

# Into the Eye of the Cytokine Storm

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## INTRODUCTION

The term “cytokine storm” calls up vivid images of an immune system gone awry and an inflammatory response flaring out of control (Fig. 1). The term has captured the attention of the public and the scientific community alike and is increasingly being used in both the popular media and the scientific literature. However, while the general concept of an excessive or uncontrolled release of proinflammatory cytokines is well known, an actual definition of what constitutes a cytokine storm is lacking. Furthermore, there is not a good understanding of the molecular events that precipitate a cytokine storm, of the contribution such a “storm” makes to pathogenesis, or of what therapeutic strategies might be used to prevent the storm or quell it once it has started.

Although the concept certainly predates the coining of the term, the first use of “cytokine storm” appears to be in an article published in 1993 on graft-versus-host disease (47). The use of the term in infectious disease research began in early 2000 in reports on cytomegalovirus (6), Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (70), group A streptococcus (15), influenza virus (154), variola virus (71), and severe acute respiratory syndrome coronavirus (SARS-CoV) (63). The term appears to have first been applied in the context of avian H5N1 influenza virus infection in 2005 (155), after which it began to appear more frequently in the scientific literature. Public interest in “bird flu” also brought the term cytokine storm into the popu-

lar media. A recent Google search for cytokine storm yielded 323,000 hits; over 170,000 were within the past year alone.

Cytokine storms are associated with a wide variety of infectious and noninfectious diseases and have even been the unfortunate consequence of attempts at therapeutic intervention (136). Previous reviews have centered on the advent of the concept (29) or its role in graft-versus-host disease (46), multiple sclerosis (89), pancreatitis (94), or multiple organ dysfunction syndrome (145). Though the term was not explicitly stated, recent reviews have addressed potential cellular and molecular mechanisms contributing to the cytokine storm in viral disease (66, 81), some of which specifically focused on influenza (114, 115). In this review, we focus on the cytokine storm in the context of infection, with particular emphasis on respiratory viruses. We also highlight how high-throughput genomic methods are revealing new insights into the cytokine storm. These methods are especially useful for obtaining global views of the complex and intertwined molecular events that attend the upregulation of multiple cytokines.

Our goals in this review are to better define the concept of a

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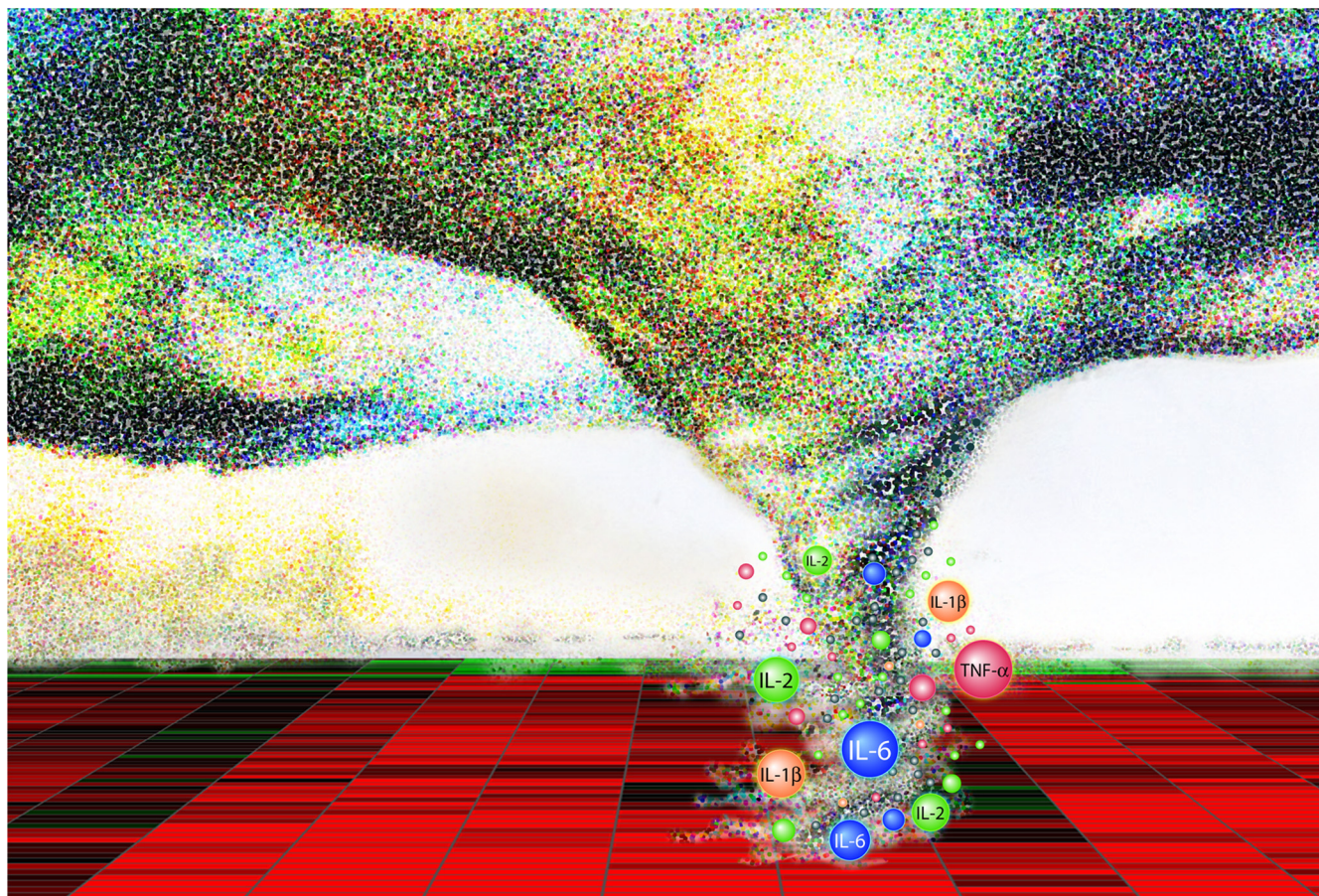


FIG 1 Imagery of a cytokine storm.

cytokine storm and the biological consequences of cytokine overproduction. We will also look at the cytokine storm through the lens of genomics, which is revealing the importance of the kinetics of cytokine gene expression and the remarkable degree of redundancy and overlap in cytokine signaling. Finally, we will address evidence for and against the role of the cytokine storm in the pathology of clinical and infectious disease and discuss why it has been so difficult to use knowledge of the cytokine storm and immunomodulatory therapies to improve the clinical outcomes for patients with severe acute infections.

## CYTOKINES

Cytokines are a diverse group of small proteins that are secreted by cells for the purpose of intercellular signaling and communication. Specific cytokines have autocrine, paracrine, and/or endocrine activity and, through receptor binding, can elicit a variety of responses, depending upon the cytokine and the target cell. Among the many functions of cytokines are the control of cell proliferation and differentiation and the regulation of angiogenesis and immune and inflammatory responses (Table 1).

Many cytokines have multiple and sometimes unrelated functions that depend on the target cell or on the presence or absence of other cytokines. Some have limited sequence similarity and engage distinct receptors yet transduce signals through common intracellular pathways (e.g., type I and type III interferons [IFNs]). In part because of this diversity of structure and function, the

classification and naming of cytokines have been a challenge. The complex network of the cytokine response is best considered a series of overlapping networks, each with a degree of redundancy and with alternate pathways. This combination of overlap and redundancy has important implications with respect to identifying the key steps in the cytokine response to infection and in targeting specific cytokines for therapeutic intervention. While many infections are characterized by broadly similar cytokine profiles, their clinical presentations can be quite different. Prior to delving into a discussion of cytokine storms, it is worth taking a brief look at the cytokines at the heart of the cytokine storm.

