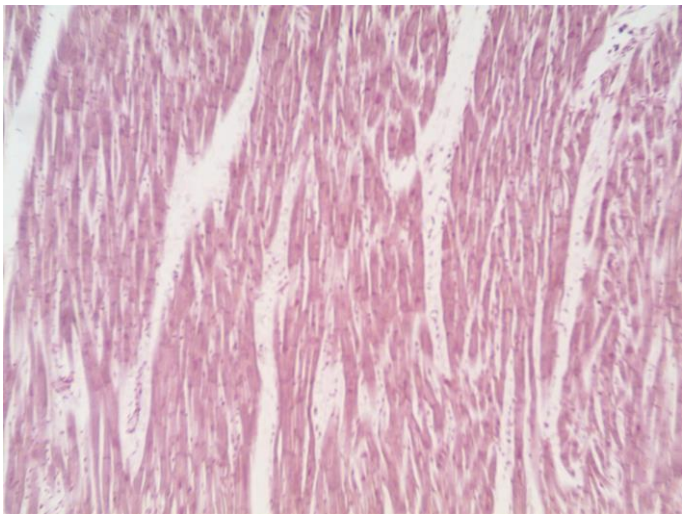

COVID-19: MULTI ORGAN SEQUELLAE

ABSTRACT

COVID-19 is a complex viral infection with an acute phase targeting the pulmonary epithelium. However, it has been seen to have multi-organ sequellae. In this report we begin to examine these sequellae particularly in the context of the impact of the innate immune system, namely natural killer cells. In particular we focus on the myocarditis which appears after such infections.

TERRENCE MCGARTY

TGL 182, DECEMBER, 2020

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1 INTRODUCTION

The COVID-19 virus has a multiplicity of secondary morbidities. It initiates via the nasopharynx and then to the lungs. The initial response is a compromising lung infiltration which is often acerbated by the immune response. However, the virus also has the tendency to move from the pulmonary confines to multiple other organs. The mechanism for this transmission is not fully understood but intra and extra vasation is most likely a mechanism.

In this report we examine some possible mechanisms for the spread to other organs of the COVID-19 virus and the possible impact on them. There has been the appearance of such spread with the higher incidence of myocarditis in young athletes who have been previously infected. The virus had been thought to be confined to pulmonary epithelial cells but it has become clear that it has the ability to infiltrate a variety of organs from heart to brain and most everything in between. We argue herein that this may be a natural sequella effect due to the response of the immune system and we focus on the natural killer cells, NK, which have characteristics of both innate and adaptive immune cells. Furthermore, the NK cells can be overly aggressive in fighting the virus and infected cells and this cytokine aggressive behaviour may be a putative pathway to secondary organ involvement. The analysis contained herein is not dispositive but we feel has probative value.

1.1 TARGETED SEQUELLA

We focus on myocarditis in this report. Myocarditis resulting from COVID-19 is not common but it does present a possible lingering sequella. We have seen low grade myocarditis with limited left ventricular expansion, modest but present, especially in some younger patients who may have suffered a mild version of the viral infection. Most cases reported have been acute myocarditis which has high mortality and are often seen in already compromised patients. However there are reports of mortality amongst young patients as young at 17 years of age.

As Cao et al have noted:

Our results demonstrate that the Spike protein of SARS-CoV-2 alone can induce cellular pathology, e.g. activating macrophages and contributing to induction of acute inflammatory responses… The molecular mechanisms of COVID-19 pathogenesis have just begun to be elucidated. In this study, we aim to investigate whether the S protein of SARS-CoV-2 interacts with macrophages and induce acute lung inflammations in vivo using a S protein-pseudotyped (Spp) lentivirus…

The S protein is thought to be the crucial glycoprotein on the surface of SARS-CoV-2 to mediate the viral entry of host cells. It can bind to ACE2 in the host cells, followed by an aid of TMPRSS2 cleavage, leading to endocytosis of viral/receptor complex into the host cells. ACE2 is present in type II alveolar cells, macrophages and endothelial cells in the lungs, which makes them targeted by SARS-CoV-2. Indeed, SARS-CoV-2 has been found in pneumocytes and endothelial cells from autopsy studies of COVID-19 patients. In this study, we showed that Spp lentivirus was mainly present in LDLr+ type II alveolar cells and macrophages in the lungs.

Those cells also express ACE2. As ACE2 receptors in rodents have shown a much lower affinity to the SARS Spike protein as compared with human ACE2 receptor, the uptake of Spp lentivirus is less likely solely through the endogenous ACE2 in the lungs of mice. We speculate that other receptors or co-factors may facilitate the S protein-mediated viral entry of host cells via an ACE2-dependent or independent pathway for example NRP1-mediated entry pathway. These data provide insight into the possibility of multiple factors/pathways involving SARS-CoV-2 entry of various target cells. The Spp lentivirus will allow us to investigate the mechanisms of viral entry into target cells in further detail in future studies

1.2 VACCINES AND SEQUELLA

Vaccines are notoriously difficult and tricky. Let us take a brief look at the mRNA vaccine coming out this week.

1. The intent of a vaccine is to induce the production of antibodies from the adaptive immune system.

2. The adaptive path usually takes several weeks after it is presented with an antigen.

3. When the process of antibody generation is complete against an antigen then when faced with a virus expressing that antigen in the future the adaptive system has been prime to go into action.

4. However it does not mean that all virions are killed off, just the opposite, an infected person who has been immunized will have their immune system attack the virus and start to kill it off but it still persists and may be able to be contagious. So much for herd immunity.

5. But, let us go back to the initial infection. When we get infected with the virus and it starts to spread we get the innate immune system to respond almost immediately. The innate system is a sledge hammer as compared to the scalpel.

6. Macrophages may pick up the virus, present some protein to a natural killer cell, NK, and then the NK is like a Rambo cell, hacking and killing its way about. It is sending out a massive amount of cytokines and the like in a rapid fire attempt to stop this infection. Think of a common cold and the runny nose and sore throat.

7. Sometimes it works but sometimes one gets a massive immune response attacking other organs such as the heart, liver, kidneys.

8. Thus the mRNA is the antigen we hope creates an antibody. But while doing this the innate system is not sitting idle. It may see this antigen as an assault and return in kind.

9. Thus, long term we may expect innate immune responses which can result in damage.

1.3 OVERVIEW

In this Report we examine the putative sequellae of COVID-19. Specifically, COVID-19 has an acute phase which we have seen mortality rate of between 0.2 to 1.5% in normal populations. In long term care facilities, however., we see mortality rates of in excess of 15%, mostly due in our opinion to the gross negligence of Government entities forcing ill patients into such closed facilities with already compromised individuals.

However, what has not yet been examined is the sequellae to this virus. We have seen such secondary effects as myocarditis, kidney failure, hepatic lesions and even neurological defects. The questions then is; why do these occur and how can they be mitigated.

As such we examine two issues. First the impact of the innate immune system, particularly the natural killer, NK, cells. These are aggressive T cell related innate elements which come to the fore almost immediately upon viral infections. They release a variety of cytokines to fight the virus yet in so doing may initiate a chain of reactions that damage normal cells. Second we examine one of the many sequellae that are attributed to this infection, namely, myocarditis. In the case of myocarditis we see a chronic like condition that results in diffuse infiltration of the myocardium with fibroblasts resembling an infarct yet no localized to a specific arterial blockage but impacting the entire myocardium. This may result in a longer term chronic morbidity, not readily diagnosed.

In a recent paper by Jiang et al they have noted:

COVID-19 has emerged as a severe infectious disease and is affecting the whole world, threatening human health and life. Lymphocytes are core components of the immune system to fight against virus intrusion, especially of CD8+ T and NK cells which play a vital role in the clearance of virus.

However, it's still largely unclear about the characteristic of lymphocyte subsets, especially of CD8+ T and NK cells, in COVID-19 patients.

In this study, we detected the characteristic of lymphocyte subsets in 32 COVID-19 patients with different severity. ***We found a significant reduction of circulating total lymphocytes in COVID-19 patients compared to healthy controls, which was consistent with previous studies****. We also found that the decreased lymphocytes in COVID-19 patients might mainly due to the reduction of CD8+ T and NK cells.* ***And with the progression of COVID-19, CD8+ T and NK cells continued to decline, this phenomenon was particularly evident in the Critical group. The loss of CD8+ T and NK cells suggests that specific and non-specific immunity is suppressed to a certain extent during COVID-19.***

Namely there was a dramatic drop in NK cells as the virus progressed in a patient but the remaining NK cells became more aggressive in their response. It does raise the question of why to both issues. Generally we would expect an increase in NK cells as the infection expanded. They continue:

… These results suggest that although the number of CD8+ T cells decreases during the progression of COVID-19, their immune status may have a compensatory activation, especially in the critical period.

This may be somewhat surprising.

Perforin is a key effector molecule for T and NK cells to mediate cytolysis. It could lyse cells by coordinating the delivery of GrA and GrB to target cells. The perforin/ granzyme cytolytic pathway is a two edged sword in viral defense, which could result in viral clearance and host cell damage.

The above observation may account for the sequellae we are now beginning to see. If indeed the impact of the NK defense elements does damage systemic elements then the oft heard argument for herd immunity via infection may have a dark side as these sequellae emerge. They continue:

Therefore, we speculate that as the COVID- 19 progression, the over-activated immune status of cytotoxic CD8+ T and NK cells might mediate pathological damage and lead to continuous deterioration of patients.

… However, the ***expression of perforin and granzymes had no better diagnostic value than total lymphocyte and NK cell counts in the identification of critical COVID-19 patients****, although GrA expression on NK cells had a good specificity for critical patients. In summary, as the course of disease progression,* ***the declined peripheral lymphocyte subsets in COVID-19 patients might lead to compensatory activation of CD8+ T and NK cells. GrA+CD8+ T and perforin+ NK cells might be used as meaningful indicators for assisting diagnosis of COVID-19****. These findings might provide a new perspective for the diagnosis and treatment of COVID-19 patients.*

The new perspective may very well include the impact on the sequellae.

1.4 SUMMARY

As such we examine the following herein:

1. The general systemic impact of viral infections. This is a well know area but worth summarizing.

2. NK cells and their functions. We believe that NK cells can have a lasting effect. It is well known that NK cells have a form of memory. As Zhang et al have noted:

Because NK cells lack gene-recombination machinery and are thought to be relatively short-lived, it is unclear whether NK cells can mount long-term effective recall responses to reinfections by diverse pathogens. In this article, we report that FcRg-deficient NK cells, which we recently identified and termed g2NK cells, possess distinct memory features directed by FcR-mediated Ab dependent target recognition. The presence of g2NK cells was associated with prior human CMV (HMCV) infection, yet g2NK cell responses were not restricted to HCMV-infected

target cells. In the presence of virus specific Abs, g2NK cells had greatly enhanced functional capabilities, superior to conventional NK cells, and were highly responsive to cells infected with either HCMV or HSV-1. Remarkably, the g2NK cell subset persisted long-term at nearly constant levels in healthy individuals. Therefore, FcRg deficiency distinguishes an Ab-dependent memory-like NK cell subset with enhanced potential for broad antiviral responses.

It may very well be this memory capability that continues the negative impact of NK cells on other organs[1].

3. We review the COVID-19 elements which we have done previously.

4. We then consider other factors related to the immune system especially the impact of IL-6 and NF-kB.

5. Finally we use the specific case of myocarditis and COVID-19.

[1] See Soleimanian and Yaghobi as relates to COVID-19 applications

2 VIRAL INFECTIONS

Viral infections are often highly transmissible and can infect multiple organs. The impact is not only from the viral invasion itself but often through the impact of the immune system attacking the virus. The results are both acute and chronic, immediate and long lasting. COVID-19 seems to have both acute and chronic responses and the better this virus is understood the better one can understand and hopefully manage the chronic effects and sequellae. The danger from this virus is not only the acute threat of death, albeit often quite low, 0.2-1.2%, but more likely the chronic manifestations.

2.1 ACTIONS

Let us begin by briefly focusing on the immune system and viruses. From Abbas et al we have the following:

Viruses typically infect various cell types by receptor-mediated endocytosis after binding to normal cell surface molecules. Viruses can cause tissue injury and disease by any of several mechanisms. ***Viral replication interferes with normal cellular protein synthesis and function and leads to injury and ultimately death of the infected cell.*** *This result is one type of cytopathic effect of viruses, and the infection is said to be lytic because the infected cell is lysed. Viruses can stimulate inflammatory responses that cause damage to tissues. Viruses may also cause latent infections, discussed later.*

The viral infections fundamentally capture the cells infrastructure to use it for the replication of the virus. The net result all too often is the death of the cell along with a release of a multiplicity of new virions. Now from Kumar et al we have:

Viruses can directly damage host cells by entering them and replicating at the host's expense. The manifestations of viral infection are largely determined by the tropism of the virus for specific tissues and cell types. Tropism is influenced by a number of factors[2].

i. *Host receptors for viruses. Viruses are coated with surface proteins that bind with high specificity to particular host cell surface proteins. Entry of many viruses into cells commences with binding to normal host cell receptors. For example, HIV glycoprotein gp120 binds to CD4 and CXCR4 and CCR5 on T cells and macrophages. Host proteases may be needed to enable binding of virus to host cells; for instance, a host protease cleaves and activates the influenza virus hemagglutinin.*

The binding issue is critical since it is the point at which the virus can then capture the cell's own infrastructure to enter the cell itself. A virus needs to find some binding point to assit it in breaking through the cell membrane. As we will not the ACEe receptor plays this role in COVID.

[2] Just for reference a tropism is the turning of part of an organism towards the assistance of the external element, in this case the virus.

ii. *Specificity of transcription factors. The ability of the virus to replicate inside particular cell types depends on the presence of lineage-specific transcription factors that recognize viral enhancer and promoter elements. For example, the JC virus, which causes leukoencephalopathy, replicates only in oligodendroglia in the CNS because the promoter and enhancer DNA sequences regulating viral gene expression are active in glial cells, but not in neurons or endothelial cells.*

Transcription is part of the process of viral replication. Once inside the cell the virus must unpack its shell and then capture the cell's own replication process, using the RNA elements to reproduce itself again and again, including the shell that protects it when released.

iii. *Physical characteristics of tissues. Host environment and temperature can contribute to tissue tropism. For example, enteroviruses replicate in the intestine in part because they can resist inactivation by acids, bile, and digestive enzymes. Rhinoviruses infect cells only within the upper-respiratory tract because they replicate optimally at the lower temperatures characteristic of this site.*

The physical factors are key. Rhinoviruses can replicate in the nasopharynx. The temperature of 94F allows for that replication. In contrast Corona viruses require 98F and thus require movement into the pulmonary system.

Once viruses are inside host cells, they can damage or kill the cells by a number of mechanisms

i. *Direct cytopathic effects. Viruses can kill cells by preventing synthesis of critical host macromolecules, by producing degradative enzymes and toxic proteins, or by inducing apoptosis. For example, poliovirus blocks synthesis of host proteins by inactivating cap-binding protein. HSV produces proteins that inhibit synthesis of cellular DNA and mRNA and other proteins that degrade host DNA. Viral replication also can trigger apoptosis of host cells by cell-intrinsic mechanisms, such as perturbations of the endoplasmic reticulum during virus assembly, which can activate caspases that mediate apoptosis.*

ii. *Anti-viral immune responses. Viral proteins on the surface of host cells may be recognized by the immune system, and lymphocytes may attack virus-infected cells. Cytotoxic T lymphocytes (CTLs) are important for defense against viral infections, but CTLs also can be responsible for tissue injury. Hepatitis B infection causes CTL-mediated destruction of infected hepatocytes, a normal response that is attempting to clear the infection.*

iii. *Transformation of infected cells. Different oncogenic viruses (e.g., HPV, EBV) can stimulate cell growth and survival by a variety of mechanisms, including hijacking the control of cell cycle machinery, anti-apoptotic strategies, and insertional mutagenesis (in which the insertion of viral DNA into the host genome alters the expression of nearby host genes).*

Similarly, from Abbas et al we have a list of ways cells may attempt to evade the immune system shown below:

Mechanism of Immune Evasion	Examples
Antigenic variation	Influenza, rhinovirus, HIV
Inhibition of antigen processing Blockade of TAP transporter Removal of class 1 molecules from the ER	HSV CMV
Production of "decoy" MHC molecules to inhibit NK cells	Cytomegalovirus (murine)
Production of cytokine receptor homologues	Vaccinia, poxviruses (IL-1, IFN-y), Cytomegalovirus (chemokine)
Production of immunosuppressive cytokine	Epstein-Barr (IL-10)
Infection and death or functional impairment of immune cells	HIV
Inhibition of complement activation Recruitment of factor H Incorporation of CD59 in viral envelope	HIV vaccinia, human CMV
Inhibition of innate immunity Inhibition of access to RIG-I RNA sensor Inhibition of PKR (signaling by IFN receptor)	Vaccinia, HIV, HCV, HSV, polio

2.2 Cell Damage

Cells can be damaged, yet remain viable. Damaged cell clusters often develop an infusion of fibroblasts and then fibrin. The fibroblasts are non-functional elements of the cell damage. The fibroblast functions can be described from NCBI where we have[3]:

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

Fibroblasts are the most common cell type represented in connective tissue. These cells produce a diverse group of products including collagen type I, III, and IV, proteoglycans, fibronectin, laminins, glycosaminoglycans, metalloproteinases, and even prostaglandins. In the adult body,

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

fibroblasts remain in a quiescent form until stimuli activate protein synthesis and contractile mechanisms.

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

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

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

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

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

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

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

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

Fibroblasts are spindle-shaped, non-epithelial (cytokeratin negative, E-cadherin negative), non-endothelial (CD31 negative) and non-immune (CD45 negative) cells of a mesenchymal lineage origin (vimentin+). In normal tissue, fibroblasts are usually considered as resting/ quiescent cells with negligible metabolic and transcriptional activities, but with the ability to respond to growth factors to become activated.

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

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

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

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

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

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

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

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

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

The ability of tissues to repair themselves is determined, in part, by their intrinsic proliferative capacity. In some tissues (sometimes called labile tissues), cells are constantly being lost and must be continually replaced by new cells that are derived from tissue stem cells and rapidly proliferating immature progenitors. These types of tissues include hematopoietic cells in the bone marrow and many surface epithelia, such as the basal layers of the squamous epithelia of

the skin, oral cavity, vagina, and cervix; the cuboidal epithelia of the ducts draining exocrine organs (e.g., salivary glands, pancreas, biliary tract); the columnar epithelium of the gastrointestinal tract, uterus, and fallopian tubes; and the transitional epithelium of the urinary tract. These tissues can readily regenerate after injury as long as the pool of stem cells is preserved.

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

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

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

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

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

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

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

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

d. ***The combination of proliferating fibroblasts, loose connective tissue, new blood vessels and scattered chronic inflammatory cells, forms a type of tissue that is unique to***

healing wounds and is called granulation tissue. This term derives from its pink, soft, granular gross appearance, such as that seen beneath the scab of a skin wound.

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

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

2.3 CELL DEATH

Cells die in a variety of ways. Green lists three types. They are:

1. Apoptosis
2. Autophagy
3. Necrosis

Apoptotic death results in cells being left in an enclosed membrane. Apoptosis is what could be called normal cell death. Autophagy (see Hayat) is a less well understood death and results in a cell eating itself up. Necrosis is a cell somewhat blowing itself apart. Cell destruction by viruses is generally considered apoptosis.

After a cell dies off the remains must be eliminated. In autophagy there is a bit of self-elimination whereby the other cells get to reuse what has been left behind. Other cell death gets cleaned up by macrophages and other immune cells.

3 NK CELLS

We discuss Natural Killer (NK) cells as a precis to understanding COVID-19 and the response to infection. The prime reason is that NK cells are part of the innate immune system and thus are the first to battle. Yet the NK cells are often an axe and not a scalpel. They can result in a great deal of collateral damage if not properly confined. NK cells result in cell death. But as we shall note, they also have the ability to sustain themselves and adapt. They have a quasi memory like capability and unlike the other innate system cells at time act in an adaptive manner.

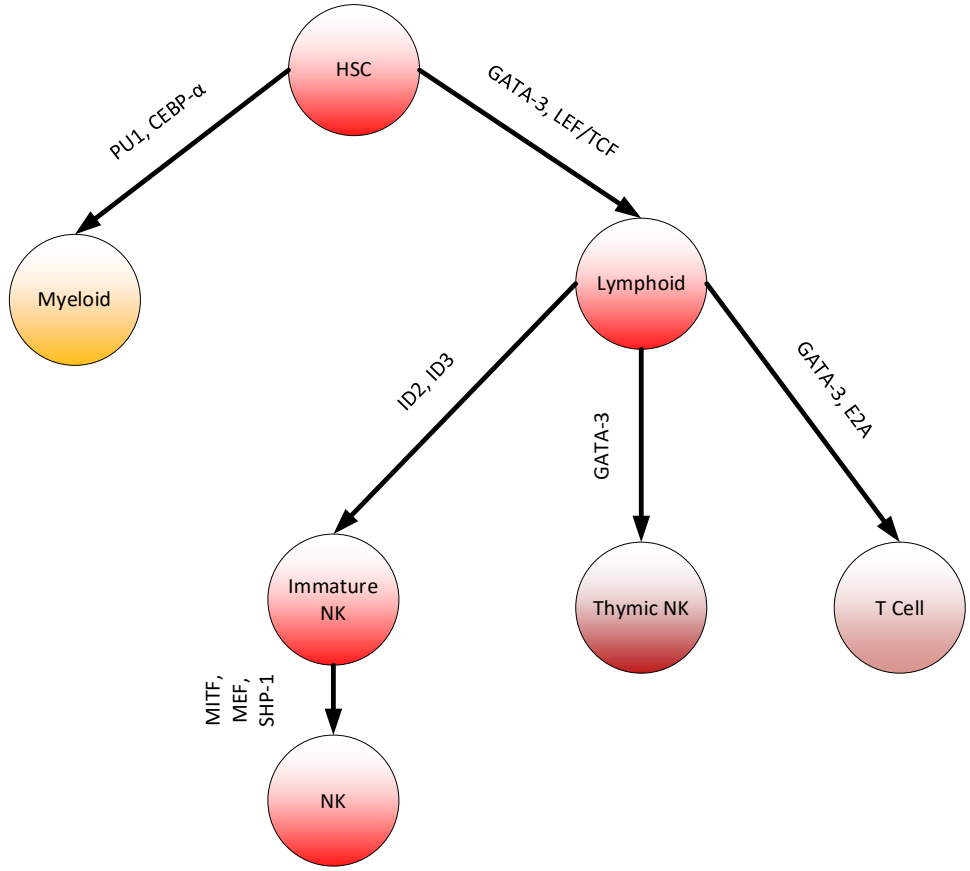
It is for these reasons that we try to focus on the importance of the NK cells, especially in their involvement with other organ inflammations and degradations.

3.1 NK CELLS AND DEVELOPMENT AND DISTRIBUTION

We again begin with Abbas et al who note:

The two principal types of reactions of the innate immune system are ***inflammation and antiviral defense****.* ***Inflammation consists of the accumulation and activation of leukocytes and plasma proteins at sites of infection or tissue injury. These cells and proteins act together to kill mainly extracellular microbes and to eliminate damaged tissues. Innate immune defense against intracellular viruses is mediated mainly by natural killer (NK) cells, which kill virus-infected cells, and by cytokines called type I interferons, which block viral replication within host cells.***

NK cells are part of the T cell family. The development of the NK cell from a fundamental hematopoietic stem cell, HSC, is demonstrated below:



As Abel et al have noted regarding NK cells:

Natural killer cells represent 5–20% of circulating lymphocytes in humans.

The percentages of NK cells among lymphocytes ranges between about 2–5% in the spleens and BMs of inbred laboratory mice and about twice that number in wild-caught mice.

