Lessons learned and concepts formed from study of the pathogenesis of the two negative-strand viruses lymphocytic choriomeningitis and influenza

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This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2008.

Contributed by Michael B. A. Oldstone, December 20, 2012 (sent for review December 5, 2012)

Viruses have unique lifestyles. To describe the pathogenesis and significance of viral infection in terms of host responses, resultant injury, and therapy, we focused on two RNA viruses: lymphocytic choriomeningitis (LCMV) and influenza (Flu). Many of the currently established concepts and consequences about viruses and immunologic tolerance, virus-induced immunosuppression, virus-induced autoimmunity, immune complex disease, and virus–lymphocyte and virus–dendritic cell interactions evolved through studies of LCMV in its natural murine host. Similarly, the mechanisms, aftermath, and treatment of persistent RNA viruses emerged, in large part, from research on LCMV. Analysis of acute influenza virus infections uncovered the prominent direct role that cytokine storm plays in the pathogenesis, morbidity, and mortality from this disease. Cytokine storm of influenza virus infection is initiated via a pulmonary endothelial cell amplification loop involving IFN-producing cells and virus-infected pulmonary epithelial cells. Importantly, the cytokine storm is chemically treatable with specific agonist therapy directed to the sphingosphine 1 phosphate receptor 1, which is located on pulmonary endothelial cells, pointing to the endothelial cells as the gatekeepers of this hyperaggressive host immune response.

viral persistence | immunosupression

The question of whether a significant component of disease instigated by viral infection is or is not an autoimmune disorder is essentially one of semantics. Certainly, such diseases are autoimmune in the sense that the host's own immunologic response makes the victim sick. The stimulant, in this case a virus, usually provokes both the innate and adaptive arms of the immune system under control of the host's relevant genes. The innate immune system consists of natural killer (NK) cells, peripheral blood lymphocytes, macrophage/monocyte inflammatory cells, cytokines/chemokines, and complement; its counterpart, the effector adaptive immune system, comprises T and B cells and antibodies.

A relationship between virus infection and the immune response that results in tissue injury (immunopathology) was noted more than 200 y ago by Edward Jenner. In fact, beginnings of virology and immunology can be traced to Jenner's observations of inflamed lesions after he inoculated cowpox virus into humans (1). More than 100 y after Jenner's report, Loeffler and Frosh (2) presented the initial characterization and evidence that the foot and mouth disease virus caused an acute disease in animals. Correspondingly, Beijerinck (3) and Ivanovski (4) also identified a virus, tobacco mosaic virus, as the cause of a plant disease. Over the last 100 or so years, it has become clear that viruses not only cause acute infections but are also major sources of chronic and degenerative diseases (5, 6) and more than 20% of cancers in humans (5–7).

Immunopathology and Anatomy of Persistent Virus Infection: Lymphocytic Choriomeningitis Virus Model

Infection by a virus offers many opportunities for the development of tissue injury and disease caused by the host's immune response. The virus is a self-replicating agent that provides a supply of macromolecular antigens (immunogens) and, in most if not all instances, elicits in its hosts innate and adaptive immune responses. With acute viral infections, an imbalance between the immune response and viral replication causes termination of either the infection or the host. That is, either components of the immune system conquer and clear the infection, or the infection overcomes and kills the host. With chronic or persistent virus infections, the time scale is lengthened to include additional and exceedingly complex events. First, the infected host mounts both a continuous immune response against the virus and ongoing viral replication. That response generates humoral antiviral antibody, but this occurs in virus (antigen) excess. Those virions and/or viral antigens circulate in the serum and interact with antiviral antibodies to form virus-antiviral antibody (V-Ab) complexes, which subsequently deposit in a variety of tissues (reviewed in ref. 8). V-Ab immune complexes are ever-present signatures of persistent infections. Additionally, in several persistent viral infections, for example, hepatitis B, excessive deposition of such complexes results in the immune complex diseases arteritis and nephritis (8). Second, although antiviral cytotoxic CD8 and helper CD4 T cells initially form at the onset of a persistent infection, the virus co-ops the host's normal negative regulators of the immune response to dampen and render ineffective the antiviral T-cell response, leading to so-called T-cell exhaustion (9, 10). Basically, when an immune response is generated, a give-and-take follows between two opposed pathways: positive immune regulators (e.g., IL-6, IL-12, IL-27) that drive a fruitful and robust immune response and negative regulators [e.g., the programmed death-1 (PD-1) molecule, IL-10, TGF-β, LAC-2, TIM-3) that function to limit or blunt excessive positive immune responses. By blunting the immune response, the latter factors minimize the host's ability to produce severe immunopathologic injury or autoimmune disease. Viruses that persist have learned to generate and manipulate several negative immune regulators, leading to T-cell exhaustion and to the induction and maintenance of persistent infection. The principal negative regulators are IL-10 and PD-1. The genetic deletion of either IL-10 or PD-1 prevents a persistent infection, whereas the use of specific antibodies to neutralize either IL-10 or PD-L1 during a persistent infection restores anti-viral T-cell function sufficiently to purge virus and terminate the persistent infection (11, 12). Thus, both of these negative immunoregulators independently exhaust or suppress the antiviral T-cell function (13). T-cell function is absolutely required to purge the infecting agent and terminate the persistent infection. However, the negative immune response factors can blunt this necessary attack by CD8

