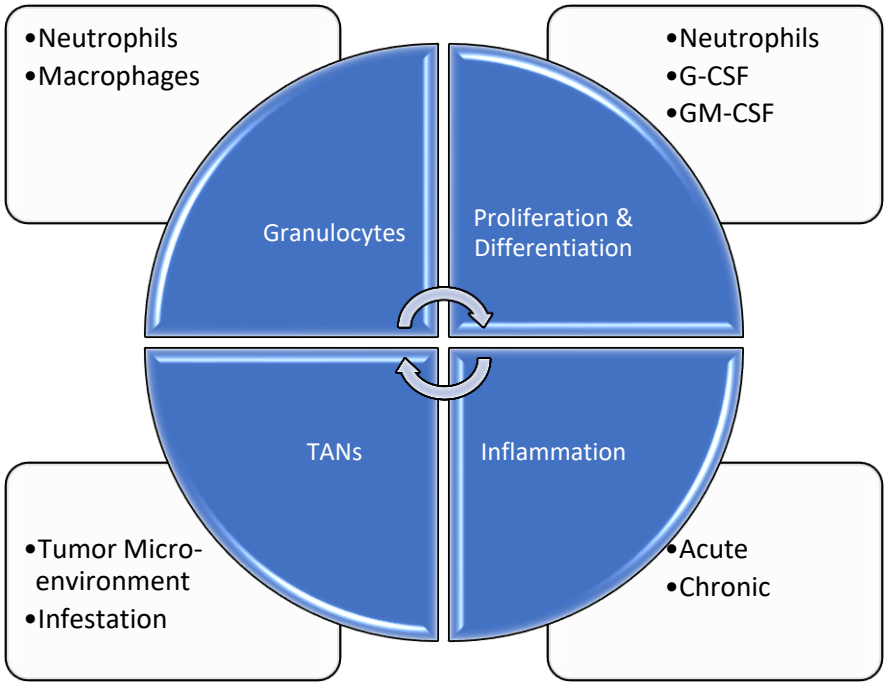
1. An irritant initiates the process say at the knee joint. Let us say the cartilage is broken down so we have a bone on bone irritation. This irritation may generate inflammasomes or cytokines which begin a process[2].
2. The signalling from the injured cells then activates macrophages that are resident.
3. The macrophages then produce G-CSF which enters the blood stream and thus the bone marrow.
4. G-CSF increases and thus it increases the proliferation of neutrophil predecessors. Ultimately neutrophil counts increase.
5. The neutrophils then are targeted to the inflamed region where damaged synovial cells are eliminated.
6. Therapeutics usually are NSAIDS which reduce some inflammation and may then reduce the driver to the neutrophil counts.

[2] See Zoete et al. They note : *Inflammasomes are large cytosolic multiprotein complexes that assemble in response to detection of infection- or stress-associated stimuli and lead to the activation of caspase-1- mediated inflammatory responses, including cleavage and unconventional secretion of the leaderless proinflammatory cytokines IL-1b and IL-18, and initiation of an inflammatory form of cell death referred to as pyroptosis. Inflammasome activation can be induced by a wide variety of microbial pathogens and generally mediates host defense through activation of rapid inflammatory responses and restriction of pathogen replication. In addition to its role in defense against pathogens, recent studies have suggested that the inflammasome is also a critical regulator of the commensal microbiota in the intestine. Finally, inflammasomes have been widely implicated in the development and progression of various chronic diseases, such as gout, atherosclerosis, and metabolic syndrome. In this perspective, we discuss the role of inflammasomes in infectious and noninfectious inflammation and highlight areas of interest for future studies of inflammasomes in host defense and chronic disease.*

It is this paradigm which we will try to fill out in this note.

1.3 Overview

Our approach is graphically shown below:



We proceed through the above topics focusing on the recent literature. Our goal is to attempt to assemble the system paradigm based upon documented experimental evidence.

1.4 Key Observations

We have made several key observations. We present some of them here.

1.4.1 Complexity of Inflammation

Inflammation is a highly complex process. Unlike the well know steps of bacterial or viral attacks on the immune system and the resulting immune system responses, we have a multiplicity of paths at play. Multiple elements of the innate system are responsive as well as the injured cells themselves. As we shall note, in the simple case of osteoarthritis, the driving factors may be disparate and complex.

1.4.2 Complexity of Proliferation

The process of proliferation of cells, such as the increase in neutrophils, occurs before differentiation yet contains a putative different element. We know that G-CSF promotes proliferation but proliferation solely of the neutrophil demands a differentiation almost concurrently. The details of this process are yet to be well defined.

1.4.3 Complexity of Differentiation

In a similar manner once proliferation occurs one must understand the differentiation into specific cell lines. However, the mechanism of differentiation appears better understood as the specific line matures. We examine this in detail.

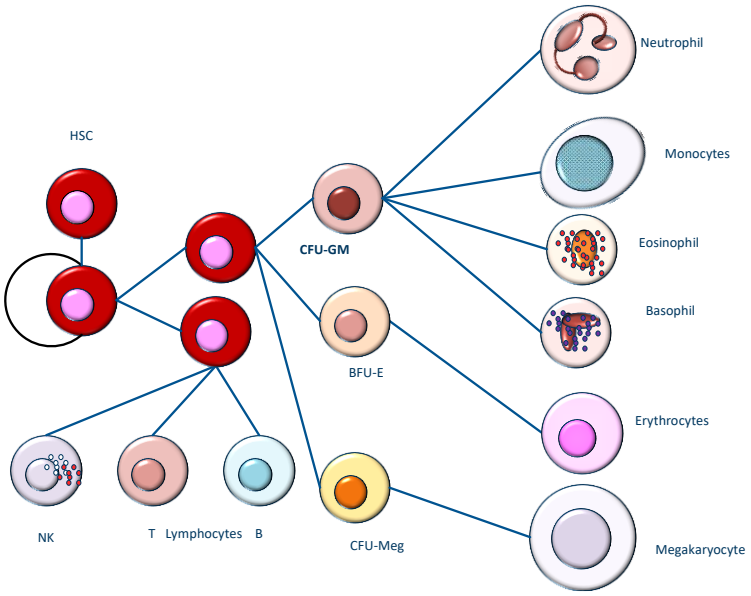
1.4.4 Putative Therapeutic Targets

Using the example of osteoarthritis we see a case for low level neutrophils to clean up the cellular detritus due to the chronic inflammation but also a set of steps wherein one may find therapeutic targets. Synovial cells do have a regenerative capability and balancing a chronic inflammatory immune response with such synovial regeneration may prove useful

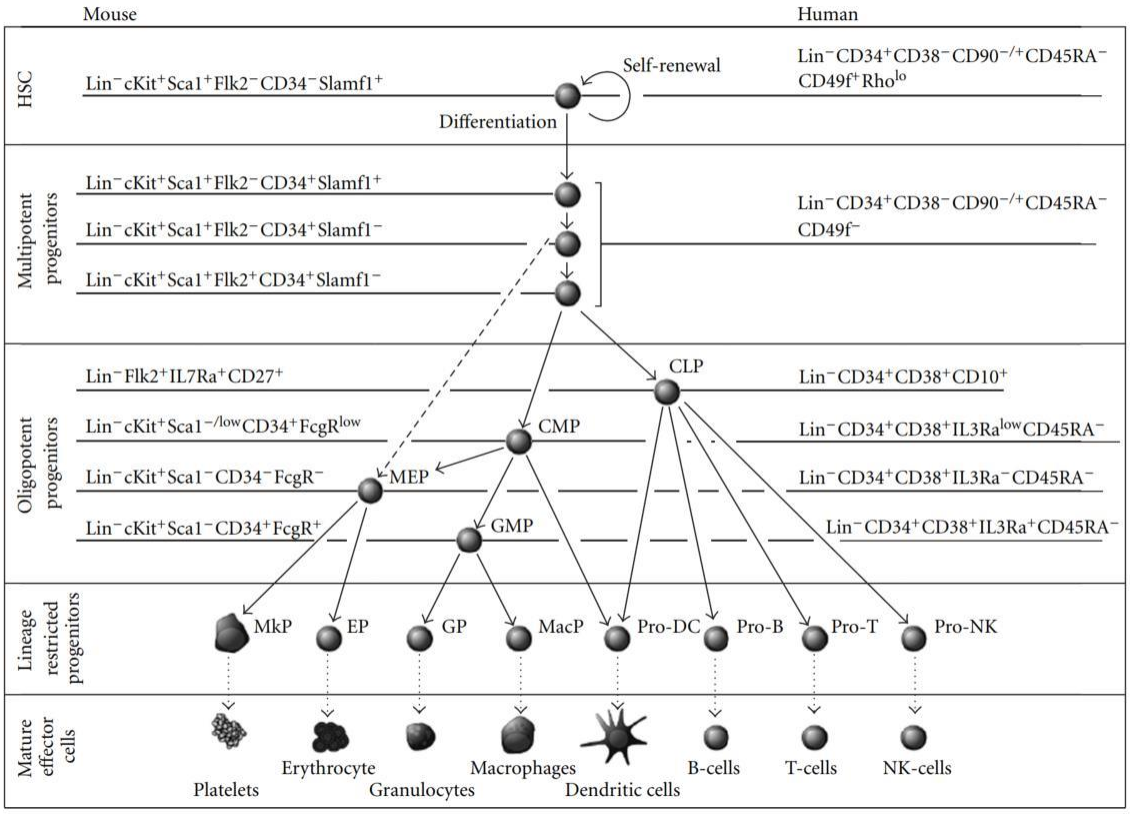
2 NEUTROPHIL GENERAL PATHWAYS

In this section we examine the neutrophil pathways that begin in the bone with a stem cell, HSC, and then progress to circulating mature neutrophils. The focus here is what causes the change from an HSC to a mature cell. It does not address the issue of what the cause of the causes are, namely the system dynamics of neutrophil production. In many ways this is a classic approach of a compartmentalized analysis, examining the system in a closed manner and deflecting the causes. We address the full system in the next section.

The graph below is the standardized form of cellular differentiation. It starts with a hematopoietic stem cell, HSC, and then the process of proliferation and differentiation occurs.



There is substantial complexity as these cells differentiate. Chotinantakul and Leeanansaksiri have a chart which shows the cell markers as they mature and we reproduce it below:



Our focus is on neutrophils and in that context there is even more complexity as we shall demonstrate.

2.1 NEUTROPHILS

Our focus herein is on neutrophils and thus this will be our initial discussion. All too often we consider them to be associated with infections. Yet they respond to a multiplicity of cellular disfunctions especially inflammation. Furthermore as we shall discuss later they play a role in multiple cancers as a tumor associated neutrophil, TAN.

As Abbas et al note :

Phagocytes, including neutrophils and macrophages, are cells whose primary function is to ingest and destroy microbes and remove damaged tissues. The functional responses of phagocytes in host defense consist of sequential steps: recruitment of the cells to the sites of infection, recognition of and activation by microbes, ingestion of the microbes by the process of phagocytosis, and destruction of ingested microbes. In addition, through direct contact and by secreting cytokines, phagocytes communicate with other cells in ways that promote or regulate immune responses.

Blood neutrophils and monocytes, which differentiate into macrophages after entering tissues, are produced in the bone marrow, circulate in the blood, and are recruited to sites of

inflammation. Although both are actively phagocytic, they differ in significant ways. The neutrophil response is more rapid and the lifespan of these cells after they enter tissues is short, whereas macrophages in tissues can live for long periods so that the macrophage response may last for a prolonged time.

Neutrophils mainly use cytoskeletal rearrangements and enzyme activation to mount rapid, transient responses, whereas macrophage responses rely more on induced gene transcription and protein expression. In addition, as we discuss later, there are subsets of macrophages that normally reside in healthy tissues, but neutrophils do not. The functions of phagocytes are important in innate immunity and also in the effector phase of some adaptive immune responses.

Neutrophils circulate as spherical cells approximately 12 to 15 μm in diameter with numerous membranous projections. The nucleus is segmented into three to five connected lobules. Because of their nuclear morphology, neutrophils are also called polymorphonuclear leukocytes (PMNs), to contrast them with mononuclear cells (macrophages and lymphocytes), whose nuclei are not multilobed. The cytoplasm contains two types of membrane-bound granules.

The majority of these granules, called specific granules, are filled with enzymes, such as lysozyme, collagenase, and elastase. These granules do not stain strongly with either basic or acidic dyes (hematoxylin and eosin, respectively), which distinguishes neutrophils from two other types of circulating leukocytes with cytoplasmic granules, called basophils and eosinophils. The remainder of the granules of neutrophils, called azurophilic granules because they are stained by azure A dyes, contain enzymes (e.g., myeloperoxidase) and microbicidal substances, including defensins and cathelicidins… Neutrophils are produced in the bone marrow and arise from precursors that also give rise to circulating monocytes.

Production of neutrophils is stimulated by granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). An adult human produces more than 1×10^{11} neutrophils per day, each of which circulates in the blood for a few hours or up to 5 days before dying. Neutrophils may migrate to sites of infection rapidly after the entry of microbes. After entering tissues, neutrophils function for only 1 to 2 days and most of them then die.

The above is a high level discussion of neutrophils. A key observation is that neutrophils have a short lifetime and a significant generation rate.

We shall examine such questions as to where the CSFs come from and what activates the sources of proliferation and differentiation. We also will examine the details of the process of proliferation and differentiation.

2.1.1 Neutrophil Complexity

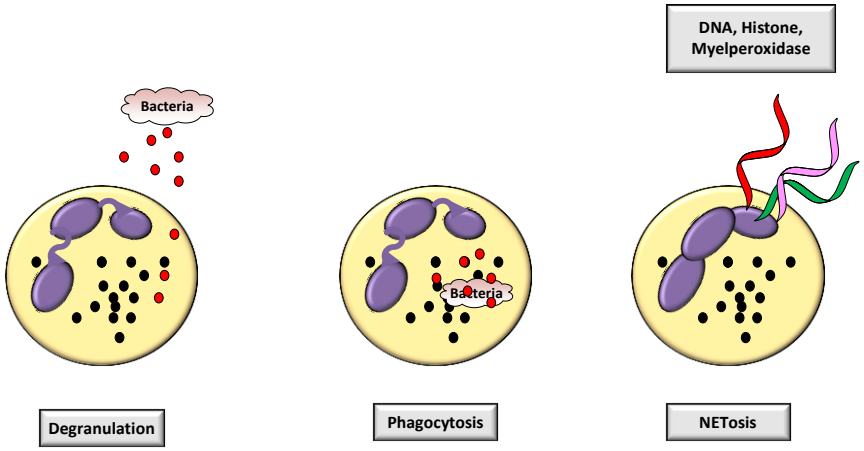
Neutrophils have a complexity comparable to many other HSC descendants. Understanding this complexity and what elements affect what cells in what environments is essential. Now Rosales has noted :

Neutrophils are the most abundant leukocytes in the circulation, and have been regarded as first line of defense in the innate arm of the immune system. They capture and destroy invading microorganisms, through phagocytosis and intracellular degradation, release of granules, and formation of neutrophil extracellular traps after detecting pathogens. Neutrophils also participate as mediators of inflammation. The classical view for these leukocytes is that neutrophils constitute a homogenous population of terminally differentiated cells with a unique function.

However, evidence accumulated in recent years, has revealed that neutrophils present a large phenotypic heterogeneity and functional versatility, which place neutrophils as important modulators of both inflammation and immune responses. Indeed, the roles played by neutrophils in homeostatic conditions as well as in pathological inflammation and immune processes are the focus of a renovated interest in neutrophil biology. In this review, I present the concept of neutrophil phenotypic and functional heterogeneity and describe several neutrophil subpopulations reported to date. I also discuss the role these subpopulations seem to play in homeostasis and disease

The above sets the stage for trying to understand the complexity of neutrophils. The classic view was one of a common neutrophil with a well understood path of proliferation and differentiation. We shall see that it is much more complex than that.

Rosales does also note in the case of bacterial attacks that neutrophils act in a variety of ways as shown below:



It is then noted that NETosis is defined as:

Three main antimicrobial functions are recognized for neutrophils: phagocytosis, degranulation, and the release of nuclear material in the form of neutrophil extracellular traps (NETs).

These functions were considered, until recently, the only purpose of neutrophils.

However, current research by investigators in several fields of neutrophil cell biology has revealed that neutrophils possess a much diverse repertoire of functional responses that go beyond the simple killing of microorganisms.

Neutrophils respond to multiple signals and respond by producing several cytokines and other inflammatory factors that influence and regulate inflammation and also the immune system.

Nowadays it is recognized that neutrophils are transcriptionally active complex cells that produce cytokines, modulate the activities of neighboring cells and contribute to the resolution of inflammation, regulate macrophages for long-term immune responses, actively participate in several diseases including cancer, and even have a role in innate immune memory. The multitude of neutrophil functional responses is induced by transcriptional activation and by changes in expression of surface molecules or activity. …

NETosis, the process for producing NETs can be activated by multiple types of microorganisms.

Yet, the capacity of neutrophils to undergo NETosis can vary with physiological states, suggesting a neutrophil diversity that could be clinically relevant.

In fact, several reports indicate that NETs can influence thrombosis and vascular inflammation, cancer and autoimmunity.

As mentioned before some metabolic conditions associated with states of ***chronic inflammation****,* ***can increase neutrophil predisposition to form NETs****. Hence, neutrophils from diabetic patients and from systemic lupus erythematosus (SLE) patients have been shown to be more prone to NET formation.*

Our focus is chronic inflammation. Thus the operation of NETosis is a critical one in understanding how neutrophils operate in such an environment such as osteoarthritis.

