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Excitotoxicity (Immunoexcitotoxicity) as a Critical Component of the Cytokine Storm Reaction in Pulmonary Viral Infections, Including SARS-Cov-2

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ABSTRACT

A hyperimmune state secondary to dysregulation of the immune system during lower pulmonary viral infections, sepsis and in some cases non-infectious disorders, is now considered to be the principle event leading to clinical deterioration and eventual death in these patients. While most studies have attributed the pathological damage to the lung to be primarily due to high levels of cytokines and chemokines along with massive infiltration of principally neutrophils and macrophages, there is compelling evidence that overactivation of glutamate receptors is also playing a significant, if not critical role in this process. Functional glutamate receptors, along with two important glutamate transport systems, have now been described in epithelial and endothelial cells in the pulmonary airways as well as all involved immune cells. Experimental studies using cytokine models have shown considerable protection against pathological damage to pulmonary tissues by reducing the activation of these glutamate receptors.

Keywords: *activation of glutamate receptors, chemokine storm, cytokine models, cytokine storm, dysregulated hyperimmune response, excitotoxic amino acid transport, excitotoxicity, glutamate transport, hyperimmune response, ibotenate, inflammation, immunoexcitotoxicity reaction, infiltration of immune cells, N-Methyl- D-aspartate, NMDA*

Introduction

The concept of a "cytokine storm", in most cases a dysregulated hyperimmune response to an infectious organism, was first described in a 1993 article as related to the graft-vs-host disease (Ferrara et al. 1993). Since then, most cases have been in reference to viral infections such as cytomegalovirus, EB-virus, influenza viruses, SARS, MERS, H1N1, H5N1 and the new SARS CoV-2 virus (Yurn & Wong, 2005).

While the extensive lung tissue damage is most often attributed solely to the pro-inflammatory reaction and its association with high concentrations of reactive oxygen and nitrogen species, and lipid peroxidation products, newer evidence suggest that a second arm of the damage may arise from

overstimulation of glutamate receptors found both on lung tissues and immune cells. It may be that this excitotoxic reaction is the primary mode of damage to these tissues. This remains to be demonstrated.

That pro-inflammatory cytokines, by themselves, may not be sufficient to cause the pathological damage that we see in cases of cytokine storm is suggested by the observation that no level of TNF-alpha elevation can cause tissue damage in the CNS, rather the damage appears to be from the excitotoxic arm of an immunoexcitotoxicity reaction (Blaylock & Maroon 2011; Blaylock, 2013). While activation of pro-inflammatory cytokines and chemokines is an early event in the cytokine storm reaction, animal studies have shown that knock out models or inhibition of major cytokines, such as IL-6, TNF-alpha and CCL2, did not offer significant protection against the cytokine storm reaction (Salomon, Hoffmann & Webster., 2007; Paquette et al. 2012)

As regards this aberrant hyperimmune state, we know that there exists an extensive variability in innate immune responses among even healthy humans, which includes hyper-responders and under-responders (Tisoncik et al. 2012). An intense immune induced inflammatory response as seen with the cytokine storm phenomenon can cause severe damage to lung tissue of a widespread nature (Peris et al. 2004).

Genomic studies of the 1918 influenza virus, demonstrated a sustained hyperimmune reaction with dramatic upregulation of cytokine/chemokine genes controlling IL-6, IL-8, CCL2 and CCL5 in the lungs of infected animal models (Kash et al. 2006). A similar microarray analysis of lung tissue from H1N1 infected animals demonstrated prolonged expression of genes controlling CCL2, CXCL9 and CLCL10 (Baskin et al. 2009).

Attempts to discover why only certain individual appear to be susceptible to these hyperimmune reactions has been only partially successful. Critical to innate immune reactions are the toll-like receptors (TLRs), which can vary in their reactivity based on genetic influences. For example, Wurfel et al. in examining a large population of septic patients, found that those with a single nucleotide polymorphism (SNP) as a marker for a hyperfunctioning variant of the TLR1, experienced increased organ damage and morbidity following a Gram-positive bacterial infection (Wurfel et al. 2008). It has also been shown that variants in TLR4 reactivity, the principle receptor for lipopolysaccharide (LPS), can predispose an individual to sepsis reactions when exposed to bacterial endotoxins (Michael et al. 2003).