TABLE 1 Major types and actions of cytokines

Type	Actions
Interferons	Regulation of innate immunity, activation of antiviral properties, antiproliferative effects
Interleukins	Growth and differentiation of leukocytes; many are proinflammatory
Chemokines	Control of chemotaxis, leukocyte recruitment; many are proinflammatory
Colony-stimulating factors	Stimulation of hematopoietic progenitor cell proliferation and differentiation
Tumor necrosis factor	Proinflammatory, activates cytotoxic T lymphocytes



## Cytokines Associated with the Cytokine Storm

**Interferons.** The interferons (IFNs) are a family of cytokines that play a central role in innate immunity to viruses and other microbial pathogens (45, 75). They are classified into three major types (types I, II, and III) on the basis of their receptor specificity. Type I IFNs (IFN- $\alpha$  and IFN- $\beta$ ) signal through a heterodimeric receptor complex, IFNAR1/IFNAR2, whereas type II IFN (IFN- $\gamma$ ) signals through IFN- $\gamma$ R1/IFN- $\gamma$ R2. Lambda IFNs are a new class of IFN with antiviral properties (127), protecting mice against influenza A virus (106). IFN- $\lambda$ 1, - $\lambda$ 2, and - $\lambda$ 3 (also referred to as interleukin-29 [IL-29], IL-28a, and IL-28b) bind receptor complex IL-28R/IL-10R $\beta$  yet are functionally similar to type I IFNs in that both transduce signals through the Jak-STAT signaling pathway. Receptor binding results in the initiation of downstream signaling cascades, the result of which is the activation of transcription factors and the induction of hundreds of IFN-stimulated genes. These genes encode protein products with antiviral, anti-proliferative, or immunomodulatory properties. Such effects have led to the therapeutic use of IFNs (often in combination with other drugs) in the treatment of viral diseases such as hepatitis C and hepatitis B, certain types of leukemia and lymphoma, and multiple sclerosis (17, 51).

**Interleukins.** In contrast to the IFNs, the interleukins are a diverse family of immune system regulators that function primarily in immune cell differentiation and activation. They may be either pro- or anti-inflammatory and, like all cytokines, elicit a wide variety of responses. The interleukin designation was originally coined to refer to cytokines produced by leukocytes that function in intercellular communication; however, interleukins are now known to be produced by a wide variety of cell types. Although a common nomenclature system has been adopted, the designation and naming of interleukins continue to be confusing (18). IL-1, for example, is actually a family of cytokines encoded by 11 genes (132). As new functions are elucidated for some IL-1 family members, new interleukin designations have been proposed (40).

IL-1 $\alpha$  and IL-1 $\beta$  are proinflammatory cytokines that mediate the host response to infection through both direct and indirect mechanisms (41). Among their biological functions, these cytokines increase acute-phase signaling, trafficking of immune cells to the site of primary infection, epithelial cell activation, and secondary cytokine production. Inflammasomes are cytosolic macromolecular complexes comprised of members of the nucleotide-binding domain and leucine-rich-repeat-containing receptor (NLR) family (the NLRP3 inflammasome is one the best characterized) that produce IL-1 $\beta$  and IL-18 in defense against pathogens, and their activity is regulated by type I IFN (57). The acute-phase response to infection results in a wide range of local effects and systemic alterations that are evidenced in changes that are generally proinflammatory, such as the increase in specific cytokine production, and which can be linked to viral clearance, such as increases in complement (53). IL-1 receptor signaling is responsible for acute lung immunopathology but increases the survival of mice infected with influenza virus by enhancing IgM antibody responses and recruiting CD4<sup>+</sup> T cells to the site of infection (124).

**Chemokines.** The largest family of cytokines is the chemokines, with 44 members (and increasing) that bind to one or more of 21 G-protein-coupled receptors (33). These small secreted proteins are classified into four types (CXC, CC, C, and CX<sub>3</sub>C), depending

upon the spacing of their first two cysteine residues. Chemokines function as chemoattractants to control the migration of cells, particularly those of the immune system, and contribute to such diverse processes as embryogenesis, innate and adaptive immune system development and function, and cancer metastasis (119). For example, differential expression of IFN- $\gamma$ -regulated CXCR3 ligands, CXCL9, CXCL10, and CXCL11, appears to regulate T-cell function and infiltration in the periphery during inflammation (reviewed in reference 56). The majority of chemokines are considered to be proinflammatory, and they are released by a variety of cells in response to virus (or other microbial) infection. The release of proinflammatory chemokines results in the recruitment of immune system cells (neutrophils, monocytes/macrophages, and lymphocytes) to the site of infection. Whereas most cytokines have pleiotropic effects, chemokine recruitment of immune cells can be highly selective for specific cell types. For example, CXCL8 (IL-8), CCL2 (monocyte chemoattractant protein 1 [MCP-1]), and CCL11 (eotaxin) are major chemoattractant factors for neutrophil, monocyte, and eosinophil immune cells, respectively. Chemokines and their receptors have been heavily targeted by the pharmaceutical industry, but with limited success (54).

**CSFs.** Colony-stimulating factors (CSFs), such as granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and granulocyte colony-stimulating factor (G-CSF), stimulate hematopoietic progenitor cell proliferation and differentiation. Colony-stimulating factors are also associated with inflammation, and there is evidence that these factors may be part of a mutually dependent proinflammatory cytokine network that includes IL-1 and tumor necrosis factor (TNF) (58). It is thought that by functioning to increase the number of cytokine-producing macrophages at a site of inflammation, colony-stimulating factors may be part of an amplification cascade that serves to perpetuate inflammatory reactions.

**TNFs.** Tumor necrosis factor (TNF) is perhaps the best known and most intensely studied of the proinflammatory cytokines, and it plays a prominent role in the cytokine storm literature. The name “tumor necrosis factor” was first used in 1975 for a cytotoxic serum factor capable of inducing tumor regression in mice (23), which soon thereafter was reported to play a role in the pathogenesis of malaria and sepsis (14, 30, 31). TNF is now considered a central cytokine in acute viral diseases, including those caused by influenza virus, dengue virus, and Ebola virus. TNF is expressed by a variety of immune cells, and its primary receptor, TNFR1, appears to be expressed by all cell types, ensuring widespread effects of this cytokine. The pleiotropic effects of TNF are further amplified by the existence of a superfamily of TNF proteins that consists of 19 members that signal through 29 receptors (2). Excess TNF production is associated with a number of chronic inflammatory and autoimmune diseases, and TNF inhibitors have been approved for the treatment of inflammatory bowel disease, psoriasis, and rheumatoid arthritis (79, 126). In contrast, the use of TNF inhibitors for the treatment of sepsis has not been successful (49), possibly due to the early release and short circulating half-life of the cytokine (30).

## Cytokine Dynamics

We still do not understand the complex nature of the immune response and have probably underestimated its dynamic nature during acute infection. For example, while TNF promotes IL-1 generation, inducing changes in endothelial cell physiology within

the local microenvironment (109), increased TNF can also exert broad systemic effects beyond the site of infection. An experimental study of rabbits with severe *Pseudomonas* pneumonia and systemic sepsis provided definitive evidence that TNF instilled into the lungs could pass into the systemic circulation, providing direct communication between the lungs and the bloodstream (80). Nevertheless, we have relied for too long on clinical studies using intermittent sampling from one compartment (typically the peripheral blood), although many of the critical responses are likely to be far more localized in tissue. For instance, in severe infections affecting the deep tissues of the respiratory tract (as has been described in avian influenza or severe primary influenza virus infections), it is probable that it is the immunological cascade that takes place directly in the deep tissues that is crucial to immunopathology rather than what can be measured as a “spillover” in the peripheral blood (28, 82, 128). It is important to also consider the compartmentalization of tissue-specific microenvironments. For example, within the lung, the respiratory epithelium and alveolar macrophages normally maintain homeostasis by limiting activation of innate immunity in airways and alveolar spaces (61). In mice, lymphoid tissue around airways also can dampen initial responses to viral challenge but can be induced to provide efficient stimulation of cellular and humoral immune responses with more severe influenza virus infection (107). Similar compartmentalization is likely to be important in infections of the central nervous system (i.e., bacterial and tuberculous meningitis, encephalitis, and fungal infections) and perhaps in more subtle ways in infections such as dengue, in which the clinical syndrome is dominated by capillary permeability and plasma leakage (130, 131).