Likewise, Abbas et al note :

Neutrophils are produced in the bone marrow and arise from precursors that also give rise to circulating monocytes. Production of neutrophils is stimulated by granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). An adult human produces more than 1 × 1011 neutrophils per day, each of which circulates in the blood for a few hours or up to 5 days before dying. Neutrophils may migrate to sites of infection rapidly after the entry of microbes. After entering tissues, neutrophils function for only 1 to 2 days and most of them then die. …

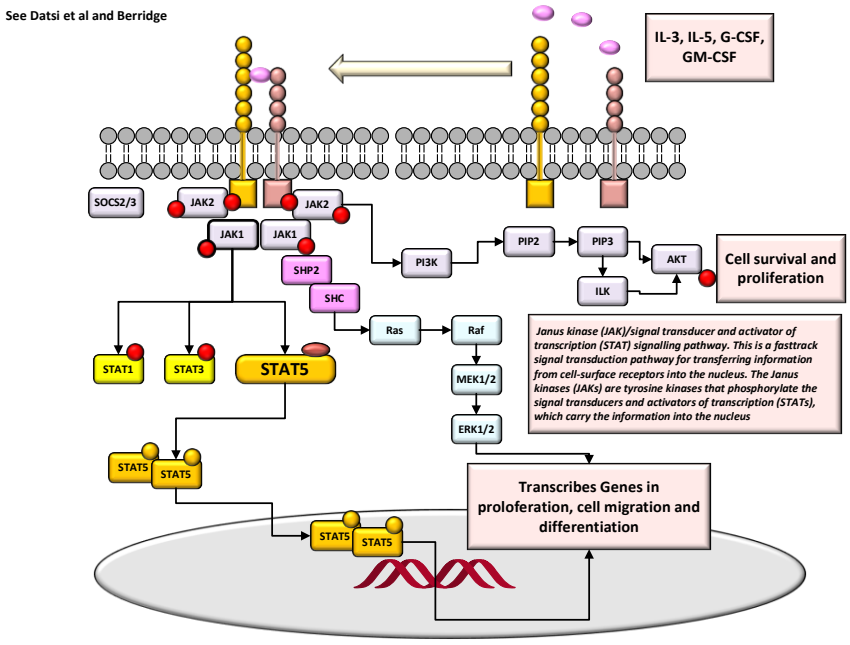
Macrophages promote the repair of damaged tissues by stimulating new blood vessel growth (angiogenesis) and synthesis of collagen-rich extracellular matrix (fibrosis). These functions are mediated by cytokines secreted by the macrophages that act on various tissue cells.

This then leads to the two key topics. Specifically we now consider the control elements from a system perspective. The previous section examined the understanding of neutrophil proliferation and differentiation in situ, independent of the other forces effecting those changes. Here we consider the process in toto. First the process of proliferation and then differentiation.

As we shall note, proliferation is a key element. One needs many neutrophils in the event of an infection or inflammatory process. Yet the neutrophils must abate when the infection or inflammations dissipates. As we noted neutrophils have a short lifetime and thus their response to activate must be fast and likewise for deactivation. However what we attempt to do here is to lay out the process of how an infection or inflammation drives proliferation and then how it is abated.

2.1.2 Proliferation

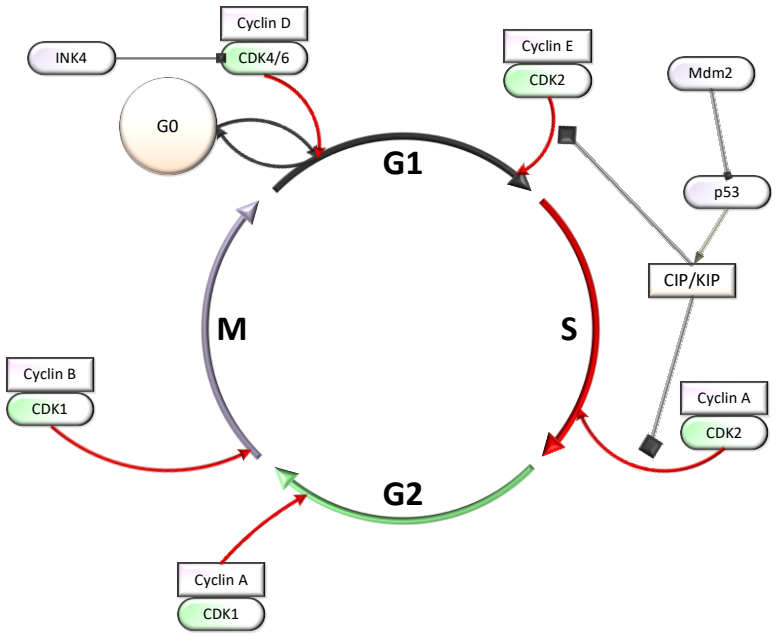
Proliferation is the expansion of the number of cells. In the following we show how G-CSF and other cytokines work through a cell to activate proliferation. The chart below is based on Datsi et al. The challenge is to understand why the progenitors of a mature neutrophil get activated. This chart focuses on the JAK/STAT paradigm, and we shall see variants of this.



How well this applies to the proliferation process details may still be an open issue. However understanding this detail is essential for many therapeutic approaches.

Recall the details of the cell cycle are as below[3]:

[3] See Morgan for an excellent overview of all cell cycle issues.



Now c-Myc plays a key role here. Myc or specifically c-Myc, is a powerful gene element which induces cell growth. c-Myc is so strong promoter of cell proliferation and growth. c-Myc is a transcription factor which is essential in the growth and expansion of the cell.

As Pelengaris et al note regarding the impact of c-MYC :

The proto-oncogene c-MYC encodes a transcription factor that is implicated in various cellular processes, cell growth, proliferation, loss of differentiation and apoptosis.

c-MYC activates a variety of known target genes as part of a heterodimeric complex with the protein MAX.

For example, cyclin D2 and CDK2 are essential for cell-cycle progression, and translation initiation factors eIF4 and eIF2 are important in cell growth.

MYC/MAX heterodimers regulate gene activation through chromatin remodelling: association with co-activator TRRAP, which contains HAT activity, leads to acetylation of nucleosomal histones.

c-MYC inhibits the differentiation of many cell types. Conversely, MAD/MXI1 transcription factors promote differentiation by antagonizing c-MYC function by forming dimers with MAX. MAD?MAX dimers recruit corepressors (such as SIN3) and HDACs to target DNA, leading to histone deacetylation and subsequent repression of MYC target genes. c-MYC sensitizes cells to a wide range of pro-apoptotic stimuli in vitro via cytochrome c release from mitochondria and subsequent formation of the apoptosome with APAF1 and procaspase-9.

Our interest in c-MYC is in its role in neutrophil proliferation. It has been found to be an essential step in terms of its activation. We shall discuss this later in some further detail.

As Madden et al note further details on c-MYC and its activation:

Myc is a transcription factor that belongs to the basichelix-loop-helix-leucine zipper (bHLHZip) family present in the cell nucleus, where it acts to regulate cell growth, differentiation, metabolism and death, and is frequently dysregulated in many human cancers. It is the prototype member of the Myc family that also encompasses N-Myc and L-Myc proteins in mammalian cells, all of which are highly homologous but distributed differently. c-Myc is ubiquitous and highly abundant in proliferating cells, whereas N-Myc and LMyc display more restricted expression at distinct stages of cell and tissue development. Myc proteins exist within the Myc/Max/Mxd network.

To fold and become transcriptionally active cMyc must first heterodimerize with Max, a process governed by the coiling of their bHLHZip domains. Once dimerized, the c-Myc/Max complex acts as a master transcriptional regulator by binding via its basic region to a specific DNA consensus sequence CANNTG, known as the Enhancer-box (E-box). Within the network, c-Myc can only heterodimerize with Max, whereas Max is more promiscuous and able to homodimerize or heterodimerize with other factors that share a bHLHZip motif. These include proteins of the Mxd family (Mxd1-Mxd4, formally called Mad proteins) as well as Mnt (a protein distantly related to Mxd-family), and the much larger Mga, an unusual protein that contains both a bHLHZip motif and a T-domain DNA-binding motif. …

c-Myc is a master regulator of immunometabolism and its dysregulation is implicated in inflammatory, autoimmune, metabolic and other non-cancerous disorders, although it remains poorly understood. The lack of an effective inhibitor that directly targets cMyc compromises studies investigating the potential of c-Myc inhibition as a therapeutic strategy to treat chronic diseases. Nevertheless, recent reports using indirect inhibitors or transgenic mice have shown some potential.

It was recently verified that c-Myc expression is upregulated in group 2 innate lymphoid cells (ILC2s) in the blood of asthma patients. Using a mouse model of allergic inflammation, it was found that inhibition of c-Myc repressed ILC2 activity, causing reduction in airways inflammation and other pathogenic responses

In the paper by Iwata et al the authors examine its influence during the development of PIN in the prostate. They state:

Lo-MYC and Hi-MYC mice develop prostatic intraepithelial neoplasia (PIN) and prostatic adenocarcinoma as a result of MYC overexpression in the mouse prostate[1]. However, prior studies have not determined precisely when, and in which cell types, MYC is induced. Using immunohistochemistry (IHC) to localize MYC expression in Lo-MYC transgenic mice, we show that morphological and molecular alterations characteristic of high grade PIN arise in luminal epithelial cells as soon as MYC overexpression is detected.

These changes include increased nuclear and nucleolar size and large scale chromatin remodeling. Mouse PIN cells retained a columnar architecture and abundant cytoplasm and appeared as either a single layer of neoplastic cells or as pseudo-stratified/multilayered structures with open glandular lumina—features highly analogous to human high grade PIN.

Also using IHC, we show that the onset of MYC overexpression and PIN development coincided precisely with decreased expression of the homeodomain transcription factor and tumor suppressor, Nkx3.1. Virtually all normal appearing prostate luminal cells expressed high levels of Nkx3.1, but all cells expressing MYC in PIN lesions showed marked reductions in Nkx3.1, implicating MYC as a key factor that represses Nkx3.1 in PIN lesions.

To determine the effects of less pronounced overexpression of MYC we generated a new line of mice expressing MYC in the prostate under the transcriptional control of the mouse Nkx3.1 control region. These ''Super-Lo-MYC'' mice also developed PIN, albeit a less aggressive form. We also identified a histologically defined intermediate step in the progression of mouse PIN into invasive adenocarcinoma. These lesions are characterized by a loss of cell polarity, multi-layering, and cribriform formation, and by a ''paradoxical'' increase in Nkx3.1 protein. Similar histopathological changes occurred in Hi-MYC mice, albeit with accelerated kinetics.

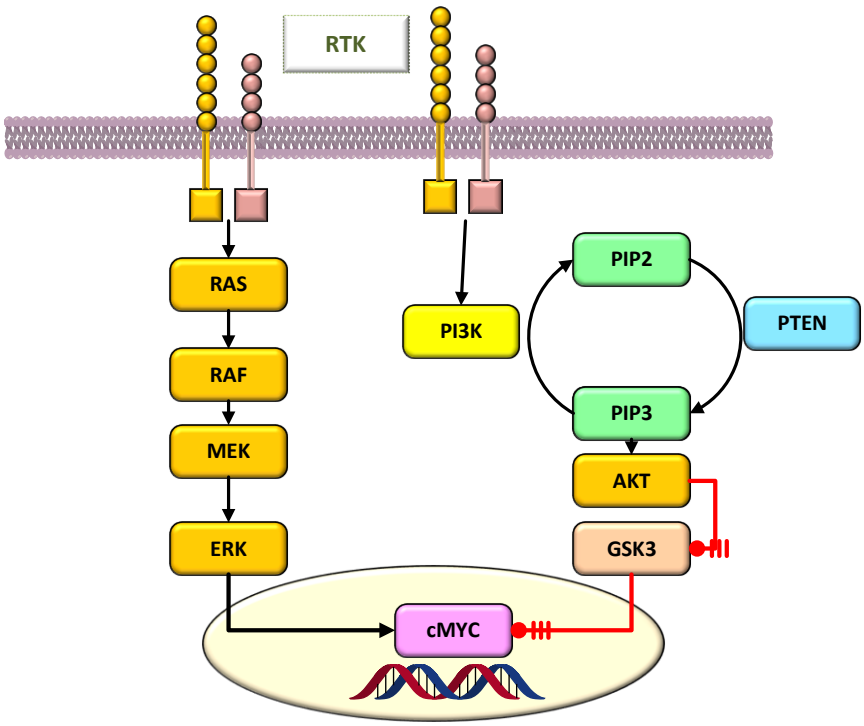
Our results using IHC provide novel insights that support the contention that MYC overexpression is sufficient to transform prostate luminal epithelial cells into PIN cells in vivo. We also identified a novel histopathologically identifiable intermediate step prior to invasion that should facilitate studies of molecular pathway alterations occurring during early progression of prostatic adenocarcinomas.

In the following graphic we depict the influence elements on c-Myc. This is a complex system of interlinking genes which when expressed in the correct manner can slow cell over expansion. The chart below is a modification from Bunz (p. 203) and it shows the gross characteristics of this control path. PTEN is a key element in control. What this does not show are two key elements, and indirectly a third.

First it does not show the fact that these are protein concentrations at work, one influencing the other and so forth. There is a feedback mechanism missing.

Second, it does not portray the temporal elements, namely this is a static gross representation of the influencing factors as if done in some generic snapshot. I fact the concentrations are time varying and it is this time variation which when combined with the feedback loops renders certain instabilities leading to malignancy, namely uncontrolled growth.

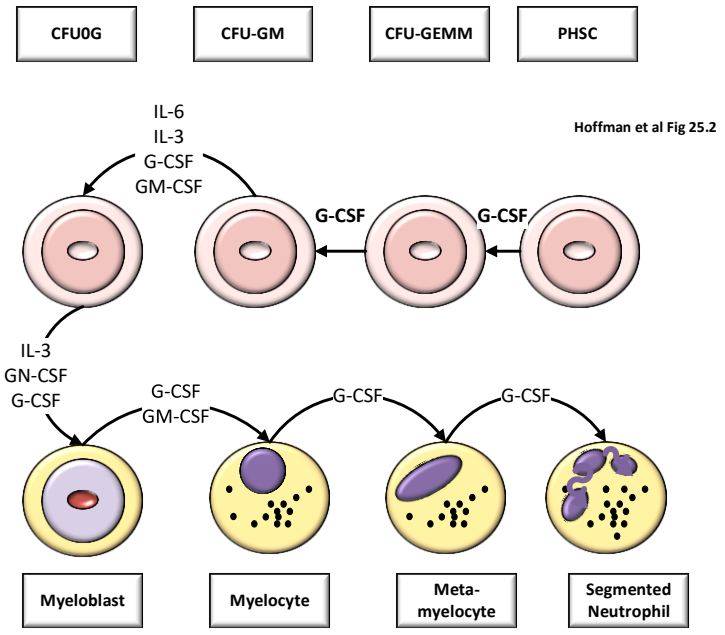
Third, it fails to show the other genes and specifically the feedback mechanism of these genes. Namely PTEN is influence by these.



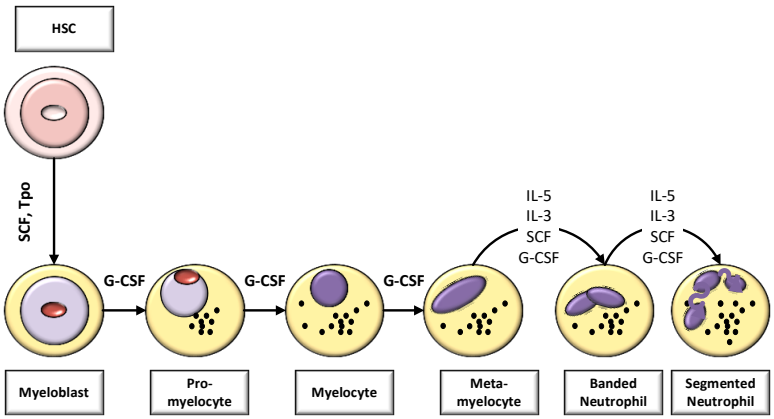
Thus cMYC plays a key role but it is a role that is played in may environments. Specifically the cell cycle and the drivers of that cycle can be initiated by cMYC actions.

2.1.3 Differentiation

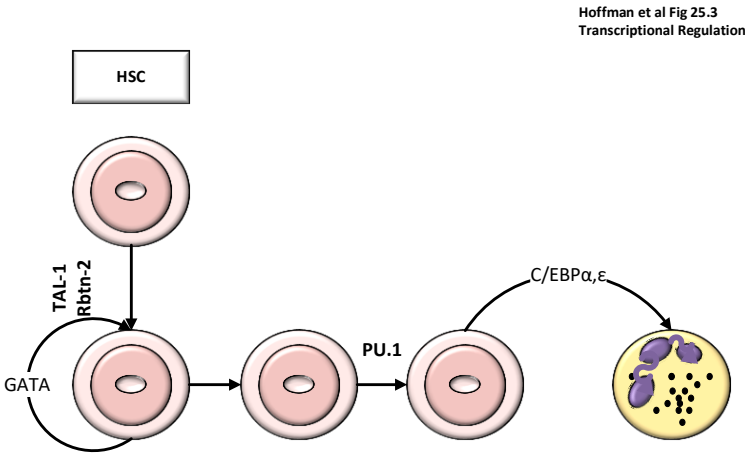
Now the details of differentiation seem better understood. One starts with the HSC and then goes through certain expression and morphological changes. The flow described below provides some insight. This is a modified version as presented by Hoffman. It should be noted that there are a multiplicity of steps in differentiation leading to the release of multi-lobed neutrophils.



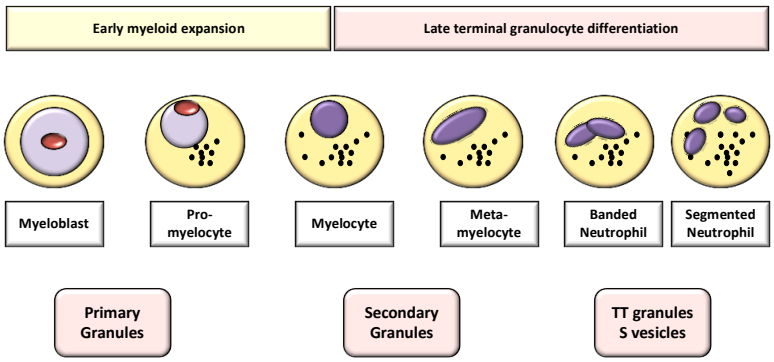
The diagram below presents another view of the process but focusing on the more mature version. Again G-CSF plays the controlling element. We will focus on G-CSF later in this section.



Further delineation is presented in the following figure where we see the development of the granulation. As these processes move forward a variety of CD surface markers can be used to ascertain the progress.