One would intuitively expect an intense hyperimmune reaction to increase killing of the initiating micro-organisms, yet the opposite appears to be true (Neilli et al. 2012). Despite a robust cytokine response, viral titers are not reduced. Failure to eliminate the invading organism further stimulates the hyperimmune reaction.

Pathological Findings in the Cytokine Storm Reaction

In patients with serious lower lung infections, such as with various influenza viruses, SARs-CoV, MERS and newly engineered SARS-CoV-2 virus, we see high viral loads in the lower lungs, which rapidly initiate local immune cell activation in the distal airways and alveoli as well as a triggering of massive migration of immune cells, principally neutrophils and macrophages, to these same areas of the lungs. (Peiris et al. 2004; de Jong et al. 2006; Kash et al. 2006; Baskin et al. ,2009). While high

viral loads initially may initiate a cytokine storm, some studies have found no relation between the viral load and the intensity of the cytokine response (Liu Q, Zhou & Yang, 2016).

While neutrophils and macrophages play the major role in lung pathology in severe viral infections of the lungs, $CD4^+$ and $CD8^+$ T-cells are the principle cells involved in viral clearance. La Gruta et al. 2007; Clark 2017). Massive influx of these immune cells, especially macrophages, are thought to play a critical role in the pathology of these viral infections, such as apoptosis of airway epithelium and the cellular structure of the alveoli, pulmonary edema, thickening of alveolar walls, microhemorrhages, thrombosis in smaller vessels and hyaline membrane formation. (Nicholls et al. 2003). The primary pathological damage is within the alveoli with diffuse alveolar damage being prominent (DAD). Several cytokines, such as TNF-alpha, enhance monocyte and neutrophil transmigration across the infected endothelium and epithelium (Wijburg OL et al. 1997; Kidney & Proud 2000). Because of a mandate from the CDC, very few autopsy studies have been performed on SARS-CoV-2 fatalities (Xu et al. 2010). From the few that have been done the pathological finding are quite similar to that seen with fatal pathogenic influenza infections.

The key cells involved in the cytokine storm reaction include epithelial cells, endothelial cells and macrophages (Nelli et al. 2012) While all three cell types can produce proinflammatory cytokines, the highest TNF-alpha levels are induced by macrophage activation, some 3 orders of magnitude higher than human epithelial cells (Nelli et al. 2012). As the process advances, the inflammation can spill over into the general circulation, triggering pathological damage in several organs, such as the kidneys and liver (Tisoncik et al. 2012).

The Immunoexcitotoxic Process Explained

I coined the term immunoexcitotoxicity in 2009 in an article concerning the pathophysiology of autism spectrum disorders, but the actual process had been described by others previously (Dzenko et al. 1993; Gelbard et al. 1993; Chao & Hu 1994; Takeuchi et al. 2006; Leonoudakis, Zhao & Beattie 2008; Blaylock RL 2009). Lucas and Newhouse in 1957, before glutamate was known as a neurotransmitter, described destruction of neural retinal cells in animals exposed to monosodium glutamate (Lucas & Newhouse 1957). Twelve years later Olney coined the name excitotoxicity to describe the process (Olney & Sharpe 1969). Subsequent studies established glutamate as the major neurotransmitter in the CNS and described several subtype glutamate receptors in the central nervous system. Recently, researchers discovered similar functional glutamate receptors on a number of peripheral tissues and cell types, including the GI tract, pancreas, kidneys, liver, megakaryocytes, endothelial cells, male lower urogenital tract and testes and a number of immune cells (Erdo 1991; Skerry & Genever 2001).

Yawata et al. demonstrated that both LPS and TNF-alpha stimulated macrophages could induce robust neurotoxicity, which was completely blocked by the N -Methyl- D -aspartate (NMDA) receptor antagonist MK-801 (Yawata et al. 2008). Others have also shown a critical link between inflammation and excitotoxicity (Shijie et al. 2009). Morimoto et al. found that co-injection of LPS plus the NMDA agonist ibotenate (ibotenic acid, a neurotoxin from *Amanita muscaria* mushrooms and an analogue of glutamate), led to significant neuronal damage, but blocking the excitotoxicity arm of the reaction prevented any tissue damage despite substantial inflammatory microglial activation (Morimoto, Murasugi & Oda 2002). Further, if the ibotenate was given one day after the

LPS, severe tissue damage was elicited. If this is also true in inflamed lung tissues, it may be that all or most of the tissue damage is secondary to excitotoxicity rather than damage by pro-inflammatory cytokines. This could result in a need for a change or at least an addition in treatment direction. Further demonstration of this immunoexcitotoxic effect is in the demonstration that adding subtoxic concentrations of LPS to subtoxic concentration of glutamate (released by rotenone) become fully toxic when combined (Gao 2003).