To date, most studies have focused on direct measurements of a few cytokines and chemokines in the peripheral blood compartment and have failed to interrogate the whole of the immune cascade in the context of the infecting pathogen and the rapidly changing immune environment in tissues. Interestingly, while the peripheral blood may not provide an accurate picture of the cytokine profiles in a tissue, in the lungs, the location of the initial infection does not seem to be a determinant of the severity of local and systemic cytokine storms. For example, influenza viruses infect and destroy the ciliated epithelial cells of the conducting airways, whereas SARS-CoV infects type II pneumocytes in the alveolar walls and hantavirus particles infect microvascular endothelial cells in the alveolar walls, yet all can lead to indistinguishable clinical syndromes of acute lung injury (ALI) with respiratory failure, sepsis, and a cytokine storm. Bacterial exotoxins, such as the *Pseudomonas* type III exotoxin and staphylococcal enterotoxins, are directly lytic to alveolar cells and destroy the gas exchange parenchyma of the lungs (48, 80, 112). Although bacterial, viral, and fungal products drive inflammation in the lungs via interactions with surface Toll-like receptors (TLR) and intracellular receptors for DNA and RNA (NOD-like and RIG-I-like receptors), endogenous products such as oxidized phospholipids and matrix breakdown products also drive inflammation through TLR4 and probably other TLRs, providing a mechanism for perpetuating inflammation when microbial products are being cleared from the lungs (68, 72). Mitochondrial membrane proteins and cellular ATP also have been implicated in driving endogenous inflammatory responses (156).

## Regulation of Pro- and Anti-Inflammatory Cytokines

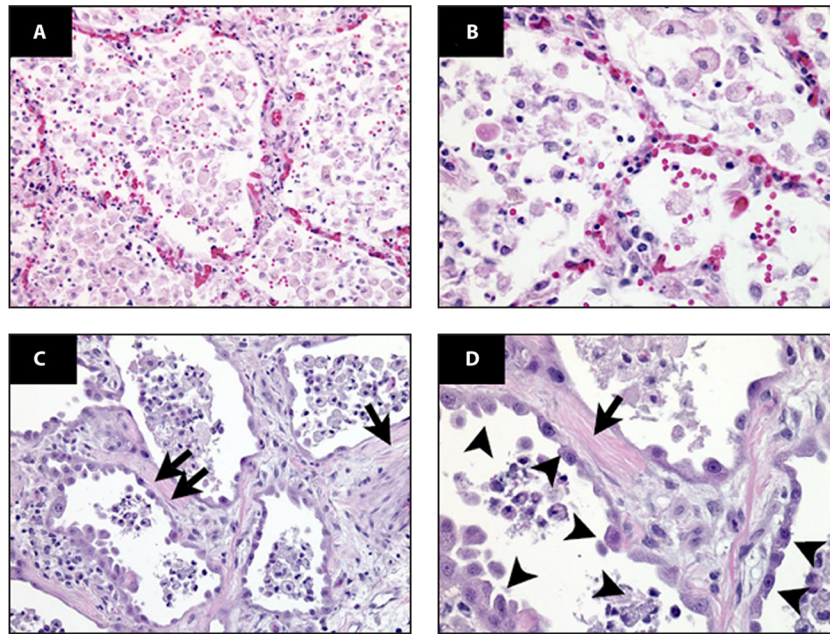
The intensity of the inflammatory response in the lungs reflects a balance between proinflammatory cytokines (e.g., TNF and IL-1 $\beta$ ) and their cognate soluble receptors or inhibitors (TNFR1, TNFR2, and IL-1RA), which inhibit the activity of these inflammatory cytokines in the aqueous phase of alveolar fluid (113). One mechanism to dampen lung inflammation is by regulating the activation of specific cell types. For example, CD200R expression on alveolar macrophages helps resolve lung inflammation during influenza virus infection by restraining macrophage activity (133). Negative regulators, such as IL-1 receptor-associated kinase (IRAK-M), suppressor of cytokine signaling 1 (SOCS1), phosphoinositide-3-OH kinase (PI3K), Toll-interacting protein (TOLLIP), and zinc finger protein A20 (34), also help maintain innate immune processes by preventing aberrant TLR activation. The production of anti-inflammatory cytokines, mainly IL-10 by macrophages and certain types of T cells (Th2 and regulatory T cells) and B cells (105), represents another mechanism involved in regulating proinflammatory responses. Although IL-10 is most commonly recognized as an anti-inflammatory cytokine, recent evidence has linked IL-10 to a potential role in fibrosis, where increased IL-10 expression was reported to induce collagen production and fibrocyte recruitment into the lung (135). In contrast, interactions between IL-6 and its soluble receptor enhance the activity of IL-6 on target cells, providing a mechanism for enhancing the activity of TNF and IL-1 $\beta$  when the concentrations of soluble TNF receptors and IL-1RA are very high (113). As the balance of pro- and anti-inflammatory mechanisms is critical to maintaining lung immune homeostasis, it is conceivable that if one or more of these regulatory mechanisms are absent or aberrantly regulated, then the outcome may contribute toward a cytokine storm.

## THE CYTOKINE STORM

### Cytokine Storm Pathology

Inflammation associated with a cytokine storm begins at a local site and spreads throughout the body via the systemic circulation. Rubor (redness), tumor (swelling or edema), calor (heat), dolor (pain), and “functio laesa” (loss of function) are the hallmarks of acute inflammation. When localized in skin or other tissue, these responses increase blood flow, enable vascular leukocytes and plasma proteins to reach extravascular sites of injury, increase local temperatures (which is advantageous for host defense against bacterial infections), and generate pain, thereby warning the host of the local responses. These responses often occur at the expense of local organ function, particularly when tissue edema causes a rise in extravascular pressures and a reduction in tissue perfusion. Compensatory repair processes are initiated soon after inflammation begins, and in many cases the repair process completely restores tissue and organ function. When severe inflammation or the primary etiological agent triggering inflammation damages local tissue structures, healing occurs with fibrosis, which can result in persistent organ dysfunction.

Acute lung injury (ALI) is a common consequence of a cytokine storm in the lung alveolar environment and systemic circulation and is most commonly associated with suspected or proven infections in the lungs or other organs (121). In humans, ALI is characterized by an acute mononuclear/neutrophilic inflammatory response followed by a chronic fibroproliferative phase marked by



**FIG 2** Human ARDS. Photomicrographs from the lungs of 2 different patients with ARDS, stained with hematoxylin and eosin (H&E) are shown. The alveolar spaces are filled with a mixed mononuclear/neutrophilic infiltrate, the alveolar walls are thickened, and the septae are edematous. Note the presence of cellular debris and proteinaceous material in the air spaces (A [magnification,  $\times 200$ ] and B [magnification,  $\times 400$ ]). In later stages (C [magnification,  $\times 200$ ] and D [magnification,  $\times 400$ ]), there is a fibroproliferative response with collagen deposition in the alveolar walls (arrows). Note that the alveolar epithelium has been replaced with cuboidal cells (arrowheads). (Figure and legend reprinted from reference 96 with permission of the publisher.)

progressive collagen deposition in the lung (Fig. 2) (reviewed in reference 96). Pathogen-induced lung injury can progress into ALI or its more severe form, acute respiratory distress syndrome (ARDS), as seen with SARS-CoV and influenza virus infections. IL-1 $\beta$  is a key cytokine driving proinflammatory activity in bronchoalveolar lavage fluid of patients with lung injury (118). Intense inflammation in the lungs also can have other systemic effects on other organs, as the combination of severe HCl injury in the lungs and mechanical ventilation in rabbits leads to renal dysfunction and evidence of apoptosis in renal tubular epithelial cells (69).

The cytokine storm is best exemplified by severe lung infections, in which local inflammation spills over into the systemic circulation, producing systemic sepsis, as defined by persistent hypotension, hyper- or hypothermia, leukocytosis or leukopenia, and often thrombocytopenia (84). Viral, bacterial, and fungal pulmonary infections all cause the sepsis syndrome, and these etiological agents are difficult to differentiate on clinical grounds. In some cases, persistent tissue damage without severe microbial infection in the lungs also is associated with a cytokine storm and clinical manifestations that mimic sepsis syndrome. In addition to lung infections, the cytokine storm is a consequence of severe infections in the gastrointestinal tract, urinary tract, central nervous system, skin, joint spaces, and other sites.