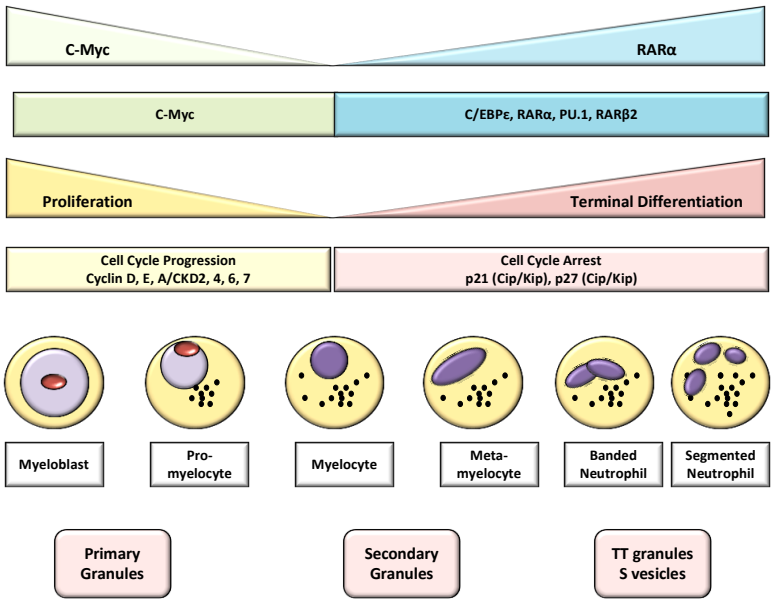


Another view is from Abdel-Azim et al. Here the authors delineate the proliferation and differentiation steps in some detail.



Now the following details the change from Proliferation to Differentiation. It is the activation of c-Myc that plays a key role in proliferation. Significant cell cycle activation allows for the proliferative process whereas that is tampered down with differentiation taking over on the proliferated cells.

From Abdel-Azim et al we have the following progression detailed.



These steps represent a complex set of interactions that are still a work in progress. These steps also show that there are multiple variants understood along the way. It further argues that proliferation seems to be a distributed process. If so then one may ask how if there is an excess drive on neutrophils why there will not be a commensurate proliferation of other common pathway granulocytes.

There is a question of uniqueness in proliferation that seems to have been unanswered. If G-CSF drives neutrophils from the Myeloblast stage forward, one should expect that all resulting myeloid lines should proliferate. If we have neutrophilia alone then there must be some complex set of factors that differentiate at the same time as proliferation. Thus if that is the case then the above graphic displays a dichotomy which does not exist. What then is the actual process?

As von Vietinghoff and Ley have summarized some minor details:

Hematopoietic cytokines promote neutrophil progenitor proliferation and differentiation acting in a complex network. The major cytokine for neutrophil proliferation and survival is GCSF. Mice and humans deficient in either G-CSF or its receptor suffer from profound neutropenia. G-CSF currently is the major therapeutic agent for neutropenia of iatrogenic as well as genetic and various other origins.

Extensive basic science and clinical data exist on the role of other granulopoietic cytokines such as M-CSF, GM-CSF, interleukin (IL)-6, IL-3, IL-17 and, most recently, IL-22 that have been reviewed elsewhere in detail. Genetic modification of intracellular messengers downstream of GCSF in mice elucidated their stage-specific roles.

For example, both STAT3 and SOCS3 deficiency resulted in neutrophilia and an increased pool of late stage progenitors in the bone marrow thus implicating an inhibitory role.

The role of transcription factors and microRNA in neutrophilic differentiation has recently been reviewed. A number of monogenic defects associated with rare forms of congenital neutropenia in humans are known.

Maturation arrest and increased cell death of neutrophil progenitor proliferation have been observed in humans with elastase gene mutations, but also in genes encoding a number of transcription factors such ***as Growth factor independent 1 (GFI 1[4]), HCLS1[5] associatied protein X-1 (HAX1[6]), and lymphoid enhancer factor-1 (LEF-1[7])…***

The above is a complex set of adjuvant genes and their protein elements. The detailed interaction amongst these products is specified but not well defined. They continue:

Stable neutrophil blood counts are the result of a highly dynamic feedback system. The study of genetically altered mice and monogenic diseases in humans has given insight into some of the involved mechanisms. However, neutrophil counts in healthy humans are regulated by a variety of environmental and genetic factors, most of which remain currently unknown. As elevated counts within the normal range are associated with excess mortality, elucidation of factors involved in steady state neutrophil regulation might have clinical relevance.

2.2 MACROPHAGES

[4] As NCBI notes: *This gene encodes a nuclear zinc finger protein that functions as a transcriptional repressor. This protein plays a role in diverse developmental contexts, including hematopoiesis and oncogenesis. It functions as part of a complex along with other cofactors to control histone modifications that lead to silencing of the target gene promoters. Mutations in this gene cause autosomal dominant severe congenital neutropenia, and also dominant nonimmune chronic idiopathic neutropenia of adults, which are heterogeneous hematopoietic disorders that cause predispositions to leukemias and infections. Multiple alternatively spliced variants, encoding the same protein, have been identified for this gene.*

[5] As NCBI notes: *Enables RNA polymerase II-specific DNA-binding transcription factor binding activity and protein kinase binding activity. Involved in several processes, including positive regulation of intracellular signal transduction; positive regulation of protein phosphorylation; and regulation of transcription, DNA-templated. Located in cytosol; nucleus; and plasma membrane. Part of transcription regulator complex*.

[6] As NCBI notes: *The protein encoded by this gene is known to associate with hematopoietic cell-specific Lyn substrate 1, a substrate of Src family tyrosine kinases. It also interacts with the product of the polycystic kidney disease 2 gene, mutations in which are associated with autosomal-dominant polycystic kidney disease, and with the F-actin-binding protein, cortactin. It was earlier thought that this gene product is mainly localized in the mitochondria, however, recent studies indicate it to be localized in the cell body. Mutations in this gene result in autosomal recessive severe congenital neutropenia, also known as Kostmann disease. Two transcript variants encoding different isoforms have been found for this gene*.

[7] As NCBI notes: *This gene encodes a transcription factor belonging to a family of proteins that share homology with the high mobility group protein-1. The protein encoded by this gene can bind to a functionally important site in the T-cell receptor-alpha enhancer, thereby conferring maximal enhancer activity. This transcription factor is involved in the Wnt signaling pathway, and it may function in hair cell differentiation and follicle morphogenesis. Mutations in this gene have been found in somatic sebaceous tumors. This gene has also been linked to other cancers, including androgen-independent prostate cancer. Alternative splicing results in multiple transcript variants*

We now move to the macrophages. As Abbas et al describe them:

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

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

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

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

Macrophages also recognize and engulf apoptotic cells before the dead cells can release their contents and induce inflammatory responses. Throughout the body and throughout the life of an individual, unwanted cells die by apoptosis as part of many physiologic processes, such as development, growth, and renewal of healthy tissues, and the dead cells are eliminated by macrophages. Macrophages are activated by microbial substances to secrete several different cytokines that act on endothelial cells lining blood vessels to enhance the recruitment of more monocytes and other leukocytes from the blood into sites of infections, thereby amplifying the protective response against the microbes. Other cytokines act on leukocytes and stimulate their migration to tissue sites of infection or damage.

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

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

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

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

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

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

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

1. M1 macrophages (classically activated cells) originate upon encounter with IFN[8]-and microbial stimuli such as LPS and are characterized by IL-12high and IL-23 production and consequent activation of polarized type I T cell response, cytotoxic activity against phagocytozed microorganisms and neoplastic cells, expression of high levels of ROI, and good capability as APCs.

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

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

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

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

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

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

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

As Quaranta and Schmid note:

*Macrophages originate from **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 u**ndergo a continuous turnover in adulthood by recruitment of circulating monocytes which differentiate into macrophages upon tissue infiltration**.*

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

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

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

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

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

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

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

From Ruffell and Coussens:

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

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

VEGF antagonists induce vascular normalization, and several studies have reported increased uptake of chemotherapeutics associated with this process, likely because of reduced vessel leakiness and interstitial fluid pressure. Although macrophages are not necessarily a dominant source of VEGF-A in all tumor tissues, specific deletion of VEGF-A in macrophages via lysozyme M promoter-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…

2.3 GRANULOCYTES

The other granulocytes have been examined and present other elements of the innate immune system. As Abdel-Azim et al note :

Neutrophil granulocytes, generally referred to as neutrophils, constitute up to 70% of total circulating leukocytes. They may be subdivided into banded and segmented neutrophils that form part of the polymorphonuclear cell family, together with basophils (~1% of leukocytes) and eosinophils (~3% of leukocytes).

Neutrophils, the most abundant type of leukocyte, function as the major phagocytes and the final effector cells to form an essential part of human innate immune system against invading microorganisms. The development of neutrophil innate immunity is dependent on neutrophil granulocytic differentiation, during which the production of stage-specific neutrophil granules are essential for the ability of neutrophils to fulfill their functions through coordinated direct and alternative antimicrobial.

Granulopoiesis, the process of producing sequential stage-specific granules, starts from the myeloblast stage of committed neutrophilic precursors. It gives rise to several morphologically distinct stages that are readily identified by their characteristic nuclear shape and granule contents.

At least four subtypes of granules, including primary (azurophil) granules, secondary (specific) granules, tertiary (gelatinase) granules, and secretory vesicles, have been characterized.

These granules are essential for neutrophils to fulfill their role as key effector cells of innate immunity against infection. The sequential formation of different granules has been characterized as two stages of granulocytic differentiation toward maturation of neutrophils, in which early myeloid expansion is associated with formation of primary granules, whereas the productions of secondary, tertiary, and secretory granules occur at the late terminal granulocytic differentiation stage.

Myeloblasts derived from GMP are the first neutrophilic precursors committed to granulopoiesis. Granule formation within the developing neutrophil begins between the myeloblast and promyelocyte stages, which acquires primary granule and forms a round nucleus.

During this early development, both myeloblasts and promyelocytes are still actively proliferating cells.

The next maturation sequence starts from myelocytes that feature the appearance of secondary granules and a round nucleus. Although myelocytes still retain some proliferative capacity, their cell division terminates at transition to the stage of metamyelocytes with a kidney-shaped nucleus. After this irreversible cell cycle exit from proliferation, metamyelocytes differentiate into banded neutrophils where tertiary granules are formed, showing a horse-shoe shaped nucleus.

Granulopoiesis is completed with the development of secretory vesicles, in which neutrophils acquire a characteristic segmented nucleus …

C/EBPα is the predominant C/EBP isoform expressed in immature myeloid cells during the developmental stages of myeloblast and promyelocyte. Absence of C/EBPα ceases neutrophil development, and C/EBPα is involved in regulating expression of primary myeloperoxidase and neutrophil elastase granules. Expression of C/EBPα also results in production of neutrophilic cells that express mRNAs encoding specific lactoferrin and collagenase. Of note in early stage of granulocytic differentiation, both C/EBPα and RARα can induce transcription of PU.1, a hematopoietic transcription factor that modulates the formation of myeloperoxidase, neutrophil elastase, lysozyme, and proteinase-3 primary granules.

c-Myc regulates myeloid expansion by promoting cell growth in actively proliferating myeloblasts and promyelocytes during early. c-Myc induces most of the critical positive cell cycle regulatory genes to promote cell proliferation, including E2F transcription factors, cyclin-dependent kinases (CDKs), and cyclins. In E2F−/− cells, c-Myc fails to induce S-phase, and transcription of E2F genes by c-Myc allows E2F to activate transcription of target genes promoting cell cycle progression.

In proliferating myeloid cells, Myc-Max heterodimers induce Ebox-containing genes and repress RARα-target genes, leading to a block of differentiation while cells are proliferating.

To promote proliferation,

c-Myc also <u>inhibits the transcription of CDK inhibitor p21Cip/Kip</u> by interacting with the initiator-binding transcription factor Miz-1,

and <u>down-regulates CDK inhibitor p21Cip/Kip by inducing SKP2</u>, a protein involved in degradation of p21Cip/Kip.

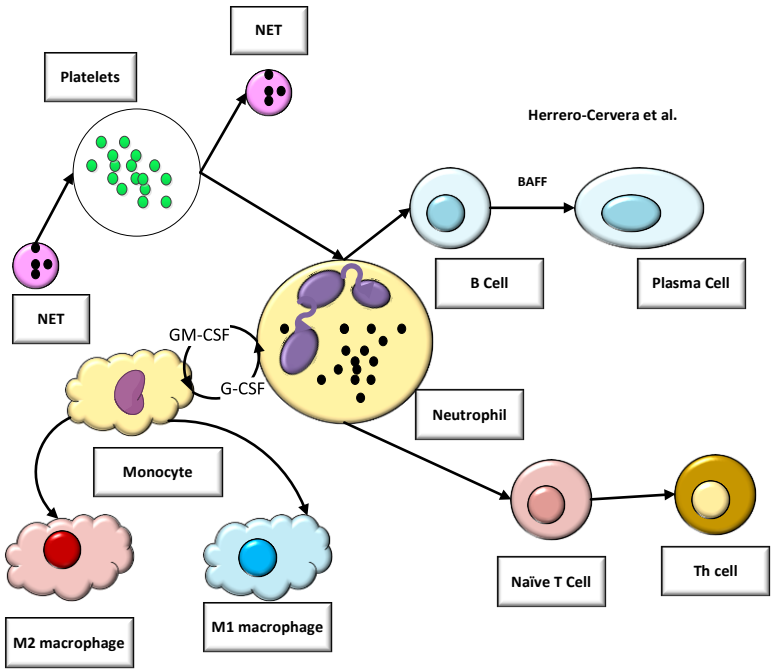
RARα, upon completion of early myeloid expansion and primary granule formation, plays essential role in promoting the transition from myeloid proliferation to late terminal granulocytic differentiation by down-regulating c-Myc expression.

At the myelocyte stage, RA-induced suppression of c-Myc expression is the first critical event required for immature myeloid cells to commit to terminal differentiation.

Downregulation of c-Myc by RA occurs via its effect on transcript elongation. Both RA treatment and blocking c-Myc mRNA significantly inhibit c-Myc expression and induce granulocytic differentiation. Importantly, phosphorylated c-Myc by Pak2 kinase following RA stimulation forms a complex with RARα on RAREs of RA-responsive genes to induce transcription of RARα-target genes, leading to irreversible cell cycle arrest and terminal granulocytic differentiation. Coordinately, C/ EBPα also down-regulates c-Myc expression to promote terminal granulocytic differentiation.

2.4 LOCAL INTERCOMMUNICATIONS

There is a complex set of local intercommunications between the resident neutrophil and other immune cells. Herrero-Cervera et al. Note this process as graphically shown below (as modified):



The authors then note:

Neutrophil crosstalk with cells in the circulation.

Neutrophil interactions with platelets occur by direct adhesion through PSGL-1, integrin αLβ2, and CD40 on neutrophils and P-selectin, ICAM-2 and CD40L on platelets.

S100A8/A9 secreted by neutrophils promotes megakaryocyte proliferation and platelet production.

Platelet secretion by PEVs containing chemokines and the release of chemokines from platelet granules activate neutrophils and promote NET formation.

Conversely, NET proteins activate the coagulation cascade.

Neutrophils promote B cell survival and differentiation to plasma cells through the secretion of BAFF, which binds to BCMA on B cells.

Neutrophils can act as APCs to promote T cell differentiation in effector T cells through MHC molecules or inhibit T cell proliferation.

Monocytes can extend the neutrophil lifespan and promote neutrophil recruitment through the secretion of GM-CSF and G-CSF. Monocyte recruitment is mediated by granule proteins released from neutrophils.

Lactoferrin, azurocidin, S100A9, HPN1–3, LL-37 and NETs induce M1 macrophage polarization, while NETs, LL-37 and Anx1 induce M2 polarization. Anx1 annexin 1, APCs antigen presenting cells, BAFF B cell activating factor; BCMA, B cell maturation antigen, CD40L CD40 ligand, HNP1–3 human neutrophil peptides 1–3, ICAM intercellular adhesion molecule, MHC major histocompatibility complex, NET neutrophil extracellular trap, PEVs platelet extracellular vesicles, PSGL-1 P-selectin granulocyte ligand 1 …

Recently, it has become clear that the neutrophil population is not homogenous and is now considered a heterogeneous population. Neutrophil heterogeneity includes phenotypic plasticity, which affects several immunoregulatory neutrophil functions.

The understanding of the lack of homogeneity is a key factor in understanding the effect of neutrophils on chronic inflammation.

A question can be asked; do the multiplicity of neutrophils change character as the inflammation changes and if so what is that process? There does not seem to be significant insight at this time. The authors continue:

To study neutrophil heterogeneity, neutrophils can be isolated from peripheral blood by density gradient centrifugation. In healthy donors, normal-density neutrophils (NDNs) are found on top of erythrocytes after density gradient centrifugation. However, patients with acute or chronic inflammation exhibit a heterogeneous population of mature and immature neutrophils in the mononuclear cell fraction.

This population is known as ***low-density neutrophils (LDNs)***.

The LDN population was first described in SLE and RA and has now been well characterized in both diseases. The LDN population includes granulocytic/ polymorphonuclear-myeloid-derived suppressor cells (PMNMDSCs) with immunosuppressive properties and low-density granulocytes (LDGs), which are characterized as having proinflammatory effects. The PMN-MDSC population has been extensively investigated in cancer patients in whom PMN-MDSCs are expanded compared to healthy subjects.

The PMN-MDSC population suppresses T cell proliferation via the release of ROS and arginase-1. Another neutrophil subset found in cancer is ***tumor-associated neutrophils (TANs)****, which are neutrophils that have infiltrated tumor tissue and that can exert pro- or antitumor effects. In this review, we will focus on the subsets that have been studied in selected chronic inflammatory disease conditions. For more information about neutrophil heterogeneity in cancer, we refer to other papers*

From Abbas et al we have a list of the principal driving cytokines, their sources and their effects.

Cytokine	Size	Principal Cellular Sources	Principal Immature Cell Targets	Principal Cell Populations Induced
Stem cell factor (c-KIT ligand)	24 kD	Bone marrow stromal cells	HSCs	All
Interleukin-7 (IL-7)	25 kD	Fibroblasts, bone marrow stromal cells	Immature lymphoid progenitors	T lymphocytes
Interleukin-3 (IL-3)	20–26 kD	T cells	Immature progenitors	All
GM-CSF	18–22 kD	T cells, macrophages, endothelial cells, fibroblasts	Immature and committed myeloid progenitors, mature macrophages	Granulocytes and monocytes, macrophage activation
M-CSF	Dimer of 70–90 kD; 40-kD subunits	Macrophages, endothelial cells, bone marrow cells, fibroblasts	Committed progenitors	Monocytes
G-CSF	19 kD	Macrophages, fibroblasts, endothelial cells	Committed granulocyte progenitors	Granulocytes
FLT-3 ligand	30 kD	Bone marrow stromal cells	HSCs, DC and B cell progenitors	Classical and plasmacytoid DCs, B cells

We will now focus on G-CSF as a major player.