They are distinguished by their unique functions and expression of surface antigens. ***NK cells lack the clonotypic T cell receptor (TCR) of T and NKT cells*** *and its associated signal-transducing adaptor, CD3ε.*

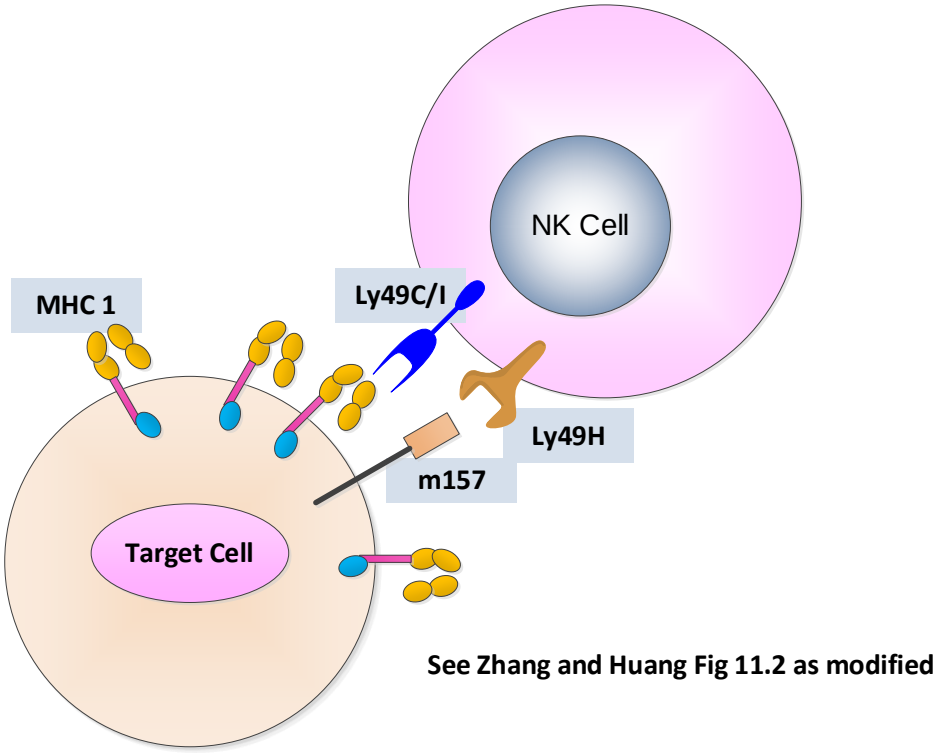
In humans, subsets of NK cells express the activating Fc receptor, ***CD16 and most express CD56*** *[neural cell adhesion molecule (NCAM) or Leu-19]. …NK cells are most similar to a group of lymphocytes known as innate lymphoid cells (ILCs).*

ILCs are further categorized into three distinct groups and are present in both humans and mice. ***NK cells are related to group 1 ILCs as both produce interferon-gamma (IFN-γ) and tumor necrosis factor (TNF)-α upon stimulation****. However, unlike Group 1 ILCs, NK cells have cytolytic functions that resemble those of CD8+ cytotoxic T lymphocytes*

Also NK cells have a life span of about two weeks.

3.2 NK Cells and Virus

The interaction between an NK and a virus infected cells is depicted below. Here we have the self-recognition on the MHC1 surface receptor via Ly49H/C and the detection of the m157 antigen representing the viral content by the Ly49H receptor. Note that the NK cell has a multiplicity of MHC1 receptors on its surface as well as m157 receptors as well.



See Zhang and Huang Fig 11.2 as modified

Grossman et al describe the Ly49 receptor actions as follows:

Natural killer (NK) cells show some features of adaptive immunity but have not been studied at the clonal level. *Here, we used retrogenic color-barcoding and single-cell adoptive transfers to track clonal immune responses to murine cytomegalovirus (MCMV) infection, derived from individual NK cells expressing activating receptor Ly49H. Clonal expansion of single NK cells varied substantially, and this variation could not be attributed to the additional presence or absence of inhibitory Ly49 receptors.*

Instead, single-cell-derived variability correlated with distinct surface expression levels of Ly49H itself. Ly49Hhi NK cell clones maintained higher Ly49H expression and expanded more than their Ly49Hlo counterparts in response to MCMV. *Thus, akin to adaptive processes shaping an antigen-specific T cell receptor (TCR) repertoire, the Ly49H+ NK cell population adapts to MCMV infection. This process relies on the clonal maintenance of distinct Ly49H*

expression levels, generating a repertoire of individual NK cells outfitted with distinct reactivity to MCMV

3.3 KIR

Natural killer cells, NK, are elements of the innate immune system. They often are the first cells on the task of attacking aberrant cells. Natural killer (NK) cells are a subset of bone marrow–derived lymphocytes. The NK cells are totally distinct from B or T cells. The NK cells function in innate immune system and they respond to kill microbe-infected cells by direct lytic mechanisms and by secreting IFN-γ. NK cells do not express clonally distributed antigen receptors like Ig receptors or T Cells Receptors and their activation is regulated by a combination of cell surface stimulatory and inhibitory receptors, the latter recognizing self MHC molecules[4].

From Lorenzo-Herrero et al:

Natural Killer (NK) cells are cytotoxic immune cells with an innate capacity for eliminating transformed cells in a non-major histocompatibility complex (MHC) and non-tumor antigen-restricted manner.

The activation of NK cells depends on a balance of signals provided by inhibitory and activating receptors that detect changes in the patterns of expression of their ligands on the surface of tumor cells. Inhibitory NK cell receptors recognize self-proteins and transmit inhibitory signals that maintain tolerance to normal cells. Killer cell immunoglobulin-like receptors (KIRs) *and the heterodimer CD94-Natural Killer Group 2A (NKG2A)* ***are inhibitory receptors that recognize self-MHC class I molecules****, whereas other inhibitory receptors, such as T cell immunoreceptor with Ig and ITIM domains (TIGIT) receptor, bind to other self-molecules. Transformed cells frequently downregulate MHC class I molecules, thereby avoiding recognition by CD8+ cytotoxic T cells, but concomitantly inducing the activation of NK cells by missing self-recognition.*

The KIR receptors will play this important role of activation control as we discuss latter. The authors continue:

Activating receptors, including, but not limited to, killer cell lectin-like receptor K1 (KLRK1—best known as NKG2D), DNAX accessory molecule-1 (CD226—best known as DNAM-1) and the natural cytotoxicity receptors NKp46, NKp44, and NKp30, recognize stress-inducible ligands on tumor cells that are scarcely expressed in their normal counterparts. Natural killer group 2D (NKG2D) is a particularly relevant activating receptor, which recognizes a group of stress-inducible molecules termed MHC class I polypeptide-related sequence A and B (MICA and MICB) and UL16 binding protein molecules (ULBP1-6), which are restrictedly expressed on stressed and transformed cells.

Thus, by this complex pattern of receptors, NK cells may kill a broad range of cancer cells. Indeed, the engagement of activating receptors by tumor-expressed ligands, along with a lack of

[4] See Abbas et al 4th Ed

co-engagement of an appropriate number of inhibitory receptors, results in the exocytosis of cytotoxic granules containing perforin and granzymes that induce apoptotic cell death of the target cells.

NK cells have a strong potential for cancer attack. The concern is that when they do attack they do so in a rather ruthless manner, but effectively. As part of the innate immune system their response once activated is immediate.

Additionally, NK cells can eliminate target cells through Fas ligand and tumor necrosis factor (TNF)-related apoptosis-inducing signals. Finally, NK cells may also kill tumor cells bound by specific IgG antibodies through Fc RIII receptors (also named as CD16s), a process known as antibody-dependent cellular cytotoxicity (ADCC).

The latter is a relevant process underlying the therapeutic activity of certain monoclonal antibodies. NK cells also regulate the innate and adaptive immune response through the secretion of cytokines with potent antitumor activity, such as interferon-gamma (IFN-γ).

As Bassani et al have recently noted regarding the TME and the NK cells:

Immune cells, as a consequence of their plasticity, can acquire altered phenotype/functions within the tumor microenvironment (TME). Some of these aberrant functions include attenuation of targeting and killing of tumor cells, tolerogenic/immunosuppressive behavior and acquisition of pro-angiogenic activities. Natural killer (NK) cells are effector lymphocytes involved in tumor immunosurveillance. In solid malignancies, tumor-associated NK cells (TANK cells) in peripheral blood and tumor-infiltrating NK (TINK) cells show altered phenotypes and are characterized by either anergy or reduced cytotoxicity.

Here, we aim at discussing how NK cells can support tumor progression and how induction of angiogenesis, due to TME stimuli, can be a relevant part on the NK cell-associated tumor supporting activities. *We will review and discuss the contribution of the TME in shaping NK cell response favoring cancer progression. We will focus on TME-derived set of factors such as TGF-β, soluble HLA-G, prostaglandin E2, adenosine, extracellular vesicles, and miRNAs, which can exhibit a dual function.*

This rather strange action of the NK cells is also a feature in macrophages as well. The TME seems to be a fertile ground for not only cancer cell growth but the adoption of what would be cancer killing cells as supportive ones instead. Whether this becomes another set of targets has been considered by others and we believe that it has substantial merit. They continue:

On one hand, these factors can suppress NK cell-mediated activities but, on the other hand, they can induce a pro-angiogenic polarization in NK cells. Also, we will analyze the impact on cancer progression of the interaction of NK cells with several TME-associated cells, including macrophages, neutrophils, mast cells, cancer-associated fibroblasts, and endothelial cells. Then, we will discuss the most relevant therapeutic approaches aimed at potentiating/restoring NK cell activities against tumors. ***Finally, supported by the literature revision and our new findings on NK cell pro-angiogenic activities, we uphold NK cells to a key host cellular paradigm in***

controlling tumor progression and angiogenesis; thus, we should bear in mind NK cells like a TME-associated target for anti-tumor therapeutic approaches.

As Lopez-Soto notes:

NK cells can exert robust antimetastatic functions independent of MHC-mediated antigen presentation via at least three pathways:

(1) the release of PRF1- and GZMB-containing pre-formed granules;

(2) the secretion of IFNG; and

(3) the exposure of death receptor ligands, including FASLG and TRAIL.

Thus, at odds with T lymphocytes (which require priming from antigen-presenting cells) NK cells are continuously poised to kill damaged, infected, or (pre)malignant cells. Such a potent cytotoxic activity is mainly regulated by the interplay between inhibitory and activatory signals originating at the plasma membrane of NK cells from NKIRs and NKARs, respectively. NKIRs keep the effector functions of NK cells at bay upon interaction with ligands expressed by normal and healthy cells.

Conversely, ***NKARs promote the effector functions of NK cells as they recognize a wide panel of ligands that are specifically upregulated in response to potentially detrimental perturbations of homeostasis, including DNA damage and viral infection.***

NKIRs and NKARs virtually operate as mutual antagonists as they contain intracellular domains that inhibit or activate the phosphorylation-dependent signal transduction cascade leading to NK cell activation*…*

In vitro, NK cells have been shown to kill cancer cell lines of different histological origin, virtually irrespective of derivation (primary tumors versus metastatic lesions), including malignant cells with stem-like features. Accordingly, Klrk1/ mice develop transgene-driven lymphomas and prostate carcinomas at increased incidence compared with WT mice. Moreover, transgene-driven overexpression of NKG2D ligands renders multiple murine cancer cells that normally form tumors upon inoculation into immunocompetent syngeneic hosts sensitive to rejection. …

Finally, NK cells generally represent a minor fraction of the immunological infiltrate of most established solid tumors in humans and have limited prognostic value compared with other tumorinfiltrating lymphocytes such as CD8+ CTLs or CD4+CD25+FOXP3+ TREG cells

We seem to understand that albeit NK presence but the most facilitating cells may be the macrophages. KIR are receptors, transmembrane proteins. Abbas et al (9th Ed) note:

Many of the NK cell–activating receptors are called ***killer cell immunoglobulin (Ig)-like receptors (KIRs)*** *because they contain a structural domain named the immunoglobulin (Ig) fold, first identified in antibody (also known as Ig) molecules,*

Killer immunoglobulin–like receptors (KIR) are described by Vilches and Parham as follows:

KIR genes have evolved in primates to generate a diverse family of receptors with unique structures that enable them to recognize MHC-class I molecules with locus and allele-specificity. Their combinatorial expression creates a repertoire of NK cells that surveys the expression of almost every MHC molecule independently, thus antagonizing the spread of pathogens and tumors that subvert innate and adaptive defense by selectively downregulating certain MHC class I molecules. The genes encoding KIR that recognize classical MHC molecules have diversified rapidly in human and primates; this contrasts with conservation of immunoglobulin- and lectin-like receptors for nonclassical MHC molecules.

As a result of the variable KIR-gene content in the genome and the polymorphism of the HLA system, dissimilar numbers and qualities of KIR:HLA pairs function in different humans. This diversity likely contributes variability to the function of NK cells and T-lymphocytes by modulating innate and adaptive immune responses to specific challenges.

Beziat et note, KIR have a complex set of actions:

Human natural killer (NK) cells are functionally regulated by killer cell immunoglobulin like receptors (KIRs) and their interactions with HLA class I molecules. As KIR expression in a given NK cell is genetically hard-wired, we hypothesized that KIR repertoire perturbations reflect expansions of unique NK-cell subsets and may be used to trace adaptation of the NK-cell compartment to virus infections.

By determining the human "KIR-ome" at a single-cell level in more than 200 donors, we were able to analyze the magnitude of NK cell adaptation to virus infections in healthy individuals. Strikingly, infection with human cytomegalovirus (CMV), but not with other common herpesviruses, induced expansion and differentiation of KIR-expressing NK cells, visible as stable imprints in the repertoire.

Education by inhibitory KIRs promoted the clonal-like expansion of NK cells, causing a bias for self-specific inhibitory KIRs. Furthermore, our data revealed a unique contribution of activating KIRs (KIR2DS4, KIR2DS2, or KIR3DS1), in addition to NKG2C, in the expansion of human NK cells. These results provide new insight into the diversity of KIR repertoire and its adaptation to virus infection, suggesting a role for both activating and inhibitory KIRs in immunity to CMV infection.

As Pittari et al note, the KIR fall into multiple classes:

The function of NK cells is governed by a set of germline- encoded activating or inhibitory receptors referred to as killer immunoglobulin-like receptors (KIRs).

The extracellular domain determines which HLA class I molecule NK cells recognize, whereas the intracytoplasmic domain transmits either an activating or an inhibitory signal.

KIRs are monomeric receptors with either 2 (KIR2D) or 3 (KIR3D) immunoglobulin-like domains, and are further subdivided into those with long (L) cytoplasmic tails (KIR2DL and KIR3DL) and short (S) cytoplasmic tails (KIR2DS and KIR3DS). Long-tail KIRs generate an inhibitory signal through the recruitment of the SH2-domain- containing tyrosine phosphatase 1 protein (SHP1).

Short-tail KIRs possess truncated portions that transduce activating signals via tyrosine phosphatase of DAP12 and other proteins.

The NK receptors are also a key element for potential immunotherapy. The KIR receptors are especially significant in this case.

Paul and Lal describe the human equivalents as follows:

Human express functionally equivalent homolog of Ly49 member which are known as ***killer cell immunoglobulin-like receptor (KIR) family of proteins***[5]*. KIRs are type I transmembrane protein with two or three IgG-like domains and a short or long cytoplasmic tail. KIRs bind to HLAA, -B, and -C molecules. In contrast to Ly49 family, KIRs bind to the peptide-binding region of HLA molecules.*

The heterogeneity of KIR repertoire expression among different individuals is due to the difference in the expression of KIR molecules on individual NK cells as well as allelic variation in KIR genes. Inhibitory KIRs include KIR2DL1–3, KIR2DL5, and KIR3DL1–3. Both KIRs and inhibitory Ly49 receptors contribute to NK cell tolerance to self-tissues. CD94-natural-killer group 2, member A (NKG2A) is another C-type lectin family of the inhibitory receptor that expresses as a heterodimer and contain ITIM.

This receptor specifically recognizes non-classical MHC molecules on target cells and protect host cell against inappropriate NK cell activation. Human NKG2A recognizes non-classical MHC molecule HLA-E while mouse counterpart interacts with the Qa1 molecule. There are several cytokines present in the tissue microenvironment that can modulate the expression of NKG2A and affect NK cell function

Now as Abbas notes:

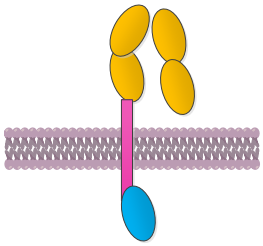
Two major families of NK cell inhibitory receptors are the killer cell immunoglobulin-like receptors (KIRs)*, so called because they share structural homology with Ig molecules, and* ***receptors consisting of a protein called CD94 and a lectin subunit called NKG2****.*

[5] From Abbas: Killer immunoglobulin-like receptors (KIRs) Ig superfamily receptors expressed by NK cells that recognize different alleles of HLA-A, HLA-B, and HLA-C molecules. Some KIRs have signaling components with ITIMs in their cytoplasmic tails, and these deliver inhibitory signals to inactivate the NK cells. Some members of the KIR family have short cytoplasmic tails without ITIMs but associate with other ITAM-containing polypeptides and function as activating receptors.

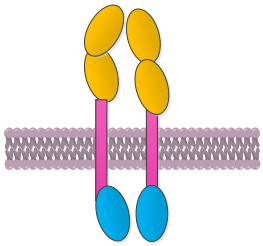
Both families of inhibitory receptors contain in their cytoplasmic domains structural motifs called ***immunoreceptor tyrosine-based inhibitory motifs (ITIMs),*** *which become phosphorylated on tyrosine residues when the receptors bind class I MHC molecules. The phosphorylated ITIMs bind and promote the activation of cytoplasmic protein tyrosine phosphatases. These phosphatases remove phosphate groups from the tyrosine residues of various signaling molecules, thereby counteracting the function of the ITAMs and blocking the activation of NK cells through activating receptors.*

Therefore, when the inhibitory receptors of NK cells encounter self MHC molecules on normal host cells, the NK cells are shut off. Many viruses have developed mechanisms to block expression of class I molecules in infected cells, which allows them to evade killing by virus-specific CD8+ CTLs. When this happens, the NK cell inhibitory receptors are not engaged, and if the virus induces expression of activating ligands at the same time, the NK cells become activated and eliminate the virus-infected cells.

The MHC molecules are either MHC1 or MHC2 as shown below. MHC1 are found on all nucleated cells and provide for self-recognition.



MHC I
All Cells
When something
NOT OK IN Cell



MHC II
APC Cells Only
Tells something
NOT OK Outside
Cell

As regards to the MHC interaction, Sullivan et al note:

Murine natural killer (NK) cells are regulated by the interaction of Ly49 receptors with major histocompatibility complex class I molecules (MHC-I). Although the ligands for inhibitory Ly49 were considered to be restricted to classical MHC (MHCIa), we have shown that the non-classical MHC molecule (MHCIb) H2-M3 was a ligand for the inhibitory Ly49A.

Here we establish that another MHC-Ib, H2-Q10, is a bona fide ligand for the inhibitory Ly49C receptor. H2-Q10 bound to Ly49C with a marginally lower affinity (5 M) than that observed between Ly49C and MHC-Ia (H-2Kb/H-2Dd, both 1 M), and this recognition could be prevented by cis interactions with H-2K in situ. To understand the molecular details underpinning Ly49 MHC-Ib recognition, we determined the crystal structures of H2-Q10 and Ly49C bound H2-Q10.

Unliganded H2-Q10 adopted a classical MHC-I fold and possessed a peptide-binding groovethat exhibited features similartothose found inMHC-Ia, explaining the diverse peptide binding repertoire of H2-Q10. Ly49C bound to H2-Q10 underneath the peptide binding platform to a region that encompassed residues … Our data provide a structural basis for Ly49MHC-Ib recognition and demonstrate that MHC-Ib represent an extended family of ligands for Ly49 molecules.

Paul and Lal describe the process of NK attack on target cells:

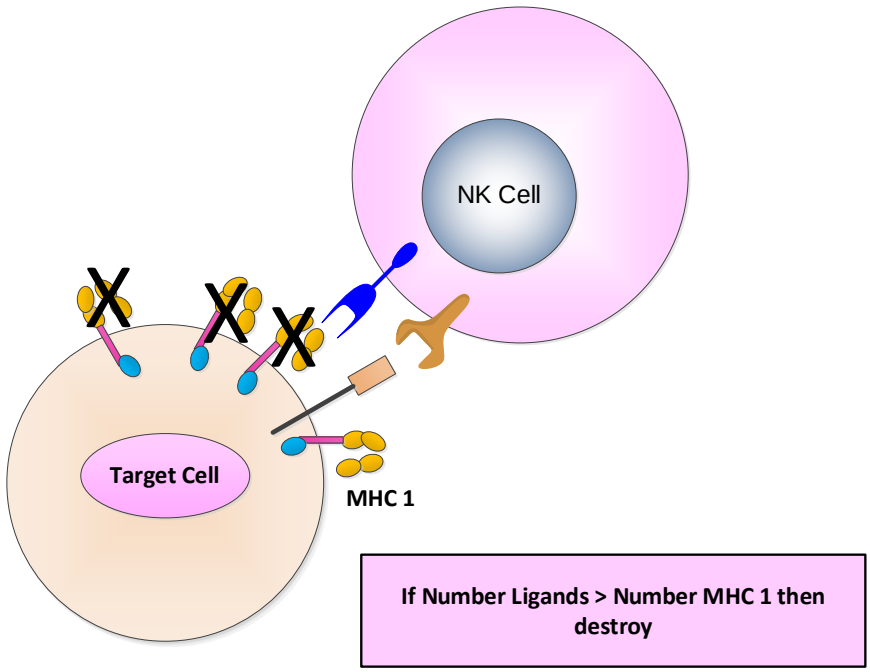
The cytotoxic response of natural killer (NK) cells. The NK cell cytotoxic response is tightly regulated in four discrete stages.

(a) Step 1: Recognition of target cells by NK cell results in the reorganization of actin cytoskeleton and the formation of immunological synapse, and clustering of cell adhesion molecules such as lymphocyte function-associated antigen 1 (LFA-1) and CD2.

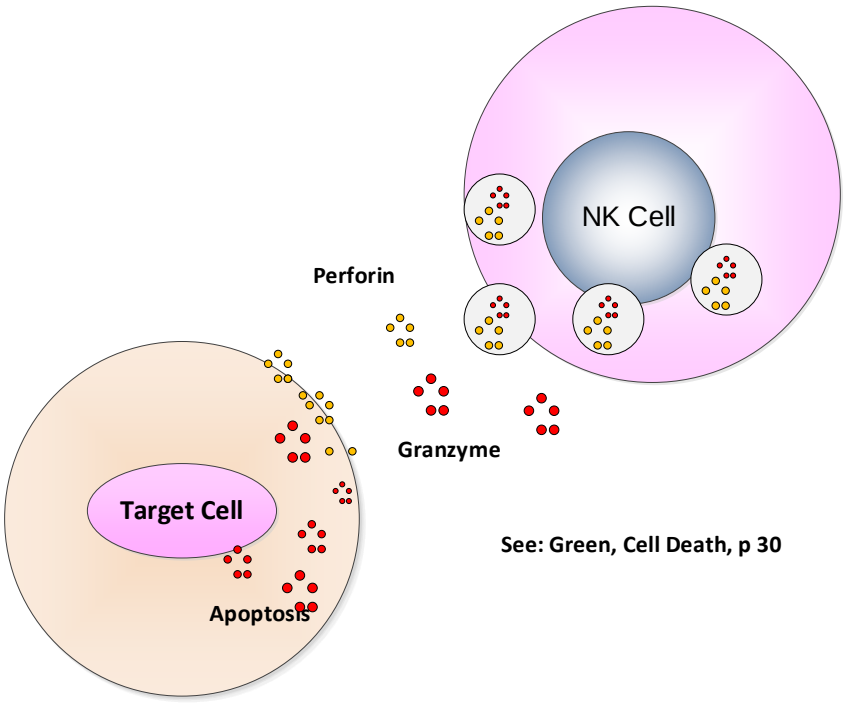
(b) Step 2: microtubule organizing center (MTOC) and secretory lysosome polarize toward the immunological synapse.