Studies of patients with severe sepsis due to pulmonary or non-pulmonary infections show characteristic plasma cytokine profiles, which change over time. The acute-response cytokines TNF and IL-1 $\beta$  and the chemotactic cytokines IL-8 and MCP-1 appear in the early minutes to hours after infection, followed by a more sustained increase in IL-6. The anti-inflammatory cytokine IL-10 appears somewhat later, as the body attempts to control the acute systemic inflammatory response. Plasma samples from a labora-

tory worker who developed septic shock following the deliberate injection of a large amount of bacterial endotoxin (in an attempt to treat a recently diagnosed tumor) provided insight into this sequence of cytokines following entry of bacterial products into the systemic circulation (138). A similar picture appeared in six healthy volunteers treated with an activating antibody against CD28 during a phase 1 clinical trial (136). IL-6 concentrations in peripheral blood have been used to assess the intensity of systemic cytokine responses in patients with sepsis, because IL-6 production is stimulated by TNF and IL-1 $\beta$ , providing an integrated signal of these two early-response cytokines (1).

Systemic production of IL-10 following the onset of a cytokine storm is a marker of a counter-anti-inflammatory response that has been termed “immunoparalysis,” in that it is associated with downregulation of neutrophil and monocyte function in the systemic circulation (32, 42, 49). Downregulation of systemic inflammation might be conceptually beneficial in controlling systemic responses to local infections (108). However, it has been suggested that patients who survive the initial cytokine storm but subsequently die may be those who do not recover from immunoparalysis. Patients with persistent downregulation of HLA-DR (a marker of immunosuppression) on monocytes 3 to 4 days after the onset of severe sepsis and cytokine storm have a high mortality rate, suggesting a rationale for therapy to reverse immunosuppression under such circumstances (104).

### Host Susceptibility to the Cytokine Storm

One of the challenging clinical questions about the cytokine storm is why some individuals seem particularly susceptible yet others seem relatively resistant, and there has been a great deal of interest in identifying underlying genetic mechanisms (149). Recent stud-



ies have shown a vast amount of variability in the innate immune responses of healthy humans, as reflected by the intermediate phenotype of whole-blood cytokine responses to bacterial products (151). Hyper- and hyporesponders to bacterial products are identifiable in the healthy population, which is explainable in part by genetically determined differences in the structure and function of TLR receptors, particularly TLR1 (150). In a large population of septic patients, those with a single nucleotide polymorphism (SNP) marking a hyperfunctioning variant of TLR1 had increased organ dysfunction and morbidity from Gram-positive bacteremia (150). Other genetic polymorphisms also contribute to the severity of the host response in sepsis and the cytokine storm, but the TLR1 polymorphism has a particularly strong relationship to Gram-positive infections (149).

Variants of TLR4, the principal receptor for lipopolysaccharide (LPS), can predispose individuals to sepsis, as evidenced by increased markers of systemic inflammation, including C-reactive protein (CRP), LPS-binding protein, and peripheral leukocytes in healthy humans exposed to endotoxin (99). Genome-wide association studies (GWAS) have associated TLR4 polymorphisms with increased susceptibility to pathogens and severity of disease. For example, TLR4 Asp299Gly occurred at a high frequency in Ghanaian children with severe malaria (100), and the polymorphism was also associated with manifestation of malaria during pregnancy in Ghanaian women (101). A recent GWAS identified multiple polymorphisms in cytokine-inducible SRC homology 2 (SH2) domain protein (CISH), a SOCS family member that controls IL-2 signaling, that were associated with increased susceptibility to bacteremia, tuberculosis, and severe malaria in persons in Gambia, Hong Kong, Kenya, Malawi, and Vietnam (77). Several GWAS have also identified variants of IFN- $\lambda$ 3 that were associated with spontaneous resolution and successful treatment of hepatitis C virus (HCV) infection (55, 140). The potential of polyethylene glycol (PEG)-IFN- $\lambda$ 1 as a novel therapeutic in the treatment of HCV is currently being investigated, though we will likely become less reliant on PEG-IFN therapies with the development of new HCV drugs such as the protease inhibitor telaprevir.

Because genetics play an important role in differential disease phenotypes, new mouse resources such as the Collaborative Cross are being utilized to further investigate host genetics and the biological pathways involved in microbial control. The Collaborative Cross is a recombinant inbred mouse resource (5) designed to capture the genetic heterogeneity of the human population, supporting systems genetics and predictive biology. Studies investigating host responses to influenza among genetically distinct recombinant inbred mice, such as BXD RI lines derived from crosses between DBA/2J and C57BL/6J mice, have demonstrated increased transcriptional responses associated with inflammation and antiviral immunity in mice that are more highly susceptible to infection (3, 16). Future investigations will likely reveal underlying genetic variants influencing host responses that contribute toward a cytokine storm during infection.

## GENOMIC VIEWS OF THE CYTOKINE STORM

Functional genomics lends itself to a deeper understanding of infectious disease by encompassing both the pathogen and the host response. Microarray technologies provide a global view of gene expression changes induced by a variety of stimuli, enabling us to simultaneously profile tens of thousands of transcriptional changes from an organ or tissue compartment. The functional

associations among these gene expression patterns show perturbations in cellular signaling pathways and cellular networks, with the implication that their differential regulation may contribute toward the resolution of infection or, alternatively, have detrimental consequences leading to a fatal outcome. The compilation of cytokine and chemokine genomic data from influenza, SARS-CoV, and dengue studies provides important insight into our understanding of the cytokine storm. In particular, the dynamic transcriptional responses among the molecular components involved in cytokine and chemokine gene expression, including their kinetic properties and the timing of gene activation, are beginning to detail the events surrounding the cytokine storm.

## Cytokine Gene Expression Kinetics

The paradigm of “hit hard and hit early” popularized for the treatment of AIDS also appears to apply to the way H5N1 avian influenza virus causes tissue damage in human infections. The rapid and intense nature of the host inflammatory response is the suspected cause of severe lung damage (116). In a case study conducted in Ho Chi Minh City, Vietnam, comparing 18 people infected with H5N1 virus in 2004 and 2005 to 8 people infected with seasonal H1N1 influenza virus, elevated levels of MCP-1 (known as CCL2), IFN- $\gamma$ -inducible IP-10 (CXCL10), MIG protein (CXCL9), and IL-8 were observed in H5N1 virus-infected patients who progressed to severe lung injury (38). Over the past decade, compelling genomic evidence from animal model systems indicates that highly pathogenic influenza viruses aberrantly regulate cytokine and chemokine transcriptional responses, leading to a cytokine storm.

The first genomic study to analyze host transcriptional responses to the 1918 pandemic influenza virus was led by Kash and colleagues. Extensive lung damage in mice infected with 1918 virus was accompanied by highly upregulated cytokine and chemokine gene expression (73). Transcriptional activation of innate immune genes was observed as early as 1 day postinfection and remained sustained through the course of infection. These genomic data suggested that the host response enhances 1918 virus pathogenesis by initiating a cytokine storm that contributes to increased disease severity. An overly aggressive innate immune response marked by early expression of proinflammatory cytokine genes was also observed in 1918 virus-infected macaques (28). For example, strong upregulation of IL-6, IL-8, CCL2, and CCL5 cytokine and chemokine gene expression in the lungs was mirrored by elevated levels of IL-8, CCL2, and CCL5 in the sera of infected animals (78). Microarray analysis of lung tissue from macaques infected with H5N1 virus revealed prolonged expression of CCL2, CXCL10, and CXCL9 genes among a gene set of 45 significantly differentially expressed cytokine and chemokine genes (7). The strong interferon and inflammatory transcriptional responses early in infection combined with histopathologic findings for H5N1-infected type II pneumocytes likely account for irreversible lung damage caused by inflammation. IFN signaling appears to play a critical role in restricting highly pathogenic influenza viruses to the lung microenvironment, as IFNAR deficiency in mice results in dissemination of the 1918 virus to brain and spleen (27).