2.5 G-CSF

We now discuss the properties and processing of G-CSF. G-CSF, the granulocyte stimulation factor, is a pervasive element in the development of neutrophils. We see it from the macrophage at the site of an inflammation to the multiple steps in proliferation and differentiation. As Goldberg et al note:

Granulocyte colony-stimulating factor (G-CSF) is a regulator of neutrophil production, function, and survival. …

Granulocyte colony-stimulating factor (G-CSF) is a major regulator of steady-state neutrophil production and survival, and granulopoiesis is reduced in G-CSF- and G-CSF receptor (G-CSF-R deficient mice.

During infection or inflammation, neutrophil production and export increases, under the influence of G-CSF, in a response known as emergency granulopoiesis. G-CSF can be produced by multiple cells throughout the body, including macrophages, bone marrow (BM) stromal cells, and endothelial cells.

Homeostatic regulation of the neutrophil pool is complex, with proposed feedback mechanisms related to clearance of apoptotic neutrophils in BM, spleen, liver, and mucosal sites. Regulation of C-X-C chemokine receptors (CXCRs) 2 and 4 and their ligands is critical to neutrophil retention and release from the BM. CXCR4-deficient mice exhibit constitutive neutrophil mobilization.

It is this complexity that makes it difficult to be specific on many of the steps. Regrettably many of the exiting paradigms are more hand waving that based upon well establish pathways. The authors continue:

Myelokathexis, a congenital cause of human neutropenia, is caused by a gainof-function mutation in CXCR4[9]*, increasing BM neutrophil retention. CXCR2 is also necessary for neutrophil mobilization, and CXCR2-deficient neutrophils are selectively retained in the BM. G-CSF regulates CXCR2 and CXCR4 expression on BM neutrophils and increases production of chemokines CXCL1 (mediated via thrombopoietin) and CXCL2*[10]*. Conversely, G-CSF administration reduces CXCR4 and CXCL12 expression in the BM. G-CSF also influences neutrophil function outside of the BM compartment.*

Neutrophil phagocytosis and oxidative burst are increased by G-CSF, as is neutrophil survival, the latter mediated by induction of Mcl-1[11] *or A1. G-CSF enhances migration into peripheral tissues via induction of CXCR2 ligands. G-CSF up-regulates expression of integrins, such as CD11b/CD18, promoting adhesion and transmigration, and stimulates glycosylation of myeloperoxidase (MPO), converting MPO to a cell surface ligand for E-selectin.*

Serum G-CSF is elevated during infection and in sterile inflammatory conditions, and G-CSF and G-CSF-R are elevated in the ocular tissue and blood of patients with uveitis.

The understanding that G-CSF is present in sterile conditions allows for a basis for neutrophil presence and increase as well. They continue:

[9] CXCR4 as NCBI notes: *This gene encodes a CXC chemokine receptor specific for stromal cell-derived factor-1. The protein has 7 transmembrane regions and is located on the cell surface. It acts with the CD4 protein to support HIV entry into cells and is also highly expressed in breast cancer cells. Mutations in this gene have been associated with WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome. Alternate transcriptional splice variants, encoding different isoforms, have been characterized*.

[10] CXCR2 as NCBI notes: *The protein encoded by this gene is a member of the G-protein-coupled receptor family. This protein is a receptor for interleukin 8 (IL8). It binds to IL8 with high affinity, and transduces the signal through a G-protein activated second messenger system. … This receptor mediates neutrophil migration to sites of inflammation. The angiogenic effects of IL8 in intestinal microvascular endothelial cells are found to be mediated by this receptor. … This gene, IL8RA, a gene encoding another high affinity IL8 receptor, as well as IL8RBP, a pseudogene of IL8RB, form a gene cluster in a region mapped to chromosome 2q33-q36.*

[11] MCL1 as NCBI notes: *This gene encodes an anti-apoptotic protein, which is a member of the Bcl-2 family. Alternative splicing results in multiple transcript variants. The longest gene product (isoform 1) enhances cell survival by inhibiting apoptosis while the alternatively spliced shorter gene products (isoform 2 and isoform 3) promote apoptosis and are death-inducing.*

In patients with active Behçet's disease, peripheral blood (PB) mononuclear cell G-CSF mRNA, CXCR2 expression on circulating neutrophils, and serum CXCL1/growth-regulated oncogene a (GROa) increase. The neutrophil chemoattractant CXCL8/IL-8 also increases in uveitis patients.

Administration of G-CSF to patients can induce or exacerbate preexisting inflammatory conditions, including uveitis. We have previously reported that G-CSF-deficient mice are resistant to collagen-induced arthritis. Wild-type (WT) mice treated with anti-G-CSF also had reduced collagen induced arthritis, even when antieG-CSF was administered therapeutically. In this study, we investigated the role of endogenous G-CSF and neutrophils in EAU. Our data show a strong relationship between neutrophilia and elevated G-CSF in PB, and in the eye, in WT mice.

In contrast, G-CSF deficient mice did not develop EAU associated neutrophilia and were markedly protected from disease, as were WT mice treated with a neutralizing anti-G-CSF monoclonal antibody (mAb; anti-G-CSF). Analysis of the ocular infiltrate revealed a neutrophil-rich infiltrate in WT mice with EAU, which was almost absent in G-CSF-deficient mice and in WT mice treated with antieG-CSF. Furthermore, expression of CXCR2 on circulating neutrophils was markedly decreased in G-CSF-deficient mice and anti-G-CSF-treated mice with EAU.

Lower CXCR2 expression was associated with reduced neutrophil actin polymerization and reduced in vitro and in vivo neutrophil migration. Intriguingly, decreased activation of the neutrophil compartment impaired the differentiation of pathogenic Th17 cells, linking innate and adaptive immunity. These results reveal a fundamental contribution of endogenous G-CSF and neutrophils to the pathogenesis of EAU.

We now examine a specific case of osteoarthritis and the cells and their functions related thereto. As van den Bosch et al noted:

Synovial inflammation is observed in a large subgroup of patients with osteoarthritis (OA) and it is believed to contribute to OA pathology.

The synovial intima layer consists of mainly 2 cell types:

the macrophage-like (type A) and

fibroblast-like synoviocytes (type B).

Although fewer synovial macrophages are present in OA compared with rheumatoid arthritis (RA), they are crucial for the production of proinflammatory cytokines, such as interleukin (IL) 6 and IL-8, and cartilage matrix-degrading enzymes, matrix metalloproteases (MMP) 1, MMP-3, and MMP-93. Previous studies showed that selective depletion of synovial macrophages during experimental OA largely reduces cartilage damage and osteophyte formation, 2 major hallmarks of OA.

Multiple types of macrophages can be distinguished, where the distinction into classically activated M1 and alternatively activated M2 macrophages is often used6,7. M1-like macrophages can produce numerous proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IL-1β, and express MHC class II and CD86 receptors, whereas M2-like macrophages are characterized by the production of antiinflammatory cytokines, such as IL-10 and IL-1Ra, and have been shown to express scavenger receptor CD163 and mannose receptor (CD206).

Activation of the synovium during OA induces the release of large amounts of the alarmins S100A8[12] and S100A9, which can easily be measured in synovial fluid as well as in serum.

S100A8 and S100A9 proteins are proinflammatory mediators produced by myeloid cells, such as monocytes and activated macrophages, and have been shown to stimulate cells through Toll-like receptor 4 (TLR4).

They are implicated in multiple rheumatic diseases. Previously, our group mainly studied the involvement of synovial S100A8 and S100A9 production in the development of OA. Using in vivo models of OA, we observed that S100A8 and/or S100A9 promote synovial activation, cartilage degradation, and osteophyte formation. Treatment with the quinoline-3-carboxamide paquinimod (ABR-215757), which can inhibit the binding of S100A9 to its TLR4 and receptor for advanced glycation endproducts (RAGE), reduced the OA pathology.

Finally, we described that the alarmins S100A8 and S100A9 induce canonical Wnt signaling, which has been shown to be detrimental for cartilage. …

In our study, we show that S100A8 and S100A9 are preferentially expressed and produced by GM-CSF–differentiated macrophages, even as high as monocytes, known to be potent producers of S100A8 and S100A9 proteins. In line with our results, a recent mouse study … also showed that murine bone marrow-derived M1-like macrophages expressed more S100A8 and S100A9 mRNA than M2-like cells. Moreover, they showed that S100A9 –/– bone marrow cells preferentially differentiate into arginase 1-positive M2-type macrophages, suggesting that the absence of S100 proteins skews macrophages to an M2-like phenotype34.

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[12] As NCBI notes on S100A8: *The protein encoded by this gene is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100 genes include at least 13 members which are located as a cluster on chromosome 1q21. This protein may function in the inhibition of casein kinase and as a cytokine. Altered expression of this protein is associated with the disease cystic fibrosis. Multiple transcript variants encoding different isoforms have been found for this gene.*

As NCBI notes on S100A9: *The protein encoded by this gene is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100 genes include at least 13 members which are located as a cluster on chromosome 1q21. This protein may function in the inhibition of casein kinase and altered expression of this protein is associated with the disease cystic fibrosis.*

Moreover, we did not observe S100A8 and S100A9 expression in fibroblasts. This is in contrast with a previous study that showed S100A8 and S100A9 mRNA expression and S100A8/A9 protein expression by isolated OA and RA fibroblasts. However, isolated cells from human synovial tissue were used between passages 3 and 9, which could imply that other cell types (e.g., fibrocytes) could be responsible for the low S100A8/A9 expression.

Therefore, in our study, fibroblasts isolated from human OA synovium were not used before passage. Moreover, the protein levels that were produced were very low (500-fold lower compared with our GM-CSF–differentiated macrophages). We also show that S100A9 acts most potently on GM-CSF–differentiated macrophages, but also stimulates the expression of proinflammatory cytokine in macrophages differentiated with M-CSF, yet to a lesser extent.

Proinflammatory M1 and reparative M2 macrophages represent the 2 extremes of the spectrum of macrophage activation, with a lot of intermediate activation states. It is generally accepted that M1 macrophages are involved in proinflammatory responses. It has been shown that mainly M1 macrophages have critical roles in host defense against infection, but these cells are also implicated in many inflammatory diseases, such as atherosclerosis, obesity, and RA. In contrast to macrophages, we did not observe significant effects of S100A9 on synovial fibroblasts.

***This difference in potency between macrophages and fibroblasts might be due to the different expression levels of TLR4, as we show by qRT-PCR. However, this does not explain the differences observed between GM-CSF– and M-CSF–differentiated* macrophages.**

As Christopher et al note :

Granulocyte colony-stimulating factor (G-CSF), the prototypical mobilizing cytokine, induces hematopoietic stem and progenitor cell (HSPC) mobilization from the bone marrow in a cell non-autonomous fashion. This process is mediated, in part, through suppression of osteoblasts and disruption of CXCR4/CXCL12 signaling.

The cellular targets of G-CSF that initiate the mobilization cascade have not been identified. We use mixed G-CSF receptor (G-CSFR)– deficient bone marrow chimeras to show that G-CSF–induced mobilization of HSPCs correlates poorly with the number of wild-type neutrophils. We generated transgenic mice in which expression of the G-CSFR is restricted to cells of the monocytic lineage. G-CSF– induced HSPC mobilization, osteoblast suppression, and inhibition of CXCL12 expression in the bone marrow of these transgenic mice are intact, demonstrating that G-CSFR signals in monocytic cells are sufficient to induce HSPC mobilization. Moreover, G-CSF treatment of wild-type mice is associated with marked loss of monocytic cells in the bone marrow. Finally, we show that bone marrow macrophages produce factors that support the growth and/or survival of osteoblasts in vitro. Together, these data suggest a model in which G-CSFR signals in bone marrow monocytic cells inhibit the production of trophic factors required for osteoblast lineage cell maintenance, ultimately leading to HSPC mobilization.

As Bajrami et al note :

Neutrophil homeostasis is maintained, in part, by their regulated release from the BM.

The chemokine CXCL12[13] (stromal cell–derived factor 1), by interacting with its major receptor CXCR4, plays a critical role in controlling neutrophil mobilization and homeostasis under both basal and stress granulopoiesis conditions.

In addition to CXCR4, CXCL2/CXCR2 signaling is considered to be a second chemokine axis required for neutrophil mobilization. CXCR2 ligands stimulate neutrophil mobilization in a VAL4-dependent.

Based on a proposed tug-of-war model, CXCL2/CXCR2 signals and CXCL12/CXCR4 signals act antagonistically to regulate neutrophil retention in and release from the BM. In the current study, we show that CXCR2 ligands MIP-2 and KC were quickly expressed during acute inflammation and were responsible for initial rapid neutrophil mobilization. The concentrations of CXCR2 ligands continued to increase during inflammation; however, the speed of neutrophil mobilization slowed after the initial acute fast phase.

We demonstrate that this slowing of neutrophil release is caused by suppression of CXCR2-mediated signaling by G-CSF. G-CSF is a prototypical neutrophil-mobilizing cytokine.

G-CSF–induced down-regulation of CXCR4 expression is one mechanism for mobilization of myeloid cells. Humans and mice treated with AMD3100, a selective CXCR4 antagonist, or CXCR4-blocking antibodies undergo rapid PB neutrophilia.

The CXCR4 antagonist plerixafor can also correct leukopenia in patients with warts, hypogammaglobulinemia, infections, and WHIM (warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis) syndrome. It is noteworthy that a recent study indicated that plerixafor-mediated CXCR4 inhibition does not mobilize neutrophils from the BM. Instead, it elicits neutrophilia by promoting neutrophil release from the marginated pool present in the lung and preventing neutrophil circulation back to the BM.

This demargination is likely to be caused by increased neutrophil blood velocities and decreased endothelium–neutrophil interactions. A recent study also suggested that autophagy plays a role in G-CSF–elicited stem cell mobilization

The above is a clear demonstration of the complexity of even the current understanding. Thus it is critical that as more is understood the process must be better defined.

Finally from Kohler et al we note:

[13] As NCBI notes on CXCL12: *This antimicrobial gene encodes a stromal cell-derived alpha chemokine member of the intercrine family. The encoded protein functions as the ligand for the G-protein coupled receptor, chemokine (C-X-C motif) receptor 4, and plays a role in many diverse cellular functions, including embryogenesis, immune surveillance, inflammation response, tissue homeostasis, and tumor growth and metastasis. Mutations in this gene are associated with resistance to human immunodeficiency virus type 1 infections. Multiple transcript variants encoding different isoforms have been found for this gene*.

Emergency mobilization of neutrophil granulocytes (neutrophils) from the bone marrow (BM) is a key event of early cellular immunity.

The hematopoietic cytokine granulocyte-colony stimulating factor (G-CSF) stimulates this process, but it is <u>unknown</u> how individual neutrophils respond in situ.

We show by intravital 2- photon microscopy that a systemic dose of human clinical-grade G-CSF rapidly induces the motility and entry of neutrophils into blood vessels within the tibial BM of mice.

Simultaneously, the ***neutrophil-attracting chemokine KC (Cxcl1[14])*** *spikes in the blood. In mice lacking the KC receptor Cxcr2, G-CSF fails to mobilize neutrophils and antibody blockade of Cxcr2 inhibits the mobilization and induction of neutrophil motility in the BM. KC is expressed by megakaryocytes and endothelial cells in situ and is released in vitro by megakaryocytes isolated directly from BM.*

This production of KC is strongly increased by thrombopoietin (TPO). Systemic G-CSF rapidly induces the increased production of TPO in BM. Accordingly, a single injection of TPO mobilizes neutrophils with kinetics similar to G-CSF, and mice lacking the TPO receptor show impaired neutrophil mobilization after short-term G-CSF administration. Thus, a network of signaling molecules, chemokines, and cells controls neutrophil release from the BM, and their mobilization involves rapidly induced Cxcr2-mediated motility controlled by TPO as a pacemaker.

[14] From NCBI on CXCL1 we have: *This antimicrobial gene encodes a member of the CXC subfamily of chemokines. The encoded protein is a secreted growth factor that signals through the G-protein coupled receptor, CXC receptor 2. This protein plays a role in inflammation and as a chemoattractant for neutrophils. Aberrant expression of this protein is associated with the growth and progression of certain tumors. A naturally occurring processed form of this protein has increased chemotactic activity. Alternate splicing results in coding and non-coding variants of this gene. A pseudogene of this gene is found on chromosome 4.*

3 INFLAMMATION

We now consider the process of inflammation. There are many inflammatory response that are distinct from infections which one generally associates with the classic immune system response. Inflammation may for the most part be a sterile process. It is generally in response to the body being placed into some form of stress. In sterile inflammatory responses the immune system is activated to eliminate the generated byproducts resulting from the inflammatory process. We shall see that in such a case as osteoarthritis we have destroyed synovium from which we see an immune response to clean that up.

3.1 ACUTE

As Hannoodee and Nasuruddin have noted[15]:

Acute inflammation is an immediate, adaptive response with limited specificity caused by several noxious stimuli, such as infection and tissue damage (tissue necrosis). The controlled inflammatory response is generally beneficial, and this can be seen clearly in providing protection against infectious organisms, including mycobacterium tuberculosis, protozoa, fungi, and other parasites. However, it can become detrimental if not regulated, such as seen in septic shock. The inflammatory pathway consists of a sequence of events involving inducers, sensors, mediators, and effectors.

The process will initiate in the presence of inducers, which can be infectious organisms or non-infectious stimuli such as foreign bodies and signals from necrotic cells or damaged tissues. This will, in turn, activate the sensors, which are specialized molecules. The sensors will then stimulate the mediators, which are endogenous chemicals that can induce pain, activate or inhibit inflammation and tissue repair, and can activate the effectors, which are the tissues and cells. These players can act together and give rise to multiple alternative pathways in the inflammatory process, depending on the type of stimuli. The goal of the inflammatory process is to restore homeostasis regardless of the cause.

3.1.1 Microbial

There are two classes of microbial inducers. The first class is pathogen-associated molecular patterns (PAMPs), which are carried by all microorganisms. The second class is virulence factors restricted to pathogens. Virulence factors trigger the inflammatory response due to the effects of their activity. Examples include enzymatic activity produced by helminths and exotoxins produced by bacteria, which will be sensed by known or unknown sensors.

3.1.2 Non Microbial

[15] https://www.ncbi.nlm.nih.gov/books/NBK556083/

Causes include allergens, toxic compounds, irritants, and foreign bodies that are too large to be digested or cause phagosomal damage in macrophages. Examples of foreign bodies include silica and asbestos.