Author contributions: M.B.A.O. designed research, performed research for several experiments discussed, contributed new reagents/analytic tools, analyzed data, and wrote the paper.

The author declares no conflict of interest.

See Profile on page 4155.

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www.pnas.org/cgi/doi/10.1073/pnas.1222025110

cytotoxic T cells (CTLs), halt their release of inflammatory cytokines, and prevent the production/release of inflammatory cytokines/chemokines from CD4 T helper cells. These cytokine/chemokines are primarily IFN-γ, TNF-α, and IL-2. Effector CTLs act to destroy virus-infected cells, the factories that make progeny virus, whereas IFN-γ and TNF-α limit viral replication and spread. By these means, the source (viruses) of persistent infection is removed. Studies using the lymphocytic choriomeningitis virus (LCMV) performed in my laboratory and others were instrumental in documenting the concept, molecular mechanism, and consequences of V-Ab immune complex activities and attributing T-cell unresponsiveness to negative immune regulators (reviewed in refs. 8, 14, and 15).

In addition, viruses that persist often infect cells that are essential for presenting viral antigens in the context of MHC molecules. Dendritic cells (DCs) are among the most professional (effective) presenters of viral antigens. These cells express MHC/viral peptides, which function to arm, generate, and expand antiviral T cells. Viruses, by infecting DCs, disrupt those functions. As first noted with acute measles virus infection (16, 17), DCs play a prominent role in persistent viral infections, and this position has been championed by experimental observations with LCMV (15, 18–22). Fibroblast reticular cells (FRCs) are the network in secondary lymphoid tissues that provide the cellular matrix through which naïve T cells migrate to encounter DCs. Antigen expression and function of FRCs are similarly altered during persistent infections with LCMV (23, 24).

Initially the dogma pioneered by Burnet and Fenner was that, during a persistent virus infection initiated in utero or congenitally, a clonal deletion of T and/or B cells promoted immunologic tolerance (25). Burnet and Fenner suggested that the lifelong symptomless state characterized by continuous high titers of LCMV in blood and tissues and the absence of detectable anti-LCMV antibody (studies done before the discovery of antiviral T cells) (reviewed in ref. 26) of adult mice infected congenitally or by LCMV injection of neonates involved immunologic tolerance of the infectious agent introduced early in life. Thus, immunologic tolerance in the LCMV model was defined by (*i*) resistance of normal newborn or LCMV congenitally infected mice to a viral dose lethal for immunologically mature adults, (*ii*) continuous presence of high titers of virus in blood and organs of adults infected in utero or during the neonatal period, (*iii*) resistance of such adult mice to ordinarily lethal LCMV challenge, and (*iv*) absence of detectable anti-LCMV antibodies or anti-LCMV T cells. However, our experiments with LCMV (27) and subsequently with murine leukemia viruses (28, 29) proved that no such clonal deletion of virus-specific antibodies occurs in persistent infections. Rather, the infecting microbial agent generates antiviral antibody that combines with the infecting virus and its antigens to form immune complexes (V-Ab) that deposit in and injure arteries, glomeruli, and the choroid plexus of the brain (8). Further, by adaptive transfer of genetically marked effector anti-LCMV T cells to initially terminate the persistent virus infection followed by an acute LCMV challenge, Jamison et al. (30) and Kim and Ahmed (31) generated LCMV-specific effector T cells in adult mice whose persistent infection was initiated early in life, again documenting that clonal deletion of antiviral T cells did not occur. When persistent infection was initiated in individuals with a competent immunologic system, virus-specific effector T and B lymphocytes were generated, but those cells became hyporesponsive or exhausted in part owing to virus antigen excess. An important and highly significant result from studies in which the persistent infection initiated in adult mice can be reversed by instilling antibody to neutralize IL-10 or PD-L1 (11-13) is that therapeutic intervention for humans undergoing persistent infection may be possible, thereby providing a road map for reversing the persistent viral state. Although each of the many DNA and RNA viruses that persist in humans has its own signature, most of the basic observations using LCMV in a murine model can be or have been translated almost exactly as duplicates of human viral infections and of many bacterial and parasitic infections (reviewed in refs. 10, 32, and 33).