(C) Step 3: docking which involves moves close to the plasma membrane of NK cell at the synapse.

(D) Step 4: secretory lysosome fuse with the target cell plasma membrane and releases the cytotoxic granules into the target cell.



The result of this activation of the attached NK cell is the release of granzymes which will break down the target cell and perforins which allow for the granzymes to enter the cell. We demonstrate this below.



3.4 NK CELLS AND COVID-19

NK cells are often attacking viral infected targets. Thus one would suspect that we would see a proliferation of NK cells in a COVID environment. However several authors have noticed a somewhat counterintuitive result.

Jiang et al have noted:

The morbidity and mortality of infectious diseases can be caused by direct damage to the host by the pathogen or collateral damage to the host tissue by the excessive immune response to the pathogen. After infected with COVID-19, whether for clinical or basic research, the patients' immune status both need to be clarified urgently. The guideline of new edition for COVID-19 as mentioned above suggests that the progressive decline of peripheral lymphocytes is one of the clinical early warning indicators for severe and critical cases in adults, and many studies have also reported lymphopenia, mainly for the reduction of CD8+ T cells, in COVID-19 patients.

In an autopsy report about one died case with COVID-19, Fu-Sheng Wang and his colleague found that ***the count of peripheral CD8+ T cells was substantially reduced, while cell immune status was hyperactivated; meanwhile, CD8+ T cells were found to harbor high concentrations of cytotoxic granules including perforin and granzyme****.*

It is well known that as primary cytotoxic lymphocytes, CD8+ T and NK cells play a vital role in the control of pathogen infection by mediating cellular immunity and cytotoxic function. However, the immune status of CD8+ T and NK cells in COVID-19 patients with different severity is still largely unclear…

We then detected the cytotoxic potential of NK cells, and found an increase of GrA and perforin expression on NK cells but a decrease of GrB expression in COVID-19 patients. Besides, we found that GrA expression on NK cells was significantly increased in the Mild and Severe groups, however, as the disease progressed, GrA expression on NK cells was significantly decreased in the Critical group, which might due to a compensatory enhancement in the mild and severe stages, but when the disease reached a critical stage, the cell immune status was significantly suppressed and couldn't be compensated.

To some extent, this could be confirmed by the fact that the absolute number of NK cells in the Critical group decreased significantly compared to the other three groups.

Perforin is a key effector molecule for T and NK cells to mediate cytolysis. *It could lyse cells by coordinating the* ***delivery of GrA and GrB (granzyme) to target cells****. The perforin/ granzyme cytolytic pathway is a two-edged sword in viral defense, which could result in viral clearance and host cell damage. Therefore, we speculate that as the COVID- 19 progression, the over-activated immune status of cytotoxic CD8+ T and NK cells might mediate pathological damage and lead to continuous deterioration of patients.*

Namely in COVID patients there was a significant drop in NK cells and a dramatically lower number in severely infected patients. The reasons seem to be unknown. As Maucourant et al have similarly noted:

NK cells are innate effector lymphocytes that are typically divided into cytokine-producing CD56bright NK cells and cytotoxic CD56dim NK cells. Evidence for a direct role of NK cells in protection against viral infections comes from patients with selective NK cell deficiencies, as these develop fulminant viral infections, predominantly with herpes viruses. *Human NK cells have also been shown to rapidly respond during the acute phase of infections in humans with hantavirus, tick-borne encephalitis virus, influenza A virus (IAV), and dengue virus, as well as after vaccination with live-attenuated yellow fever.*

NK cells not only have the capacity to directly target and kill infected cells but also can influence adaptive T cell responses. *The degree of NK cell activation can function as a rheostat in regulating T cells, where a certain level of NK cell activation might promote infection control, while another degree of activation is related to immunopathology. Compared with peripheral blood, the human lung is enriched in NK cells. Human lung NK cells display a differentiated phenotype and also have the capacity to respond to viral infections such as IAV.* ***In COVID-19,***

emerging studies have reported low peripheral blood NK cell numbers in patients with moderate and severe disease.

Two recent reports assessing the single cell landscape of immune cells in bronchoalveolar lavage (BAL) fluid of COVID-19 patients have suggested that NK cell numbers are increased at this site of infection. However, a detailed map of the NK cell landscape in clinical SARS-CoV-2 infection has not yet been established. Given the important role of NK cells in acute viral infections, their relatively high presence within lung tissue, as well as the links between NK cell activation, T cell responses, and development of immunopathology, we here performed a detailed assessment of NK ells in patients with moderate and severe COVID-19 disease.

Using strict inclusion and exclusion criteria, patients with moderate and severe COVID-19 disease were recruited early during their disease, sampled for peripheral blood, and analyzed by 28-color flow cytometry to assess NK cell activation, education, and presence of adaptive NK cells. The results obtained are discussed in relation to the role of NK cell responses in acute SARS-CoV-2 infection and associated COVID-19 disease immunopathogenesis….

NK cells are known to rapidly respond during diverse acute viral infections in humans including those caused by dengue virus, hantavirus, tick-borne encephalitis virus, and yellow fever virus. Although a similarly detailed analysis of NK cells has not been performed in acute SARS-CoV-2 infection causing COVID-19, early reports from the pandemic have indicated low circulating NK cell numbers in patients with moderate and severe disease. Those reports are in line with what is reported here. Transiently reduced NK cell numbers during the acute phase of infections have also been shown in SARS-CoV-1 infection and in acute hantavirus infection.

The magnitude of the present detected NK cell response, with a quarter of the circulating cells either displaying signs of proliferation or activation, mirrors the responses observed during acute dengue fever and in acute hantavirus infection*. The present in-depth phenotypic assessment of NK cells in moderate and severe COVID-19 patients revealed an activated phenotype with up-regulated levels of effector molecules and chemokines, activating receptors, and nutrient receptors. We also observed signs of inhibitory immune checkpoint receptor up-regulation through increased levels of TIGIT and Tim-3. Hence, the present results confirm and extend, at protein level, what was recently reported for peripheral blood NK cells using scRNA-seq.*

Human NK cells are enriched in the lung compared with peripheral blood. COVID-19 patients display significant immune activation in the respiratory tract, where moderate disease is associated with a high number of T cells and NK cells, whereas neutrophils and inflammatory monocytes are enriched at these sites in patients with severe disease. *By analyzing publicly available scRNAseq data on BAL NK cells from COVID-19 patients), we found these cells to be strongly activated.*

The interferon response appeared stronger in BAL NK cells from moderate COVID-19 patients. his is in line with severe COVID-19 associating with a blunted interferon response. We further confirmed that a similarly activated phenotype of NK cells was present in BAL fluid, as was observed in peripheral blood, including high expression of effector molecules and chemokines.

NK cell homing to the site of infection is important for pathogen clearance in murine models, where several chemokine receptors have been shown to mediate NK cell tissue homing. The reduced numbers of NK cells that we observed in circulation might, to some extent, reflect a redistribution to the lung.

Chemotaxis was another gene ontology module that, together with cytotoxicity, was enriched in BAL NK cells of severe COVID-19 patients. In addition, BAL fluid from COVID-19 patients contains elevated levels of chemokines that potentially could attract NK cells, including CCL3, CCL3L1, CCL4, CXCL9, CXCL10, and CXCL11…. Overall, we observed a robust NK cell response toward SARS-CoV-2 infection and specific features unique to severe COVID-19 and hyperinflammation, providing a base for understanding the role of NK cells in patients with COVID-19.

3.5 NK Cells and Inflammation

Inflammation is a process whereby cells react to rid the invaders. The result is that any of these cells are placed under significant stress and may be terminally damaged. As Jiang and Chess note:

Natural killer T cells are a distinctive population of T cells. They have properties of natural killer cells but express α/β T-cell receptors that consist of an invariant alpha chain (Vα24–JαQ) paired preferentially to various Vβ chains. These cells specifically recognize glycolipids related to the glycolipid α-galactosylceramide that often occurs in pathogenic microorganisms and tumor cells. The α-galactosylceramide binds to CD1d, an MHC molecule on antigen-presenting cells. The CD1d– glycolipid complex triggers natural killer T cells to lyse targets and secrete cytokines.

Mammalian antigens such as isoglobotrihexosylceramide and bacterial glycosphingolipid antigens are structurally related to α-galactosylceramide and can stimulate natural killer T cells.

Natural killer T cells were originally thought to mediate the innate immune responses that lyse tumor cells and pathogens, but they also are involved in autoimmune diseases.

When stimulated by contact with antigen, natural killer T cells develop heightened killer-cell activity and secrete large amounts of interleukin-4, interferon-γ, TGF-β, and interleukin-10, all known to be involved in the activation of cells that mediate inflammation, innate immunity, and Th2-type immunity. Natural killer T cells influence a variety of autoimmune diseases in animal models. Prominent among these are murine models of insulin dependent diabetes and multiple sclerosis, which involve primarily Th1 cells. In these diseases the secretion by natural killer T cells of the Th2-favoring cytokines interleukin-4 and interleukin-10 is probably an important inhibitory mechanism.

In the nonobese diabetic mouse, injection of cell populations enriched for natural killer T cells prevents type 1 diabetes, whereas depletion of natural killer T cells early in the evolution of diabetes accelerates the onset of diabetes. In murine models of inflammatory bowel disease and multiple sclerosis, depletion of natural killer T cells accelerates the onset of disease, while

activation of natural killer T cells by treatment with α-galactosylceramide ameliorates or prevents disease. These effects are abrogated in mice that are deficient in CD1d.

Natural killer T cells have also been implicated in human autoimmune diseases. In monozygotic twins that are discordant for type 1 diabetes, the twin with diabetes has fewer Vα24–JαQ natural killer T cells than the twin without diabetes,61 suggesting protection against the disease by natural killer T cells.

However, studies that compared patients who had diabetes with healthy controls, including discordant twins, found that the numbers of natural killer T cells and the production of interleukin-4 are unaltered during the course of type 1 diabetes. These results do not necessarily refute the hypothesis that natural killer T cells defend against type 1 diabetes — they may indicate that development of the autoimmune disease is controlled by several subgroups of immunoregulatory cells acting in concert.

From Abbas et al:

The principal mechanisms of innate immunity against viruses are inhibition of infection by type I interferons and NK cell–mediated killing of infected cells. Infection by many viruses is associated with production of type I interferons (IFNs) by infected cells, and by dendritic cells, especially of the plasmacytoid type, responding to viral products (see Chapter 4). Several biochemical pathways trigger IFN production. These include recognition of viral RNA and DNA by endosomal TLRs and activation of cytoplasmic RIG-like receptors and the STING pathway by viral RNA and DNA, respectively. These pathways converge on the activation of protein kinases, which in turn activate the IRF transcription factors that stimulate IFN gene transcription. Type I IFNs function to inhibit viral replication in both infected and uninfected cells.

NK cells kill virus-infected cells and are an important mechanism of immunity against viruses early in the course of infection, before adaptive immune responses have developed. Class I MHC expression is often shut off in virus-infected cells as an escape mechanism from CTLs. This enables NK cells to kill the infected cells because the absence of class I releases NK cells from a normal state of inhibition. Viral infection may also stimulate expression of activating NK cell ligands on the infected cells.

They continue:

NK cells, often considered the first known ILC, are cytotoxic cells that play important roles in innate immune responses, mainly against viruses and intracellular bacteria. The "natural killer" designation derives from the fact that their major function is killing infected cells, similar to the adaptive immune system's killer cells, the cytotoxic T lymphocytes (CTLs), and they are ready to do so once they develop, without further differentiation (hence natural). NK cells also secrete IFN-γ and are sometimes referred to as a type of ILC1. Unlike the cytokine-producing ILCs discussed previously, which are found in peripheral tissues but are rare in the blood and lymphoid organs, NK cells constitute 5% to 15% of the mononuclear cells in the blood and spleen.

They are rare in other lymphoid organs but are more abundant in certain organs such as the liver and placenta. NK cells in the blood appear as large lymphocytes with numerous cytoplasmic granules. NK cells do not express diverse, clonally distributed antigen receptors typical of B and T cells. Rather, they use germline DNA-encoded receptors (discussed later) to distinguish pathogen-infected cells from healthy cells. They can be identified in the blood by expression of CD56 and the absence of the T cell marker CD3. Most human blood NK cells also express CD16, which is involved in recognition of antibody-coated cells…

The effector functions of NK cells are to kill infected cells and to produce IFN-γ, which activates macrophages to destroy phagocytosed microbes. The mechanism of NK cell–mediated cytotoxicity is essentially the same as that of CD8+ CTLs, which we will describe in detail in Chapter 11. NK cells, like CTLs, have granules that contain proteins that mediate killing of target cells. When NK cells are activated, granule exocytosis releases these proteins adjacent to the target cells. One NK cell granule protein, called perforin, facilitates the entry of other granule proteins, called granzymes, into the cytosol of target cells. The granzymes are proteolytic enzymes that initiate a sequence of signaling events that cause death of the target cells by apoptosis.

By killing cells infected by viruses and intracellular bacteria, NK cells eliminate reservoirs of infection. Early in the course of a viral infection, NK cells are expanded and activated by recognition of activating ligands on the infected cells and by the cytokines IL-12 and IL-15, and they kill infected cells before antigen-specific CTLs can become fully active, which will usually take 5 to 7 days. NK cells may also be important later in the course of viral infection by killing infected cells that have escaped CTL-mediated immune attack by reducing expression of class I major histocompatibility complex (MHC) molecules. Some tumors, especially those of hematopoietic origin, are targets of NK cells, perhaps because the tumor cells do not express normal levels or types of class I MHC molecules, which inhibit NK cell activation, discussed later.

Natural killer (NK) cells are a class of lymphocytes that recognize infected and stressed cells and respond by killing these cells and by secreting the macrophage-activating cytokine IFN-γ. ***NK cells make up approximately 10% of the lymphocytes in the blood and peripheral lymphoid organs. NK cells contain abundant cytoplasmic granules and express some unique surface proteins, but do not express immunoglobulins or T cell receptors, the antigen receptors of B and T lymphocytes, respectively.***

On activation by infected cells, NK cells empty the contents of their cytoplasmic granules into the extracellular space at the point of contact with the infected cell. The granule proteins then enter infected cells and activate enzymes that induce apoptosis. The cytotoxic mechanisms of NK cells, which are the same as the mechanisms used by cytotoxic T lymphocytes, result in the death of infected cells. ***Thus, as with CTLs, NK cells function to eliminate cellular reservoirs of infection and eradicate infections by obligate intracellular microbes, such as viruses.***

Activated NK cells also synthesize and secrete the cytokine interferon-γ. IFN-γ activates macrophages to become more effective at killing phagocytosed microbes. Cytokines secreted by macrophages and dendritic cells that have encountered microbes enhance the ability of NK cells

to protect against infections. Three of these NK cell–activating cytokines are interleukin-15 (IL-15), type I interferons (type I IFNs), and interleukin-12 (IL-12). IL-15 is important for the development and maturation of NK cells, and type I IFNs and IL-12 enhance the killing functions of NK cells. Thus, NK cells and macrophages are examples of two cell types that function cooperatively to eliminate intracellular microbes: Macrophages ingest microbes and produce IL-12, IL-12 activates NK cells to secrete IFN-γ, and IFN-γ in turn activates the macrophages to kill the ingested microbes.

The activation of NK cells is determined by a balance between engagement of activating and inhibitory receptors. The activating receptors recognize cell surface molecules typically expressed on cells infected with viruses and intracellular bacteria, as well as cells stressed by DNA damage and malignant transformation. Thus, NK cells eliminate cells infected with intracellular microbes as well as irreparably injured cells and tumor cells. One of the well-defined activating receptors of NK cells is called NKG2D; it recognizes molecules that resemble class I major histocompatibility complex (MHC) proteins and is expressed in response to many types of cellular stress. Another activating receptor, called CD16, is specific for immunoglobulin G (IgG) antibodies bound to cells. The recognition of antibody-coated cells results in killing of these cells, a phenomenon called antibody-dependent cellular cytotoxicity (ADCC). NK cells are the principal mediators of ADCC.

Activating receptors on NK cells have signaling subunits that contain ***immunoreceptor tyrosine-based activation motifs. (ITAMs) in their cytoplasmic tails****. ITAMs, which also are present in subunits of lymphocyte antigen receptor–associated signaling molecules, become phosphorylated on tyrosine residues when the receptors recognize their activating ligands. The phosphorylated ITAMs bind and promote the activation of cytoplasmic protein tyrosine kinases, and these enzymes phosphorylate, and thereby activate, other substrates in several different downstream signal transduction pathways, eventually leading to cytotoxic granule exocytosis and production of IFN-γ….*

Signals generated by engagement of TLRs activate transcription factors that stimulate expression of genes encoding cytokines, enzymes, and other proteins involved in the antimicrobial functions of activated phagocytes and other cells. Among the most important transcription factors activated by TLR signals are nuclear factor κB (NF-κB), which promotes expression of various cytokines and endothelial adhesion molecules, and interferon regulatory factors (IRFs), which stimulate production of the antiviral cytokines, type I interferons.

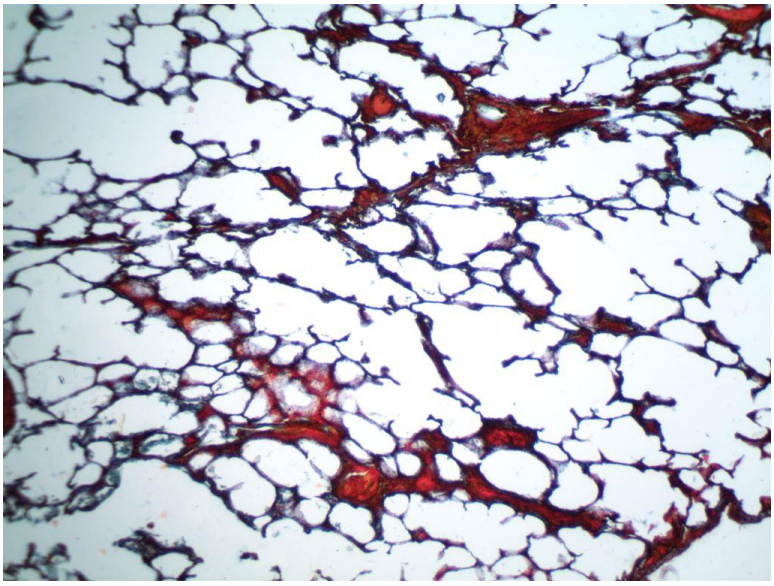
4 COVID-19

COVID-19 is a corona virus sourced from Wuhan, China. The actual transfer vector is unknown. It has been hypothesized as a bat or some other mammal but it could equally have been a result of studies in the Chinese Government Virus Studies center in that city. As we shall note, the Center has been actively modifying these class of viruses as evidenced by publications emanating therefrom. At this point, lacking an open environment for information gathering, all one can do is speculate. In this section we examine at a high level what is known regarding this virus.

4.1 LUNG PATHOLOGY

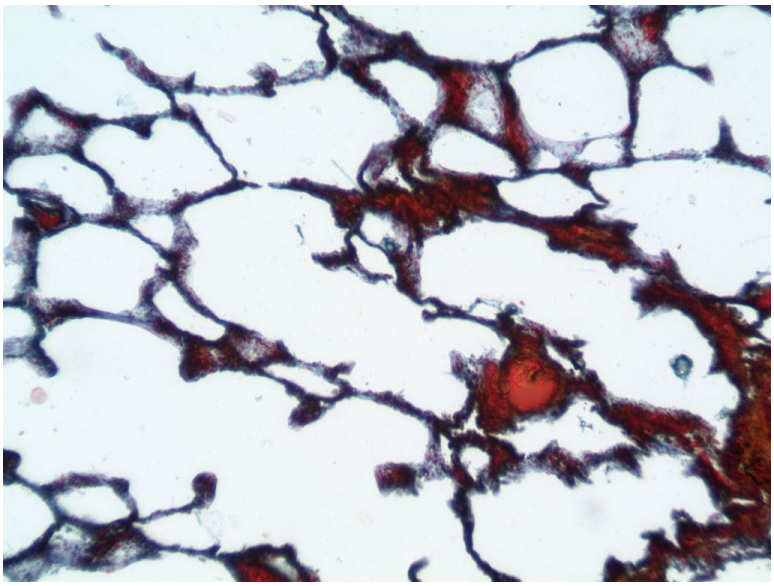
We begin with a brief discussion of the lung and its histology in a normal and inflamed state. This is useful since it clearly indicates what the virus does, namely reduce lung surface area for CO2 elimination and O2 intake. The lung is predominantly an organ filled with air and infiltrated with veins and arteries. It transfers CO2 outward and collects O2 inwards. A normal operating lung has massive amounts of transfer area for this process to occur.

First, below we show a normal lung tissue at low magnification[6]. There are almost all open spaces. This provides the maximum amount of transfer area for the loss of CO2 and gain of O2.

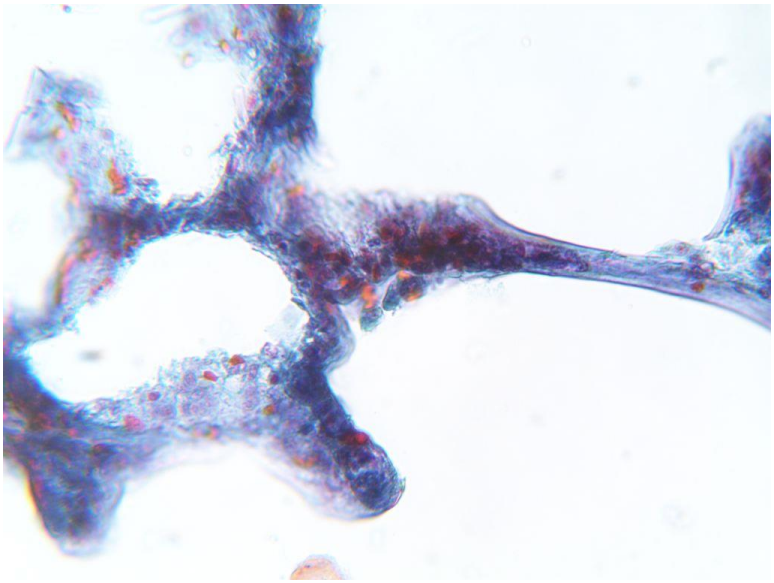


Upon higher magnification we obtain:

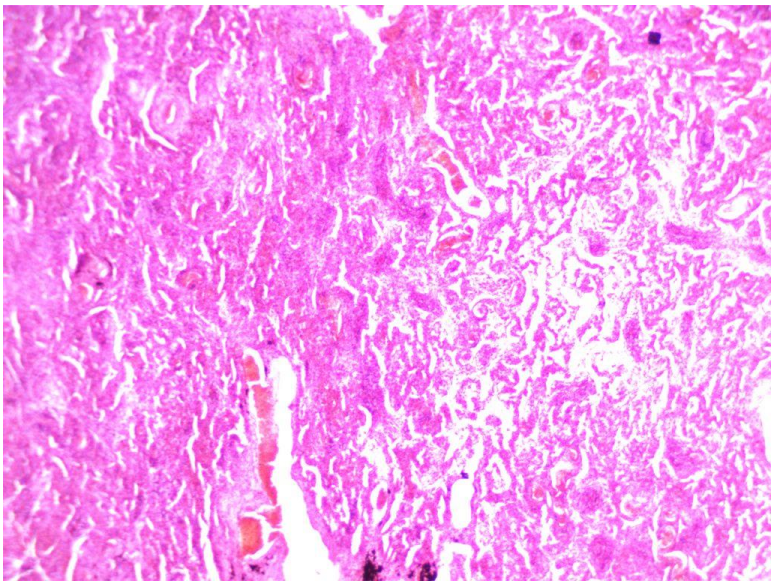
[6] These slides are from the author's collection based upon anonymized patient records.