3.2 CHRONIC

As Herrero-Cervera et al note:

The acute inflammatory process is a vital response to injury and infection that protects the organism against invading pathogens and repairs tissue damaged by trauma. Acute inflammation is self-limiting with a resolution phase that terminates the inflammatory response and initiates the reparative process. Chronic inflammation occurs if the initiating stimulus is not removed or if the resolution program is disturbed, resulting in a state of lowgrade inflammation.

Chronic inflammatory diseases, including atherosclerosis, diabetes mellitus, nonalcoholic fatty liver disease (NAFLD) and autoimmune disorders, are major causes of death worldwide.

Systemic chronic inflammation increases with age, is low-grade and persistent, and the causes include chronic infections, lifestyle and environmental factors, physical inactivity, microbiome dysbiosis, diet, psychological stress, and toxins.

Neutrophils, which are the most abundant white blood cells in humans, have been well established as first responders to acute inflammation.

Under normal conditions, neutrophils contribute to resolution and tissue repair by phagocytosing necrotic cells to stop them from attracting more immune cells, releasing mediators to promote growth and angiogenesis, and producing resolvins and protectins.

Over the past decades, neutrophils have also been shown to play a significant role in chronic inflammation.

Neutrophils are continuously recruited to the site of chronic inflammation and contribute to driving the process through the release of serine proteases and the formation of neutrophil extracellular traps (NETs), as well as the activation of other immune cells.[16]

Here, we will review neutrophil crosstalk with other cells in the context of chronic inflammation and the contribution of neutrophils to selected chronic inflammatory diseases and summarize potential strategies to interfere with the damaging effects of neutrophils in chronic diseases.

3.2.1 Obesity

[16] Also see Amulic et all. *NETosis is a type of neutrophil cell death, distinct from apoptosis or necrosis, which remains poorly characterized. It is an active process characterized by internal breakdown of nuclear and granular membranes, the mixing of the contents of these compartments in the cytosol and finally, their extracellular release via plasma membrane rupture*

Herrero-Cervera et al. note:

T2DM is a risk factor for developing NAFLD, as it accelerates the progression of liver disease. Approximately 55% of patients with T2DM have NAFLD. Moreover, T2DM and NAFLD have been linked to obesity, which is characterized by the abnormal accumulation of adipose tissue, leading to chronic low-grade inflammation and an imbalance in the immune profile, including the activation of neutrophils. Type 2 diabetes mellitus. In 2015, it was estimated that 1 in 11 adults globally (approximately 415 million people) aged 20–79 years, had diabetes, and over 90% of diabetes mellitus cases were T2DM. T2DM is a chronic disease characterized by high levels of glucose in the blood, impaired insulin secretion and/or insulin resistance due to dysregulated carbohydrate, lipid and protein metabolism. Insulin resistance occurs many years before the onset of T2DM and is caused by obesity, physical inactivity and genetic predisposition.

Neutrophil activation, which is assessed as the expression of neutrophil elastase (NE) and myeloperoxidase (MPO) in peripheral blood leukocytes, NE serum levels, or MPO plasma levels, is enhanced in obese and T2DM patients compared to lean subjects. Bariatric surgery partially decreases neutrophil activation in patients.

Furthermore, obese patients exhibit reduced levels of alpha-1-antitrypsin (A1AT), and the levels correlate negatively with body mass index, indicating that neutrophils play a role in the disease progression of T2DM. Neutrophil infiltration and the consequent release of granule proteins in adipose tissue in mice fed a high-fat diet (HFD) contribute to the development of insulin resistance. Insulin signaling is impaired by nitration of the β subunit of the insulin receptor by MPO and the degradation of insulin receptor substrate 1 by NE.

Therefore, mice with NE deficiency or MPO deficiency have reduced adipose tissue inflammation and improved carbohydrate metabolism, including improved glucose tolerance and insulin sensitivity and a reduction in insulin resistance. Neutrophil effects can also be mediated by direct interaction with adipocytes.

Neutrophil adhesion to adipocytes is mediated by the interaction of CD11b with ICAM-1 on adipocytes in vitro . Adhesion results in increased IL1β expression via the NF-κB pathway in neutrophils and contributes to the expression of chemotactic molecules and the infiltration of macrophages, amplifying adipose tissue inflammation and subsequently the occurrence of insulin resistance. In summary, neutrophils infiltrate adipose tissue in obesity and T2DM.

Within adipose tissue, neutrophils secrete NE and MPO, and in mouse models of T2DM, these factors are thought to promote the development of insulin resistance and inflammation in adipose tissue. Future investigations in patients need to be performed to examine the contribution of neutrophil protease to T2DM progression, especially with regard to MPO as a possible target. MPO deficiency in humans has been shown to increase susceptibility to fungal infections in some patient groups but does not affect inflammatory disease progression

3.2.2 Atherosclerosis

Herrero-Cervera et al. note:

Cardiovascular disease (CVD) is the leading cause of mortality worldwide. The underlying pathological process of CVD is atherosclerosis, a slowly progressing chronic disorder of large and medium-sized arteries that is characterized by the accumulation of lipids in the arterial wall, the infiltration of immune cells, and the formation of a fibrous cap composed of smooth muscle cells and collagen.

Neutrophils are implicated in the development and progression of atherosclerosis, which has been reviewed elsewhere .

However, as the field is continuously expanding, this review will summarize discoveries reported in 2020 and 2021. The Apoe−/− mouse is a hyperlipidemic mouse strain on a C57BL/6 J background that is commonly used to study the pathophysiology of atherosclerosis. Wild-type mice are protected against atherosclerosis development, as most cholesterol is carried in high-density lipoprotein (HDL) particles, which ensures cholesterol elimination through the reverse cholesterol transport pathway.

Therefore, this transgenic mouse model was developed to study atherosclerosis pathogenesis. Genetic deletion of Apoe increases plasma cholesterol levels to the range of 400–600 mg/dL, triggering spontaneous atherosclerosis development at approximately 20 weeks of age. High-fat and highcholesterol diets accelerate atheroma plaque formation as plasma cholesterol levels reach more than 1000 mg/dL. Although this mouse is the most frequently used strain to study atherosclerosis, its pathophysiology differs from that of humans. For example, human lesions occur mainly in coronary arteries, carotids and peripheral vessels, while in mice, atheroma plaques occur in the aortic root, aortic arch and innominate artery.

Therefore, the results from mouse studies cannot be directly translated to humans. One mechanism by which neutrophils contribute to atherosclerosis is through NET formation. LPS administration to Apoe−/− mice produced larger lesion sizes with the accumulation of myeloid cells and NETs. NET-associated histone 2a is responsible for monocyte adhesion to NETs in a charge-dependent manner.

Unstable plaques in patients have increased levels of peptidyl arginine deiminase 4 (PAD4) compared to carotid plaques with stable features. Additionally, coronary thrombi in patients with ST-segment elevation myocardial infarction have increased NETs. Downstream plaque regions with vulnerability features have a major numbers of neutrophils and increased expression of NE and citrullinated histone 3 (citH3) compared to upstream regions.

Serum autoantibodies against ApoA1, the main protein fraction of high-density lipoproteins, are a predictor of cardiovascular events. In patients with anti-ApoA1-positive serum, the citH3 signal does not colocalize with neutrophils, suggesting increased production of citH3 by neutrophils. Diabetes can induce or accelerate atherosclerosis, and diabetic characteristics such as higher glucose levels, dyslipidemia and chronic inflammation are involved in atherosclerosis development.

Hence, patients with both diabetes and atherosclerosis have been shown to have increased circulating markers of NET formation compared to patients with only atherosclerosis. In a model that mimics transient intermittent hyperglycemia in Apoe−/− mice, hyperglycemia increases lesion size, myelopoiesis and circulating monocytes and neutrophils compared to those in control mice. S100A8/A9 released by neutrophils in transient intermittent hyperglycemic mice induces myelopoiesis through RAGE activation. Impaired resolution of atherosclerosis in diabetic mice correlates with increased NETs, CD68+, NLRP3+ and caspase 1+ cell numbers, necrotic cores, and plaque sizes compared to those of nondiabetic mice. These parameters are reduced by treatment with DNase 1

3.2.3 Autoimmune

Herrero-Cervera et al. note:

Autoimmune diseases are a varied group of disorders in which damaging immune responses to self-antigens occur. Approximately 3–5% of the population is affected by these diseases, and this percentage is continuously increasing. Autoimmune diseases include several diseases, such as type 1 diabetes mellitus (T1DM), RA, multiple sclerosis, SLE and IBD. Neutrophils have been implicated in all of these conditions, but we will focus on T1DM and IBD. Neutrophils and RA , multiple sclerosis or SLE have recently been reviewed elsewhere.

3.2.4 COPD

Herrero-Cervera et al. note:

COPD is a major cause of chronic morbidity and was the third leading cause of death worldwide in 2015. The primary cause of COPD is exposure to CS, but other factors, such as air pollution or fumes, have been implicated. The main features of this disease are airflow limitation, emphysematous alveolar wall destruction, local and systemic chronic inflammation of the airways, alveoli and microvasculature, and recurrent infections. Overall, these disease components result in lung failure and ultimately death. Bacterial and viral infections exacerbate inflammation, hypoxia and airway damage in this Disease.

3.2.5 Osteoarthritis

Osteoarthritis is an interesting example of chronic inflammation. It is generally the result of some mechanical wear and tear on a joint and not a result of some physiological or biological cause. Thus studying this disease allows a possible single threading from cause to effects. As Wu et al have noted:

Osteoarthritis (OA) is a disease of the joint organ system characterized by the degradation of articular cartilage, inflammation of the synovium and joint fat pad, as well as alterations in bone structure. Over 27 million people are estimated to suffer from OA in the US, resulting in a tremendous socioeconomic burden.

The etiology of OA has been shown to be heterogenous, and may in fact represent a family of diseases rather than one disease. As such, several risk factors such as genetic predisposition, obesity, aging, and joint trauma have been identified for OA. Irrespective of this multifaceted nature of OA pathophysiology, it is now accepted that joint inflammation plays a major role in OA onset and progression.

Inflammation is classically regulated by a variety of immune cells such as T cells, neutrophils, and macrophages.

Macrophages are phagocytic cells that can be found in almost every tissue (including brain, liver, skin, and tissues of the joint organ system). The primary role of macrophages is to maintain tissue homeostasis and protect the host from infection.

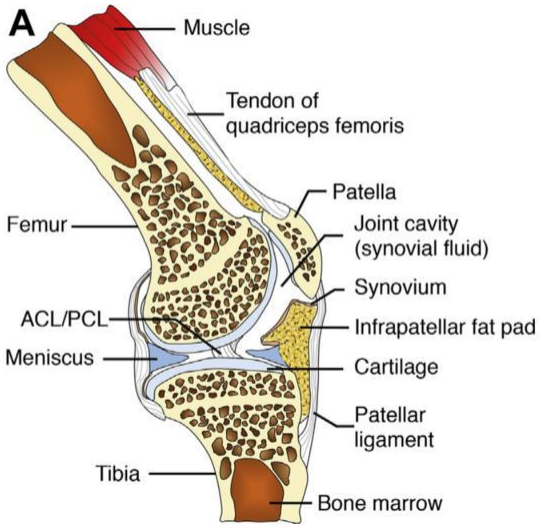
Although primarily considered to be critical components of innate immunity, macrophages are capable of bridging and instructing the response of the adaptive immune system via various secretory mediators.

Based on their interaction with T cells, macrophages have been typically dichotomized into two phenotypes:

M1-like macrophages ("classically" activated) are antimicrobial and pro-inflammatory, and are activated in response to stimuli from T helper type 1 (Th1) cells, while

M2-like macrophages ("alternatively" activated) are anti-inflammatory and pro-resolving, and are induced by Th2 cells.

The dysregulated balance between pro- and anti-inflammatory macrophages may lead to chronic low-grade inflammation and has been suggested to be critical in the development of several musculoskeletal diseases, including OA



As Zoete note regarding inflammasomes:

Osteoarthritis, which is characterized by the degeneration of cartilage, has also been shown to involve NLRP3 activation. Synovial uric acid correlated strongly with synovial fluid IL-1b and IL-18 in patients, and mice deficient in NLRP3 had significantly reduced pathogenesis in osteoarthritis mouse models. Although IL-1 blocking therapy has so far shown less promising results as compared to the treatment of gout, the above results suggests that a detailed investigation of therapies targeting the NLRP3 inflammasome or its mediators in treating osteoarthritis might be fruitful.

Furthermore Chaney et al note:

OA is a chronic degenerative disease and worldwide endemic issue leading to pain, decreased movement, and worsening joint function. While the pathogenesis of this disease is not entirely understood, there are well-established risk factors for developing osteoarthritis such as aging, obesity, female sex, and repetitive movements with excessive loading. Neutrophils contribute via multiple pro-inflammatory and degenerative mechanisms to the progression of OA and overall decline of the quality of life of OA patients. This disease affects all synovial joints and is characterized by the progressive destruction of the articular cartilage and secondary episodic synovitis.

OA most often affects the interphalangeal joints, hips, spine, knees, and feet.

It is estimated that worldwide, there are 250 million people who suffer from knee osteoarthritis alone. Predictors of disease include genetics, diet, age, sex, and obesity, with some specific occupations presenting higher rates of OA prevalence than others. In the present review, we assemble recent findings on the involvement of neutrophils in OA pathophysiology, focusing on secreted cytokines, chemokines, metalloproteinases, microRNAs, and exosomes. Understanding the mechanisms of action of neutrophils will contribute to the discovery of new therapies to inhibit the progression of OA and to reestablish joint homeostasis

Zhang et al note:

Osteoarthritis (OA) is a degenerative joint disease that seriously affects cartilage and surrounding tissues. The pathogenesis of OA results in the degeneration of chondrocytes and cartilage. Imbalances in the synthesis of articular chondrocytes, extracellular matrix, and subchondral bone is a pathological feature during degradation.

Articular cartilage is a component of the articular surface that acts as a buffer, allowing fluid flow and the dispersion of pressure. After the degeneration of cartilage, degradation products cause inflammation in the joints, which are accompanied by changes in subchondral bone and the synovium. At present, the clinical treatment of early OA includes the use of anti-inflammatory analgesic drugs, the application of chondroprotective drugs, and arthroscopic debridement, among other approaches.

However, those measures can only alleviate the clinical symptoms and cannot prevent the development of disease; thus, the effect is not satisfactory. Advanced OA is often treated with artificial joint replacement, which can relieve pain and restore patients' daily living abilities. However, complications such as the necessity to wear prostheses and loosening of artificial joints greatly limit the application of artificial joint replacement. The incidence of OA in the middle-aged population can be as high as 40%-80%, and the disability rate is greater than 50% , which imparts a substantial burden to individuals and society.

Therefore, to determine the pathogenesis of OA, effective intervention in the early stages of pathogenesis is a key step for successful treatment. Synovial inflammation is associated with the pathogenesis of OA and is significantly associated with the severity of OA.

The synovial membrane is thin, soft, loose connective tissue that is coated on the inner side of the joint capsule and forms a closed capsule around the joint cavity. The normal synovium can be divided into an intimal lining layer and a synovial sub-lining layer. The intimal lining is composed of two to three layers of synovial cells, including macrophage-like cells, fibroblast-like cells, dendrite-like synoviocytes, and a few mesenchymal stem cells.

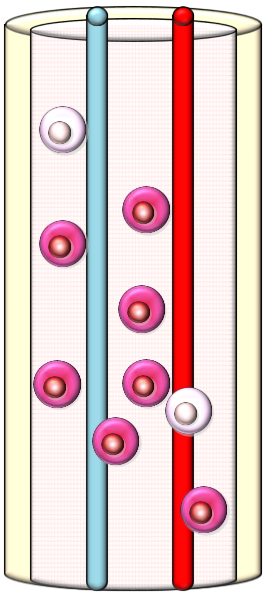
The synovial sub-lining layer consists of blood vessels, fibrous connective tissue, macrophages, and a small number of lymphocytes.

Synovitis is closely related to the polarization of synovial macrophages. The main features of synovitis are synovial tissue hyperplasia, inner macrophage aggregation, and cell secretory dysfunction.

Macrophages can be divided into two polarization states of classically activated M1 macrophages and alternatively activated M2 macrophages under different microenvironments. After being stimulated by lipopolysaccharide (LPS) and interferon-γ, M1 macrophages secrete a large number of proinflammatory cytokines, such as interleukin-1β, IL-6, and TNF-α, triggering the body's inflammatory response. However, excessive inflammation can cause damage to normal tissues of the body.

M2 macrophages have anti-inflammatory activities and mainly secrete anti-inflammatory cytokines, such as IL- 4, IL-10 and TGF-β, which can inhibit the development of inflammation and promote wound healing. Studies have shown that synovial macrophage polarization is closely related to the development of OA. … in a mouse OA model, macrophages in the synovial membrane and joint cavity aggregated, M1 synovial macrophages increased and accelerated OA progression.

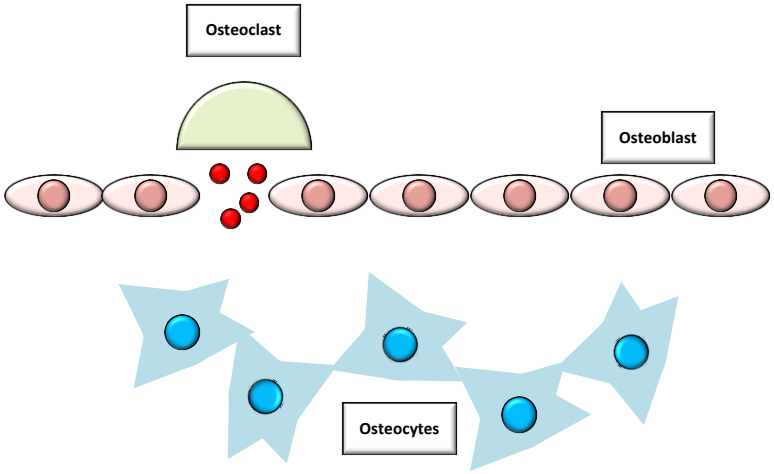
The figure below details some of the key elements that are part of bone structure and inflammation.



- Bone: Osteocytes, osteoblasts, osteoclasts
- Veins
- Arteries
- Marrow stroma: fibroblasts, adipocytes, osteoblasts (generate), osteoclasts (resorb) macrophages
- HSC stem cells

The soft, spongy tissue that has many blood vessels and is found in the center of most bones. There are two types of bone marrow: red and yellow. Red bone marrow contains blood stem cells that can become red blood cells, white blood cells, or platelets. Yellow bone marrow is made mostly of fat and contains stem cells that can become cartilage, fat, or bone cells.

