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Purple Paper

The Role of Cytokine Storm in Influenza Pathogenesis

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Key Points

- An aberrant host immune response (immunopathology) is the main cause of pandemic influenza–related deaths.
- As part of the normal host immune response to bacterial and viral infections, cells of the immune system release chemical messengers (cytokines), which control and coordinate host immune responses to invading pathogens.
- An unbalanced cytokine response (cytokine storm) can lead to damage of the vascular barrier resulting in tissue edema, capillary leakage, multiple organ failure and death.
- There is no singular mechanism when it comes to inducing cytokine storm with respect to pandemic influenza strains.
- Inhibition of individual cytokines involved in cytokine storm leads to decreased pathology of influenza infection but also impairs viral clearance.
- Treatment options should focus on the overall cytokine imbalance and associated immunopathology rather than using a more targeted approach.

The Concept of Immunopathology

The immune system has many mechanisms capable of causing damage to a variety of pathogens, as well as our own cells and tissues. In turn, an exquisite degree of specificity and a complex network of redundant, overlapping immune regulation have evolved to ensure that the damage is done to a threatening invader whether that be a virus, bacterium, fungus, or cancerous cell and not the host's normal cells and tissues. However, this regulation can fail, as demonstrated by autoimmune conditions such as lupus and diabetes, where the immune system inadvertently attacks host tissues. Another example of specificity and regulation gone awry are infectious diseases where much of the damage to the host is caused by "immunopathology": aberrant responses of the host's own immune system stimulated by the infecting agent. One such agent is the influenza virus. While normal antiviral immunity always requires the activation of inflammatory pathways by the innate and adaptive immune systems, this inflammation can also be a potent weapon that can cause severe disease if it becomes uncontrolled. This review focuses on one particular type of cytokine response, called a "cytokine storm", which has been associated with three major influenza viruses – the pandemic 1918-19 Spanish H1N1 influenza, H5N1 avian influenza and the pandemic H1N1 influenza of 2009.

The Role of Cytokines in the Immune System

Cytokines are proteins that act as chemical messengers, sending signals between many types of cells and tissues. These molecules play an essential role in almost every immunological process, such as white blood cell trafficking, activation, regulation, survival, viral clearance and cell death. Cytokines are secreted by a diverse array of cells, such as macrophages, neutrophils, epithelial cells, and T lymphocytes, usually in response to an invading pathogen. Frequently, cytokines are divided into pro-inflammatory and anti-inflammatory cytokines and the balance between the two can be an important determinant of the outcome of infection.[1] Pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN) -γ, serve to recruit cells to the site of infection, increase expression of factors that increase cell-to-cell adherence and mediate direct antiviral effects. The up-regulation of anti-

inflammatory cytokines, such as IL-10 and transforming growth factor (TGF) -β, also occurs during infection with the purpose of regulating the extent of inflammation. This and other forms of regulation are critical to contain excessive inflammation that can cause more harm than good. However, during some infections and pathological conditions, regulation fails, and an unbalanced cytokine response called a cytokine "storm" can develops, leading to uncontrolled inflammation and increased morbidity and/or mortality for the host.

Cytokines Storms and Associated Sequelae

A cytokine storm, or hypercytokinemia, has been defined as "a sudden surge in the circulating levels of pro-inflammatory cytokines, such as IL-1, IL-6, TNF, and IFN-γ."[2] There are several known causes of cytokine storms, including non-infectious causes. It has been speculated that molecules called superantigens/superagonists allow for excessive receptor cross-linking and subsequent stimulation of the inflammatory response.[3] The most famous example was a superagonist monoclonal antibody to CD28, which appeared to be a promising treatment for B cell chronic lymphocytic leukemia in preclinical studies.[3] Administration of this monoclonal antibody led to rapid induction of pro-inflammatory cytokines (within 90 minutes), followed by the early appearance of headache, nausea, myalgia and other symptoms. Within 12 to 24 hrs, all patients became critically ill, displaying signs of renal failure, disseminated coagulation and pulmonary infiltrates. All of them required ventilation, plasma infusion, steroids and other therapeutic strategies, and they recovered in two to four weeks. Though catastrophic for the patients involved, this trial serves as one of the better examples of the dynamics of a severe cytokine storm in humans.