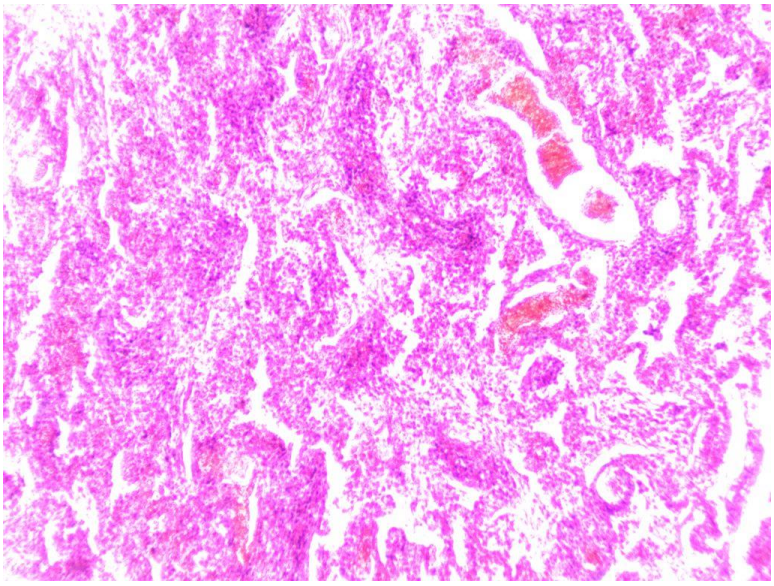
Finally, with the highest magnification we can obtain:



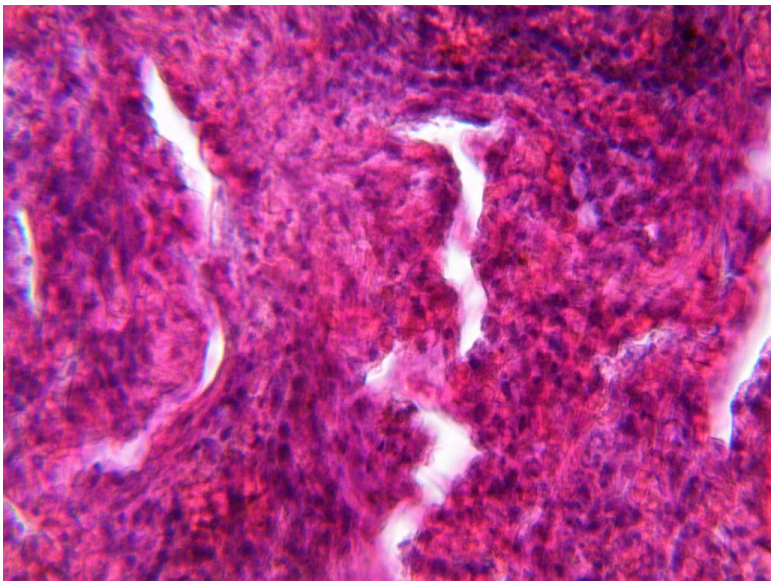
The above clearly shows a clear and healthy alveoli. That is what we would expect in a health lung. Now consider an inflamed lung with influenza as below:



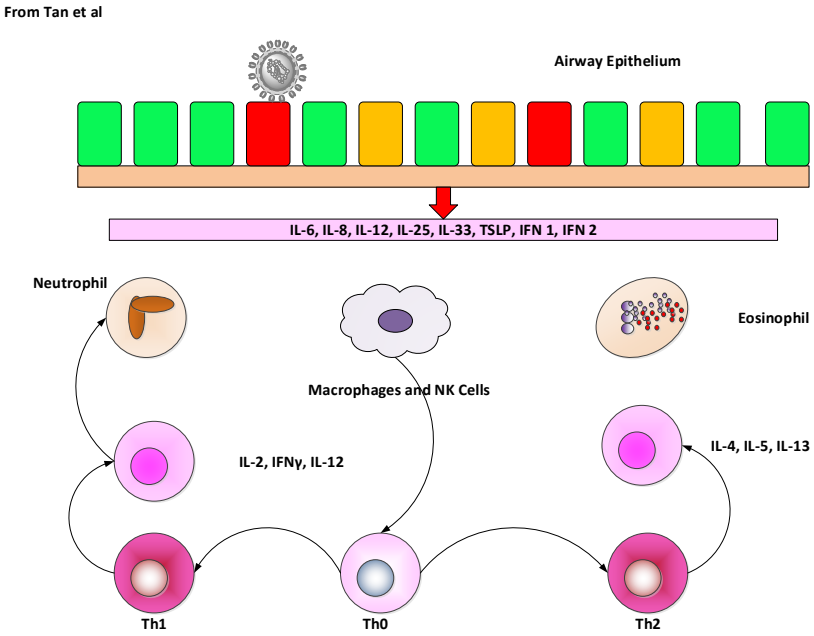
Note the massive loss of open alveoli. The surface area has almost been lost with the inclusion of inflamed cells. Closer examination is shown below.



Finally at highest magnification of the inflamed lung we obtain below:



This as seen above is what we must avoid in the viral infections we discuss herein. The mechanism for this can be seen in Tan et al:



where they note:

Current understanding of viral induced exacerbation of chronic airway inflammatory diseases. Upon virus infection in the airway, antiviral state will be activated to clear the invading pathogen from the airway. Immune response and injury factors released from the infected epithelium normally would induce a rapid type 1 immunity that facilitates viral clearance. However, in the inflamed airway, the cytokines and chemokines released instead augmented the inflammation present in the chronically inflamed airway, strengthening the neutrophilic infiltration in COPD airway, and eosinophilic infiltration in the asthmatic airway. The effect is

also further compounded by the participation of Th1 and ILC1 cells in the COPD airway; and Th2 and ILC2 cells in the asthmatic airway

They then continue:

*On the other end of the spectrum, viruses that induce strong type 1 inflammation and cell death such as IFV and certain CoV (**including the recently emerged COVID-19 virus**), may not cause prolonged inflammation due to strong induction of antiviral clearance. These infections, however, cause massive damage and cell death to the epithelial barrier, so much so that areas of the epithelium may be completely absent post infection. Factors such as RANTES and CXCL10, which recruit immune cells to induce apoptosis, are strongly induced from IFV infected epithelium.*

Additionally, necroptotic factors such as RIP3 further compounds the cell deaths in IFV infected epithelium. The massive cell death induced may result in worsening of the acute exacerbation due to the release of their cellular content into the airway, further evoking an inflammatory response in the airway. Moreover, the destruction of the epithelial barrier may cause further contact with other pathogens and allergens in the airway which may then prolong exacerbations or results in new exacerbations.

***Epithelial destruction may also promote further epithelial remodeling during its regeneration as viral infection induces the expression of remodeling genes such as MMPs and growth factors. Infections that cause massive destruction of the epithelium, such as IFV, usually result in severe acute exacerbations with non-classical symptoms of chronic airway inflammatory diseases.** Fortunately, annual vaccines are available to prevent IFV infections; and it is recommended that patients with chronic airway inflammatory disease receive their annual influenza vaccination as the best means to prevent severe IFV induced exacerbation.*

As Wu et al noted:

As reported by Huang et al,3 patients with COVID-19 present primarily with fever, myalgia or fatigue, and dry cough. Although most patients are thought to have a favorable prognosis, older patients and those with chronic underlying conditions may have worse outcomes. Patients with severe illness may develop dyspnea and hypoxemia within 1 week after onset of the disease, which may quickly progress to acute respiratory distress syndrome (ARDS) or end-organ failure.4 Certain epidemiological features and clinical characteristics of COVID-19 have been previously reported.

However, these studies were based on relatively small sample sizes, and risk factors leading to poor clinical outcomes have not been well delineated. In this study, we report the clinical characteristics and factors associated with developing ARDS after hospital admission and progression from ARDS to death in patients with COVID-19 pneumonia from a single hospital in Wuhan, China …

In this cohort study, we reported the clinical characteristics and risk factors associated with clinical outcomes in patients with COVID-19 pneumonia who developed ARDS after admission,

as well as those who progressed from ARDS to death. Patients who received methylprednisolone treatment were much more likely to develop ARDS likely owing to confounding by indication; specifically, sicker patients were more likely to be given methylprednisolone. However, administration of methylprednisolone appeared to reduce the risk of death in patients with ARDS.

These findings suggest that for patients with COVID-19 pneumonia, methylprednisolone treatment may be beneficial for those who have developed ARDS on disease progression. However, these results should be interpreted with caution owing to potential bias and residual confounding in this observational study with a small sample size. Doubleblinded randomized clinical trials should be conducted to validate these results.

Guan et al had depicted the course of the disease in February in NEJM as follows:

During the initial phase of the Covid-19 outbreak, the diagnosis of the disease was complicated by the diversity in symptoms and imaging findings and in the severity of disease at the time of presentation.

Fever was identified in 43.8% of the patients on presentation but developed in 88.7% after hospitalization. Severe illness occurred in 15.7% of the patients after admission to a hospital. No radiologic abnormalities were noted on initial presentation in 2.9% of the patients with severe disease and in 17.9% of those with nonsevere disease.

Despite the number of deaths associated with Covid-19, ***SARS-CoV-2 appears to have a lower case fatality rate than either SARS-CoV or Middle East respiratory syndrome–related coronavirus (MERS-CoV)****. Compromised respiratory status on admission (the primary driver of disease severity) was associated with worse outcomes.*

Approximately 2% of the patients had a history of direct contact with wildlife, whereas more than three quarters were either residents of Wuhan, had visited the city, or had contact with city residents. These findings echo the latest reports, including the outbreak of a family cluster, transmission from an asymptomatic patient, and the three-phase outbreak patterns. Our study cannot preclude the presence of patients who have been termed "super-spreaders."

Conventional routes of transmission of SARSCoV, MERS-CoV, and highly pathogenic influenza consist of respiratory droplets and direct contact, mechanisms that probably occur with SARS-CoV-2 as well. Because SARS-CoV-2 can be detected in the gastrointestinal tract, saliva, and urine, these routes of potential transmission need to be investigated. The term Covid-19 has been applied to patients who have laboratory-confirmed symptomatic cases without apparent radiologic manifestations.

A better understanding of the spectrum of the disease is needed, since in 8.9% of the patients, SARS-CoV-2 infection was detected before the development of viral pneumonia or viral pneumonia did not develop. In concert with recent studies,1,8,12 we found that the clinical characteristics of Covid-19 mimic those of SARS-CoV.

Fever and cough were the dominant symptoms and gastrointestinal symptoms were uncommon, which suggests a difference in viral tropism as compared with SARS-CoV, MERS-CoV, and seasonal influenza.

***The absence of fever in Covid-19 is more frequent than in SARS-CoV (1%) and MERS-CoV infection (2%)**, **so afebrile patients may be missed if the surveillance case definition focuses on fever detection**. Lymphocytopenia was common and, in some cases, severe, a finding that was consistent with the results of two recent reports.*

We found a lower case fatality rate (1.4%) than the rate that was recently reportedly, probably because of the difference in sample sizes and case inclusion criteria. Our findings were more similar to the national official statistics, which showed a rate of death of 3.2% among 51,857 cases of Covid-19 as of February 16, 2020.

Since patients who were mildly ill and who did not seek medical attention were not included in our study, the case fatality rate in a real-world scenario might be even lower. Early isolation, early diagnosis, and early management might have collectively contributed to the reduction in mortality in Guangdong.

4.2 PRIOR CORONA VIRUSES

As Wang et al have noted:

Six coronaviruses are known to infect humans and cause respiratory disease, including human coronavirus (HCoV) 229E, OC43, severe acute respiratory syndrome CoV (SARS-CoV), NL63, HKU1 and Middle East respiratory syndrome CoV (MERS-CoV).

SARS-CoV and MERS-CoV are highly pathogenic coronaviruses that caused severe and fatal respiratory infections in humans. The SARSCoV pandemic infected over 8000 people worldwide. As of 9 September 2019, 2458 MERS cases with 848 deaths (34.5% mortality) were reported to World Health Organization (WHO). HCoV-229E, OC43, NL63 and HKU1 are endemic in humans and mainly cause mild respiratory infections worldwide [8,9]. HCoV-NL63 has been prevalent worldwide for many years. The majority of HCoV-NL63 infections in human are mild, although occasionally NL63 causes pneumonia or central nervous system diseases in susceptible individuals including young children, elderly and immunosuppressed patients. HCoVNL63 primarily infects upper respiratory tract and most of HCoV-NL63 infections are acquired during childhood. Neutralizing activity directed against HCoV-NL63 is common in sera from adults and rarely in infant's serum.

During 2009 and 2016, HCoVNL63 accounted for about 0.5% (60/11399) of all acute respiratory tract infections in hospitalized pediatric patients in Guangzhou, China, most of these cases associated with HCoV-NL63 were considered to be evidence of endemic infection and no outbreaks were reported. Here, we identified a cluster of 23 hospitalized pediatric patients with severe lower respiratory tract infection caused by two subgenotypes (C3 and B) of HCoVNL63, and half of the patients were caused by a new subgenotype C3 which was first reported here. A unique mutation (I507 L) in receptor-binding domain (RBD) was detected in the new

subgenotype of HCoV-NL63 associated with increased viral entry into host cells indicating that HCoV-NL63 was undergoing continuous mutation which potentially could enhance HCoV-NL63 virulence and promote transmission.

This study showed that HCoV-NL63 had the potential to cause epidemics in humans and it may be a more important human pathogen than is commonly believed. Efforts should be paid to monitor genetic changes in HCoV-NL63 genome and also its pathogenicity and prevalence in the human population

4.3 A WUHAN VIRUS CONSTRUCT

In July 2016 the Key Laboratory of Special Pathogens, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China published a paper discussing the work they did in modifying a SARS like corona virus. The authors state:

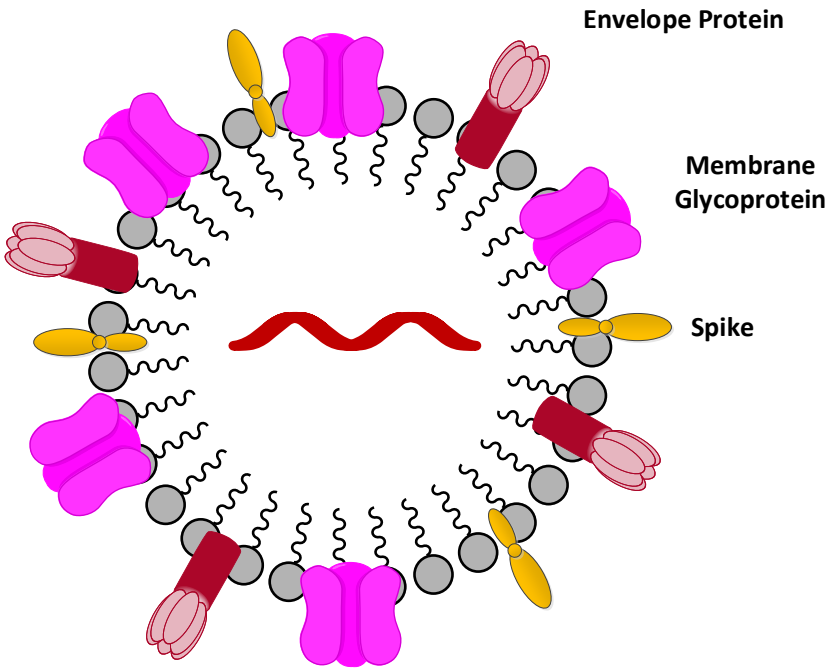
Bats harbor severe acute respiratory syndrome (SARS)-like coronaviruses (SL-CoVs) from which the causative agent of the 2002-2003 SARS pandemic is thought to have originated. However, despite the fact that a large number of genetically diverse SL-CoV sequences have been detected in bats, only two strains (named WIV1 and WIV16) have been successfully cultured in vitro. These two strains differ from SARS-CoV only in containing an extra open reading frame (ORF) (named ORFX), between ORF6 and ORF7, which has no homology to any known protein sequences.

In this study, ***we constructed a full-length cDNA clone of SL-CoV WIV1 (rWIV1), an ORFX deletion mutant (rWIV1-X),*** *and a green fluorescent protein (GFP)-expressing mutant (rWIV1-GFP-X).*

Northern blotting and fluorescence microscopy indicate that ORFX was expressed during WIV1 infection. A virus infection assay showed that rWIV1-X replicated as efficiently as rWIV1 in Vero E6, Calu-3, and HeLa-hACE2 cells.

Further study showed that ORFX could inhibit interferon production and activate NF- B. Our results demonstrate for the first time that the unique ORFX in the WIV1 strain is a functional gene involving modulation of the host immune response but is not essential for in vitro viral replication.

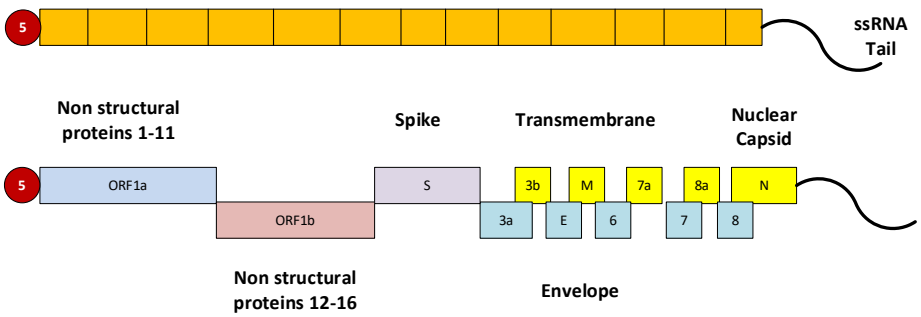
One should read through this carefully and cautiously. The mapping of the bases of this virus map well onto what is currently spreading worldwide. There is no claim other than that of coincidence. The paper is worth the read. I want to thank colleagues in California for the reference.



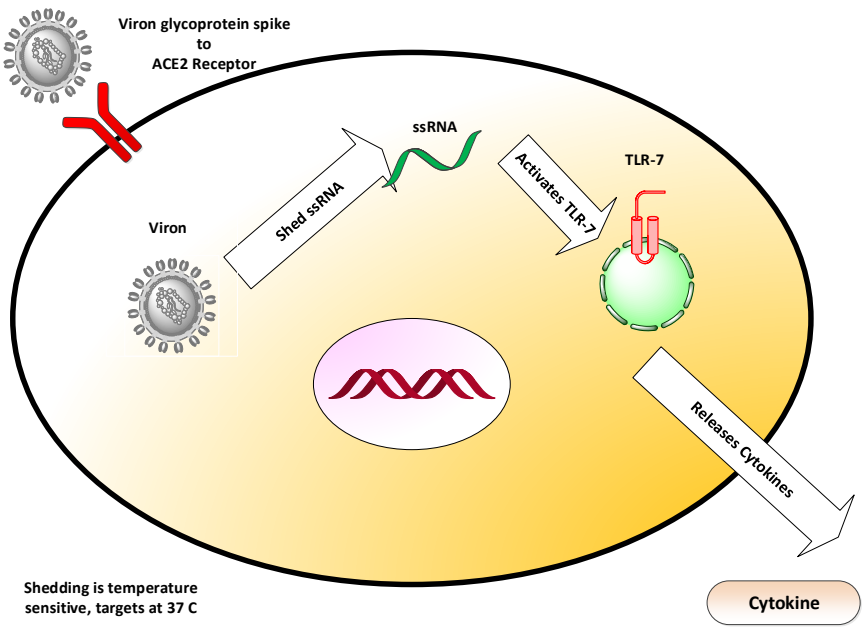
4.4 COVID-19 SPECIFICS

Now we can consider some COVID-19 specifics. The Figure below shows the process of viral infection on a cell in the lung. The cell has a receptor, ACE2, which facilitates the binding of the virion. Then it enters the cell shedding its coat, setting loose the RNA in the virion. This RNA then gets processed and replicated, then rebound in a shell and sent outwards. At the same time the TLR-7 in an endosome releases a mass of cytokines which in a sense do much of the damage we have shown previously.

The RNA is 29,903 nucleotides (see Wu et al). The specifics of this is from Wu et al as shown below:



In the above Wu et al refer to COVID-19 as WHCV.



We can now discuss further details of this process. It is important to note the importance of ACE2. As NCBI notes[7]:

The protein encoded by this gene belongs to the angiotensin-converting enzyme family of dipeptidyl carboxydipeptidases and has considerable homology to human angiotensin 1 converting enzyme. This secreted protein catalyzes the cleavage of angiotensin I into angiotensin 1-9, and angiotensin II into the vasodilator angiotensin 1-7. The organ- and cell-specific expression of this gene suggests that it may play a role in the regulation of cardiovascular and renal function, as well as fertility. In addition, the encoded protein is a functional receptor for the spike glycoprotein of the human coronavirus HCoV-NL63 and the human severe acute respiratory syndrome coronaviruses, SARS-CoV and SARS-CoV-2 (COVID-19 virus).

This may be a putative target for a therapeutic.

4.5 Entry and Activation

The entry of the COVID virus has been demonstrated by Sungnak et al as follows:

The SARS-CoV-2 coronavirus, the etiologic agent responsible for COVID-19 coronavirus disease, is a global threat. To better understand viral tropism, we assessed the RNA expression of the coronavirus receptor, ACE2, as well as the viral S protein primingprotease TMPRSS2 thought to govern viral entry in single-cell RNA-sequencing (scRNAseq) datasets from healthy individuals generated by the Human Cell Atlas consortium. We found that ACE2, as well as the protease TMPRSS2, are differentially expressed in respiratory and gut epithelial cells. In-depth

[7] https://www.ncbi.nlm.nih.gov/gene/59272

analysis of epithelial cells in the respiratory tree reveals that nasal epithelial cells, specifically goblet/secretory cells and ciliated cells, display the highest ACE2 expression of all the epithelial cells analyzed. The skewed expression of viral receptors/entry-associated proteins towards the upper airway may be correlated with enhanced transmissivity.

Finally, we showed that many of the top genes associated with ACE2 airway epithelial expression are innate immune-associated, antiviral genes, highly enriched in the nasal epithelial cells. *This association with immune pathways might have clinical implications for the course of infection and viral pathology, and highlights the specific significance of nasal epithelia in viral infection. Our findings underscore the importance of the availability of the Human Cell Atlas as a reference dataset. In this instance, analysis of the compendium of data points to a particularly relevant role for nasal goblet and ciliated cells as early viral targets and potential reservoirs of SARS-CoV-2 infection. This, in turn, serves as a biological framework for dissecting viral transmission and developing clinical strategies for prevention and therapy*

Now Holbrook et al have noted the temporal viability of the virus as follows:

We evaluated the stability of SARS-CoV-2 and SARS-CoV-1 in aerosols and on various surfaces and estimated their decay rates using a Bayesian regression model. SARS-CoV-2 nCoV-WA1-2020 (MN985325.1) and SARS-CoV-1 Tor2 (AY274119.3) were the strains used. Aerosols (<5 μm) containing SARS-CoV-2 (105.25 50% tissue-culture infectious dose [TCID 50] per milliliter) or SARS-CoV-1 (106.75-7.00 TCID 50 per milliliter) were generated with the use of a three-jet Collison nebulizer and fed into a Goldberg drum to create an aerosolized environment.

The inoculum resulted in cycle-threshold values between 20 and 22, similar to those observed in samples obtained from the upper and lower respiratory tract in humans. Our data consisted of 10 experimental conditions involving two viruses (SARS-CoV-2 and SARS-CoV-1) in five environmental conditions (aerosols, plastic, stainless steel, copper, and cardboard). All experimental measurements are reported as means across three replicates. SARS-CoV-2 remained viable in aerosols throughout the duration of our experiment (3 hours), with a reduction in infectious titer from 103.5 to 102.7 TCID 50 per liter of air.

This reduction was similar to that observed with SARS-CoV-1, from 104.3 to 103.5 TCID 50 per milliliter.