We follow the above with a more detailed characterization of the three bone cell types and their functioning.



As McCauley et al note :

Fracture repair is a complex process requiring heterotypic interactions between osteogenic cells and immune cells. Recent evidence indicates that macrophages are critically involved in fracture repair. Polarized macrophage populations differentially promote and regulate inflammation in other tissues, but little is known about the various macrophage subtypes and their signaling activities following a bone fracture.

The authors hypothesized that classically activated (M1 subtype) and alternatively activated (M2 subtype) macrophages are active during the early repair process to initiate and regulate the inflammatory response.

To test our hypothesis, bone marrow was collected from intact femurs (naïve group), contralateral and fractured femurs of mice on days 0, 1, 2, 4, and 7 postfracture. Macrophages were isolated from the bone marrow and macrophage subtypes were identified using flow cytometry with antibodies to F4/80, MHC II, CD86, CD11c, and CD40. Bone marrow cytokine levels were measured using xMAP. Flow cytometry revealed dynamic changes in M1 subtype (F4/80+/MHC II+/CD86+), M2 subtype (F4/80+/MHC II −/CD86−), and dendritic cell (DCs; MHCII+/CD11c+/CD40+) populations following fracture as compared to naïve controls. M1 subtype levels were correlated with IL-1α, IL-1ß, IL-2, IL-17, Eotaxin, and MCP-1, while DCs were correlated with IL-6, G-CSF, LIF, KC, and VEGF-A.

The results indicate that M1 and M2 subtypes and DCs are recruited to the fracture site early during the repair process and consequently may work in tandem to regulate the inflammatory response required to recruit osteogenic cells needed for later stages of repair.

As Akeson and Malemud note:

Interleukin-6 (IL-6) is one of several pro-inflammatory cytokines present at elevated levels in the synovial fluid of individuals with confirmed clinical diagnosis of rheumatoid arthritis (RA) and osteoarthritis (OA).

The mechanism of action of IL-6 was shown to involve its capacity to interact with a membrane-bound IL-6 receptor (mIL-6Rα), also known as the "classical" IL-6 pathway, or through its interaction with a soluble IL-6 receptor (sIL-6R) termed the "trans-signaling" pathway. Activation of downstream signaling is transduced via these IL-6 receptors and principally involves the Janus Kinase/Signal Transduction and Activators of Transcription (JAK/STAT) signaling pathway that is further regulated by glycoprotein-130 (gp130) interacting with the IL-6/mIL-6R complex.

Phosphorylation of STAT proteins via JAK activation facilitates STAT proteins to act as transcription factors in inflammation. However, the biological function(s) of the sIL-6R in human chondrocytes requires further elucidation, although we previously showed that exogenous sIL-6R significantly suppressed the synthesis of neutrophil gelatinase-associated lipocalin (NGAL) in the immortalized line of human chondrocytes, C28/I2. NGAL was shown to regulate the activity of matrix metalloproteinase-9 (MMP-9), whose activity is crucial in OA for the destruction of articular cartilage.

The "shedding" of sIL-6R from the plasma membrane is carried out by a family of enzymes known as A Distintegrin and Metalloproteinase (ADAM), which are also elevated in OA. In this paper, we have systematically reviewed the role played by IL-6 in OA. We have proposed that sIL-6R may be an important target for future drug development in OA by ameliorating cartilage extracellular protein degradation….

The inflammatory component of OA, as evidenced by chronic synovitis, is associated with a modulation of the chondrogenic phenotype.

These changes include the upregulation of proinflammatory cytokine gene expression; the upregulation of matrix metalloproteinase (MMP) gene expression combined with a skewing of the ratio of the level of tissue inhibitor of metalloproteinases (TIMPs) to MMPs towards MMPs has also been considered as relevant; elevated expression of a disintegrin and metalloproteinases with thrombospondin motif (ADAMTS) genes; and a disintegrin and metalloproteinase (ADAM) genes [20], the production of alarmins and Toll-like receptors, and an increased frequency of chondrocyte apoptosis.

These changes are likely to be arise from aberrations in signal transduction involving the mitogen-activated protein kinase (MAPK) and Janus Kinase/Signal Transduction and Activators of Transcription (JAK/STAT) pathways, negative regulators of JAK/STAT , and by those cytokines that activate the nuclear factor-κB (NF-κB) pathway .

Interleukin-6 (IL-6), in addition to other cytokines belonging to the IL-6 family of proteins, which include oncostatin M and adiponectin (a member of the adipokine family), are among the most prominently elevated cytokines involved in the inflammatory response in OA.

In that regard, it will be imperative that we further our understanding of the molecular mechanisms underlying the interaction between IL-6-type cytokines with the membrane form of the IL-6 receptor known as mIL-6Rα/gp130 and the soluble IL-6R form (sIL-6), as well as other respective membrane-bound receptors. ...

Interleukin-6 (IL-6) is one of several cytokine regulators of inflammation.

At present, there are two principal mechanisms by which IL-6 is known to interact with its target cells. The "classical" pathway of IL-6 signaling involves membrane-bound IL-6 receptors (mIL-6R/ mIL-6Rα) which associate with membrane-bound gp130 . Gp130, when engaged by IL-6 bound to IL-6R, serves as a locus for a tyrosine kinase cascade, resulting in the activation of JAK/STAT and Src-family kinase signaling pathways, as well as ERK and PI3K/Akt/mTOR signaling.

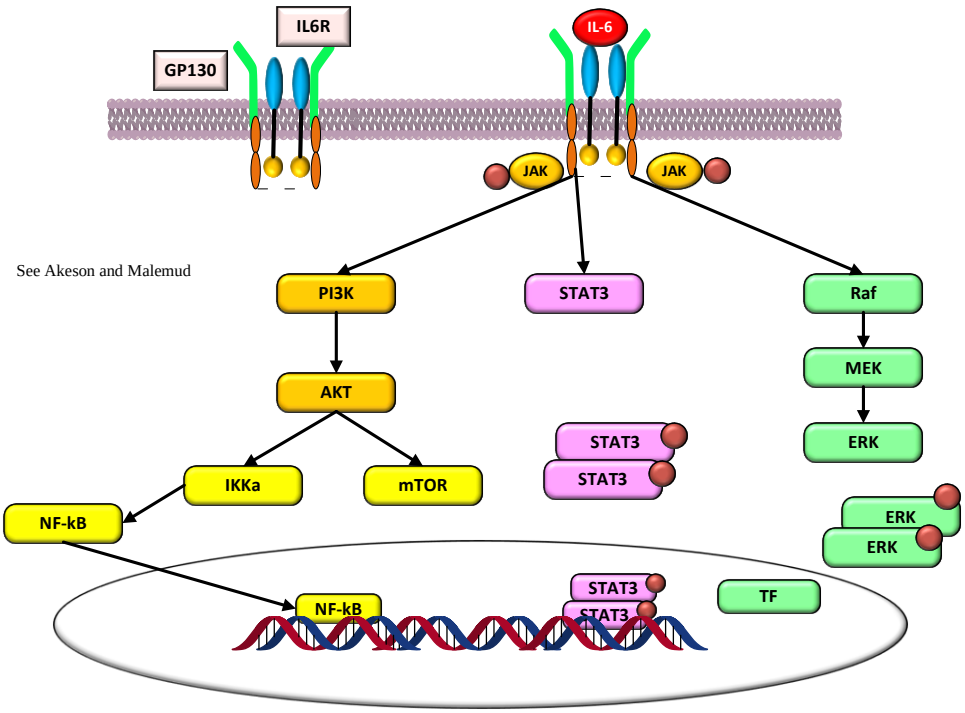
However, only a limited number of cell types express membrane-bound mIL-6R, including hepatocytes, neutrophils, monocytes, macrophages, as well as naive and memory T-cells. IL-6R is also known to interact with ciliary neurotrophic factor. IL-6R does not contain a signal-transduction domain. Gp130 serves as the signal transducer for IL-6R, and gp130 is a target for small-molecule inhibition of inflammatory pathways. Circulating gp130 can bind to IL-6/sIL-6R complex, inactivating the complex, as well as sequestering the signaling molecules. This mechanism creates an IL-6 buffer. Thus, for classical or trans-signaling to occur, the concentration of IL-6 must be high enough so that the signal is not diluted by functional cytokine loss due to circulating gp130. This observation led to a therapeutic development with soluble gp130 employed as an IL-6 inhibitor.

Cells expressing membrane-bound IL-6R are the source of soluble IL-6R (sIL-6R), and this soluble receptor is the mediator of the IL-6-trans signaling pathway. Approximately 80% of sIL-6R is produced by proteolytic cleavage of the membrane-bound IL-6R via ADAM 17, and direct synthesis of the soluble receptor contributes to 20% of the circulating level of sIL-6R. Once mIL-6R is released, IL-6 can bind to sIL-6R.

This receptor-ligand pair interacts with membrane-bound gp130 which is expressed by a majority of cell types. In that regard, once engaged, the gp130/sIL-6R/IL-6 complex induces protein kinase activity within the cell and activation of the JAK/STAT pathway, among other protein kinase pathways. IL-6 does not require an IL-6 specific membrane-bound receptor to induce a response. This means that gp130 serves as the signal-transducing domain for both the classical- and transsignaling pathways.

However, a novel mechanism of IL-6 signaling (termed "cluster signaling") has been described for the development of Th17 cells in this pathway, membrane-bound IL-6/IL-6R/gp130 complex that is found on dendritic cells binds to and activates membrane-bound Gp130 on T-cells, promoting FoxP3 activation, which induces Th17 differentiation. Thus, the proximal downstream response to IL-6 signaling does not seem to differ between cells based solely on the presence or absence of mIL-6R.

Accumulating evidence indicates that differences in phenotypic expression occur in response to "classical" IL-6 stimulation versus IL-6 trans-stimulation. Stimulation from the "classical" IL-6 pathway appears to primarily produce an anti-inflammatory effect, whereas trans-IL-6-stimulation predominantly results in a pro-inflammatory effect. Of note, the homogenous nature of these signal transduction pathways can produce opposite phenotypes because the genomic targets of IL-6 signaling can vary based on cell type.



Chaney et al note:

Neutrophils and macrophages are detected within osteoarthritic joints. Macrophages secrete metalloproteinases and inflammatory cytokines while neutrophils secrete degradative proteases including neutrophil elastase (NE), which can cause damage to joint cartilage over time, contributing to the progression of OA.

One study showed that NE was not detectable in human OA synovia with no synovitis but was detectable in samples in which "slight" or "moderate" synovitis was present [7]. With regards to abundance in OA joints, macrophages, neutrophils, and T cells can all be found in the synovial fluid and synovial tissue.

Macrophages are the most abundant population in synovial tissue, followed by T cells.

Neutrophils were the least abundant cell type found within the synovial tissue, found in 35% of patient samples, with a much greater presence in synovial fluid, i.e., a mean of 26% in fluid vs. 8% of total cells in tissue.

Another study found that neutrophils made up 8% of the cells in synovial fluid, with no differences in the percentage of cells between men and women.

In addition to the presence in synovial fluid, the neutrophil to lymphocyte ratio (NLR) has been associated with OA progression, with a significantly higher NLR seen in patients with severe knee OA compared to those with mild to moderate knee OA Further investigation is needed to

elucidate the role that neutrophils, and other immune modulators, play into the progression of OA.

They then detail the degradation process as below:

The compensatory actions of chondrocyte hypertrophy and ECM synthesis occur during the natural process of endochondral ossification and are abnormally activated during the progression of OA. This change in the overlying articular cartilage has been associated with subchondral bone remodeling; however, it is unclear if the changes in bone occur prior to cartilage changes or a result of it. Under osteoarthritic conditions, two subpopulations of osteoblasts have been described, i.e., low and high osteoarthritic osteoblasts, which are characterized by low or high secretion of prostaglandin E2 (PGE2) and IL-6.

The amount of PGE2 and IL-6 secreted is positively correlated with osteoprotegerin (OPG) expression and negatively correlated with RANKL expression[17].

The ultimate effect is that low osteoblasts allow higher rates of bone resorption compared to high osteoblasts. Inflammatory mediators released from immune cells, such as neutrophils that have extravasated to the site of injury, play a role in these morphologic changes as well. Neutrophils contribute to this bone resorption by activating osteoclasts through increased RANKL expression on neutrophils and induction of RANKL secretion from osteoclast precursors.

Neutrophilic RANKL expression is induced by Toll-like receptor 4 (TLR4) activation, while osteoclast precursor RANKL secretion is induced by the neutrophil chemoattractant chemokine (C-X-C motif) ligands 2 (CXCL2).

In late-stage OA, unrestricted chondrocyte differentiation, proliferation, and hypertrophy results in the calcification and sclerosis of subchondral bone accompanied by articular surface fibrillation.

As OA progresses, hypertrophic chondrocytes undergo apoptosis and halt proliferation. The resultant lacunar emptying after apoptosis ... The role of neutrophils in the early as well as late stages of osteoarthritis progression. Neutrophils are recruited at the synovial capsule and contribute to the secretion of many cytokines and chemokines within synovial fluid that promote inflammation and vascular infiltration and inhibit chondrogenic progenitor cell migration. The formation of neutrophil elastase (NE) enhances cartilage degradation, chondrocytes apoptosis, unbalanced subchondral bone remodeling, and osteophyte formation.

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[17] RANKL is a *gene encodes a member of the tumor necrosis factor (TNF) cytokine family which is a ligand for osteoprotegerin and functions as a key factor for osteoclast differentiation and activation. This protein was shown to be a dentritic cell survival factor and is involved in the regulation of T cell-dependent immune response.* ***T cell activation was reported to induce expression of this gene and lead to an increase of osteoclastogenesis and bone loss.*** *This protein was shown to activate antiapoptotic kinase AKT/PKB through a signaling complex involving SRC kinase and tumor necrosis factor receptor-associated factor (TRAF) 6, which indicated this protein may have a role in the regulation of cell apoptosis. Targeted disruption of the related gene in mice led to severe osteopetrosis and a lack of osteoclasts. The deficient mice exhibited defects in early differentiation of T and B lymphocytes, and failed to form lobulo-alveolar mammary structures during pregnancy.* https://www.ncbi.nlm.nih.gov/gene/8600

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The ultimate effect is that low osteoblasts allow higher rates of bone resorption compared to high osteoblasts [25]. Inflammatory mediators released from immune cells, such as neutrophils that have extravasated to the site of injury, play a role in these morphologic changes as well. Neutrophils contribute to this bone resorption by activating osteoclasts through increased RANKL expression on neutrophils and induction of RANKL secretion from osteoclast precursors. Neutrophilic RANKL expression is induced by Toll-like receptor 4 (TLR4) activation, while osteoclast precursor RANKL secretion is induced by the neutrophil chemoattractant chemokine (C-X-C motif) ligands 2 (CXCL2). In late-stage OA, unrestricted chondrocyte differentiation, proliferation, and hypertrophy results in the calcification and sclerosis of subchondral bone accompanied by articular surface fibrillation.

As OA progresses, hypertrophic chondrocytes undergo apoptosis and halt proliferation. The resultant lacunar emptying after apoptosis leads to loss of articular cartilage and eventually osteophytes. The proposed mediator causing irreversible cartilage degradation in OA is overactivated MMP-13.

Neutrophils play a role in activating latent pro-MMP-13 through the release of NE. Even at low concentrations, NE has been shown to degrade cartilage collagen quickly in vitro. An in vivo murine model demonstrated that it only took 4 h for NE incubated with cartilage to compromise its structure and cause significant pain development. NE also inhibits chondrocyte proliferation and promotes apoptosis. This is shown by reduced survival of chondrocytes exposed to NE in a dose-dependent manner.

NE is hypothesized to induce apoptosis by means of caspase 3 activation, inducing DNA degeneration, increasing free calcium levels, disrupting mitochondrial membrane potential, and increasing intracellular reactive oxygen species (ROS) production.

As receptive insult to the joint and the continued weakening of cartilage continues, fibrillation and micro-fractures occur and cause damage of the underlying subchondral bone. This leads to inflammation, bone remodeling, unresolved edema, and eventually, osteosclerosis. There is no proven mechanism of inflammation inducing osteosclerosis. B cells and macrophages have shown to be significantly increased in sclerotic bone. However, when studies used macrophage-depleted murine models in post-trauma OA, the injured joint was not recovered.

In fact, this led to higher levels of systemic inflammation and increased levels of T cells and neutrophils extravasated into the joint. This suggests that macrophages have a somewhat

protective, anti-inflammatory phenotype to inhibit the injury inflicted by neutrophilic activity. Sclerotic bone ultimately leads to osteophyte formation, as an attempt to distribute the burden of stress on the joint by increasing the surface area.

NE was implicated in the development of osteophytes through the activation of proteinase-activated receptor 2 (PAR2). A murine study showed that PAR2 deficiency significantly reduces the presence of osteophytes, further exposing that OA pathology requires neutrophilic activity. However, there have been contradicting results from clinical OA studies that used colchicine to inhibit neutrophil activity. One randomized control trial of OA patients found no significant difference in inflammatory markers between the colchicine and placebo treated groups.

This suggests that neutrophils are necessary for recovery, but in OA pathology, they exhibit erroneous activity. This erroneous activity of neutrophils may be mediated by increased mechanosensory signals within the joint, such as shear stress, that lead to activation via mechanotransduction of neutrophils and subsequent degeneration of cartilage.

They continue:

Neutrophils contribute to the many cytokines and chemokines released by immune cells within synovial fluid that promote OA progression.

Neutrophilic cytokines found in the synovial fluid of OA joints include interleukin (IL)-1β, IL-6, IL-21, IL-22, IL-23, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β.

Increased levels of IL-7 have also been found in synovial tissue, which is known to precipitate the recruitment of neutrophils. The different functions of these cytokines include proinflammatory (IL-1β, IL-6, IL-22, and TNF-α) and immunoregulatory (IL-21, IL-23, and TGF-β). The presence of these 2 opposing classes of cytokines reveals the complex balance of pro- and anti-inflammatory activity that gives rise to the characteristic lowgrade inflammation in OA and permits the combination of restorative and degradative processes.