A number of other circumstances can also lead to cytokine storms, including those that occur during infections (discussed below). Common to most cytokine storms is that the release of cytokines results in an influx of macrophages, neutrophils and T cells from the peripheral blood into the tissue where the storm occurs.[4] As noted above, these cytokine storms have destructive effects on host physiology, leading to destabilization of endothelial cell-to-cell interactions, damage of the vascular barrier resulting in tissue edema, capillary leakage, multiple organ failure and ultimately death.[5] Important characteristics of this type of immune response are positive feedback loops, whereby the presence of inflammation rapidly drives further inflammation. While this "ramping up" is a necessary biological process to combat infections, in the absence of regulation, the effects can be devastating.

Acute respiratory distress syndrome (ARDS)…is a major cause of mortality in pandemic influenza. Although the exact mechanism of ARDS is not fully understood, the production of inflammatory cytokines is considered to be one of the main contributing factors.

The Immune Response to Influenza

Influenza virus can infect a wide range of animals although the site of infection differs depending on the species. Influenza virus predominately causes a respiratory infection in mammals including humans and pigs. There are many different types and strains of influenza. The major surface glycoproteins of the virus that induce protective host antibody responses are called hemagglutinin (HA) and neuraminidase (NA). When a new strain emerges, often with different HA and NA molecules, populations may not have pre-existing immunity, and the possibility of an influenza pandemic increases. For example, individuals with cross-reactive antibodies to the 2009 pandemic H1N1 virus were primarily over the age of 30.[6] This is most likely because older individuals had been exposed to that particular strain of influenza previously and therefore had protective antibodies remaining from that exposure. A similar mechanism has been proposed as an explanation for the lower mortality observed in elderly during the 1918-19 Spanish influenza pandemic. In that pandemic, peak mortality was

observed in individuals between the ages of 20 and 40 years.[7]

Influenza mainly targets epithelial cells of the respiratory tract, but also infects alveolar macrophages. Immunity to influenza is highly complex, involving both innate and adaptive or acquired (antibody and T cell) arms of the host response. Innate immunity provides immediate protection against invading pathogens. When activated by microbial components, the cells of the innate immune system (monocytes, natural killer cells, etc.) release multiple cytokines that can have direct inhibitory effects on the virus or act to recruit additional immune cells to the site of infection. Antibody responses are the main correlates of protection for influenza, while cell-mediated ($CD4^+$ and $CD8^+$ T cells) responses play an important role in successfully clearing the virus. Production of pro-inflammatory cytokines by epithelial and immune cells increases vascular permeability, allowing the additional cells of the immune system to pass through the endothelial barrier and reach the infected tissue. Continuous viral replication leads to a constant influx of immune cells into the site of infection and increased production of cytokines. Without control mechanisms, this positive feedback loop between cytokines and immune cells results in hypercytokinemia, ultimately causing severe damage to the host.

Cytokine Storms in Pandemic Influenza

Why certain strains of influenza emerge to cause acute pandemics has long been a topic of debate, and the reason for it is likely due to many factors. Some of these include the prevailing social conditions and state of public health (both thought to play a role in the spread of "Spanish flu" in 1918-19), virulence factors of particular influenza strains (including cell tropism),[8] the presence of pre-existing humoral immunity in a given population,[9] and host response factors. Risk factors for seasonal influenza are usually characterized by underlying conditions such as immunosuppression, and therefore are more common in populations such as the elderly. In the case of 2009 pH1N1, however, almost half of patients who were hospitalized or died had no underlying medical conditions, and pregnant women seemed to be at particularly high risk.[10] Situations where seemingly healthy individuals are infected and succumb to a novel pandemic influenza strain are obvious causes for major clinical and public health concern.