In summary, the strength of the LCMV model rests on three unique foundations. First, the virus, per se, in its natural murine host or in cultured cells is not cytolytic. This quality allows two experimental advantages. The first is a clear separation of effects caused by viruses from those caused by the host immune system. Thus, tissue injury emanating from such infection introduced in vivo is caused by the host's immune response against the virus and that response can be proven, dissected, quantified, and manipulated. Second, the persistent infection in vivo or in vitro of highly differentiated cells as those of the nervous, endocrine, or immune system, etc., allows one to study how continuously replicated foreign genes, in the absence of cell lysis, alter those cells' function and biochemistry, thereby disturbing homeostasis and promoting disease (14, 34, 35). Further, when a secondary infection with an infectious agent that contains cross-reacting proteins occurs, autoimmune disease can be introduced by the mechanism of molecular mimicry (36). Third, the LCMV genome is relatively simple in that the virus contains but four genes, two each placed on two distinct pieces of genomic RNA (14). Therefore, one can use a genetic approach in which gene reassortment or reverse genetics is applied to dissect the biology of LCMV infections (37, 38). This technology is extraordinarily useful, because the LCMV gene is 10.7 kb and the virus generates a plethora of variants that differ by as few as five to six nucleotides and two to three amino acids, yet display enormous biologic diversity. Some examples are immunosuppression and viral persistence vs. antiviral immune responsiveness and termination of acute infection. Additionally, cell and tissue tropisms differ, as do the range of quantifiable defects caused by various variants for a broad spectrum of diseases that mimic human illnesses like diminished cognitive and learning functions in the central nervous system resulting from diminished transcription of GAP43, causation of type 1 diabetes due to decreased insulin, or failure to grow and develop due to diminished growth hormone production (reviewed in ref. 14).

Immunopathology Associated with Acute Influenza Virus Infection

The outcome of a viral infection is determined by a balance between the virulence of the microbe and the resistance of the host. Tissue injury that causes morbidity and mortality during severe influenza virus infections reflects the intrinsic properties of the virus complemented by the host's antiviral immune response. The extent of virulence specific to particular influenza virus strains depends on receptor use, replication potential, and direct cytopathic effects on pulmonary epithelial cells lining the respiratory tract and endothelial cells of the alveolar sacs where oxygen is exchanged. For example, the genetically reconstructed H1N1 1918/1919 influenza virus, one of the strains that caused the worst known loss of human life from an environmental agent, was responsible for an estimated 50,000,000 deaths (35, 39, 40). In laboratories, this strain replicated at an enormously heightened rate (a 1,000–10,000 greater factor of virus replication) in cultured cells, inoculated eggs, and experimental animals over that observed with other H1N1 influenza virus isolates (41). Using reverse genetics to shuffle individual genes from the virulent H1N1 1918/1919 influenza strain to a less virulent H1N1 Texas/36191 strain mapped a critical role for virulence to the polymerase PB1, hemagglutinin, and the neuraminidase genes (42–44).