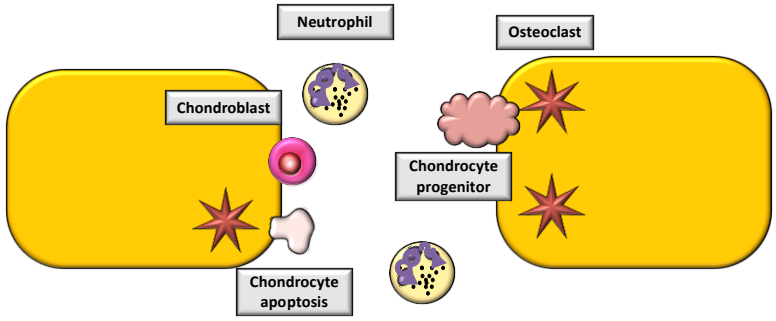
Thus, the inhibition of just one cytokine may not be enough to ameliorate the pathology of OA. For example, TNF-α is readily present in the joints of OA patients and incites chondrocyte catabolism in vitro. However, the use of TNF inhibitors does not significantly improve pathology. Nonetheless, some OA patients reported melioration of pain and function.

This suggests that utilizing cytokines as therapeutic targets will require a complete understanding of the balance and interactions of all cytokines involved in OA pathogenesis. Chemokines are involved in the recruitment of immune cells and activation of signaling cascades in the synovium and synovial fluid of patients with OA synovitis. Molnar et al. suggests that the most substantial chemokine families affiliated with OA are C-C motif chemokine ligand (CCL)2, CCL3, CCL4, CCL5, C-X-C motif chemokine ligand (CXCL)8 (IL-8) and CXCL12.

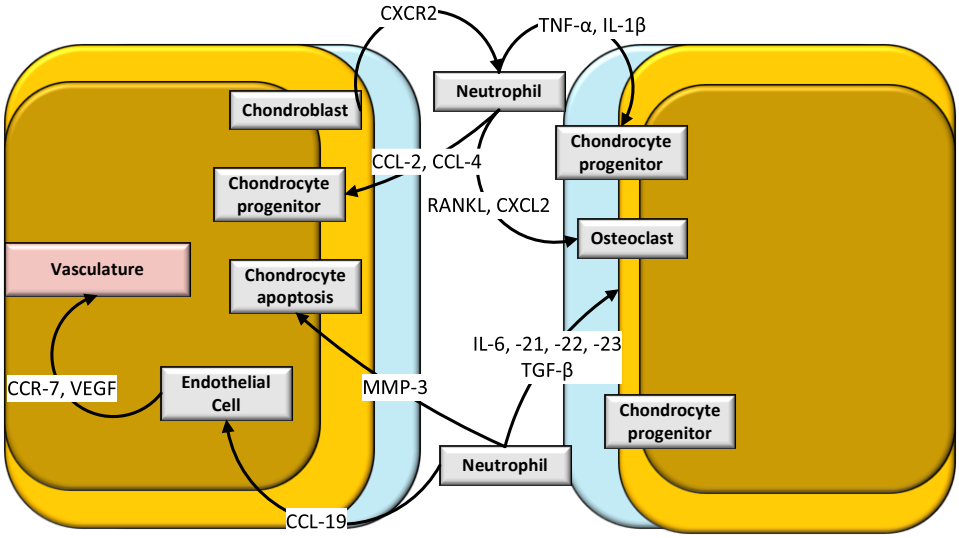
Neutrophils are known to produce all of these except CCL5 and CXCL12.

Chemokines like monocyte chemoattractant protein (MCP-1) (CCL2) and macrophage inflammatory protein (MIP-1β) (CCL4) have been linked to joint pain, possibly due to proteoglycan loss in articular cartilage, which is caused by an upregulation of MMP-3. MCP-1 and MMP-3 are significantly correlated with the presence of neutrophils and macrophages. … CCL19, CCL21, C-C motif chemokine receptor (CCR)7, and C-X-C motif chemokine receptor (CXCR)2 involved in OA synovitis.

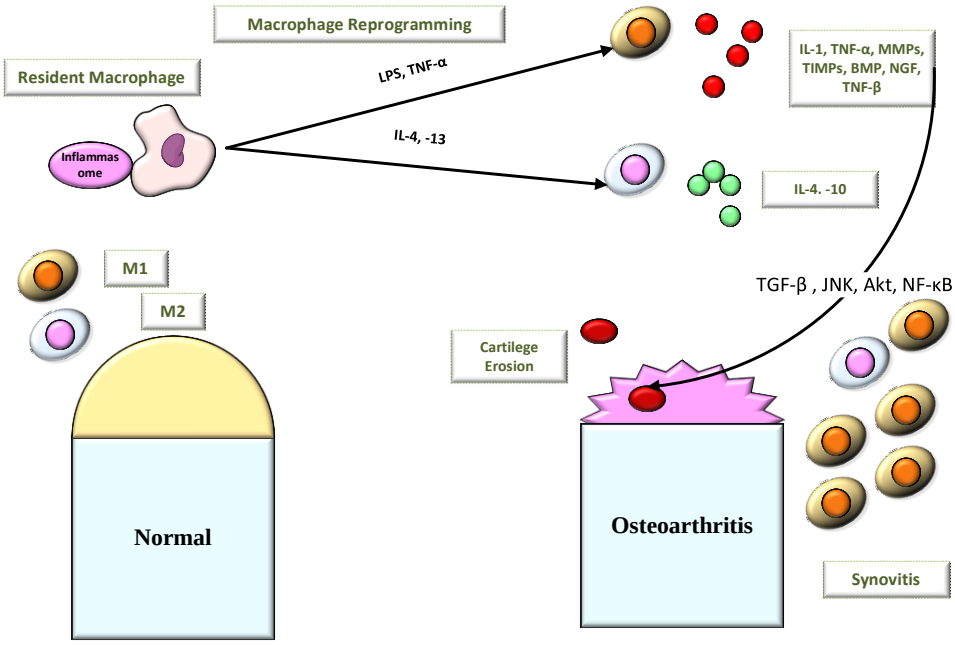
CCL19 and CCR7 are correlated with more severe symptoms and are markers of early synovitis. CCL19 activates CCR7 expression on synovial fibroblasts which then stimulates the release of vascular endothelial growth factor (VEGF) leading to neoangiogenesis in synovial tissue.



Where the detailed signalling is shown below:



From Zhang [1] we have the following conceptualization of the progression to osteoarthritis:



Where the authors note:

Mechanisms of macrophages in the progression of osteoarthritis. PAMPs, DAMPs and inflammasome act as microenvironment stimuli promoted synovial macrophages activation and polarization, which are also regulated by mTOR, NF-kB, JNK, PI3K/Akt and other signaling pathways. Enhanced M1-polarized macrophages in the OA synovium secrete cytokines, growth factors, MMPs and TIMPs, among other factors that lead to inflammation and subsequent cartilage degradation and osteophyte formation.

Aside from autocrine interactions, polarized macrophages alter the intercellular signaling pathways in chondrocytes including TGF-b, JNK, Akt, NF-kB and b-catenin signaling, promoting the degradation of extracellular matrix (ECM) components. ECM acts as DAMPs and further stimulates macrophages activation and polarization, resulting in a repeating cycle of inflammation and cartilage degradation. Polarized synovial macrophages and macrophage reprogramming appear to be suitable therapeutic targets for the prevention and early treatment of OA.

They further summarize macrophage actions as follows:

1. ***Macrophages are activated and polarized during osteoarthritis***
2. ***Macrophages secrete cytokines and matrix metalloproteinases to promote OA development***
3. ***Macrophages and chondrocytes interact during OA development***

And they conclude:

Synovial macrophages acting as immune cells are of critical importance in the symptomology and structural progression of OA. Activated macrophages generate proinflammatory mediators, as well as multiple tissue-degrading enzymes that escalate the inflammatory milieu and

contribute to the destruction of cartilage and bone. Studies have revealed that apart from their autocrine effects, paracrine interactions between macrophages and chondrocytes cause additional feedback loops and enhance synovitis and cartilage degradation.

Targeting synovial macrophages relieves pain, and protects from synovitis, cartilage damage, and osteophyte formation during OA development. Considering the high plasticity of macrophages, significant progress has been made in delineating the ontogeny and function of the various subsets of synovial macrophages in OA; however, sufficient details of which remain to be elucidated.

No specific M1/M2 markers have been identified for in vitro or in vivo studies of human and mouse macrophage polarization. Although macrophages accumulated and tended to display the M1 polarized phenotype in OA synovium, the complete depletion of both M1 and M2 macrophages did not inhibit the development of OA. Those data indicated that the failure to transform from the M1 to M2 subtype may play a larger role in the progression of OA, than does the quantity of activated macrophages.

Previous studies have shown that synovial hyperplasia and the effect of macrophages were more prominent in an OA model with high synovial activation (collagenase-induced osteoarthritis, CIOA), compared with low synovial activation (DMM), suggesting that the role of macrophages in OA is related to synovitis. Different synovial tissue environments modulated macrophage function and led to the characterization of OA into two distinct subgroups, the inflammatory-like OA subgroup and the classical OA subgroup (characterized by cartilage remodeling genes), indicating that macrophage-mediated inflammation is involved in a specific subtype of OA.

Apart from synovial tissues, macrophages were expressed abundantly in the synovial fluid and peripheral blood. The ratio of M1/M2 macrophages was significantly associated with the K-L grading system in knee OA, indicating the heterogeneity of this disease and the M1/M2 ratio was a potential predictor of OA severity.

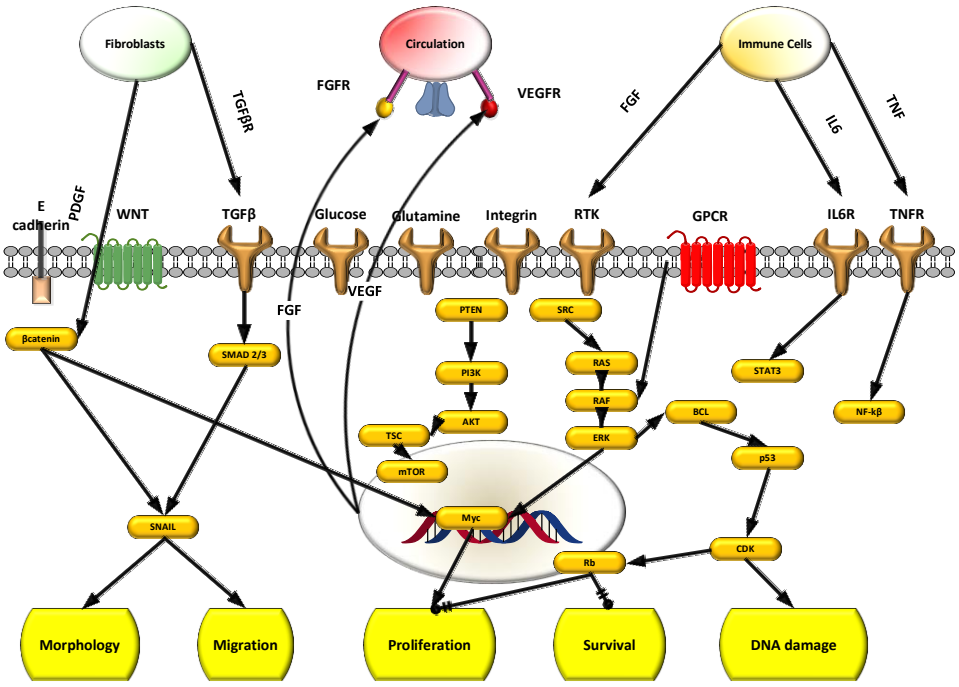
Further identifying synovial macrophages and better understanding the potential functions are needed for future stratification of OA patients for treatments targeting synovial inflammation. Additional research is required to fully elucidate the underlying mechanisms leading to the imbalance of M1 and M2 macrophages in OA, and to explore suitable approaches to manage macrophage reprogramming. OA is a complex disease that affects the whole joint, and thus, therapies targeting single cells or molecules may be insufficient or problematic.

Further studies aimed at understanding the crosstalk between joint tissues at the cellular level within the innate immune network, and the impacts on the disease process at different stages of progression will lead to the development of new therapeutic strategies. Collectively, a multifunctional agent (drug or biomaterial) with immunomodulatory effects on macrophage reprogramming that can skew the inflammatory microenvironment towards a pro-chondrogenic atmosphere would be a potential therapeutic for the treatment of OA and other immune diseases.

4 TUMOR ASSOCIATED NEUTROPHILS

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

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



The above is a simplified attempt to demonstrate the complexity of the cell, the extracellular matrix[18], the immune system[19] and the circulatory system[20].

Cancer is a complex set of diseases. The immune system often attempts to mitigate against new cancer cells. However the same immune system can take part in promoting and protecting those very same cells.

As Masucci et al note :

Tumor Associated Neutrophils (TANs) are engaged into the tumor microenvironment by cytokines and chemokines, can be distinguished according to their activation and cytokine status

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

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

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

and effects on tumor cell growing in N1 and N2 TANs. N1 TANs exert an antitumor activity, by direct or indirect cytotoxicity. N2 TANs stimulate immunosuppression, tumor growth, angiogenesis and metastasis by DNA instability, or by cytokines and chemokines release. In tumor patients, either a high number of TANs and Neutrophil-to-Lymphocyte Ratio (NLR) do correlate with poor prognosis, and, so far, TAN counts and NLR can be regarded as biomarkers.

Owing to the pivotal role of TANs in stimulating tumor progression, therapeutic strategies to target TANs have been suggested, and two major approaches have been proposed:

(a) targeting the CXCL-8/CXCR-1/CXCR-2 axis, thereby blocking TANs or

(b) targeting substances produced by polymorpho-nuclear cells that promote tumor growth.

Many studies have been accomplished either in vitro and in animal models, whereas clinical studies are restrained, presently, due to the risk of inducing immunosuppression. In this review, we deeply discuss the anti-tumorigenic or pro-tumorigenic activity of TANs. In particular, TANs relevance in tumor prognosis and in vitro therapeutic strategies are widely described. On-going clinical trials, aimed to inhibit neutrophil recruitment into the tumor are also accurately debated …

By now, it has been assessed that almost every tumor type produces chemokines regulating the PMN content in microenvironment of solid tumors. Human chemokines, such as IL-8, macrophage inflammatory protein-1α (MIP-1α/CCL3) and human granulocyte chemotactic protein-2 (huGCP-2/CXCL6), murine chemokines, like MIP-1α, GCP-2 and KC (15–17), "behave as potent chemoattractants and neutrophil activators".

Chemokines produced by MG-63 osteosarcoma cells recruit mononuclear cells, lymphocytes and neutrophils . Chemokines are well regulated: Ras upregulates CXC chemokines, causing an accumulation of neutrophils, in mice (24, 25). The cytokines "IL-1, IL-2, IL-4, IL-7, IL-10, IL-12, IFN-α, IFN-β, G-CSF, and TNF-α," cause chemokines release in vitro and granulocytosis in vivo (26). In humans, CXCL5 (epithelial neutrophil-activating peptide-78) recruits neutrophils in Hepato-Cellular Carcinoma (HCC) promoting cancer growth and metastasis. CXCL5 appertains to a small family of secreted proangiogenic chemokine, whose expression increases in metastatic HCC cell lines and in HCC patients. CXCL5 activates the PI3K-Akt and ERK1/2 signaling pathways in HCC cells.

Furthermore, it acts as a chemoattractant, in vitro. CXCL5 over-expression in human HCC samples does well correlate with high neutrophil content, shorter OS, and tumor recurrence.

In tumor bearing mice, MMP-9 promotes HCC, lung and pancreatic cancer angiogenesis by promoting neutrophil recruitment. In mice bearing mammary tumors, IL-17 has been demonstrated to recruit neutrophils: Interleukin (IL)-1β induces IL-17 expression from γδ T cells, which results in a G-CSF-dependent neutrophil expansion and polarization. These tumor-induced neutrophils become able to suppress cytotoxic CD8 T lymphocytes, which limit the establishment of metastases. In mouse models of spontaneous breast cancer metastasis, the neutralization of IL-17 or G-CSF and the absence of γδ T cells prevented neutrophil

accumulation and down-regulated the T-cell-suppressive phenotype of neutrophils, the absence of γδ T cells or neutrophils reduces pulmonary and lymph node metastases without influencing primary tumor progression.

Increased IL17 levels, produced by tumor-infiltrating T lymphocytes have been shown in metastatic invasive ductal breast carcinoma (IDC). IL17 neutralization inhibits tumor cell growth and prevents neutrophil and tumor cell migration and metastasis. Pro- tumor neutrophils induce progression disease and release of CXCL1, MMP9, VEGF, and TNFα, whose reduction suppresses tumor growing. Thus, IL17 plays a pivotal role in tumor progression as emphasized by the fact that high IL17 levels do associate with shorter disease-free survival and poor prognosis in IDC patients.

In 238 HCC patients, the presence of neutrophils in peritumoral stroma was demonstrated. Pro-inflammatory IL-17 promotes neutrophil recruitment in peritumoral HCC tissues, via chemokines production or by activation of IL-17 producing γ δT cells (32). Besides blood, it has been also assessed that spleen is an important reservoir of TANs. Indeed, during tumor progression, neutrophil precursors shift from spleen to the tumor stroma. On the contrary, the surgical removal of spleen delays tumor growing by reducing the number of infiltrating neutrophils, as found in a mouse model of lung adenocarcinoma presenting activation of K-RAS and inactivation of p53 (33)

Wu et al note:

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

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

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

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

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

Additionally, the higher neutrophil to lymphocyte ratio (NLR) indicates a worse prognosis for those patients. There are also studies regarding neutrophils establishing a pre-metastatic niche for the malignant tumor cells. These studies indicate the overall pro-tumor functions of neutrophils in multiple cancer types.

5 THERAPEUTICS

Therapeutics for the disorders discussed herein are multiple yet many result in limited improvement. Sharma has provided an excellent update as to current approaches. They range from exercise, topical NSAIDS, oral NSAIDS, and ultimately surgery.

In contrast there is a multiplicity of potential targeted therapies. As Sun et al have noted:

Osteoarthritis (OA) is a complicated degenerative disease that affects whole joint tissue. Currently, apart from surgical approaches to treat late stage OA, effective treatments to reverse OA are not available. Thus, the mechanisms leading to OA, and more effective approaches to treat OA should be investigated.