Although strong pre-existing antibody responses with specificity to seasonal influenza strains were present in [patients aged 20 to 50 years], the antibodies did not bind the [2009 pandemic] H1N1 virus effectively, leading the immune complex formation and severe inflammation in the lungs.

Evidence from research involving monkeys suggests that the severity of the early inflammatory response is the key to distinguishing pandemic influenza strains from regular circulating strains.[11] During serious influenza infection, it is believed that massive epithelial cell infection in the respiratory tract causes apoptosis (programmed cell death) and necrotic death, triggering the overproduction of pro-inflammatory cytokines.[2, 3, 12] Acute respiratory distress syndrome (ARDS) leading to low oxygen levels in the blood is a major cause of mortality in pandemic influenza. Although the exact mechanism of ARDS is not fully understood, the production of inflammatory cytokines is considered to be one of the main contributing factors.[13] Studies in mice with 1918 influenza have shown significantly increased amounts of more than 10 inflammatory cytokines and chemokines in the lungs.[7, 14] Recent research in which monkeys were infected with 1918 influenza showed that IL-6 was up-regulated 6- to 19-fold by day six of infection. However, in the same study, IFN-α genes, as well as other pathways needed to activate antiviral responses, were drastically reduced as compared to the control virus.[15] This suggests that type I IFNs, such as IFN-α, could be important in viral clearance and lethal infection.[15] H5N1 and 1918 pandemic influenza viruses are associated with excessive and early macrophage and neutrophil recruitment to the lungs leading to

increased cytokine production (IL-1β, TNFα, IFN-γ, etc).[16] H5N1 virus has been shown to infect cells of the innate immune system including dendritic cells (DCs) and natural killer (NK) cells; this may also contribute to the viral dissemination and impaired immune response.[17] When compared to human H1N1, H5N1 viruses are more potent inducers of pro-inflammatory cytokines in primary human respiratory epithelial cells, and this hyperinduction of cytokines is likely to contribute to the disease severity of H5N1.[18]

It is important to emphasize that it is not the absence or the presence of either pro- or anti-inflammatory cytokines, but rather the balance between the two that determines the outcome of the infection.

Specific mutations in proteins produced by influenza have also been linked to the propensity for induction of cytokine storms. All influenza strains contain the protein NS1, which is known to attenuate type I IFN responses, leading to influenza viruses with increased capacities for replication. A study of the NS1 gene from the avian H5N1 strain demonstrated that a novel mutation, as compared to seasonal influenza strains, drastically increases this strain's resistance to antiviral cytokines [19]. Novel mutations like these could contribute to the ability of pandemic strains to cause a lethal cytokine storm. A decreased antiviral response leads to increased viral replication and tissue damage, altering the cytokine balance causing cytokine storm. These findings suggest a potential association between cytokine storms and the attributes of certain strains of influenza viruses.

Defects in host immunity can also lead to cytokine storms. A recent study aimed to explain why the 2009 H1N1 influenza was so deadly to relatively healthy people between the ages of 20 and 50, while it was apparently less virulent in the young and elderly.[20] Although strong pre-existing antibody responses with specificity to seasonal influenza strains were present in these patients, the antibodies did not bind the H1N1 virus effectively, leading to immune complex formation and severe inflammation in the lungs. Since the young were less likely to have acquired these antibodies, and the elderly would have more specific antibodies to the pandemic H1N1 strain, these groups were less prone to this complication. Based on histological findings in the lungs of 1957 pandemic influenza casualties, this pathology may be relevant to more than one pandemic. Therefore, high titres of non-protective antibodies and immune complex-induced inflammation might be one explanation for increased pro-inflammatory cytokines associated with pandemic influenza strains and cytokine storm.