In general, the host's immune response balances the virus's virulence by ameliorating viral replication and spread. However, with respect to an influenza virus, that agent can by itself enhance morbidity and mortality by eliciting an excessively exaggerated immune response termed a cytokine storm. A cytokine storm as

the direct effect of acute influenza viral infection causes severe pulmonary injury resulting in a poor clinical outcome (reviewed in refs. 45 and 46). Cytokine storms comprise factors from both innate and adaptive immune responses but are most severe during the early innate immune response, usually days 1–3 postinfection. A cytokine storm is characterized by the massive production of proinflammatory cytokines/chemokines and recruitment and activation of inflammatory leukocytes, NK cells, and monocytes/macrophages into the virally infected lung. The latter adaptive immune response by CD8 and CD4 T cells (days 6–8 postinfection) mediates additional tissue injury caused by virus-specific T cells acting to contain the influenza virus infection by destroying the infected cells and releasing chemokines that then attract cells of the myeloid lineage to the infected area.

According to experimental results using the reconstructed 1918/1919 influenza virus inoculated into mice, ferrets, and subhuman primates (44, 47), cytokine storms were likely major contributors to the morbidity and mortality of humans during the 1918–1919 pandemic. Such experimental studies complement recent clinical and epidemiologic evaluation of cytokine storm-related deaths during outbreaks of the H5N1 bird flu and the 2009 H1N1 influenza virus pandemic (46, 48). In these and related reports, humans requiring hospitalization and having the highest mortality rates suffered the worst cytokine storms. For example, in one study of hospitalized patients infected with 2009 H1N1, those who recovered had viral titers equal to those of patients who died, but the survivors had little or very modest evidence of cytokine storms compared with severe cytokine storms occurring in the deceased (46). Recently, the first evidence of a direct role for cytokine storm, rather than a bystander-associated event during severe influenza virus infection, was provided by experiments and results from my and Hugh Rosen's laboratories (45, 49–51).

We showed that the human H1N1 swine influenza viral infection was amenable to specific drug therapy with several spingosphine-1-phosphate (S1P) receptor agonists such as AAL-R, RP-002, and CYM5442. These agonists blocked cytokine storms without acting on the infecting influenza virus, i.e., did not reduce viral titers (45, 49–51). However, the S1P agonists significantly protected experimental mice (50, 51) and ferrets from the pulmonary tissue injury and clinical disease that ordinarily accompanies influenza virus infection. Further, protection from death and limitation of tissue injury were significantly better after blunting cytokine storms with specific S1P therapies than by using the antiviral drug oseltamivir (Tamiflu) (50, 51), which lowers the virus' activity and replication. Importantly, however, in both mice and ferrets, the greatest benefit resulted from combining the two therapies: one to blunt the host-mediated cytokine storm and the other to dampen influenza virus replication.

Our adventure with the pathogenic effects of influenza virus began when we determined the kinetics and numbers of virus-specific CD8 and CD4 T cells that entered the infected lungs. To accomplish this task, our collaborator, Kawaoka (49), inserted the H-2^b (D^b and IA^b) restricted immunodominant LCMV CD8T cell (GP33-41:KAVYNFATC) and CD4 T-cell (GP65-76: PDIYKGVYQFSV) epitopes into the neuraminidase stalk of the influenza gene and then used reverse genetics to incorporate the LCMV/neuraminidase chimera into an infectious influenza virus particle. We used this recombinant virus to infect H-2^b mice that 2 d earlier received 1×10^4 RFP-labeled LCMV-specific CD8 T cells selected to recognize the LCMV CD8 T-cell epitope GP33-41. Additionally, we used GFP-labeled LCMV-specific CD4 T cells that recognized the LCMV CD4 T-cell epitope GP65-76. Six days after H1N1 influenza virus infection of mice, virus-specific T cells were detected in the lungs, and their numbers peaked by the eighth day (49).

Chemical modulation of the S1P signaling system proved useful in the study of immune cell trafficking (52–54) and led to our application of S1P reagents to probe H1N1 influenza virus infection in the mouse model. S1P is a signaling lipid present at concentrations of 1–3 μM in plasma and ~100 nM of lymph. The vast majority of S1P in plasma is bound to high-density lipoproteins, leaving a free concentration of 15–45 nM in blood. S1P is generated by phosphorylation of sphingosine by the action of two intracellular sphingosine kinases, and its level is tightly controlled to maintain normal physiologic function. The biology of the S1P system is complex, because five separate S1P receptors are coupled to different G proteins. These proteins regulate many downstream signaling pathways and affect numerous cellular and organ targets (53, 54). Agonist and antagonist chemical probes to the various S1P receptors are available. With their use and the accessibility of mice in which a specific S1P receptor(s) has been genetically knocked out or knocked in while linked to an indicator GFP probe, the role of S1P receptors in normal or exaggerated immune responses can be gauged, and immune cell trafficking into the lung parenchyma can be studied.