SARS-CoV-2 was more stable on plastic and stainless steel than on copper and cardboard, and viable virus was detected up to 72 hours after application to these surfaces, although the virus titer was greatly reduced … The stability kinetics of SARS-CoV-1 were similar.

On copper, no viable SARS-CoV-2 was measured after 4 hours and no viable SARS-CoV-1 was measured after 8 hours.

On cardboard, no viable SARS-CoV-2 was measured after 24 hours and no viable SARSCoV-1 was measured after 8 hours (Fig. 1A). Both viruses had an exponential decay in virus titer across all experimental conditions, as indicated by a linear decrease in the log10TCID50 per liter of air or milliliter of medium over time.

The half-lives of SARS-CoV-2 and SARS-CoV-1 were similar in aerosols, with median estimates of approximately 1.1 to 1.2 hours and 95% credible intervals of 0.64 to 2.64 for SARS-CoV-2 and 0.78 to 2.43 for SARS-CoV-1.

The half-lives of the two viruses were also similar on copper. On cardboard, the halflife of SARS-CoV-2 was longer than that of SARSCoV-1.

***The longest viability of both viruses was on stainless steel and plastic; the estimated median half-life of SARS-CoV-2 was approximately 5.6 hours on stainless steel and 6.8 hours on plastic**.*

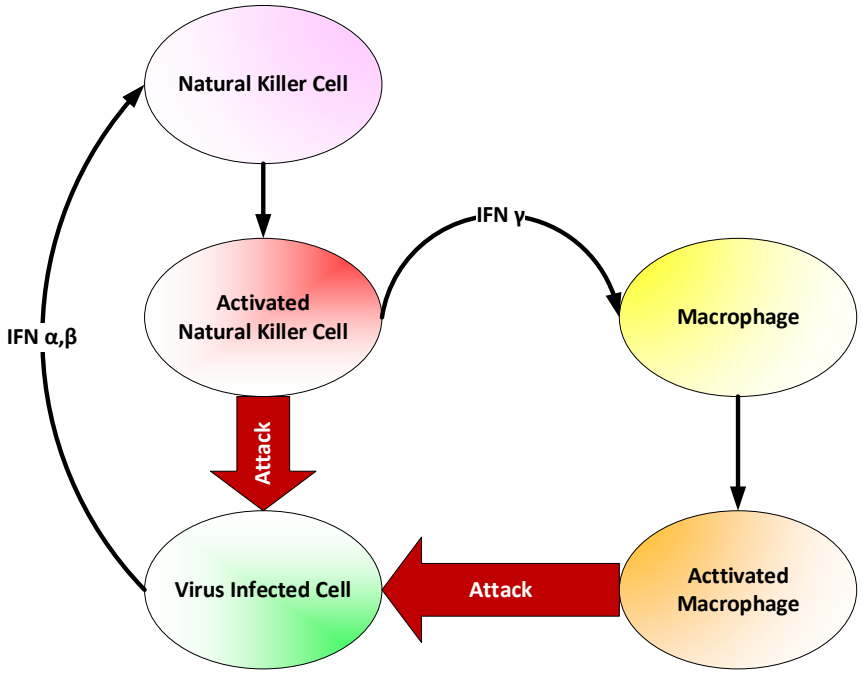
Estimated differences in the halflives of the two viruses were small except for those on cardboard. Individual replicate data were noticeably "noisier" (i.e., there was more variation in the experiment, resulting in a larger standard error) for cardboard than for other surfaces (Fig. S1 through S5), so we advise caution in interpreting this result.

These factors are essential if one is to establish a transmission potential.

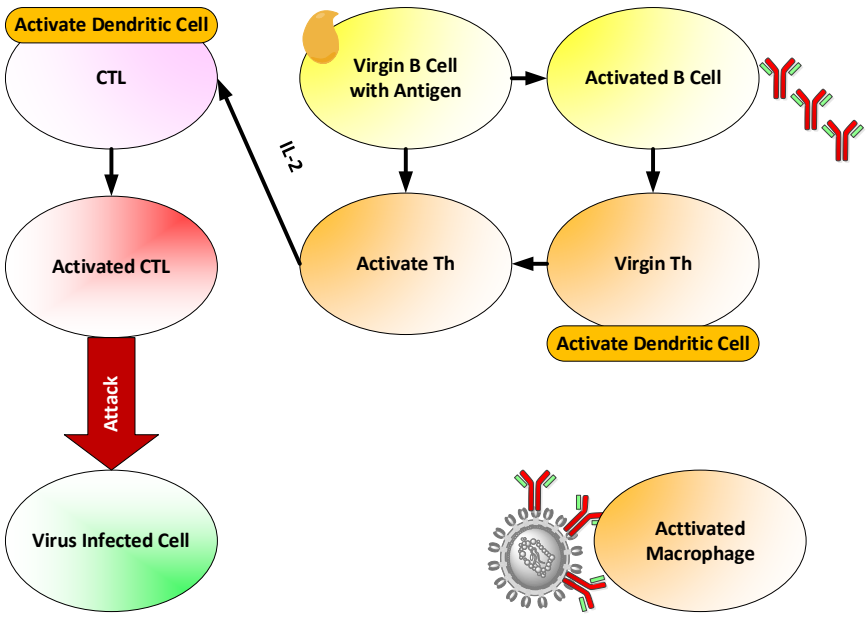
5 SECONDARY FACTORS

We examine the impact of IL-6 as a prime driver of the cytokine storm. We first review the key elements of the immune system, then consider IL-6 specifically and finally consider the impact on NF-kB, a significant control element.

As a simple summary we present below the overall innate system:



Followed below by the adaptive system. It should be noted, however, that the NK cells do provide some interlinkage which we shall explore.



The above depicts what appear to be two disconnected systems. However the nexus as we have discussed is the NK cell complex.

5.1 Immune System Issues

As Abbas et al note regarding Toll Like Receptors, TLRs, and their control capabilities in the immune system. TLRs are found on the cell surface and on intracellular membranes and are thus able to recognize microbes in different cellular locations. The authors then note:

TLRs 1, 2, 4, 5, and 6 are expressed on the plasma membrane, where they recognize various PAMPs in the extracellular environment. Some of the most potent microbial stimuli for innate immune responses bind to these plasma membrane TLRs, such as bacterial LPS and lipoteichoic acid, which are recognized by TLRs 4 and 2, respectively.

In contrast, TLRs 3, 7, 8, and 9 are mainly expressed inside cells on endoplasmic reticulum and endosomal membranes, where they detect several different microbial nucleic acids. These include double-stranded RNA, which binds to TLR3; single-stranded RNA, which binds to TLR7 and TLR8; and unmethylated CpG motifs in DNA, which bind to TLR9. Single- and double-stranded RNA are not unique to microbes, but their location in endosomes likely reflects origin from microbes. This is because host cell RNA is not normally present in endosomes, but microbial RNA may end up in endosomes of neutrophils, macrophages, or DCs when the microbes are phagocytosed by these cells.

TLR7 seems to be the primary TLR for this virus. We shall assume that but it does not seem to make a material difference. No literature seems to exist on this fact as of this time.

Enzymatic digestion of the microbes within endosomes will release their nucleic acids so these are able to bind TLRs in the endosomal membrane. Thus, the endosomal TLRs may distinguish

nucleic acids of normal cells from microbial nucleic acids on the basis of the cellular location of these molecules. A protein in the endoplasmic reticulum called UNC-93B is required for the endosomal localization and proper function of TLRs 3, 7, 8, and 9.

Genetic deficiency in UNC-93B leads to susceptibility to certain viral infections, especially herpes simplex virus encephalitis, demonstrating the importance of the endosomal location of TLRs for innate defense against viruses.

TLR recognition of microbial ligands results in the activation of several signaling pathways and ultimately transcription factors, which induce the expression of genes whose products are important for inflammatory and antiviral responses (Fig. 4.3). The signaling pathways are initiated by ligand binding to the TLR at the cell surface or in the endoplasmic reticulum or endosomes, leading to dimerization of the TLR proteins. Ligand-induced TLR dimerization is predicted to bring the TIR domains of the cytoplasmic tails of each protein close to one another. This is followed by recruitment of TIR domain–containing adaptor proteins, which facilitate the recruitment and activation of various protein kinases, leading to the activation of different transcription factors.

***The major transcription factors that are activated by TLR signaling pathways are nuclear factor κB (NF-κB)**, activation protein 1 (AP-1), interferon response factor 3 (IRF3), and IRF7. NF-κB and AP-1 stimulate the expression of genes encoding many of the molecules required for inflammatory responses, including inflammatory cytokines (e.g. tumor necrosis factor [TNF] and IL-1), chemokines (e.g., CCL2 and CXCL8), and endothelial adhesion molecules (e.g., E-selectin) (discussed later). IRF3 and IRF7 promote production of type I interferons (IFN-α and IFN-β), which are important for antiviral innate immune responses.*

NF-kB is a major player in activating a variety of genes both for viral as well as malignant paths. We shall discuss this later in this section.

The endosomal TLRs 7 and 9, which are most highly expressed in plasmacytoid Dendritic Cells, signal through a MyD88-dependent, TRIF-independent pathway that activates both NF-κB and IRFs. Therefore, TLR7 and TLR9, like TLR4, induce both inflammatory and antiviral responses.

1. *The innate immune system uses cell-associated pattern recognition receptors, present on plasma and endosomal membranes and in the cytosol, to recognize structures called PAMPs, which are shared by microbes, are not present on mammalian cells, and are often essential for survival of the microbes, thus limiting the capacity of microbes to evade detection by mutating or losing expression of these molecules. In addition, these receptors recognize molecules made by the host but whose expression or location indicates cellular damage; these are called DAMPs.*

2. *TLRs, present on the cell surface and in endosomes, are the most important family of pattern recognition receptors, recognizing a wide variety of ligands, including bacterial cell wall components and microbial nucleic acids. Cytosolic pattern recognition receptors exist that recognize microbial molecules. These receptors include the RLRs, which recognize viral*

RNA, CDSs which recognize microbial DNA, and NLRs, which recognize bacterial cell wall constituents and also serve as recognition components of many inflammasomes.

3. *Pattern recognition receptors, including TLRs, NLRs, and RLRs, signal to activate the transcription factors NF-κB and AP-1, which stimulate expression of cytokines, costimulators, and other molecules involved in inflammation, and the IRF transcription factors, which stimulate expression of the antiviral type I interferon genes.*

4. *The inflammasome, a specialized caspase-1 containing enzyme complex that forms in response to a wide variety of PAMPs and DAMPs, includes recognition structures, which are often NLR family proteins, an adaptor, and the enzyme caspase-1, the main function of which is to produce active forms of the inflammatory cytokines IL-1 and IL-18.*

We believe that the immune system is a major player in this disease. To date most of the effort seems to be focused by infectious disease experts such as those at NIH. However immunological understanding we believe is critical.

5.2 IL-6 Details

IL-6 is a powerful cytokine. It has been linked closely to cytokine storms and is often activated via viral infections. As NCBI notes[8]:

This gene encodes a cytokine that functions in inflammation and the maturation of B cells. In addition, the encoded protein has been shown to be an endogenous pyrogen capable of inducing fever in people with autoimmune diseases or infections. The protein is primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through interleukin 6 receptor, alpha. The functioning of this gene is implicated in a wide variety of inflammation-associated disease states, including suspectibility to diabetes mellitus and systemic juvenile rheumatoid arthritis. Alternative splicing results in multiple transcript variants.

As Puel and Casanova have recently observed:

The time has come for IL-6 to begin to reveal its true nature. IL-6, first identified as B cell stimulatory factor 2, or BSF2, was cloned in 1986 and has been one of the most intensively studied cytokines ever since.

Its pleiotropy is legendary. It is produced by and acts on many cell types by binding to a receptor composed of the transmembrane IL-6R (or its soluble form, sIL-6R) and the transmembrane GP130 protein. The result is a tremendous diversity of effects, in cis and trans, on the development and function of many leukocyte subsets and various other cell types.

IL-6–producing cells include bone marrow stromal cells, T cells, macrophages, dendritic cells, fibroblasts, synovial cells, endothelial cells, glia cells, and keratinocytes, whereas IL- 6–

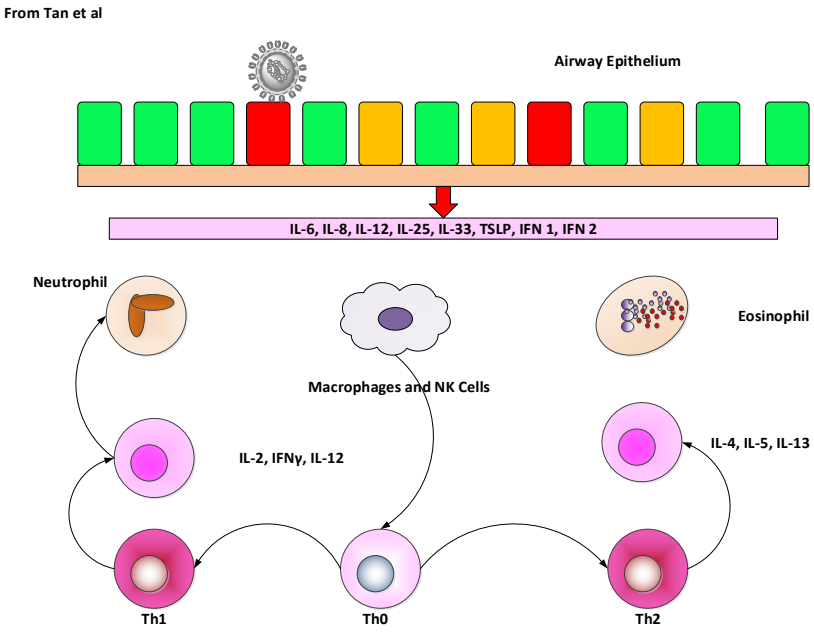
[8] https://www.ncbi.nlm.nih.gov/gene/3569

responsive cells include B cells, T cells, hepatocytes, monocytes, vascular endothelial cells, and synoviocytes.

Studies of mouse IL-6 at the whole-organism level have revealed multiple roles, the most frequently studied of which relates to inflammation, host defense, metabolism, bone homeostasis, and tissue regeneration. Mice with knockouts of IL-6 or IL-6R display a wide range of immunological and nonimmunological phenotypes, including impaired inflammatory responses to localized tissue damage, impaired induction of acute-phase proteins, and impaired responses to or enhanced susceptibility to various microbes (e.g., vesicular stomatitis virus, vaccinia virus, and Listeria monocytogenes).

Following these studies, therapeutic agents blocking IL-6 activity have been introduced into clinical practice for various inflammatory conditions. These studies raised questions about the essential immunological functions of human IL-6 and the most likely clinical phenotype of humans with genetic defects resulting in a lack of IL-6 immunity .

The Figure below presents a high level summary of the various immune system cells. As noted previously the driver of many of the responses includes the NK cells.



From Garbers et al we have a discussion of the interaction between IL-6 and the TLR. Specifically they note:

IL-6 is a four-helical cytokine of 184 amino acids that can be secreted by many cell types upon appropriate stimulation during infection, inflammation or cancer. IL-6 is secreted by monocytes and macrophages after engagement of Toll-like receptors (TLRs) by, for example, lipopolysaccharides (LPS); by fibroblasts, keratinocytes, astrocytes and endothelial cells after IL-1 stimulation; and by subsets of activated B cells and T cells and by microglial cells after viral infection. *IL-6 is important for regulating B cell and T cell responses and for coordinating*

the activity of the innate and the adaptive immune systems. Moreover, IL-6 is needed for regeneration of the liver.

Although under normal conditions, the IL-6 concentration in the circulation is around 1–5 pg per ml, IL-6 concentrations in the serum can easily increase into the nanograms per millilitre range in pathological states*. IL-6 is strongly induced during most, if not all, inflammatory processes, infection and cancer. In sepsis, IL-6 levels of several microgram per millilitre have been reported. In the brain, high IL-6 levels lead to astrocytosis and neurodegeneration.*

IL-6 binds to IL-6R — an 80 kDa receptor devoid of signalling capacity13 — and the complex of IL-6 and IL-6R binds to a second membrane protein, glycoprotein 130 (gp130; also known as IL-6R subunit-β), which dimerizes and initiates intracellular signalling20. Although gp130 is expressed on all cells, IL-6R is found only on a few cells, such as hepatocytes, some leukocytes and epithelial cells17. IL-6 exhibits measurable affinity only for IL-6R but not for gp130; consequently, cells expressing gp130 but not IL-6R are unresponsive to IL-6 per se. The gp130 protein has been shown to act as a signalling receptor for additional cytokines, including IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT1), leukaemia inhibitory factor (LIF) and the cardiotrophin-like cytokine factor 1 (CLCF1), which, together with IL-6, form the IL-6 family of cytokines.

In addition, gp130 is one component of the heterodimeric receptor complexes for some heterodimeric IL-12 family members, including IL-27 (which is composed of subunits p28 (IL-27p28) and IL-27 subunit-β (EBI3)) and IL-35 (composed of IL-12p35 and EBI3).

In a similar manner Su et al note:

Interleukin-6 (IL-6) is a pleiotropic cytokine that not only regulates the immune and inflammatory response but also affects hematopoiesis, metabolism, and organ development. IL-6 can simultaneously elicit distinct or even contradictory physiopathological processes, which is likely discriminated by the cascades of signaling pathway, termed classic and trans-signaling. Besides playing several important physiological roles, dysregulated IL-6 has been demonstrated to underlie a number of autoimmune and inflammatory diseases, metabolic abnormalities, and malignancies.

As Tanaka and Kishimoto note:

Interleukin-6 (IL-6), initially designated as a B cell differentiation factor, is a representative cytokine featuring redundancy and pleiotropic activity. In the early phase of infectious inflammation, IL-6 is produced by monocytes and macrophages immediately after the stimulation of Toll-like receptors (TLRs) with distinct pathogen-associated molecular patterns (PAMPs). In noninfectious inflammations, such as burn or traumatic injury, damage-associated molecular patterns (DAMPs) from damaged or dying cells stimulate TLRs to produce IL-6.

As Velazquez-Salinas et al note

IL-6 is a pleotropic cytokine produced in response to tissue damage and infections. Multiple cell types including fibroblasts, keratinocytes, mesangial cells, vascular endothelial cells, mast cells, macrophages, dendritic cells, and T and B cells are associated with the production of this cytokine.

After targeting its specific receptor, IL-6 starts a cascade of signaling events mainly associated with the JAK/STAT3 activation pathway *promoting the transcription of multiple downstream genes associated with cellular signaling processes, including cytokines, receptors, adaptor proteins, and protein kinases. …It also controls the production of proteins implicated in regulation of gene expression …. The number of genes regulated by IL-6 activity may explain the pleotropic nature of this interleukin.*

Accordingly, the biological consequences of IL-6 production have been associated with both pro- and anti-inflammatory effects, highlighting IL-6's pivotal role in the activation and regulation of the immune response. Biological activities affected by production of IL-6 include: control of the differentiation of monocytes into macrophages by regulating the expression of macrophage colony-stimulating factor, increasing B-cell IgG production by regulating the expression of IL-21, negative regulation of dendritic cell maturation by activation of the STAT3 signaling pathway, as well as the promotion of the Th2 response by inhibiting Th1 polarization.

Two different mechanisms have been described to promote the inhibition of Th1 polarization by IL-6:

(1) IL-6 stimulates CD4 T cells to secrete IL-4 and direct the response to Th2, and

(2) IL-6 affects the secretion of IFNγ by CD4 T cells, an essential interferon to promote Th1 polarization.

Thus IL-6 is truly a powerful initiator and supporter of a variety if immune elements. They continue:

As a warning signal during viral infections, different immune cellular pathogen recognition receptors, including toll-like receptors ***(TLR:2, 3, 4, 7, 8, and 9)****, nucleotide-binding oligomerization domain-like receptors, DNA receptors, and retinoic acid-inducible gene-1-like receptors, are able to sense a variety of pathogen-associated molecular patterns displayed by viruses (envelope glycoproteins, single and double-stranded RNA, and unmethylated CpG DNA), which stimulate transcription of IL-6 among other proinflammatory cytokines.*

In this context, it has been shown that specific amino acid substitutions in a TLR-like structure in the NS4B protein of a highly virulent classical swine fever virus (CSFV) strain resulted in a completely attenuated phenotype in pigs. Infection of pigs with this mutant CSFV was characterized by the sustained accumulation of IL-6 in tonsils.

Further in vitro experiments using exogenous IL-6 confirmed the ability of this cytokine to repress the replication of CSFV in swine peripheral blood mononuclear cells, the natural target cell during CSFV infection in pigs.

Similarly, evidence of the antiviral effect of IL-6 was described during in vitro studies conducted with hepatitis B virus (HBV) where the direct ability of exogenous IL-6 to suppress the replication of this virus was described. Disruption of HBV replication was characterized by a marked decrease in the number of viral genome-containing nucleocapsids, an effect mediated in an interferon-independent manner.

The above observation of the anti-viral properties of IL-6 is one of those "on the one hand, on the other hand" observations. Namely it responds well to virus intrusion but it may respond too well. They continue:

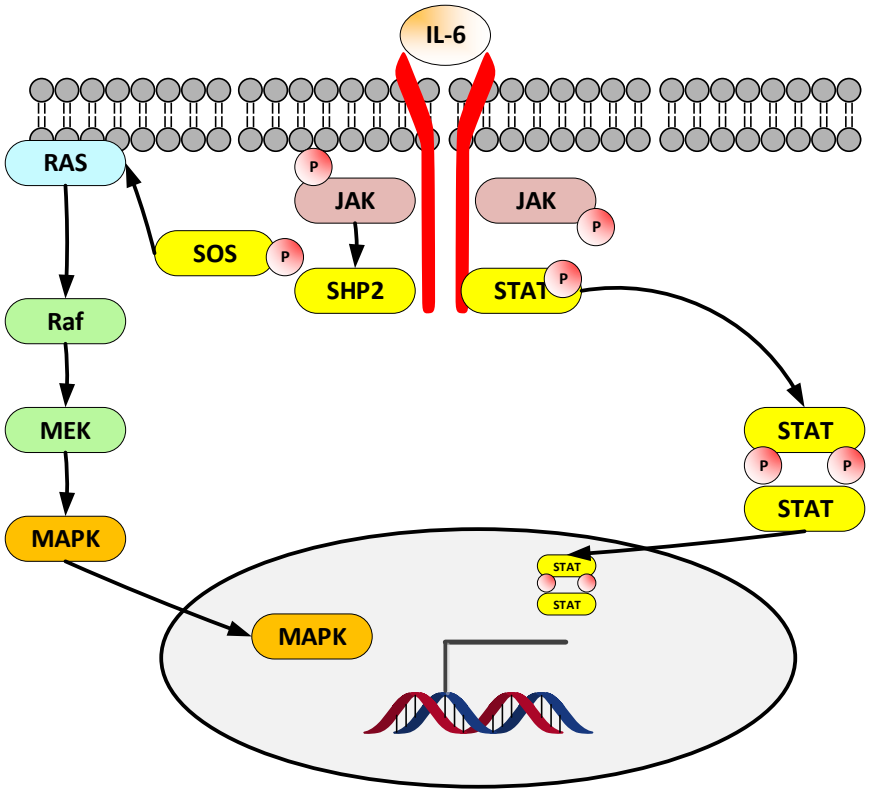
Furthermore, IL-6 was able to block HBV infection in hepatocytes by inhibiting expression of HBV receptor in the human liver, i.e., the bile acid transporter Na (+)/taurocholate co-transporting polypeptide, and effectively disrupted epigenetic control of the nuclear cccDNA mini-chromosome, inhibiting HBV transcription and the expression of hepatocyte nuclear transcription factors 1 and 4 alpha. However, experimental scientific evidence also suggests potential negative consequences that increased levels of IL-6 might have on the cellular immune response against viruses. In this context different potential mechanisms involving this cytokine might affect viral clearance, ultimately favoring the establishment of a viral persistent state in infected hosts.