Possible Therapeutic Avenues

Antiviral therapies that directly target the virus (including NA inhibitors: oseltamivir and zanamivir, and M2 inhibitors: amantadine and rimantadine) are currently the main form of influenza treatment. Viral resistance to M2 inhibitors is widespread among seasonal and pandemic influenza strains, while oseltamivir resistance is mainly observed in seasonal influenza and is not very common in pandemic influenza strains.[21] The success of oseltamivir and zanamivir lies in the fact that most influenza viruses have difficulty altering the NA site that they target, making viral escape difficult. However, a low number of oseltamivir-resistant pandemic (H1N1) 2009 as well as H5N1 viruses have been reported in several countries.[21, 22] Infection with oseltamivir-resistant H5N1 in Vietnamese patients resulted in death despite early initiation of treatment.[23] This raises concern over high rates of viral resistance to currently available drugs and points to the need for strengthening available treatment options,[24] including therapy that targets the host immune response. Anti-inflammatory agents that dampen the cytokine responses during influenza infection have been shown to decrease morbidity and mortality in influenza-infected mice.[25, 26] Antiviral therapy in combination with immunomodulatory treatment reduced the mortality in mice infected with H5N1 virus.[27] There are a number of anti-inflammatory treatments used to treat autoimmunity, including TNF blockers,

which may be of use in a cytokine storm situation during acute influenza infection.[28] One important caveat to this approach that needs to be addressed is that any suppression of immune activation can also dampen the responses required to clear the infection.

Multiple studies have addressed the question of whether inhibition of inflammatory cytokines could successfully reduce morbidity and mortality associated with influenza infection. Mice lacking IL-6 demonstrated significantly decreased pathology after influenza infection as well as delayed viral clearance, indicating both positive and negative effects.[29, 30, 31] Similar results were obtained from studies with other pro-inflammatory cytokines (including IFN-γ, IL-1α and IL-1β), supporting the conclusion that blocking cytokines involved in influenza-induced cytokine storms may reduce immunopathology but will also impair efficient viral clearance.[32] These studies highlight the inherent challenges to developing treatments that selectively down-regulate harmful host responses without interfering with the beneficial immune responses needed to clear the viral infection.

A recent study has challenged the idea that cytokine storms are the major cause of influenza-induced pathology by showing that inhibition of pro-inflammatory cytokines such as TNFα, IL-6 or CCL2 or administration of glucocorticoids (cytokine suppression) does not protect mice against lethal H5N1 virus.[33] These data indicate that the early inhibition of viral replication, by drugs that target influenza virus directly, may be more promising than the inhibition of cytokine responses. It is important to emphasize that it is not the absence or the presence of either pro- or anti-inflammatory cytokines, but rather the balance between the two that determines the outcome of the infection. Based on current evidence, treatment with immune modulators, such as corticosteroids, has not been associated with decreased mortality and morbidity in H5N1 outbreaks in Asia, and such treatment is not recommended by the World Health Organization.[22]

Recently, London *et al* demonstrated a way of combating deleterious aspects of cytokine storm by strengthening the host vascular structure through activation of specific signalling pathway.[34] This approach resulted in decreased endothelial permeability in the lung, less severe lung pathology and increased survival in mice exposed to H1N1. Enhancing vascular stability, as opposed to blocking each individual cytokine that contributes to cytokine storm, could be a more practical therapeutic approach. Because this therapy is targeting symptoms and not a particular pathway, it could potentially be used to treat a variety of conditions including sepsis, ARDS, rheumatoid arthritis and a variety of other human diseases. However, one drawback of this treatment is that it needs to be administered very early in order to be effective, otherwise the vascular damage can become too great to be repaired. Clearly, more research is required before many of these immunomodulatory concepts can move into the clinic.

Despite an increase in influenza literature over the last two years, it is still unclear what causes the severe morbidity and mortality associated with pandemic influenza strains. One potential mechanism is the induction of a cytokine storm and an imbalance of protective *versus* pathogenic immune responses. Since cytokine storms are likely to arise through various mechanisms, treatment options should focus on the overall cytokine imbalance and associated immunopathology rather than targeting specific components of the storm. While it is clear that more work is needed to fully understand the pathogenicity of pandemic influenza viruses and correlations of cytokine storms to disease severity, ongoing efforts in this area could lead to better therapeutic approaches to combat the next influenza pandemic.

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