We began probing the role of cytokine storms in the influenza infection model using a permissive nonselective S1P probe, AAL-R, that signaled via S1P receptors 1, 3, 4, and 5, but not 2 (49, 50, 52). As a control, we used the conformational isomer AAL-S that did not signal any S1P receptors. The permissive AAL-R, but not the control AAL-S isomer, significantly inhibited cytokine/chemokine production and leukocyte/macrophage infiltration into the lung. As a result, lessening of the cytokine storm significantly protected hosts from the lethal effects of influenza viral infection without altering either the kinetics or titers of neutralizing anti-influenza antibody (48–51). Numbers of influenza virus-specific CD8 or CD4 T cells decreased sufficiently to diminish the excessive immunopathologic effects, yet enough T cells remained available to purge the viruses and terminate infection. Other studies showed that the permissive S1P receptor agonist AAL-R, but not the AAL-S isomer, inhibited DC migration from the mediastinal lymph nodes to the lungs and reduced the expression of MHC and costimulatory molecules on DCs (49). The result was to lessen the numbers of virus-specific T cells that could be primed and exported. By this means, a decrease of effector T cells infiltrating the lung followed, thereby limiting the otherwise severe pulmonary injury. However, a sufficient adaptive host antiviral T-cell immune response remained to control and terminate the virus infection.

Other experiments used specific S1P receptor agonists to determine which one of the four possible S1P receptor signaling pathways, S1P1, S1P3, S1P4, or S1P5, participated in blocking the early proinflammatory cytokine/chemokine activity and innate immune cell recruitment into the lung. The results of extensive investigations indicated that S1P1 receptor signaling alone was responsible for the injurious early cytokine storm (50, 51). Interestingly, the suppression of S1P1 receptor signaling signified that cytokine/chemokine expression was dependent on type 1 IFN, but type 1 IFN was not involved in the recruitment of innate inflammatory cells into the lung (51). Thus, initial cytokine/chemokine release was separable from initial early innate inflammatory cell inflammation. Influenza viruses infect the epithelial cells lining the respiratory tract, so it was unexpected to find that S1P1 receptors were not located on epithelial cells but resided, instead, on endothelial cells (51). Thus, endothelial cells serve as gatekeepers: S1P1 signaling occurs via those endothelial cells and engages a pulmonary amplification loop to produce the cytokine storm. The IFN-α signal comes predominantly from dendritic plasmacytoid (p)DCs. Thus, the pulmonary endothelial cytokine storm loop involves signaling between viral infected epithelial cells, endothelial cells, the cells that express S1P1 receptor, and pDCs.

In summary, our findings revealed that cytokine storms play a direct role in causing pulmonary tissue injury and disease during human pathogenic H1N1 influenza virus infection. Not only does the pulmonary endothelium act as a gatekeeper of influenza virus-induced cytokine storms, but those storms are also chemically retractable with S1P1 receptor agonists. S1P agonist

represents a potentially new therapy for influenza virus infection. Last, the genetic differences in humans with respect to their regulation of S1P, especially the S1P1 pathway, may provide leads to other therapeutic targets, as well as additional means for identifying individuals susceptible or resistant to developing a cytokine storm on viral infection. Hence, genetic profiling of pulmonary endothelial cell/epithelial cell/pDC cross-talk and signaling may provide factors to predict who will succumb to and who will live through a severe influenza virus infection. Finally, the role of S1P and cytokine storms in other severe respiratory diseases, like those from Hanta or severe acute respiratory syndrome (SARS) viruses and pneumococci, remains to be determined. Such studies are currently under way by my group in association with the Rosen laboratory and colleagues using appropriate BSL/4 facilities.

ACKNOWLEDGMENTS. This work was performed over multiple years with legions of gifted postdoctoral fellows and colleagues on the faculty of The Scripps Research Institute. Especially the work with cytokine storm and S1P agonist was performed with my colleague, Hugh Rosen. The research presented here was supported by several National Institutes of Health grants, most recently Grants R01 AI-09484 (persistent infection, immune complexes, T-cell exhaustion), U01 AI 074564 (influenza), and U54 AI057160 (Midwest Regional Center of Excellence) (influenza).

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