According to available evidence, the PI3K/AKT/mTOR signaling pathway is essential for normal metabolism of joint tissues, but is also involved in development of OA. …

This review highlights the role of PI3K/AKT/ mTOR signaling in cartilage degradation, subchondral bone dysfunction, and synovial inflammation, and discusses how this signaling pathway affects development of the disease. We also summarize recent evidences of therapeutic approaches to treat OA by targeting the PI3K/AKT/mTOR pathway, and discuss potential challenges in developing these strategies for clinical treatment of OA. …

To date, the key management strategies for osteoarthritis have included non-pharmacological (e.g., education and self-management, exercises, weight loss if overweight), pharmacological (e.g., NSAIDs and intra-articular injection of corticosteroids), and surgical approaches86. Traditional therapies are effective for alleviating related clinical symptoms and improving quality of life to some extent. Nevertheless, they fail to reverse cartilage degradation, and may cause adverse events87. The targeting of key molecules and signaling pathways involved in the pathogenesis of OA has been extensively investigated. Considering the important role of PI3K/ AKT/mTOR signaling in OA, it might offer promising targets for treatment of OA (Table I).

Currently, PI3K/AKT/mTOR signaling based intervention strategies for OA can be divided into two main categories:

(1) inhibition of PI3K/AKT/mTOR signaling attenuates joint damage due to OA by restoring cartilage homeostasis, enhancing autophagy, and suppressing inflammatory responses.

(2) Activation of PI3K/AKT/mTOR signaling may play an antiarthritic role by promoting chondrocyte proliferation, and reducing apoptosis.

Some approaches and agents that could block transduction of PI3K/AKT/mTOR signaling might be beneficial to patients with the disease. For example, small-molecule inhibitors of PI3K, AKT, and mTOR (LY294002, Casodex, and rapamycin) are shown to promote autophagy of articular chondrocytes, and attenuate the inflammation response in rats with OA39. Additionally, blocking PI3K/AKT signaling with LY294002 reduces bone sclerosis in subchondral bone, and delays

post-traumatic osteoarthritis71. Apart from well characterized inhibitors, some bioactive compounds isolated from herbs could protect joints from OA via inhibition of this pathway. Leonurine, vanillic acid, and scoparone have been demonstrated to ameliorate both chondrocyte and cartilage injury in mice by promoting autophagy and/or repressing inflammatory responses.

The key downstream effectors, mTOR and NF-kB serve as master modulators responsible for initiation of autophagy and inflammation. In another way, activation of PI3K/AKT/mTOR signaling may be beneficial for patients with the disease. Activated PI3K/AKT/mTOR signaling has been found to promote chondrocyte proliferation and reduce apoptosis. Thus, development of some therapeutic approach to activate signaling for its protective aspect is a worthy pursuit.

As expected, some agents, such as 17b-estradiol (E2), FGF18, and ghrelin, which have been correlated with PI3K/AKT activation have a potential protective effect against OA by increasing chondrocyte proliferation or reducing apoptosis27,53,89. Furthermore, microRNAs play an essential role in modulating PI3K/AKT/mTOR signaling and its effects on OA development. Cai et.al. found miR-27a is a regulator of the PI3K-AKT-mTOR axis in human chondrocytes and participates in OA pathogenesis. Chondrocytes transfected with miR- 27a inhibitor reduced IL-1b-induced apoptosis via upregulation of PI3K activity90. Similarly, miR-218-5p, shown to target PIK3C2A mRNA, is a novel inducer of cartilage destruction. Its expression substantially affected expression of matrix synthesis genes, chondrocyte proliferation, and apoptosis. OA mice exposed to a miR- 218-5p inhibitor were protected from cartilage degradation91. These evidences suggest microRNAs have potential as therapeutic targets in osteoarthritis.

In addition, some molecules that affect PI3K/AKT/mTOR activity might also be considered as potential therapeutic targets. For example, peroxiredoxin 4 (PRDX4) overexpression could activate AKT, to reverse IL-1b-stimulated apoptosis mediated by increased BCL-2-associated X apoptosis regulator (BAX) levels and Caspase-3/9 activation92. Consistent with the effects of PRDX4, activated glucagon-like peptide-1 receptor exerts a similar influence on the PI3K/AKT axis and downstream cell apoptosis93.

Targeting these genes provide new insights and approaches to regulating the functions of PI3K/AKT/mTOR in OA development. As PI3K/AKT/mTOR signaling undertakes multiple functions in normal and abnormal cells, its effects are complex, making it difficult to verify the effects of this axis in OA in general, and there remain many prominent issues to be addressed.

First, does activation or inhibition contribute most to OA pathogenesis, est protects against OA progression.

In other words, the PI3K/AKT/ mTOR axis, at least in part, mediates inflammation, autophagy, proliferation, apoptosis, ECM homeostasis, and other cell processes in chondrocytes. Which are the dominant ones? What are the cross talks among them? Cross talks between prominent cellular processes related to the PI3K/AKT/mTOR pathway can occur also in joint cells in a timely manner during the OA process. Thus, more detailed research into these cellular processes is needed to clarify their connection to PI3K/AKT/mTOR signaling.

Moreover, its role in mammals varies from tissue to tissue, particularly for whole joints, which include cartilage, subchondral bone, and synovium. Hence, it is also important to target tissues precisely and correctly to achieve axis-mediated protective effects during OA treatment. As a consequence, it is not appropriate to simplistically link PI3K/AKT/mTOR to the disease, and develop modifying agents.

The authors then summarize these in a Table as shown as modified below

Inhibitor/regulator	Target cell/tissue	Target	Main findings
IGF-1	Rat endplate chondrocytes	PI3K	Induces increased expression of col2al and reduced expression of MMP13
TGF-P	Rat cartilage	PI3K/AKT	Induces decreased expression of MMP13
Leonurine	Mouse	P13K/AKT/NF-kB	Reduces IL-lp-induced inflammatory response
Scoparone	Human chondrocytes	PI3K/AKT/NF-kB	Reduces IL-ip-induced inflammatory response
LY294002 Casodex Rapamycin	Rat chondrocytes	PI3K AKT mTOR	Promotes autophagy of articular chondrocytes and attenuates inflammatory response
Vanillic acid	Rat chondrocytes	MAPK and PI3K/ AKT/NF-kB	Attenuates inflammatory response and cartilage degeneration
17beta-Estradiol	Rat OA chondrocytes	PI3K/AKT	Promotes cell proliferation
FGF18	Rat chondrocytes	PI3K/AKT	Promotes chondrocyte proliferation and migration and attenuates IL-1 p-induced apoptosis in vitro, attenuates cartilage degradation *in vivo*
Ghrelin	Human chondrocyte and cartilage	AKT	Down-regulates the production of various inflammatory cytokines, inhibits apoptosis of chondrocytes, attenuate ECM degradation
miR-218-5p	SW1353 and C28/I2 cells	PI3K/AKT/mTOR	miR-218-5p inhibitor alleviates mice cartilage degradation with reduced proteoglycan loss and reduced loss of articular chondrocyte cellularity
miR-27a	Human	P13K/AKT/mTOR	Promotes the autophagy and apoptosis of IL-1P treated-articular chondrocytes
Peroxiredoxin 4 (PRDX4)	Rat chondrocytes	AKT	PRDX4 overexpression reverses IL-ip-stimulated apoptosis
Liraglutidc	Rat chondrocytes	GLP-1R/PI3K/AKT	Protects chondrocytes against endoplasmic reticulum stress and apoptosis induced by interleukin (IL)-lp or triglycerides and attenuates rat cartilage degeneration in an OA model of knee joints *in vivo*
Platelet-derived growth factor	Human	Src/PI-3K/AKT	Rescues IL-lp-induced increases in mitochondrial-related apoptosis
Berberine	Rat chondrocytes	AKT	Ameliorates cartilage degeneration from OA by promoting cell survival and matrix production of chondrocytes
Tormentic acid	Human	PI3K/AKT	Inhibits IL-ip-induced cytotoxicity and apoptosis in chondrocytes

Inhibitor/regulator	Target cell/tissue	Target	Main findings
PPARgamma	Murine cartilage	mTOR	PPARgamma maintains articular cartilage homeostasis, in part, by regulating mTOR pathway
The hydromethanolic extract of Butea monosperma (BME)	Human	mTOR	BME has strong potential to activate autophagy and suppress IL-1 p induced expression of IL-6 and MMP-3, -9 and - via inhibition of mTOR
Rapamycin	Mouse articular cartilage and synovium	mTOR	Induces autophagy activation and alleviates synovitis and IL-1 P levels in synovial tissue in OA mouse knees.
Raptor	Preosteoblasts	mTORCl	Disruption of Raptor reduces subchondral bone formation and cartilage degeneration, and attenuated post-traumatic OA in mice Reduces BCP-induced production of the pro-inflammatory cytokines

6 OBSERVATIONS

We now make some general observations on extensions of the issues discussed herein.

6.1 TEMPORAL VARIATIONS

There are temporal dynamics to this process. Specifically the neutrophil has a short lifetime and as such can be a tool to reflect the temporal variations to the chronic inflammation. These types of inflammation may increase for example with extensive use of a joint such as the knee. In addition it may vary depending on medications such as NSAIDs that be employed. It would be useful to have the ability to examine this overall process.

6.2 SPATIAL VARIATIONS

Spatial variations are also expected. It is understood how neutrophils pass from the blood to the inflamed area. The question is; is there accurate targeting of the inflammation and the resulting irritants by the neutrophils or is the coverage just a broad cover in general.

6.3 SUPPRESSIONS VS INHIBITION

Does the intervention of the neutrophil suppress or inhibit. As we have noted the neutrophil is responding to inflammasomes as an example. Inflamed and injured synovium may be such an irritant. Then what function does the neutrophil provide in detail. We have demonstrated some efforts along that line but a more detailed study would be useful.

6.4 HSC ISSUES

As we have seen herein, there is a great deal still unknown in the ever more complex transition from the elusive HSC to a mature neutrophil as well as all other cells. As Metcalf has noted:

If hematopoietic commitment and self-generation by HSC are events controllable by nuclear transcription factors, what controls the production and action of these transcription factors? Here, we are in essentially unknown territory. It presumably must be possible to signal an HSC to influence its balance of nuclear transcription factors. Are these inducing signals of more familiarity to embryologists or are they systems such as the Tie2- Ang1 or the Notch- 1-Delta microenvironmental control system? It is less likely that these signals include the conventional hematopoietic regulators because receptor expression for these regulators would appear to be the consequence of such signaling.

However, in some experiments, growth factors were able to alter the relative concentrations of nuclear transcription factors. The comprehensive reviews to follow each gives a detailed description of the subpopulation heterogeneity in hematopoietic populations and the transcriptional controls that regulate differentiation commitment into the various hematopoietic lineages and their subpopulations.

… We take much for granted when carrying out a routine HSC transplantation, simply because matters appear to proceed with such reproducibility.

We forget the heterogeneity of the cells in use, the niches that injected cells must somehow find, and the signals needed to be generated by the hematopoietic-depleted hosts to stir the injected cells into increased proliferative activity. The control of stem cell numbers, their commitment, and progeny generation are biological questions of great clinical importance in many hematological diseases.

The facts that stem cells remain frustratingly difficult to examine and manipulate, let alone mass produce, are salutory reminders that we still have much to learn about hematopoiesis

What we have tried to summarize herein, focused on the simple clinical example, will undoubtedly be an ever progressing set of complex processes. The typical medical student is told a simple tale of neutrophil progression and representation. We see herein that such is but the tip of the iceberg, with many parts still hidden.

6.5 Malignant Transformations

We know that chronic inflammation can be associated with certain cancers. The full dynamics of this process if poorly understood. However in the case we are examining we can see what type of damage the neutrophils result in. As Maxson and Tyner have recently noted:

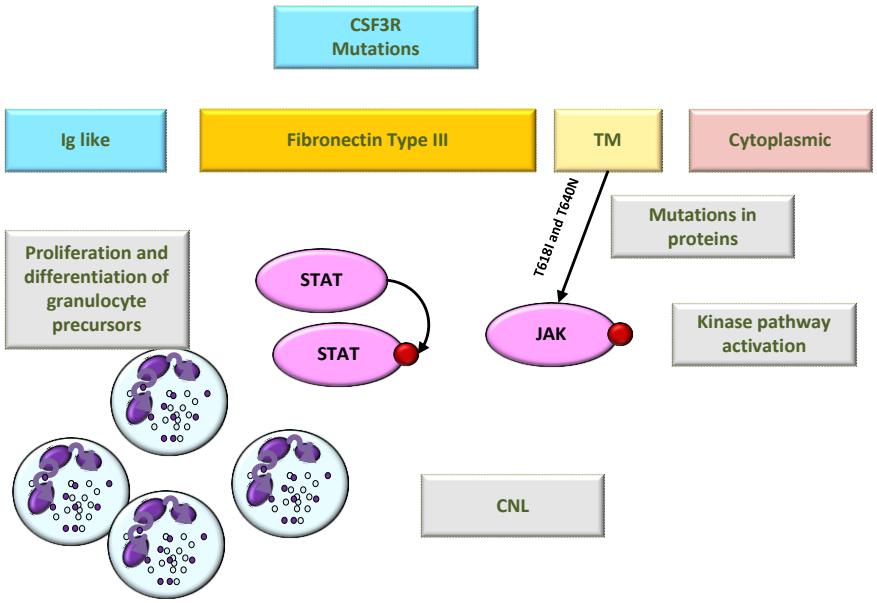
Chronic neutrophilic leukemia (CNL) is a distinct myeloproliferative neoplasm with a high prevalence (>80%) of mutations in the colony-stimulating factor 3 receptor (CSF3R). These mutations activate the receptor, leading to the proliferation of neutrophils that are a hallmark of CNL. Recently, the World Health Organization guidelines have been updated to include CSF3R mutations as part of the diagnostic criteria for CNL.

Because of the high prevalence of CSF3R mutations in CNL, it is tempting to think of this disease as being solely driven by this genetic lesion. However, recent additional genomic characterization demonstrates that CNL has much in common with other chronic myeloid malignancies at the genetic level, such as the clinically related diagnosis atypical chronic myeloid leukemia.

These commonalities include mutations in SETBP1, spliceosome proteins (SRSF2, U2AF1), and epigenetic modifiers (TET2, ASXL1). Some of these same mutations also have been characterized as frequent events in clonal hematopoiesis of indeterminate potential, suggesting a more complex disease evolution than was previously understood and raising the possibility that an age-related clonal process of preleukemic cells could precede the development of CNL.

The order of acquisition of CSF3R mutations relative to mutations in SETBP1, epigenetic modifiers, or the spliceosome has been determined only in isolated case reports; thus, further work is needed to understand the impact of mutation chronology on the clonal evolution and progression of CNL. Understanding the complete landscape and chronology of genomic events

in CNL will help in the development of improved therapeutic strategies for this patient population.



They then note:

CSF3R mutations activate kinase signaling to promote the expansion of neutrophils. CSF3R has an N-terminal extracellular domain comprising an Ig-like domain and fibronectin type-III repeats. The T618I and T615A (not shown) mutations in the extracellular domain and the T640N mutation in the transmembrane domain (purple) cause ligand-independent receptor activation. Truncation mutations in the cytoplasmic domain cause increased cell-surface expression of the receptor. CNL associated mutations in CSF3R cause activation of downstream kinase signaling pathways, such as the JAK/STAT pathway, ultimately driving neutrophil production. P, phosphorylation.

6.6 Impact of Cancer Development

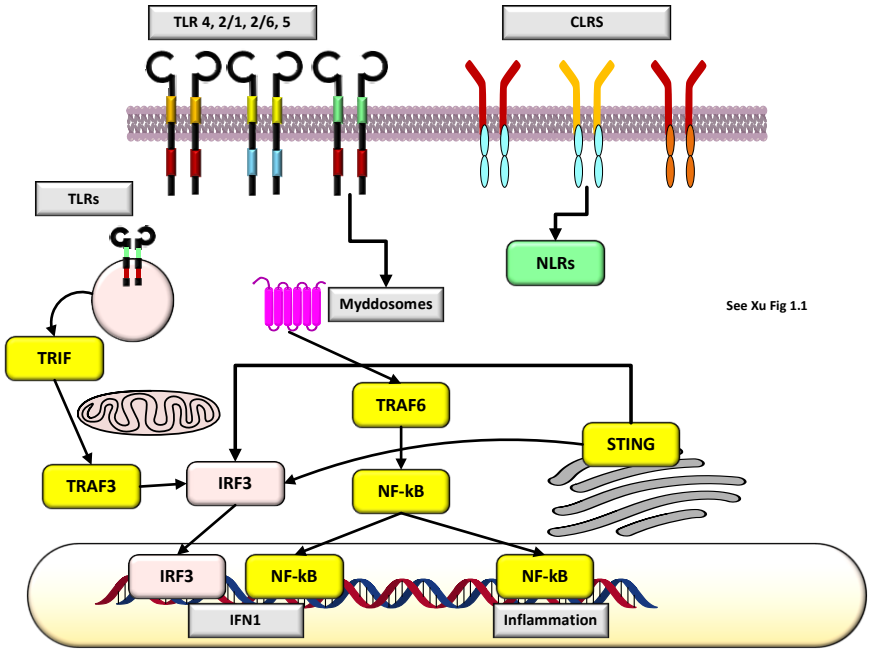
We have discussed TANs as present in an existing cancer micro environment. We have discussed neutrophils in chronic inflammation. There is the question of connectivity, namely does a chronic inflammation potentially excite a cancer process. Are neutrophils capable of facilitating a malignant transformation by the presence of strong cytokine emissions?

6.7 Filling in the Paradigm

At the beginning of this note we set out a paradigm for how an osteoarthritis may evolve from the perspective of neutrophil generation. It was somewhat speculative but we have attempted to fill in the gaps with knowledge from the literature. What we have seen is a complicated set of interactions that lead to proliferation and differentiation. We have seen that neutrophils are not all alike and that each variant may serve a different purpose.

Thus it would be useful if using such a paradigm the steps from activation to response could be detailed and articulated. It is by this understanding that therapeutic approaches may be obtained.

As Xu has indicated below (as modified) regarding the general issues of inflammation:



The linkages in inflammation can be quite complex. The above is a simplification. But there are a multiplicity of drivers and effectors as one examines various inflammatory conditions. We have tried to focus on a specific set but even here the complexity is significant and the linkages not fully explicated.

Thus attempting to “fill in the paradigm” on what is a very common benign problem has been demonstrated to be quite complex. It is a temporal and spatially varying set of transitions that result in the observed outcome. Namely the degradation of the bone tissues in a knee osteoarthritis can result in a mild neutrophilia, and in attempting to follow through on all the related genetic process has led to a highly complex system, with many uncertainties.

This is a simple example of a system analysis applied to a simple chronic inflammatory process. Taking this approach to multiple malignancies may face equal challenges.

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