Direct comparison of transcriptional responses in infected macaque bronchi showed that these viruses induce similar IFN-regulated gene expression patterns, while genes associated with inflammation were significantly differentially regulated by 1918 and H5N1 viruses. Among the inflammatory response genes dif-



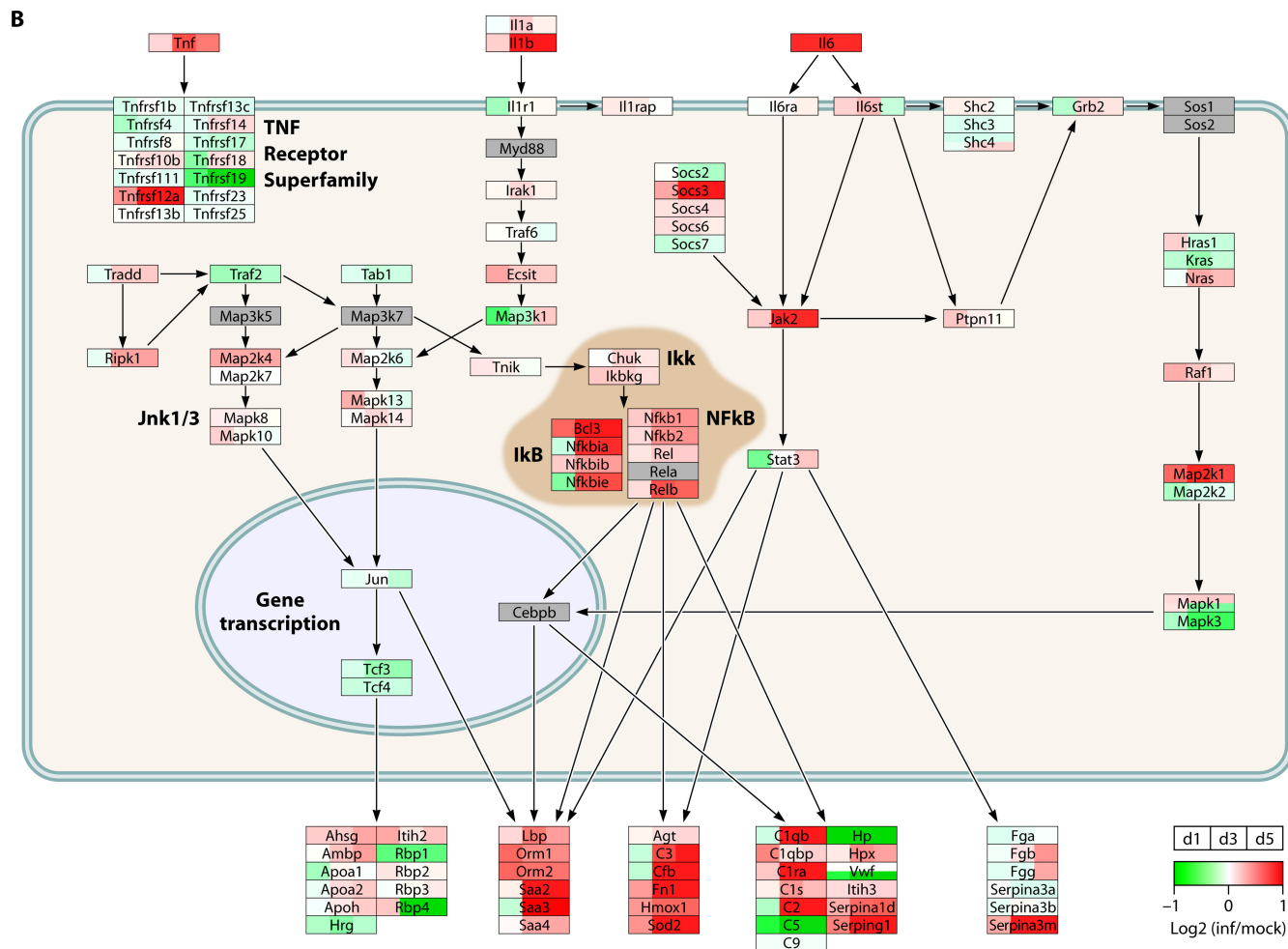


FIG 3 continued

gen recognition receptors (PRRs). As discussed above, there are several classes of cellular PRRs that activate and modulate innate immunity during infection, the specificity of which is determined by the recognized microbial motif, including TLRs that engage LPS, molecules derived from protozoan parasites, and viral RNA products (110, 152, 153), RLRs such as RIG-I, LGP2, and MDA5 (reviewed in reference 92), and NLRs such as NOD2 (122). RIG-I is critical for type I IFN transcription during viral infection (76). RIG-I-deficient cells show reduced expression of IFN- $\alpha$  and - $\beta$  genes as well as IFN-stimulated genes during influenza virus infection (91). In the absence of RIG-I signaling, mice fail to produce type I IFN in response to vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), and Sendai virus infection (74).

Infection with malarial parasites causes proinflammatory priming of TLR responses via TLR9 activation. *Plasmodium* infection initiates IL-12 and IFN- $\gamma$  production, which in turn enhances TLR expression and innate immune signaling pathways. The increased TLR expression is thought to lead to a stage of hyperresponsiveness to TLR agonists. This was observed in humans acutely infected with *Plasmodium falciparum*, resulting in enhanced activation of innate immune cells to TLR agonists. Genes involved in TLR signaling pathways for which enhanced expression was observed during *P. falciparum* infection included the

CD39, TLR4, TLR1, TLR8, and MYD88 genes. This phenomenon was also observed, in a TLR9/IL-12/IFN- $\gamma$ -dependent manner, in mice infected with *Plasmodium chabaudi* (50). Similarly, respiratory syncytial virus (RSV) infection results in increased TLR4 expression on airway epithelial cells that sensitizes cells to endotoxin by enhancing LPS-receptor engagement (103), and TLR4 variants may attenuate innate immune responses by disrupting RSV triggering of TLR4 surface expression (142). A hyperresponsive TLR state represents one mechanism by which a cytokine storm can be perpetuated.

### Differential Host Proinflammatory Responses

Swine are an important reservoir for influenza virus and have been linked to the emergence of some of the most notable historic and contemporary influenza pandemics, including the most recent swine-origin pandemic virus arising in 2009. It is not clearly understood why influenza viruses that are associated with severe or fatal infection in humans and nonhuman primates are generally nonfatal and cause less severe disease in swine (146). To understand the host response to influenza in pigs, Ma and colleagues recently profiled the gene expression in the lungs of pigs infected with recent H1N1 influenza viruses, a 2009 pandemic H1N1 human isolate, and a 2009 swine H1N1 virus and compared tran-