In a similar manner Heinrich et al noted:

Dysregulation of IL-6-type cytokine signalling contributes to the onset and maintenance of several diseases, such as rheumatoid arthritis, inflammatory bowel disease, osteoporosis, multiple sclerosis and various types of cancer (e.g. multiple myeloma and prostate cancer). IL-6-type cytokines exert their action via the signal transducers gp (glycoprotein) 130, LIF receptor and OSM receptor leading to the activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and MAPK (mitogenactivated protein kinase) cascades.

This review focuses on recent progress in the understanding of the molecular mechanisms of IL-6-type cytokine signal transduction. Emphasis is put on the termination and modulation of the JAK/STAT signalling pathway mediated by tyrosine phosphatases, the SOCS (suppressor of cytokine signalling) feedback inhibitors and PIAS (protein inhibitor of activated STAT) proteins. Also the cross-talk between the JAK/STAT pathway with other signalling cascades is discussed

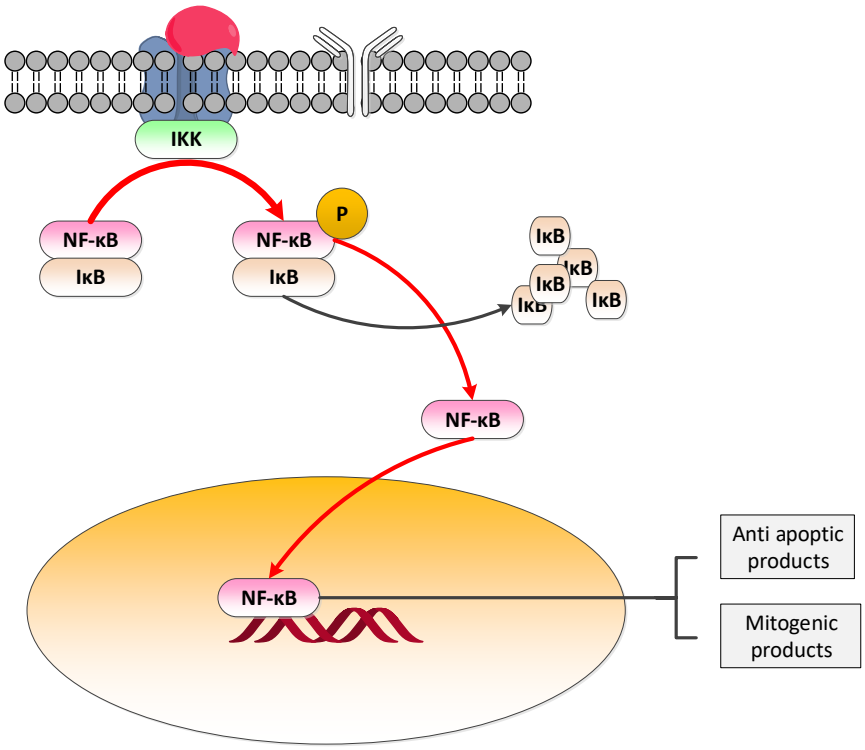
We demonstrate several of these above pathways below.



5.3 NF-KB AND ITS IMPLICATIONS

The NF-kB dimer is a powerful transcription factor that plays a role in the function of the immune system and in dealing with inflammation[9]. It also has a significant role in cancer development. We briefly summarize this significant factor and highlight the key elements that relate to the conjunction between inflammation and cancer.

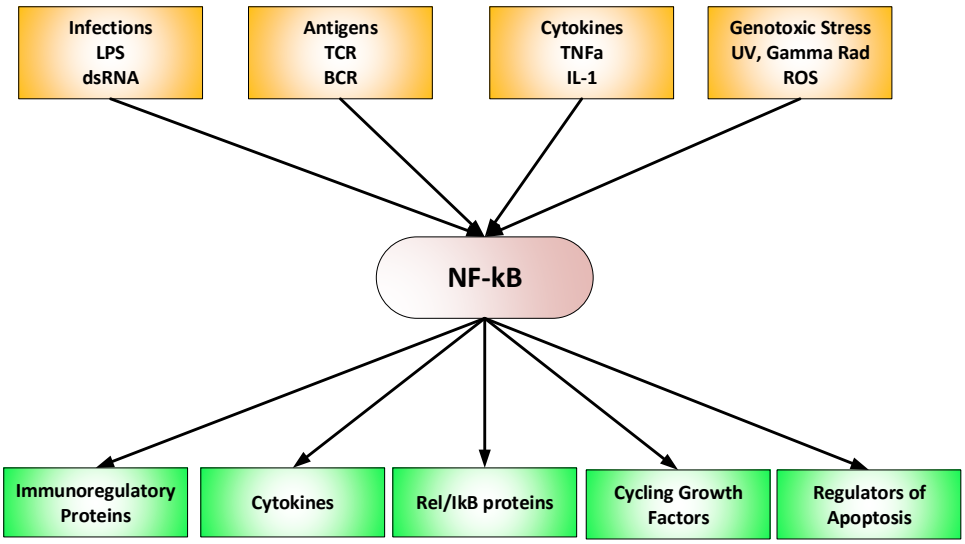
[9] See Karin and Staudt



From Gorlach et al we have:

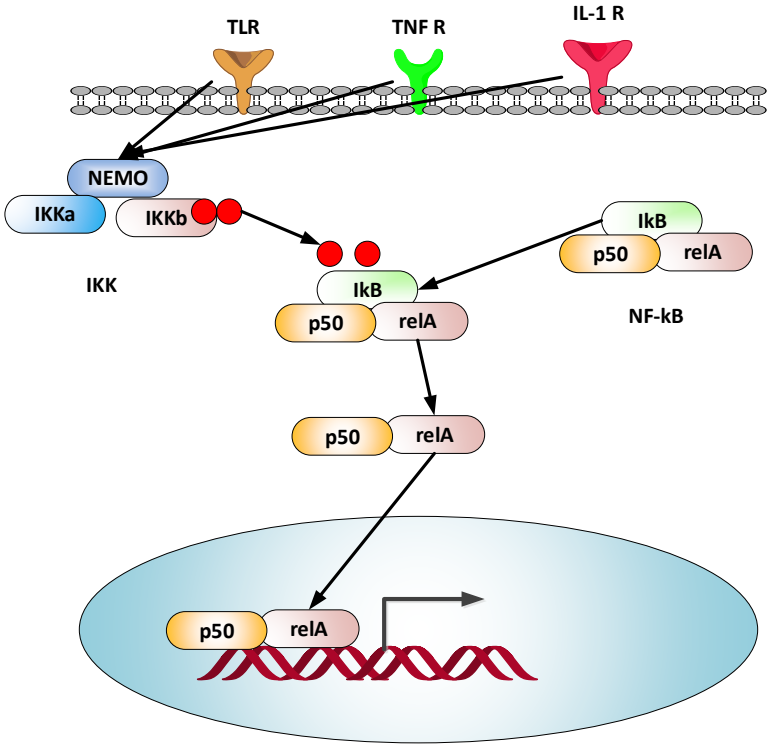
The activation of NF-κB is closely linked with ROS generation during inflammation and obesity. ROS were found to mediate inhibitor of NF-κBα (IκBα) kinase (IKKα and IKKβ) phosphorylation and release of free NF-κB dimers. Tumor necrosis factor α (TNFα), a bona fide NF-κB activator, was shown to mediate a redox- dependent activation of protein kinase A which subsequently phosphorylated Ser276 on RelA (v-rel avian reticuloendotheliosis viral oncogene homolog A). By contrast, the NF-κB member p50 was found to have reduced DNA binding activity when oxidized at Cys62.

We demonstrate the impact of NF-kB below. There are a multiplicity of drivers as well as impacts on cells.

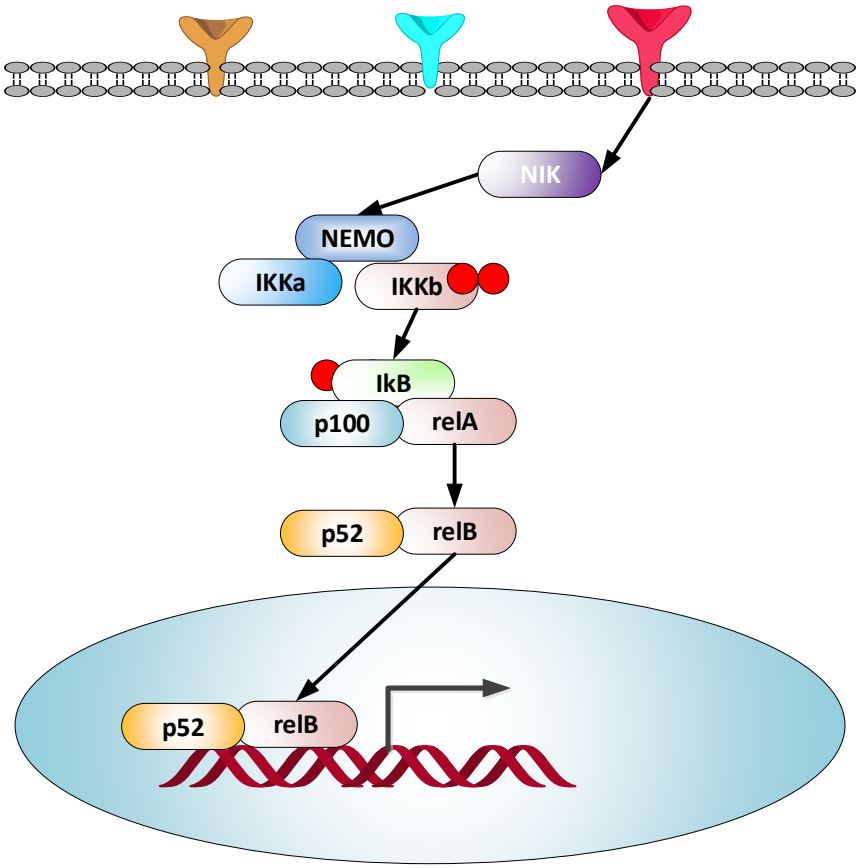


Note that NF-kB can be activated by the very things that are part of the inflammatory response. In turn, NF-kB as a promoter can then release more of this drivers, increase growth factor expression, and stop normal apoptosis. NF-kB is one of the most significant intracellular drivers that connects inflammation, the immune system, and unregulated growth.

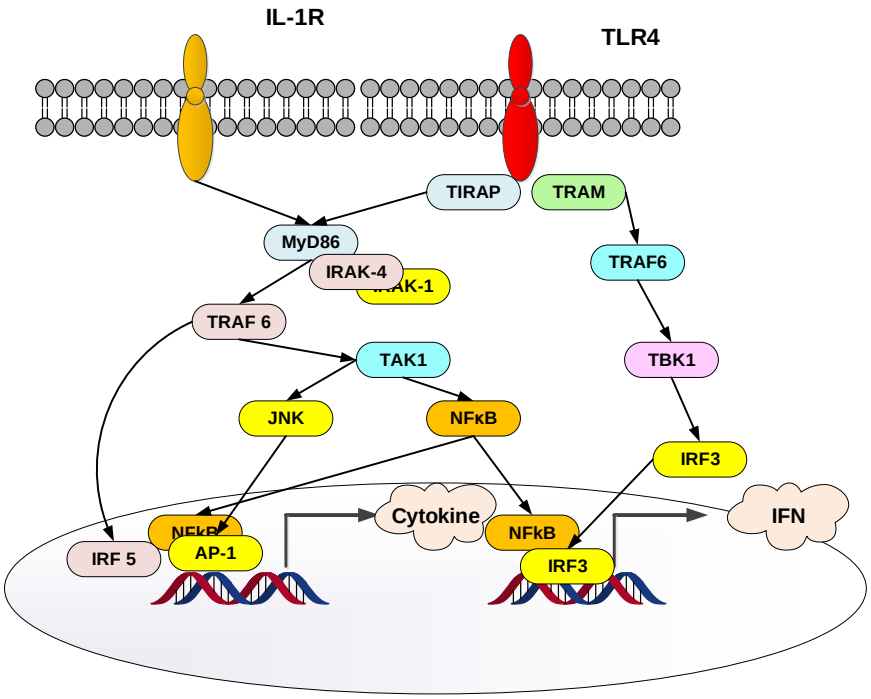
Now as we noted NF-kB is a dimer, namely a combination of two proteins which in turn, when activated, result in a molecule which in a very effective promoter. We show this below with a set of examples:



Here in the above is one of the dimer expressed, namely a relA along with a p50. Below we show the relB and the p52 expression.



The integrated combination is shown below.



To understand NF-kB we need to see how it functions as a powerful promoter. From NCBI we have the following description[10]:

NF-kappa-B is a ubiquitous transcription factor involved in several biological processes. It is held in the cytoplasm in an inactive state by specific inhibitors. Upon degradation of the inhibitor, NF-kappa-B moves to the nucleus and activates transcription of specific genes. NF-kappa-B is composed of NFKB1 or NFKB2 bound to either REL, RELA, or RELB. The most abundant form of NF-kappa-B is NFKB1 complexed with the product of this gene, RELA. Four transcript variants encoding different isoforms have been found for this gene.

From Nature we have the following description[11]:

The canonical pathway is induced by tumour necrosis factor-alpha (TNFalpha), interleukin-1 (IL-1) and many other stimuli, and is dependent on activation of IKKbeta. This activation results in the phosphorylation (P) of IkappaBalpha at Ser32 and Ser36, leading to its ubiquitylation (Ub) and subsequent degradation by the 26S proteasome. Release of the NF-kappaB complex allows it to relocate to the nucleus. Under some circumstances, the NF-kappaB–IkappaBalpha complex shuttles between the cytoplasm and the nucleus (not shown).

IKK-dependent activation of NF-kappaB can occur following genotoxic stress. Here, NF-kappaB essential modifier (NEMO) localizes to the nucleus, where it is sumoylated and then ubiquitylated, in a process that is dependent on the ataxia telangiectasia mutated (ATM)

[10] https://www.ncbi.nlm.nih.gov/gene/5970

[11] https://www.nature.com/nrm/journal/v8/n1/box/nrm2083_BX1.html

checkpoint kinase. NEMO relocates back to the cytoplasm together with ATM, where activation of IKK-beta occurs. IKK-independent atypical pathways of NF-kappaB activation have also been described, which include casein kinase-II (CK2) and tyrosine-kinase-dependent pathways.

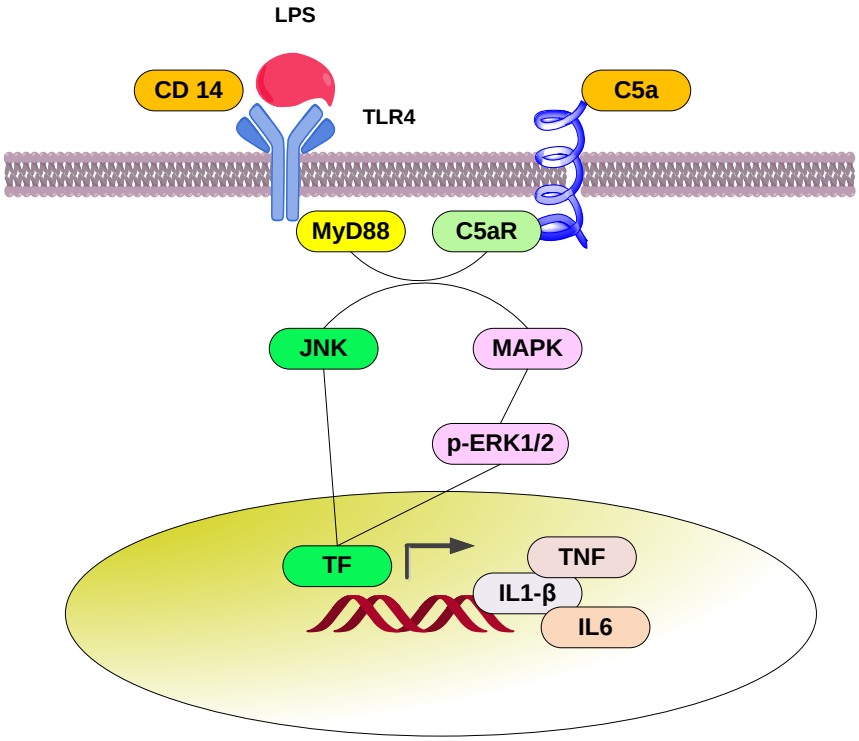
The non-canonical pathway results in the activation of IKK alpha by the NF-kappa B-inducing kinase (NIK), followed by phosphorylation of the p100 NF-kappa B subunit by IKK alpha. This results in proteasome-dependent processing of p100 to p52, which can lead to the activation of p52–Rel B heterodimers that target distinct kappa B elements. Phosphorylation of NF-kappa B subunits by nuclear kinases, and modification of these subunits by acetylases and phosphatases, can result in transcriptional activation and repression as well as promoter-specific effects.

Moreover, cooperative interactions with heterologous transcription factors can target NF-kappa B complexes to specific promoters, resulting in the selective activation of gene expression following cellular exposure to distinct stimuli.

As Merle et al discuss when examine the Complement system they state:

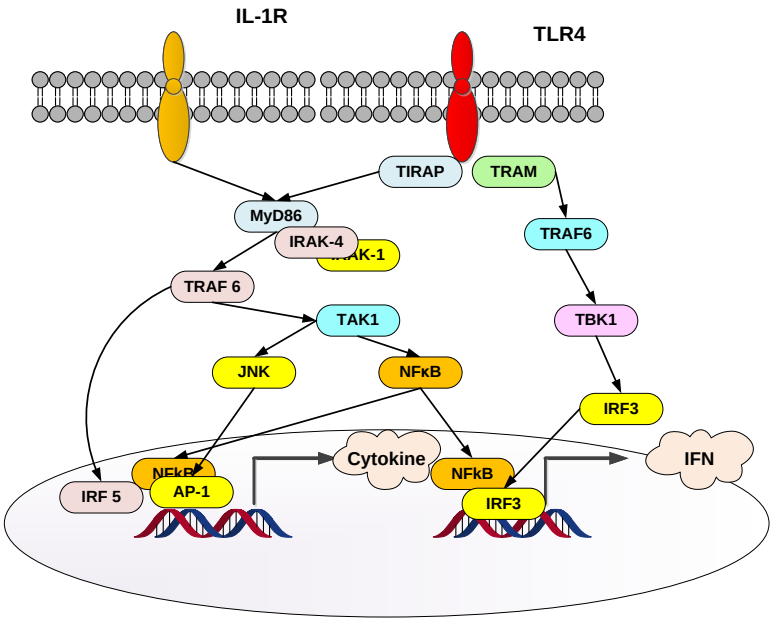
C3a and C5a are able to induce potent inflammatory pathways via their receptors C3aR and C5aR. The implication of intermediates such as NF-kB, MAPK, and c-Jun N-terminal kinase (JNK) in their transduction pathways suggests a potential crosstalk with other pathways, such as those of TLRs. Indeed, complement is involved in TLR-induced inflammation.

They show in the following Figure how this does function:



C5a/C5aR signalingpathwaycancooperatewithTLR-4activationby LPS on macrophages. Intermediate signaling pathways JNK and MAPK are activated and thus lead to proinflammatory effect by TNF-α, IL6, and IL1-β synthesis. On dendritic cells (DCs),TLR-4 and C5aR cooperate in different manner between mice and human. In vivo experiments have demonstrated an implication in Th1cells expansion, whereas in human, an anti-inflammatory role of TLR-4/C5aR collaboration has been described by an antagonized effect on IL-12 and IL-23 synthesis by DC.

Thus, when examining the effects of the complement proteins one must also examine the interactions with other receptors. Further details on this interaction are shown below. Here we show the Toll like receptors, TLR as initiations. These are powerful initiators in the innate response.



As Amiri and Richmond state:

Nuclear Factor-kappa B (NF-κB) is an inducible transcription factor that regulates the expression of many genes involved in the immune response. Recently, NF-κB activity has been shown to be upregulated in many cancers, including melanoma. Data indicate that the enhanced activation of NF- κB may be due to deregulations in upstream signaling pathways such as Ras/Raf, PI3K/Akt, and NIK. Multiple studies have shown that NF-κB is involved in the regulation of apoptosis, angiogenesis, and tumor cell invasion, all of which indicate the important role of NF-κB in tumorigenesis. Thus, understanding the molecular mechanism of melanoma progression will aid in designing new therapeutic approaches for melanoma.

They continue:

Constitutive activation of NF-κB is an emerging hallmark of various types of tumors including breast, colon, pancreatic, ovarian, and melanoma. In the healthy human, NF-κB regulates the

expression of genes involved in normal immunologic reactions (e.g. generation of immunoregulatory molecules such as antibody light chains) in response to proinflammatory cytokines and by-products of microbial and viral infections. NF-κB also modulates the expression of factors responsible for growth as well as apoptosis. However, increased activation of NF-κB results in enhanced expression of proinflammatory mediators, leading to acute inflammatory injury to lungs and other organs, and development of multiple organ dysfunctions as well as cancer.

They then summarize NF-kB's role as:

3.1. Apoptosis resistance and cell proliferation*: In processes such as tumor initiation and promotion where prolonged survival of cells is a crucial event, NF-κB plays an important role as a mediator of inhibition of apoptosis. In melanoma, NF-κB has been shown to activate expression of anti-apoptotic proteins such as tumor necrosis factor receptor-associated factor 1 (TRAF1), TRAF2, and the inhibitor-of apoptosis (IAP) proteins c-IAP1, c-IAP2, and melanoma inhibitor of apoptosis (ML-IAP), survivin as well as Bcl-2 like proteins…*

3.2. Invasion and metastasis*: In invasion and metastasis of melanoma, NF-κB may regulate the production of prostaglandins via cyclooxygenase-2 (COX-2), which has been shown to be overexpressed in melanoma [44,45]. It was shown that COX-2 is expressed in the majority of primary malignant melanoma, as well as in five human malignant melanoma cell lines….*

However, as Liu et al (2006) state:

Malignant melanoma is the most lethal skin cancer, whose ability to rapidly metastasize often prevents surgical cure.

Furthermore, the systemic treatment of melanoma is largely ineffective due to the intrinsic resistance of melanoma cells to numerous anticancer agents. Increased survival of melanoma cells is primarily attributed to the constitutive activation of the transcription factor nuclear factor kB (NF-kB), which regulates the expression of many anti-apoptotic, pro-proliferative and pro-metastatic genes.

Canonical activation of the NF-kB pathway occurs when NF-kB switches its localization from the cytoplasm, where it is maintained inactive by assembly with the inhibitor IkB protein, to the nucleus, where NF-kB regulates gene expression. NF-kB activation relies upon the phosphorylation dependent ubiquitination and degradation of IkB mediated by the IkB kinase (IKK) complex and b-Trcp E3 ubiquitin ligases.

Consequently, both IKK activity and the levels of b-Trcp regulate the extent of IkB degradation and hence NF-kB activation. The genetic basis that underlies the elevated NF-kB activity in malignant melanoma largely remains elusive.

Constitutively active IKK has been demonstrated to sustain NF-kB activation in human melanoma cells, resulting in induction of the chemokine CXCL1. CXCL1, in turn, is capable of activating IKK and NF-kB and promoting cell survival and tumorigenesis …. However, the

original genetic alterations that initiate this feed-forward mechanism in melanoma remain unclear.