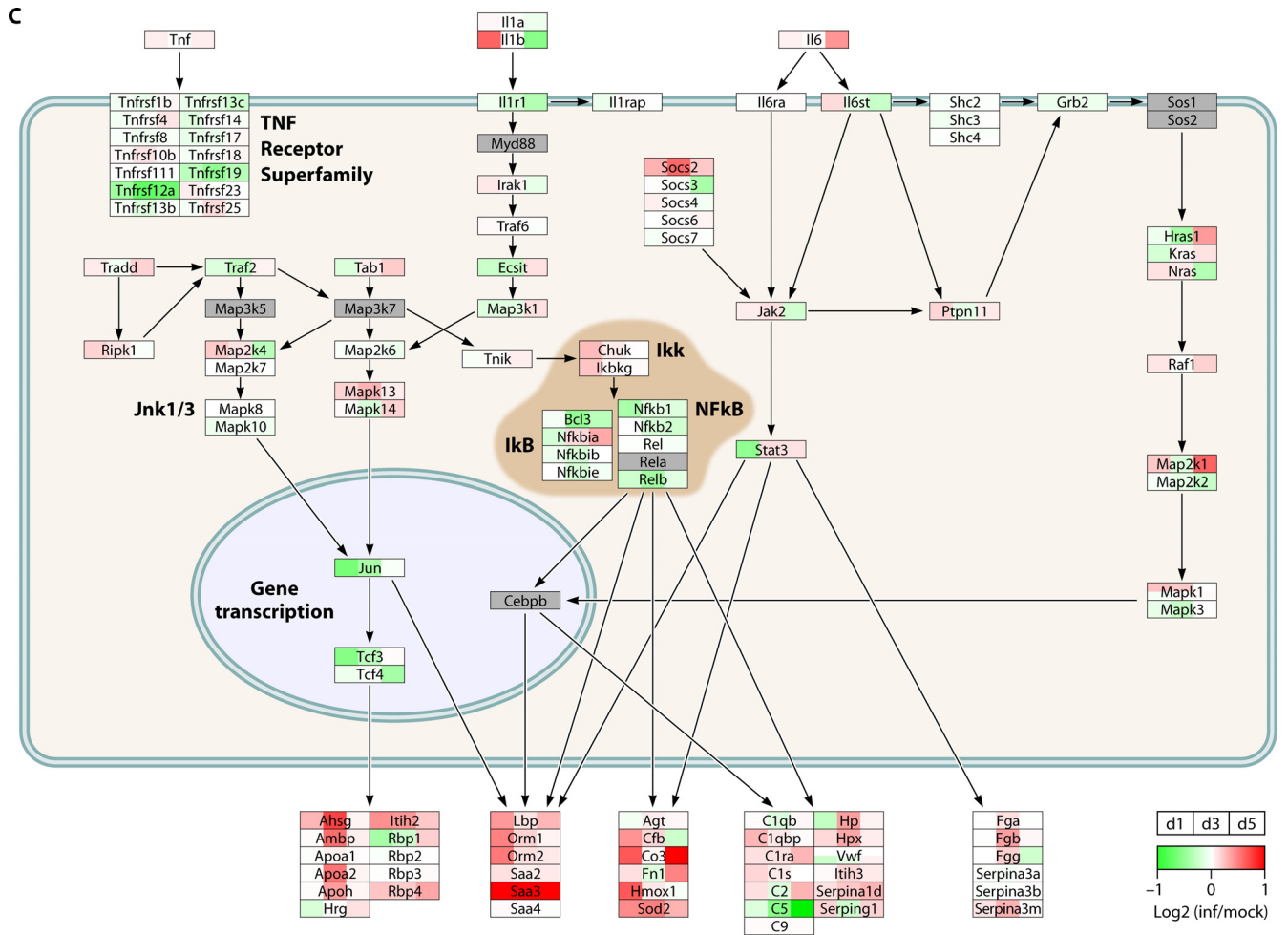


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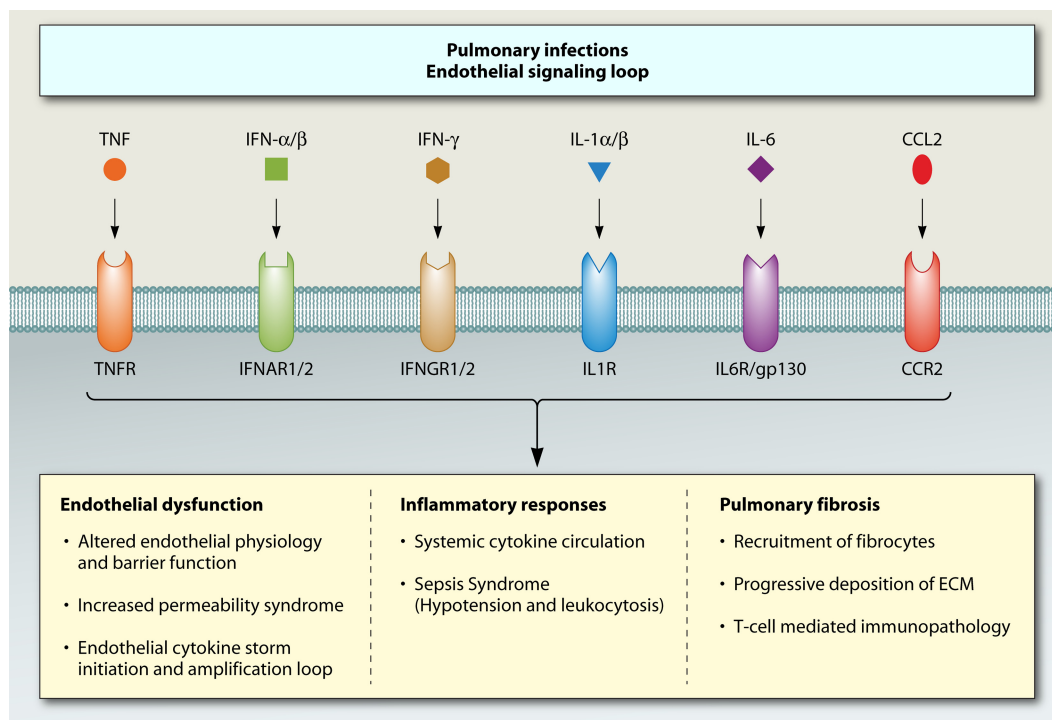
scriptional patterns with those of a historical isolate, A/swine/Iowa/1930, that causes mild clinical symptoms (93). Pigs infected with the contemporary viruses experienced a transient, early increase in immune response concurrent with greater clinical illness. This included greater expression of cytokines and chemokines such as TNF, TNFR, CXCL10, IFN- $\beta$ , IL-12 $\alpha$ , and IFN-related signaling molecules. In contrast to the case for lethal influenza virus infection in other animal models, the increased cytokine and chemokine gene expression resolved and all pigs recovered. The host lung environment regulating inflammatory responses to infection may be dependent on the species, with swine having a more controlled environment for otherwise pathogenic influenza viruses.

It is not clear why even though most human SOIV infections were mild, some individuals, particularly young, healthy adults, presented severe clinical symptoms. In a macaque infection model, clinically distinct Mexican isolates of SOIV showed an increase in IL-6, TNF, and IL-1 $\beta$  gene expression in the lungs and pronounced levels of IL-6 and MCP-1 in the plasma (123). The upregulation of these cytokines associated with the cytokine storm likely varies between pigs and macaques, potentially in the kinetics of PRR stimulation, allowing the pig to serve as a reservoir for influenza viruses with pathogenic potential. It appears that pigs

mount a protective proinflammatory response without tipping the scale to promote an adverse cytokine storm. Many of the pro-inflammatory cytokines contributing to the cytokine storm were observed to play a protective role against 2009 H1N1 pandemic influenza virus infection. Different influenza viruses may present agonists accounting for differential host proinflammatory responses to infection.

### Key Cytokine Storm Mediators

The functional role of cytokines during influenza virus infection has been pursued largely using single cytokine gene and cytokine receptor gene knockout (KO) mice, including those lacking IL-6 (137), IL-17RA (35), or CCR2 (39), or using KO mice lacking multiple cytokine receptor genes, such as IL-1R and TNFR (117). Transgenic mice with humanized immune systems present a greater opportunity to study cytokines in a more clinically relevant animal model. For example, IL-3 and GM-CSF knock-in mice (hIL-3/GM-CSF KI mice) have been used to study human innate immune responses in the lung and may provide a good resource in future cytokine storm investigations (148). The pivotal contribution of some cytokine storm mediators has been identified using KO mice, but the complete absence of a cytokine or its cognate receptor has also revealed compensation among



**FIG 4** Mediators of the cytokine storm and the associated phenotypes with infection outcome. The diagram shows the functional roles of key cytokines and chemokines and their cognate receptors in the development of the main clinical outcomes associated with the cytokine storm. The redundancy of the cytokine and chemokine signaling pathways is emphasized.

cytokine responses to infection. In a study led by Belisle and colleagues, IL-1R and TNFR KO mice infected with the 1918 virus showed a protective role for IL-1 $\alpha$ , while TNF exerted adverse cytokine storm effects. In the absence of TNF, mice showed an increase in survival that was associated with a decrease in IFN-signaling-related antiviral gene expression. The transcriptional response in IL1R KO mice during acute infection is strongly associated with an increase in TNF gene expression, revealing the interplay between IL-1 and TNF proinflammatory mediators (11). Cytokine signaling pathway redundancy is also observed with respect to type I IFN signaling, as there is strong upregulation of IFN-stimulated gene expression in IFN- $\alpha/\beta$ R KO mice infected with the 1918 virus (27). A combination of knockout mouse models and pharmacological intervention (administration of chemokine receptor small-molecule antagonists and/or cytokine antibodies) will likely provide greater insight into the cytokine storm and the redundancy that may exacerbate disease (Fig. 4). However, dampening an overactive proinflammatory response is not as straightforward as targeting the key cytokine mediators, since most chemokine receptors, for example, bind several ligands and contain small ligand binding pockets that have proved difficult to identify effective antagonists.