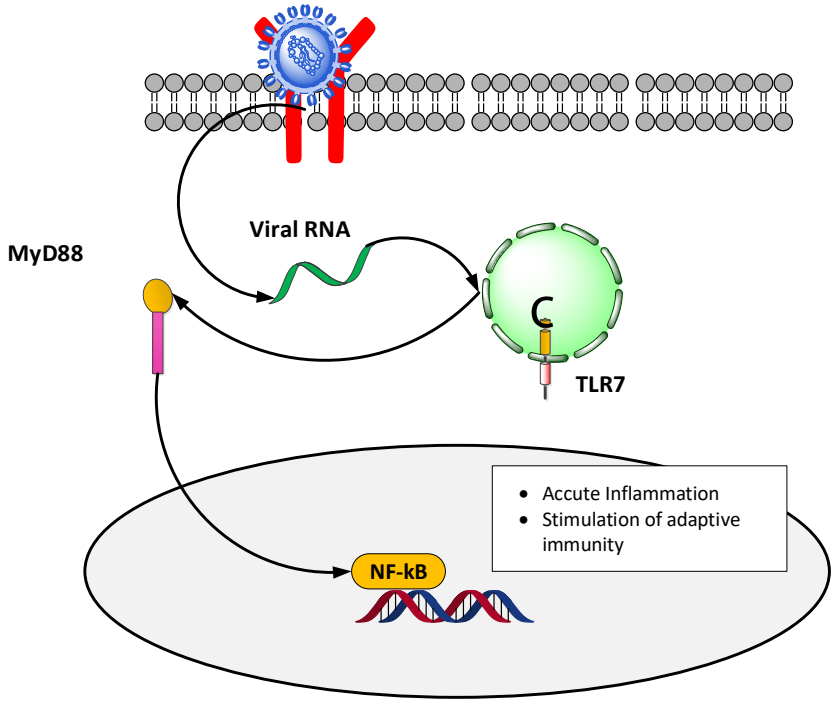
One of the major oncogenic events described in the genesis of malignant melanoma is constitutive activation of the Ras-regulated RAF-MEK-ERK mitogen-activated protein kinase (MAPK) pathway. This is achieved most frequently by activating mutations in either BRAF (e.g. V600E substitution) or, less frequently, in N-RAS … Recent evidence indicates that oncogenic BRAF activity is essential for human melanoma cell growth and survival …

However, despite prior reports that RAF can activate NF-kB …, the mechanism(s) by which $BRAF_{V600E}$ ($BRAF_{VE}$) may elicit NF-kB signaling in melanoma cells have not yet been elucidated. Activation of the canonical NF-kB pathway depends on both IKK activity, which has been shown to be elevated in human melanomas….

Liu et al conclusion is speculative but telling:

Taken together, these data support a model in which mutational activation of BRAF in human melanomas contributes to constitutive induction of NF-κB activity and to increased survival of melanoma cells.

Again we have the issue of speculation as to where and why the mutations occur. Here they speculate about the BRAF mutation resulting in the antiapoptotic control with NF-κB.



6 MYOCARDITIS

We now examine a specific possible sequella. Namely, we look at myocarditis; acute and chronic. We have seen a few cases which may be related to COVID and the literature does examine a few. However, in the chronic phase, which we often find as sequella to viral infections, the result is just fibrotic infiltration of the myocardium. There may be a diffuse thickening of the heart walls, a reduction in ejection fraction and perhaps some other secondary symptoms. However, it is important to use this as an example of the impact on multiple other organs.

Gupta et al discussed the extrapulmonary manifestations of CPVID-19. The range from neurologic, renal, hepatic, gastrointestinal, hematological, endocrinological, dermatological and cardiac. Some are more severe than others. The authors detail the cardiological as follows:

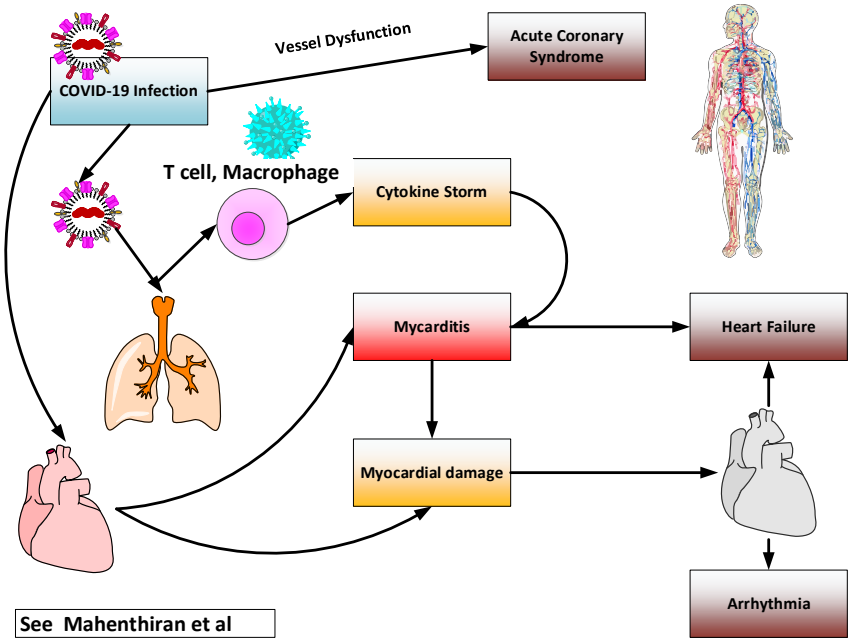
1. Clinical presentations

a) Myocardial ischemia and MI (type 1 and 2)
b) Myocarditis
c) Arrhythmia: new-onset atrial fibrillation and flutter, sinus tachycardia, sinus bradycardia, QTc prolongation (often drug induced), torsades de pointes, sudden cardiac death, pulseless electrical activity
d) Cardiomyopathy: biventricular, isolated right or left ventricular dysfunction
e) Cardiogenic shock

2. COVID-19-specific considerations

f) Do not routinely discontinue ACE inhibitors or ARBs in patients already on them at home; assess on a case-by-case basis
g) Perform an electrocardiogram or telemetry monitoring for patients at medium to high risk for torsades de pointes who are being treated with QTc-prolonging drugs138
h) Carefully consider the utility of diagnostic modalities, including cardiac imaging, invasive hemodynamic assessments, and endomyocardial biopsies, to minimize the risk of viral transmission

We now focus on myocarditis, especially the chronic presentation. This overall complex can be presented as again by Mahenthiran et al:



6.1 BASICS OF MYOCARDITIS

Let us begin with some basics of myocarditis. From Strayer and Saffitz:

Myocarditis is myocardial inflammation with myocyte necrosis. This definition specifically excludes ischemic heart disease. The true incidence of myocarditis is hard to establish, as many cases are asymptomatic. It occurs at any age but is most common in children 1 to 10 years old. It is one of the few heart diseases that can cause acute heart failure in previously healthy children, adolescents, or young adults.

Severe myocarditis can cause extensive myocardial necrosis, arrhythmias, and sudden cardiac death. Viral etiology is generally suspected, but unless special studies identify viral genomes in heart biopsies, evidence is usually circumstantial. During the second half of the 20th century, enteroviruses, especially coxsackie virus, were most commonly identified in the western world. Since then, sensitive methods to detect viral genomes have identified H1N1 strain influenza, adenovirus, cytomegalovirus, parvovirus B-19, and others in viral myocarditis.

Viral myocarditis develops in phases.

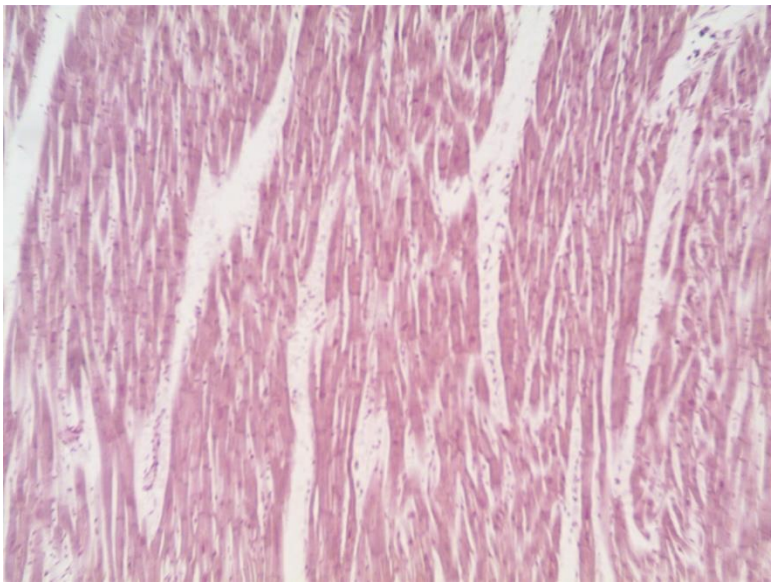
Virus first enters myocytes and activates innate immune responses. Coxsackie and adenoviruses gain entry by binding the coxsackie-adenovirus receptor (CAR). CAR belongs to the family of intercellular adhesion molecules. It is especially abundant in children, which may explain why viral myocarditis so often afflicts them. Intracellular coxsackie viruses produce proteases 2A and 3C which are crucial for viral replication but may also impair myocardial function. Protease 2A cleaves myocyte proteins such as dystrophin, which increases cell permeability and diminishes contractile function. Viral proteases may also activate myocyte apoptosis pathways by cleaving caspases.

During active viral replication, myocytes produce type 1 (i.e., virus-induced) interferons. Antibodies to viral and cardiac proteins, the latter probably arising via molecular mimicry, further contribute to tissue damage and contractile dysfunction. At this point, myocytes undergo degeneration and apoptosis, with minimal inflammation.

A second phase, develops over days to weeks, with activation of acquired immunity. Infected myocytes produce and release cytokines including TNFα, IL-1, IL-2, and IFNγ. NK cells, macrophages, and T lymphocytes accumulate at sites of infection producing the classic pathology of viral myocarditis. T cells eventually clear viruses resolving inflammation, healing by fibrosis in areas of myocyte necrosis and restored contractile function. Sometimes, impaired viral clearance and/or persistent immune activation may lead to dilated cardiomyopathy.

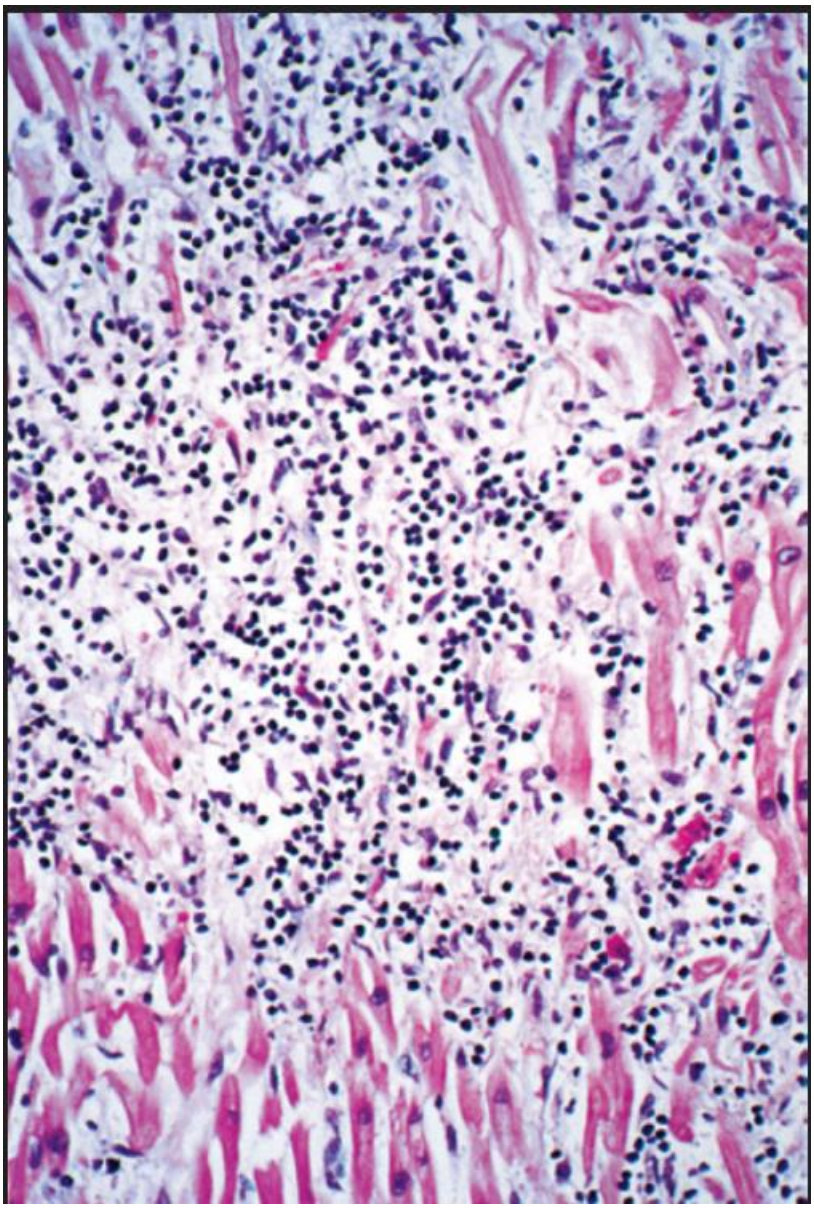
The above sequence describes common forms of myocarditis caused by viruses that infect cardiac myocytes. Other viruses can cause myocarditis by infecting cardiac endothelial cells. For example, parvovirus B-19, a frequent cardiac pathogen, stimulates production of IL-6 and TNFα and upregulates adhesion molecules, like E-selectin, in endothelial cells. Resulting endothelial injury, and accumulation of intravascular T cells, may cause enough microvascular damage to produce local ischemia and further impair cardiac function.

The slide below is a section of normal myocardial tissue[12].



Note in the above the elongated muscle cells and the lack of any significant infiltrates. Now consider the example below where we have significant infiltrates. This is an acute myocarditis sample.

[12] This is from the author's collection.



This example of viral myocarditis with infiltrates of lymphocytes and macrophages, is quite typical. The myocardial cells are the long stretched out muscle cells and the infiltrate is the dark stained set of cells.

From Kumar et al:

Myocarditis encompasses a diverse group of clinical entities in which infectious agents and/or inflammatory processes target the myocardium. *It is important to distinguish myocarditis from conditions such as IHD, where the inflammatory process is secondary to some other cause of myocardial injury.*

In the United States, viral infections are the most common cause of myocarditis, with ***coxsackieviruses A and B and other enteroviruses accounting for a majority of the cases.*** *Cytomegalovirus (CMV), human immunodeficiency virus (HIV), influenza virus, and others are less common pathogens. Offending agents can be identified by serologic studies that show rising antibody titers or through molecular diagnostic techniques using infected tissues. While some viruses cause direct cell death, in most cases the injury results from an immune response directed against virally infected cells; this is analogous to the damage inflicted by virus-specific T cells on hepatitis virus–infected liver cells. In some cases, viruses trigger a reaction against cross-reacting proteins such as myosin heavy chain.*

The nonviral infectious causes of myocarditis run the entire gamut of the microbial world. The protozoan Trypanosoma cruzi is the agent of Chagas disease. Although uncommon in the northern hemisphere, Chagas disease affects up to one half of the population in endemic areas of South America, with myocardial involvement in the vast majority. About 10% of the patients die during an acute attack; others can enter a chronic immune-mediated phase with development of progressive signs of CHF and arrhythmia 10 to 20 years later. Toxoplasma gondii (household cats are the most common vector) also can cause myocarditis, particularly in immunocompromised individuals. Trichinosis is the most common helminthic disease associated with cardiac involvement.

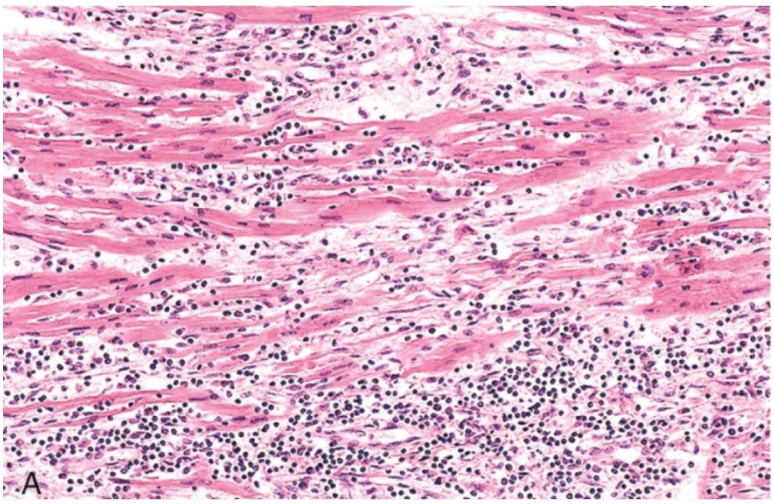
Myocarditis occurs in approximately 5% of patients with Lyme disease, a systemic illness caused by the bacterial spirochete Borrelia burgdorferi. Lyme myocarditis manifests primarily as self-limited conduction system disease, frequently requiring temporary pacemaker insertion.

Noninfectious causes of myocarditis include systemic diseases of immune origin, such as systemic lupus erythematosus and polymyositis. Drug hypersensitivity reactions affecting the heart (hypersensitivity myocarditis) may occur with exposure to a wide range of agents; such reactions typically are benign and only in rare circumstances lead to CHF or sudden death. Morphology

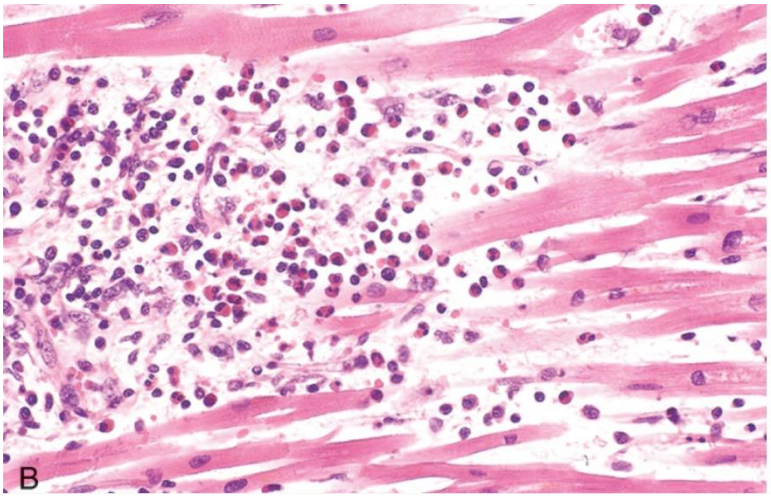
In acute myocarditis, the heart may appear normal or dilated; in advanced stages, the myocardium typically is flabby and often mottled with pale and hemorrhagic areas. Mural thrombi may be present.

Microscopically, myocarditis is characterized by edema, interstitial inflammatory infiltrates, and myocyte injury. A *diffuse lymphocytic infiltrate is most common, although the inflammatory involvement is often patchy and can be "missed" on endomyocardial biopsy. If the patient survives the acute phase of myocarditis, lesions may resolve without significant sequelae or heal by progressive fibrosis.*

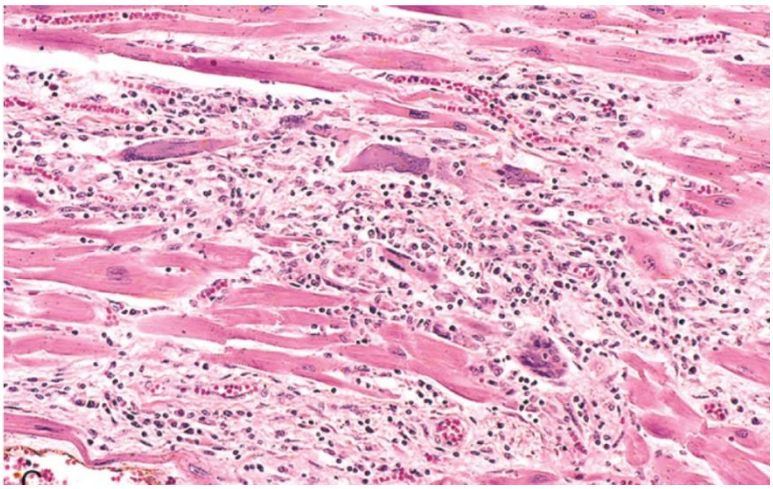
Myocarditis types consist of: (A) Lymphocytic myocarditis, with edema and associated myocyte injury. (B) Hypersensitivity myocarditis, characterized by perivascular eosinophil-rich inflammatory infiltrates. (C) Giant cell myocarditis, with lymphocyte and macrophage infiltrates, extensive myocyte damage, and multinucleate giant cells. (D) Chagas myocarditis. A myofiber distended with trypanosomes (arrow) is present, along with mononuclear inflammation and myofiber necrosis.



The above is an example of Lymphocytic myocarditis. One can see the muscle cells alons with a proliferation of stained lymphocytes.



The above is hypersensitivity myocarditis which characterized by perivascular eosinophil-rich inflammatory infiltrates. The eosinophils permeate the tissues and dramatically reduce the structure of the myocardial cells.



An example of the Giant cell myocarditis is shown above , with lymphocyte and macrophage infiltrates, extensive myocyte damage, and multinucleate giant cells.

In hypersensitivity myocarditis, interstitial and perivascular infiltrates are composed of lymphocytes, macrophages, and a high proportion of eosinophils. Giant cell myocarditis is a morphologically distinctive entity characterized by widespread inflammatory cell infiltrates containing multinucleate giant cells (formed by macrophage fusion). Giant cell myocarditis probably represents the aggressive end of the spectrum of lymphocytic myocarditis, and there is at least focal—and frequently extensive—necrosis. This variant carries a poor prognosis.

Chagas myocarditis is characterized by the parasitization of scattered myofibers by trypanosomes accompanied by an inflammatory infiltrate of neutrophils, lymphocytes, macrophages, and occasional eosinophils.

The clinical spectrum of myocarditis is broad; at one end, the disease is asymptomatic, and patients recover without sequelae. At the other extreme is the precipitous onset of heart failure or arrhythmias, occasionally with sudden death. *Between these extremes are many levels of involvement associated with a variety of signs and symptoms, including fatigue, dyspnea, palpitations, pain, and fever. The clinical features of myocarditis can mimic those o*f acute MI. *Clinical progression from myocarditis to DCM occasionally is seen.*

Acute myocarditis does present the possibility of sudden death. There has been several recent examples of young patients recovering from COVID who have demonstrated severe myocardial stress and upon examination demonstrated continuing acute myocarditis as well as thickening of the myocardium most likely the result of fibrotic change.

6.2 Clinical Presentation

It is worth a brief discussion of clinical presentation. As Chasouraki et al note:

Increases in troponin I and CK-MB, which are suggestive of myocardial injury, are frequent findings in COVID 19 disease and associated with adverse prognosis. Huang et al. reported that 31% of patients with COVID-19 hospitalised in ICU had an increase in troponin I compared to 4% of non-ICU patients. In a meta-analysis, including 341 patients, levels of troponin I were significantly increased in critically ill patients as opposed to those with milder illness

The authors then discuss putative mechanisms:

1. Direct injury through connection to ACE2 receptors that are expressed in the myocardium.

2. Indirect injury due to the systemic inflammatory response syndrome (SIRS) and the cytokine storm that the infection provokes.

3. Infection-induced vasculitis attributed either to contamination of the endothelial cells or to immunological response. Binding to ACE2 receptors is the entrance point of SARSCoV-2.

These receptors are expressed, among others, in the epithelial cells of lungs, heart and enterocytes. These same receptors were also found to be the entrance point of SARS-CoV. Both viruses seem to be able to modulate the ACE2 myocardial and pulmonary pathways, leading to inflammation of the heart, pulmonary oedema and acute respiratory failure.

Myocardial inflammation in SARS-CoV infection can also be mediated by macrophages entering the myocardial tissue and producing cytokines*… Previous experience with SARS-CoV and current reports of COVID-19 infection mandate a high level of clinical suspicion for cardiovascular involvement, myocardial injury and possibly acute myocarditis. Diagnosis of acute myocarditis as a result of COVID-19 may be challenging for clinicians.*

High levels of troponin I and NT-BNP along with ECG abnormalities and the appropriate clinical context should raise suspicion.

Cardiac magnetic resonance has proven to be a useful diagnostic tool in acute myocarditis cases.

So far, most patients have favourable outcomes but the limited data cannot allow us to draw safe conclusions.

Chowdhury et al note:

Viral myocarditis is believed to be caused by a combination of direct T cell injury and T lymphocyte mediated cardiotoxicity. *In another cohort study from Germany, the presence of SARS-CoV-2 was examined in myocardial tissue during autopsy from 39 individuals with documented infection. The virus was detected in 24 patients (61.5%), and a viral load above 1000 copies/µg RNA was documented in 16 cases (41%). Interestingly, there was no increase in inflammatory cell infiltrate, but the expression of six pro-inflammatory genes was upregulated in this population. Though the long-term consequences remain unknown at this time due to the novelty of COVID-19, the high incidence of cardiac involvement, including myocarditis, will put*

patients who recovered from COVID-19 infection at elevated risk for the development of heart failure in the future, as seen with other viral causes of myocarditis.

The SARS-CoV-2 virus uses the angiotensin converting enzyme 2 (ACE2) receptors to gain access to the cell cytoplasm. ACE2 receptors are expressed on the cell surface in most organs, including the lungs, heart, kidneys, pericytes and the vascular endothelium. Viral invasion of pericytes and vascular endothelium has been shown to initiate localized inflammation, ultimately provoking microvascular dysfunction. In addition, the cytokine storm associated with COVID-19 infection (caused by interleukins-6 & 8) may cause platelet activation, neutrophil recruitment and blood hyperviscosity. This cytokine storm, in addition to the direct viral invasion of the myocardium, is responsible for the variable cardiac presentation of COVID-19 infection that may range from minor biomarker elevation to acute cardiogenic shock.

We also note the interaction of the NK cells and the adaptive T cells responses. The NK cells continue to have a positive effect on ongoing T cell actions.

6.3 CAUSATIVE AGENTS

From Cooper we have:

Viral and postviral myocarditis remain major causes of acute and chronic dilated cardiomyopathy. Seroepidemiologic and molecular studies linked coxsackievirus B to outbreaks of myocarditis from the 1950s through the 1990s. The spectrum of viruses that were detected in endomyocardial biopsy samples shifted from coxsackievirus B to adenovirus in the late 1990s and, in the past 5 years, to parvovirus B19 and other viruses, according to reports from the United States and Germany. In Japan and in a serologic study of myocarditis in the United States, hepatitis C virus was also linked to myocarditis and dilated cardiomyopathy. Many other viruses have also been associated less frequently with myocarditis; these viruses include Epstein–Barr virus, cytomegalovirus, and human herpesvirus 6.

The large number of observations that link viruses with myocarditis have led to ongoing treatment trials of antiviral therapy in patients with virus-associated cardiomyopathy. In addition to viruses, certain other infectious causes of myocarditis should be considered in patients with acute or chronic cardiomyopathy. Myocarditis can result from infection with Borrelia burgdorferi (Lyme disease), and patients with myocarditis due to Lyme disease are occasionally coinfected with ehrlichia or babesia. Lyme myocarditis should be suspected in patients with a history of travel to regions where the disease is endemic or of a tick bite, particularly if they also have atrioventricular conduction abnormalities.

In areas of rural Central and South America, Trypanosoma cruzi infection can present as acute myocarditis or chronic cardiomyopathy, sometimes with right bundle-branch block or left anterior fascicular block. In this disorder, echocardiography or contrast ventriculography may reveal a left ventricular apical aneurysm, regional wallmotion abnormalities, or diffuse cardiomyopathy. Regional wall-motion abnormalities or perfusion defects that are not in the distribution of a coronary artery may also be seen in noninfectious disorders, such as cardiac sarcoidosis and arrhythmogenic right ventricular cardiomyopathy or dysplasia. Myocarditis is

the most common cardiac pathological finding at autopsy of patients infected with the human immunodeficiency virus (HIV), with a prevalence of 50% or more.

Cardiomyopathy in patients with HIV infection may be caused by an inhibition of cardiac contractility by HIV type 1 glycoprotein 120, coinfections, or antiviral medications. Drug-induced hypersensitivity reactions and systemic hypereosinophilic syndromes can cause a specific myocarditis that often responds to withdrawal of the offending agent or to treatment of the underlying disorder, though adjuvant corticosteroid therapy is often required. Numerous medications, including some anticonvulsants, antibiotics, and antipsychotics, have been implicated in hypersensitivity myocarditis.

Eosinophilic myocarditis is characterized by a predominantly eosinophilic infiltrate in the myocardium and may occur in association with systemic diseases, such as the hypereosinophilic syndrome, the Churg– Strauss syndrome, Löffler's endomyocardial fibrosis, cancer, and parasitic, helminthic, or protozoal infections. Eosinophilic myocarditis has been reported after vaccination for several diseases, including smallpox. Clinical manifestations of eosinophilic myocarditis include congestive heart failure, endocardial and valvular fibrosis, and endocardial thrombi. A rare disorder, acute necrotizing eosinophilic myocarditis is an aggressive form of eosinophilic myocarditis with an acute onset and a high death rate.

6.4 TREATMENT

We can now examine some of the treatment modalities for myocarditis. As Cooper notes:

Patients who present with myocarditis with acute dilated cardiomyopathy should be treated according to the current guidelines of the American Heart Association, the American College of Cardiology, the European Society of Cardiology, and the Heart Failure Society of America. The mainstay of therapy for acute myocarditis is supportive therapy for left ventricular dysfunction.

Most patients will improve with a standard heartfailure regimen that includes the administration of angiotensin-converting–enzyme inhibitors or angiotensin-receptor blockers, beta-blockers such as metoprolol and carvedilol, and diuretics, if needed. In patients whose condition deteriorates despite optimal medical management, case series suggest a role for mechanical circulatory support, such as ventricular assist devices or extracorporeal membrane oxygenation, as a bridge to transplantation or recovery. The overall rate of survival after cardiac transplantation for myocarditis is similar to that for other causes of cardiac failure.104 Since no clinical trials of therapy for heart failure have been conducted specifically in patients with myocarditis, the only relevant studies describe animal models.

Patients recovering from acute myocarditis should refrain from aerobic activity for a period of months after the clinical onset of the disease, based on studies in rodents with myocarditis in which increased death rates were associated with sustained exercise. The reintroduction of aerobic activities somewhat depends on the severity of left ventricular dysfunction and the extent of recovery. The use of candesartan improved survival in a murine model of viral myocarditis (60%, vs. 18% with no candesartan treatment).

The use of carteolol, a nonselective beta-blocker, improved histopathological results and reduced wall thickness in coxsackievirus B myocarditis. The use of nonsteroidal antiinflammatory drugs was associated with increased mortality. Taken together, these data support the application of the current heart-failure guidelines to patients with heart failure from myocarditis. In patients with acute myocarditis, therapy for arrhythmias is also supportive, since such arrhythmias usually resolve after the acute phase of the disease, which can last several weeks.

The 2006 guidelines of the American Heart Association, the American College of Cardiology, and the European Society of Cardiology recommend that arrhythmias be managed conventionally in patients with myocarditis. However, in acute myocarditis, temporary pacemakers may be required for patients with symptomatic bradycardia or complete heart block. Patients with symptomatic or sustained ventricular arrhythmias may need amiodarone and possibly an implantable cardioverter–defibrillator, even if active inflammation is still present. The prognostic importance and treatment of nonsustained ventricular arrhythmias in acute myocarditis have not been systematically evaluated.

7 OBSERVATIONS

We can now make several observations regarding the sequellae.

7.1 CYTOKINE STORM

COVID-19 appears to assert an aggressive immune response in multiple organs. The immune response results in the release of massive amounts of cytokines and thus results in damage to multiple organs. One could assume that this is in many ways akin to the cytokine storm reaction. As Fajgenbaum and June have noted however:

No single definition of cytokine storm or the cytokine release syndrome is widely accepted, and there is disagreement about how these disorders differ from an appropriate inflammatory response. The National Cancer Institute's definition, based on the Common Terminology Criteria for Adverse Events (CTCAE), is too broad, since the criteria for an inflammatory syndrome can also apply to other physiological states, and the definition of the American Society for Transplantation and Cellular Therapy is based on criteria that focus too specifically on iatrogenic causes of cytokine storm alone.

Although cytokine storm is easy to identify in disorders with elevated cytokine levels in the absence of pathogens, the line between a normal and a dysregulated response to a severe infection is blurry, especially considering that certain cytokines may be both helpful in controlling an infection and harmful to the host. The interdependence of these inflammatory mediators further complicates the distinction between a normal and a dysregulated response.

7.2 OTHER ORGANS

As we have noted, myocarditis is but one example of COVID-19 sequellae. The key questions are related to how the virus enters the organs. We understand that the spike protein attaches to epithelial cell ACE receptors. In endothelial cells and in myocardial cells the surface receptors appear to be more limited and thus one wonders if there is a second mechanism for entry.

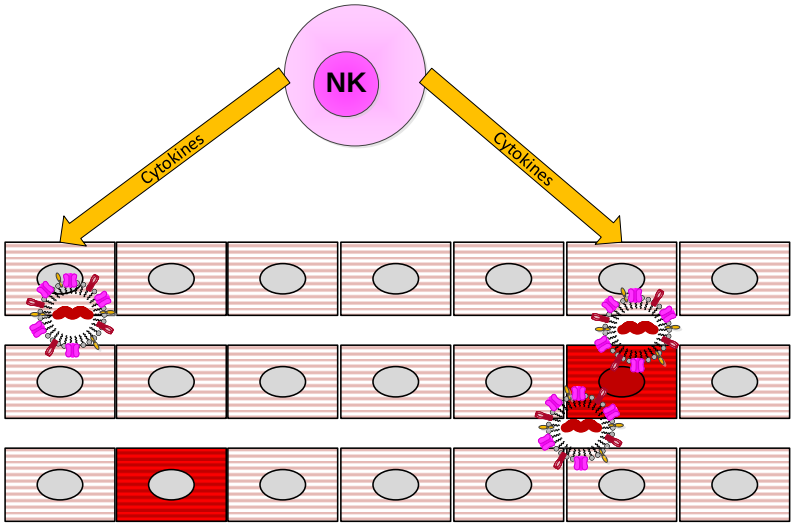
The myocarditis example is just one of many and recognition of its presence is generally not anticipated unless the patient presents with substantial symptoms[13].

In Kindermann et al they demonstrate the impact in the acute phase of cytokines on the development of myocarditis as follows:

[13] See https://www.tennessean.com/story/sports/college/vanderbilt/2020/12/07/vanderbilt-womens-basketball-player-covid-19-myocarditis-demi-washington-out-season/6484351002/ also https://www.cbsnews.com/news/keyontae-johnson-collapse-florida/ It appears that myocarditis is not as uncommon as suspected. Its presence appears when the patients heart is stressed. https://www.acc.org/latest-in-cardiology/articles/2020/11/20/15/13/cardiac-imaging-in-athletes-diagnosed-with-covid-19

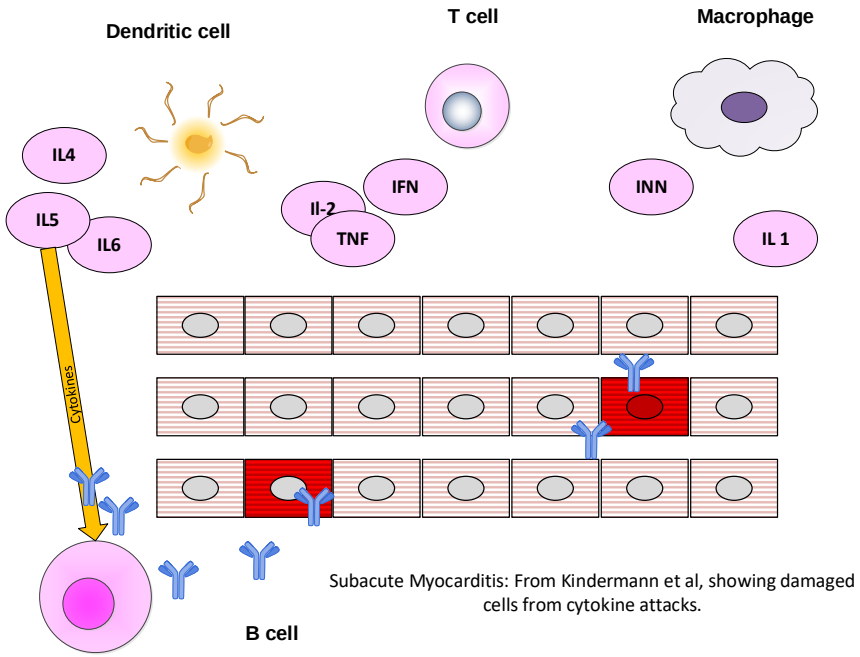
The acute phase of myocarditis takes only a few days. After the acute phase of virus-induced injury, the second phase is characterized by (auto)immune reactions. This subacute phase, which covers few weeks to several months, is defined by activated virus-specific T lymphocytes, which may target the host's organs by molecular mimicry. ***Cytokine activation (tumor necrosis factor alpha, interleukin [IL]-1 and -6) and antibodies to viral and cardiac proteins may aggravate cardiac damage and cause impairment of the contractile function. In most patients with myocarditis, immune response declines with virus elimination, and left ventricular (LV) function recovers without sequelae.*** *However, in some murine models and probably in patients, (auto)immune processes persist independently of detection of virus genome in the myocardium and lead to the chronic phase, which is characterized by myocardial remodeling and development of DCM*

We demonstrate this below showing the criticality of the NK cells.



Acute Myocarditis: From Kindermann et al, showing damaged cells from cytokine attacks.

In contrast the subacute attack appears as follows:



Subacute Myocarditis: From Kindermann et al, showing damaged cells from cytokine attacks.

Note that we have suppressed the lasting effects of the NK cells which we have seen can still produce their own cytokines. All of these cytokines continue to damage the myocardial cells.

7.3 Herd Immunity

There has been a great deal of talk about herd immunity. Some of the anti-VAX folks say that we should just all get exposed and then there will be no problem. I think that is what some say. Now there are problems here.

First, and this is serious, is the issue of multi-organ infection. For example, we are seeing, especially amongst young males, myocarditis. This is an infection of the heart muscles. Acute infections result in lots of natural killer cells, NK, and inflammation. Chronic infection results in replacement of the killed heart muscle cells with fibroblasts, creating fibrin patches like a heart attack. Namely the infection with COVID becomes a diffuse heart attack, slowly, and unknowingly debilitating your heart. We have seen cases amongst basketball players where they just drop in the court while playing. This post infection immune reaction can also affect the liver, kidney, brain. Slow but debilitating.

Second, one must remember that the vaccine stops the infection from proliferating and causing the above. However it does not prevent the infection. In fact one can be vaccinated, not get sick but be a "mini carrier". A Typhoid Mary is you will. That means those not vaccinated subject themselves to infection. Thus there is no herd immunity, only mitigation for those vaccinated. Cute little virus.

Third, we are starting to see mutations. The classic flu mutates on an annual basis and the annual flu shot addresses the best guess approach. If COVID continues to mutate then we expect that we may very well have to establish a similar annual approach. This is not unusual but expected. The

problem, of course, is that we may not have a simple spike protein to attack it with. Also we need an international monitoring system that works, not like the last bout with the grossly incompetent CDC and WHO.

This is not the beginning of the end. It is the beginning of a new approach to highly infectious diseases. We need global monitoring, Government actions, rapid testing, and vaccine production that is efficient and effective. It is the cost of being a global society. Also we may very well be facing a second pandemic of debilitating sequellae as noted above.

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9 APPENDIX TABLES

Protein	Description
GATA3	This gene encodes a protein which belongs to the GATA family of transcription factors. The protein contains two GATA-type zinc fingers and is an important regulator of T-cell development and plays an important role in endothelial cell biology. Defects in this gene are the cause of hypoparathyroidism with sensorineural deafness and renal dysplasia
LEF	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.
TCF	The protein encoded by this gene is a nuclear transcription factor which binds DNA as a homodimer. The encoded protein controls the expression of several genes, including hepatocyte nuclear factor 1 alpha, a transcription factor which regulates the expression of several hepatic genes. This gene may play a role in development of the liver, kidney, and intestines. Mutations in this gene have been associated with monogenic autosomal dominant non-insulin-dependent diabetes mellitus type I. Alternative splicing of this gene results in multiple transcript variants encoding several different isoforms
PU1	This gene encodes an ETS-domain transcription factor that activates gene expression during myeloid and B-lymphoid cell development. The nuclear protein binds to a purine-rich sequence known as the PU-box found near the promoters of target genes, and regulates their expression in coordination with other transcription factors and cofactors. The protein can also regulate alternative splicing of target genes. Multiple transcript variants encoding different isoforms have been found for this gene.
CEBP	This intronless gene encodes a transcription factor that contains a basic leucine zipper (bZIP) domain and recognizes the CCAAT motif in the promoters of target genes. The encoded protein functions in homodimers and also heterodimers with CCAAT/enhancer-binding proteins beta and gamma. Activity of this protein can modulate the expression of genes involved in cell cycle regulation as well as in body weight homeostasis. Mutation of this gene is associated with acute myeloid leukemia. The use of alternative in-frame non-AUG (GUG) and AUG start codons results in protein isoforms with different lengths. Differential translation initiation is mediated by an out-of-frame, upstream open reading frame which is located between the GUG and the first AUG start codons.

E2A	This gene encodes a member of the E protein (class I) family of helix-loop-helix transcription factors. E proteins activate transcription by binding to regulatory E-box sequences on target genes as heterodimers or homodimers, and are inhibited by heterodimerization with inhibitor of DNA-binding (class IV) helix-loop-helix proteins. E proteins play a critical role in lymphopoiesis, and the encoded protein is required for B and T lymphocyte development. Deletion of this gene or diminished activity of the encoded protein may play a role in lymphoid malignancies. This gene is also involved in several chromosomal translocations that are associated with lymphoid malignancies including pre-B-cell acute lymphoblastic leukemia (t(1;19), with PBX1), childhood leukemia (t(19;19), with TFPT) and acute leukemia (t(12;19), with ZNF384). Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene, and a pseudogene of this gene is located on the short arm of chromosome 9
ID2	The protein encoded by this gene belongs to the inhibitor of DNA binding family, members of which are transcriptional regulators that contain a helix-loop-helix (HLH) domain but not a basic domain. Members of the inhibitor of DNA binding family inhibit the functions of basic helix-loop-helix transcription factors in a dominant-negative manner by suppressing their heterodimerization partners through the HLH domains. This protein may play a role in negatively regulating cell differentiation. A pseudogene of this gene is located on chromosome 3
ID3	The protein encoded by this gene is a helix-loop-helix (HLH) protein that can form heterodimers with other HLH proteins. However, the encoded protein lacks a basic DNA-binding domain and therefore inhibits the DNA binding of any HLH protein with which it interacts.
MITF	The protein encoded by this gene is a transcription factor that contains both basic helix-loop-helix and leucine zipper structural features. The encoded protein regulates melanocyte development and is responsible for pigment cell-specific transcription of the melanogenesis enzyme genes. Heterozygous mutations in the this gene cause auditory-pigmentary syndromes, such as Waardenburg syndrome type 2 and Tietz syndrome.
MEF	This gene encodes a protein, also known as pyrin or marenostrin, that is an important modulator of innate immunity. Mutations in this gene are associated with Mediterranean fever, a hereditary periodic fever syndrome
SHP1	The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. N-terminal part of this PTP contains two tandem Src homolog (SH2) domains, which act as protein phospho-tyrosine binding domains, and mediate the interaction of this PTP with its substrates. This PTP is expressed primarily in hematopoietic cells, and functions as an important regulator of multiple signaling pathways in hematopoietic cells. This PTP has been shown to interact with, and dephosphorylate a wide spectrum of phospho-proteins involved in

	hematopoietic cell signaling. Multiple alternatively spliced variants of this gene, which encode distinct isoforms, have been reported.

Organisms	Representative.	Phagocytosis.	Neutrophils	Complement	NK Cells.
Viruses (intracellular. cytoplasmic)	Influenza virus				
	Mumps virus				
	Morbillivirus (measles, rubeola)				
	Rhinovirus				
Bacteria (intracellular)	*Listeria monocytogenes*				
	Legionella spp.				
	Mycobacteria				
	Rickettsia				
Bacteria (extracellular)	*Staphylococcus* spp.				
	Streptococcus spp.				
	Neisseria spp.				
	Salmonella typhi				
Protozoa (intracellular)	*Plasmodium malariae*				
	L. donovani				
Protozoa (extracellular)	*Entamoeba histolytica*				
	Giardia lamblie				
Fungi (extracellular)	*Candida* spp.				
	Histoplasma				
	Cryptococcus				

Mediator	*Main Cell Source*	*Type and Function*
Cytokines and growth factors		
Interleukin· 1	Macrophages, epithelial cells; pyroptotic cells	Proinflammatory alarmin cytokine; pyrogenic function, macrophage and Thl7cell activation
lnterleukin·2	T cells	Effector T-cell and regulatory T-cell growth factor
lnterleukin-6	Macrophages. T cells, endothelial cells	Proinflammatory cytokine: pyrogenic function, increased antibody production, induction of acute-phase reactants
lnterleukin·9	Th9 cells	Protection from helminth infections, activation of mast cells, association with type 1 interferon in Covid-19ˈ
lnterleukin-10	Regulatory T cells. Th9 cells	Antiinflammatory cytokine: inhibition of Thl cells and cytokine release
Interleukin-12	Dendritic cells, macrophages	Activation of the fhl pathway; induction of interferon-y from Thl cells. CTLs. and NK cells; acting in synergy with interleukin-lᵎ
Interleukin· 17	Thl7 cells. NK cells, group 3 innate lymphoid cells	Promoting neutrophilic inflammation, protection from bacterial and fungal infections
lnterleukin-18	Monocytes, macrophages, dendritic cells	Proinflammatory alarmin cytokine; activation of Thl pathway, acting in synergy witli interleukin-12
lnterleukin-33	Macrophages, dendritic cells, mast cells, epithelial cells	Proinflammatory alarmin cytokine; amplification of Thl and Th2 cells, activation of NK cells. CTLs. and mast cells
lnterferon-y	Thl cells. CTLs. group 1 innate lymphoid cells, and NK cells	Proinflammatory cytokine; activation of macrophages
Tumor necrosis factor	Macrophages. T cells. NK cells, mast cells	Increasing vascular permeability; pyrogenic function
GM-CSF	Thl7 cells	Proinflammatory cytokine
VEGF	Macrophages	Angiogenesis
Chemokines		
Interleukin-8 (CXCL8)	Macrophages, epithelial cells	Recruitment of neutrophils
MIG (CXCL9)	Monocytes, endothelial cells, keratinocytes	Interferon-inducible chemokine; recruitment of Till cells, NK cells, plasmacytoid dendritic cells
IP-10 (CXCL10)	Monocytes, endothelial cells, keratinocytes	Interferon-inducible chemokine: recruitment of macrophages. Thl cells. NK cells
MCP-1 (CCL2)	Macrophages, dendritic cells, cardiac myocytes	Recruitment of Th2 cells, monocytes, dendritic cells, basophils
MIP-lcr (CCL3)	Monocytes, neutrophils, dendritic cells. NK cells, mast cells	Recruitment of macrophages. Till cells. NK cells, eosinopliils. dendritic cells; pyrogenic function
MIP-10 (CCL4)	Macrophages, neutrophils, endothelium	Recruitment of macrophages. Thl cells. NK cells, dendritic cells
BLC (CXCL13)	B cells, follicular dendritic cells	Recruitment of B cells. CD4 T cells, dendritic cellsᵎ
Plasma Proteins		
CRP	Hepatocytes	Monomeric CRP increases interleukin-S and MCP-1 secretion: interfeukin-6 increases CRP expression

Mediator	*Main Cell Source*	*Type and Function*
Complement	Hepatocytes, other cells	Complement activation contributes to tissue damage in cytokine storm; complement inhibition can reduce immunopathologic effects of cytokine storm
Ferritin	Ubiquitous	Primary site of iron storage in cells

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