#### Strength of Chemoattractant Responses

SARS provides one example of chemokine responses that lead to a strong proinflammatory response in the lungs. One characteristic feature of SARS is the development of pulmonary fibrosis, observed with inflammation and hypercytokinemia. Atypical IFN-mediated immune responses suggest that type I IFN signaling may be important in the pathogenesis of SARS-CoV (21). Cameron

and colleagues (21) assessed gene signatures and protein profiles associated with nonsevere and severe clinical courses of SARS in humans with acute SARS-CoV infection. Gene expression signatures for type I and type II IFN responses in the lungs of SARS patients revealed persistent expression of CXCL10, CCL2, IFNAR1, IFNGR1, and CD58. High plasma levels of the IFN-stimulated chemokines CXCL10 and CCL2 were found in infected patients, and those who died from infection exhibited significantly higher levels of CXCL10 than those who recovered. In addition to playing a distinct role in the early clinical course and persistence of SARS in infected patients, differential chemokine expression is observed in viral lung disease caused by respiratory syncytial virus (RSV) (36). Genomic studies have revealed chemokine gene expression and regulation patterns that can impact Th1- and Th2-biased immunopathology.

Genomic studies of SARS-CoV infection in ferrets showed enhanced expression of chemokine ligands, in particular, CCL2, CCL4, CCL14, CCL19, and CCL25 (37). Upregulation of the fibrosis-associated chemokine CCL2 was shown to be mediated by ERK1/2 in human lung A549 epithelial cells incubated with SARS-CoV spike (S) protein or SARS virus-like particles (VLPs) (25). Characterization of a recombinant mouse-adapted SARS-CoV strain (rMA15) in STAT1 knockout mice revealed increased pathology in the absence of STAT1 signaling, including evidence of pulmonary fibrotic lesions (52). In particular, microarray profiling showed a skewing toward Th2 responses that was influenced by the absence of STAT1 signaling and likely accounted for the protein deposition in the lungs (158). Enhanced antiviral gene expression to SAR-CoV in IFNAR KO mice suggested that alter-

nate signaling pathways were compensating for the lack of type I IFN signaling through IFNAR, possibly Jak-STAT signaling activated by type III IFNs.

### Increased Capillary Permeability Syndrome

We have been discussing how cytokines are associated with increased inflammation. Another consequence of the cytokine storm in dengue virus infection is an increased capillary permeability syndrome. Dengue is an acute systemic viral illness caused by one of four serotypes of dengue virus. There are no animal models that mimic the clinical syndrome of severe dengue in humans, and therefore observational studies of patient cohorts are the main source of understanding the immunopathogenesis of dengue virus infections. Intriguingly, severe dengue occurs most commonly in individuals experiencing a second infection with a serotype distinct from that of a past exposure. In these so-called heterotypic infections, the host anamnestic immune response is postulated to mechanistically contribute to the syndrome of increased capillary permeability that characterizes severe dengue.

In the first few days of clinically apparent infection, there is an innate immune response in all patients. Gene expression studies have shown that type I IFN-inducible transcripts are the most prominent overabundant family of transcripts in blood leukocytes in the early acute phase of dengue relative to the late convalescence phase (60). Transcripts from pathways associated with signaling through TLRs, IL-27, IL-12, IFN- $\gamma$ , and the Jak-STAT pathway are also overabundant in the acute phase. Interconnecting these overabundant transcripts were transcription factors involved in IFN/NF- $\kappa$ B signaling responses, particularly STAT1, STAT2, STAT3, IRF7, IRF9, IRF1, CEBPB, and SPI1 (60, 90). Serum concentrations of IFN- $\alpha$ , IFN- $\gamma$ , IL-10, and TNF receptors are elevated in this early phase and remain elevated until defervescence occurs (86). Elevated concentrations of TNF-RII predict severe dengue in children (13). Gene expression analysis of primary human cells infected with dengue virus revealed strong upregulation of CCL8 and CXCL10, and these proteins were increased in infected patients during the febrile period of the disease (10).

The mechanism through which dengue virus elicited cytokines such as TNF might mediate endothelial dysfunction are not clear, though changes in the integrity of inter-endothelial cell junctions is a possible cause. Most permeability-inducing factors bind to endothelial cell plasma membrane receptors, activate heterotrimeric G proteins, and cause an increase in intracellular Ca<sup>2+</sup>. This results in myosin-driven endothelial contraction and opening of tight junctions. Other studies have suggested a role for vascular endothelial growth factor (VEGF) and its regulatory receptors in mediating capillary permeability. *In vivo*, plasma levels of free but not total VEGF-A are elevated in severely ill patients at the time of plasma leakage, while VEGFR2 levels are diminished (134). This, together with dengue virus-induced changes to endothelial cell VEGFR2 surface expression and responsiveness to VEGF stimulation, has led to the suggestion that the VEGF axis may be an important mechanism of plasma leakage in severe dengue.

Increased capillary permeability in dengue usually occurs between days 4 and 6 of illness, is transient, and only occasionally results in hypovolemic shock. At this stage of the illness, viremia is in steep decline and serum cytokine concentrations of IFN- $\gamma$  and IL-10 are at or near their peak levels (86). One hypothesis is that the functional phenotype of dengue virus-responsive CD8<sup>+</sup> T cells, together with the magnitude of the T-cell response, may play

a role in the development of severe disease. In particular, T-cell responses in severely ill patients are limited, in that they produce IFN- $\gamma$  and/or TNF and rarely CD107a, a marker of cytotoxic degranulation. Conversely, in patients with uncomplicated dengue, relatively more CD8<sup>+</sup> T cells display CD107a and few express only IFN- $\gamma$  and/or TNF. These and other data (102) suggest that T cells are potent contributors of cytokines to the inflammatory milieu in infected tissues and that this contributes to vasodilation during this critical phase of the illness, although not all studies have found evidence in support of this hypothesis (44).

### TARGETING THE CYTOKINE STORM

Across much of the world, infectious diseases remain a very real threat, accounting for approximately half of all deaths. Malaria, tuberculosis, HIV disease, influenza, dengue, and endemic and emerging infections all contribute to morbidity and mortality. As economies develop, urbanization and environmental degradation gather pace and the structures of societies change, creating many new challenges in the 21st century. In addition to the emergence of new diseases, the continued rise of drug resistance among all the major infections is outpacing the rate of discovery of new antibiotics. Against this backdrop of antimicrobial resistance and the emergence of new pathogens, increasing interest has focused on the development of drugs that target the immune response to infection. As we have discussed, many acute infections are characterized by a powerful and potentially destructive immune response, and it would seem logical to target this response in order to reduce the self-inflicted damage initiated by the host in response to infection (129). Yet to date, successful targeting of the immune system during acute infections has proved to be extraordinarily difficult and largely unsuccessful.

### Challenges of Current Immunotherapies

A great deal of effort has been devoted to targeting the host response with a variety of anti-inflammatory drugs and adjunct approaches in a range of acute severe infections, including treatment with corticosteroids, aspirin, monoclonal antibodies (MAbs), anti-cytokine and anti-chemokine agents, plasma exchange, and statins. Despite these efforts, none has been proved to be effective (98), and some have worsened the outcome (19). For example, in severe malaria, an infection characterized by a powerful immunological cascade, a dozen interventions targeting the host response to infection have been tried over the last 20 years, including artemisinin-based combinations and treatment with chloroquine. The majority of antimalarial drugs sold in Africa are either ineffective or accelerate malarial parasite resistance owing to the distribution of counterfeit drugs (9, 43). The capillary permeability syndrome that is characteristic of severe dengue is a result of endothelial dysfunction and compromised integrity of endothelial cell junctions. Clinical trials to test the ability of early corticosteroid therapy to attenuate the host inflammatory response in dengue, and in turn capillary permeability, are ongoing (ClinicalTrials.gov, ISRCTN39575233). Previous trials of corticosteroids have involved patients with hypovolemic shock and did not show benefit, though this could have reflected the late timing of therapy. The availability of generic anticytokine MAb therapies might open new avenues for therapy in the future. Nevertheless, despite strong evidence of a cytokine storm in many severe acute infections, it has proven difficult to intervene and improve clinical outcomes, for reasons that remain elusive.



TABLE 2 Anti-inflammatory properties of immunomodulatory drugs

Type of therapeutic	Drug(s)	Immunomodulatory effect(s) (reference[s])
COX inhibitors	Mesalamine, celecoxib	Coadministration of COX inhibitors with zanamivir diminished cellular infiltrate and improved survival of H5N1 virus-infected mice compared to antiviral treatment alone (22, 157)
CCR2 inhibitor	PF-04178903	Increased survival of mice infected with influenza virus and reduced lung immunopathology (87, 88)
Sphingosine receptor agonists		Suppresses cytokine and chemokine production; sphingosine receptors have been shown to play an important role in innate immune responses (120)
Anti-TNF agents		Mediator of pulmonary inflammation during influenza A viral pneumonia; decreased severity of pulmonary immunopathology and prolonged survival of A/PR/8-infected mice (67)
Statins	Simvastatin	Statins were not found to reduce the risk of developing severe disease in patients with pandemic influenza (H1N1) 2009 (144)
OX40	OX40-Ig fusion proteins	OX40 plays a critical role in T-cell-mediated immunopathology in the lung during viral infection (65); ligation on activated T cells reduces pulmonary eosinophilia during <i>Cryptococcus neoformans</i> infection (64)
PPAR $\alpha$ /PPAR $\gamma$ agonists	Gemfibrozil, pioglitazone, rosiglitazone, 15d-PGJ2, ciglitazone, troglitazone	15d-PGJ2, ciglitazone, and troglitazone decreased production of IL-1 $\alpha$ , IL-6, and TNF cytokines, CXCL8 and CCL5 chemokines, and ICAM-1 in RSV-infected lung epithelial cells (4); administration of gemfibrozil (intraperitoneally) on days 4 to 10 after exposure to H2N2 influenza virus and following the onset of illness significantly increased survival in mice with severe influenza (20)

### Implications for Therapeutic Strategies

Immunomodulatory drugs that diminish inflammation during infection with drug treatment show therapeutic benefit (Table 2). Sphingosine receptors play an important role in innate immune responses (120), and sphingosine analogs have shown potential for controlling the cytokine storm caused by influenza virus (95). Sphingosine-1-phosphate (S1P) receptor 1 suppresses immune cell recruitment through downregulation of cytokine and chemokine production by respiratory endothelial cells in the presence of S1P<sub>1</sub>-selective agonists, CYM-5442 and RP-002, including production of IFN- $\alpha$ , CCL2, IL-6, TNF- $\alpha$ , and IFN- $\gamma$ , (139). Teijaro and colleagues (139) demonstrated that blunted innate chemokine and cytokine responses mediated by S1P<sub>1</sub>-selective agonists protected mice from lethal infection with pandemic H1N1 influenza virus. Agonism of S1P<sub>1</sub> receptor has no effect on viral replication; however, some strategies dampening the immune response can correlate with increased susceptibility to infection. This was observed in mice treated with DNA encoding transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), which inhibited pulmonary eosinophilic responses yet increased susceptibility to *Cryptococcus neoformans*, influenza virus, and respiratory syncytial virus (147).

Peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors that act as key regulators of lipid metabolism and inflammation (reviewed in reference 12). PPAR- $\gamma$  agonists downregulate the inflammatory response to virus-induced lung inflammation (8). For example, gemfibrozil targets PPAR $\alpha$ , dampening the inflammatory response and improving the survival of mice infected with influenza H2N2 virus (20). Combination therapy consisting of zanamivir, an inhibitor of the influenza virus neuraminidase, and celecoxib and mesalazine, inhibitors of inflammation, increased the survival of mice infected with a highly pathogenic strain of H5N1 influenza virus (157). The cyclooxygenase-2 (COX-2) inhibitors celecoxib and mesalazine also reduced the mortality rate of mice infected with H5N1 influenza virus when administered in combination with zanamivir (22). These studies demonstrated that antiviral treatment was less effective alone than in combination with immunomodulatory therapies that suppressed inflammation. Once initiated, the pro-inflammatory cytokines and chemokines can continue to drive the

progression of immunopathologic events in the absence of continued viral replication.

In simple terms, it is likely that during a severe acute infection, certain elements of the immune response need enhancing at times and need suppressing at other times. What is required of the immune system early in the infection, when the pathogen is dividing rapidly and reaching high infectious loads, may be very different from what is needed later, possibly only a few hours later, when either the pathogen is at steady state or the pathogen load is falling due to either the effects of appropriate antimicrobial therapy or the clearance of the pathogen by the immune response. Treating patients with the “right” immunomodulating drug but at the “wrong” time could worsen the clinical outcome. We still do not understand the delicate nature of this rapidly changing immune response, and until we do, it is unlikely that we will develop rational therapies that target the exact phase of the immune cascade and administer those therapies at the time they are needed. It might be that within the long list of adjunct interventions that have been tried in acute severe infections, therapies exist that would be successful if only the immune dynamic were understood so that they could be used at the right time and their pharmacological profile fit the need for a potentially short-acting drug. We know that individuals respond differently to infection and that individual responses to antibiotics and adjunct therapies also vary (141). These variations in host response are likely to be complex and controlled by host and pathogen genetic determinants as well as by the immune memory of each individual.

### CONCLUDING REMARKS

We are in a potentially golden age of scientific discovery led in part by recent technological advances in next-generation sequencing (NGS). For example, NGS has unveiled a more comprehensive view of the transcriptome, enabling identification of large and small RNAs, splice isoforms, and novel transcripts from unannotated genes. Yet the mind-numbing weight of data we can now accrue brings its own challenges. Overall, the need for better methods for NGS data analysis, including RNA-Seq bias correction and improved isoform quantification, is just one of the challenges faced with using this technology. There is no agreement on

what should be measured and how it should be analyzed. An international consensus on this would help define how the field develops in the coming years. Moreover, we have split interdependent systems into silos of research such that studies of the immune cascade rarely integrate the pathogen or the host genetic makeup or attempt to interrogate the whole delicate and rapidly changing immune environment. The world of host and pathogen genetics has been revolutionized in the last decade, and through scale and increasingly sophisticated biostatistical analysis, what associations mean and how they should be interpreted will continue to be developed. The development of transcriptional signatures and proteomics to complement host and pathogen genetic studies is providing great steps forward; however, we need to standardize these integrated studies with much tighter definitions of the clinical and phenotypic data.

In this review we have focused on the biological consequences of a cytokine storm, namely, immunopathogenesis caused by SARS-CoV, influenza virus, and dengue virus infections. We have highlighted lessons learned from genomic studies within the past decade, in particular how the kinetics of cytokine and chemokine gene expression can influence cytokine overproduction, the redundancy that exists within these signaling systems that can affect the landscape of transcriptional responses, and how these early molecular events can influence later stages of disease phenotypes associated with the cytokine storm. Moving forward, the application of systems biology approaches to infectious disease research will offer further insight into the key cellular processes that either sustain the cytokine storm or contribute to its resolution. As part of a systems biology effort, the use of high-throughput technologies and analytical and computational modeling methods to identify and model the pathogen and host determinants and interactions underlying infectious disease will aid in the identification of molecular signatures of infection. For example, weighted gene correlation network analysis has been used to model the dynamic H5N1 virus-mediated transcriptional response in infected human Calu-3 bronchial epithelial cells (85), and transcriptional network inference has predicted gene behavior in mice and macaques, including key components of the NLRP3 inflammasome (CASP1, NLRP3, and PYCARD) and cytokine and chemokine genes (IL-6, CXCL10, and CCL4) relevant to cytokine storm that are conserved across species (97). Within this framework, we will be better able to predict the various facets of the circuit operation and coordination and the overall system in the resulting phenotypic outcome. This will impact how potential immunotherapies will perturb the circuitry and machinery related to the cytokine storm and likely will present key targets for therapeutic intervention and diagnostics.

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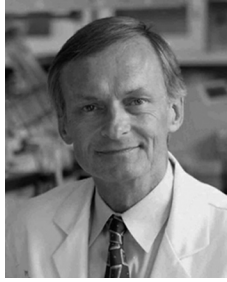


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