The effect of inflammation on excitotoxicity can be explained to some degree by molecular mechanisms. For example, it is known that TNF-alpha and less so IL-1ß, increase insertion of NMDA and AMPA receptors (GluR2 lacking) onto the cell surface (Bernardino et al. 2005; Leonoudakis, Zhao & Beattie 2008). Another way inflammatory cytokines, such as TNF-alpha, can enhance excitotoxicity is by inhibiting glutamate transporters (EAATs), which elevates extracellular glutamate levels (Zou & Crews 2005). TNF alpha and IL-1ß also stimulate glutamate production by macrophages and microglia by upregulating the activity of the cellular glutaminase enzyme, responsible for the conversion of glutamine to glutamate (Ye et al. 2013). The two processes acting in concert, inhibition of glutamate uptake and increase glutamate production, can dramatically elevate extracellular glutamate to toxic levels. This not only stimulates high levels of reactive oxygen and nitrogen species and lipid peroxidation products, but also acts as a stimulus for migration of macrophages and neutrophils from the blood (Gupta & Chattopadhyay 2009; Gupta, Palchaudhuri & Chattopadhyay 2013).

Inflammation is known to raise glutamate levels in affected tissues and because neutrophils and macrophages also release glutamate when stimulated, it can act as an autocrine/paracrine loop to further increase invasion of these immune cells into the injured lung (Lawand, McNearney & Westlund 2000). This interaction between inflammatory mediators, extracellular glutamate levels and glutamate receptors is intimately involved in such a way that it constitutes a common immunoexcitotoxic pathological mechanism for a number of disorders. It appears that inflammation rarely if ever occurs without the involvement of excitotoxicity.

Glutamate Receptors in the Lungs: Pathophysiological Considerations

Said et al. first described functional glutamate receptors in the lungs using a high concentration of NMDA in a perfusate of isolated, ventilated rat lungs (Said, Berisha & Pakbaz 1996). In a follow-up study, they found that an inhibitor of NMDA receptors greatly attenuated oxidant lung injury caused by paraquat or xanthine oxidase (Said et al. 2000). In addition, Said made a link between activation of NMDA receptors in the lungs and airway hyperresponsiveness in bronchial asthma (Said 1999).

In the Dickman et al. rat lung study, they found that NMDAR1 mRNA and protein were moderately expressed in all regions of the lung tissue and airways. NMDAR2D was predominately expressed in the peripheral gas-exchange regions of the lungs and NMDAR2D and 2C were expressed in medium-sized and larger airways. He proposed that all of these receptors were within non-neural tissues, as no neurons are present beyond the conducting airways (Van Genechten et al. 2003). In the Dickman et al. study they found no evidence of NMDAR2A or 2B in any lung compartment or trachea (Dickman et al. 2004).

Robertson et al. using autoradiography with radio-labeled MK-801, demonstrated binding sites in the rat peripheral lung, which included alveolar walls as well as bronchial epithelium and bronchial smooth muscle (Robertson et al. 1997). In addition, one observes selective expression of NMDA receptor subtype 2D in alveolar macrophages, a major immune cell involved in viral and bacterial lung inflammation (Dickman et al. 2004). The differential localization of NMDAR2D suggest that it is most closely connected to excitotoxic lung injury, especially excitotoxic-induced pulmonary edema. This includes both airway hyperresponsiveness and airway and alveolar inflammation.

Said et al. demonstrated that NMDA receptor subtypes involved in lung injury were co-localized with vasoactive intestinal peptide (VIP) and neuronal nitric oxide synthase (nNOS), and that excitotoxic lung injury was mediated by excessive generation of nitric oxide and markedly attenuated by VIP (Said, Berisha & Pakbaz, 1996).

Also, of interest, Dickman et al. found that infusing the lung with NMDA caused a marked upregulation of NMDAR-2D receptor expression, thereby increasing lung sensitivity to excitotoxic injury (Dickman et al. 2004). They suggested that these glutamate receptor subtypes may act as a positive feedback mechanism, which would allow an amplified and perpetuated excitotoxic lung injury.

Collard et al. demonstrated that injured neutrophils release glutamate into the interstitial spaces in high concentrations, which can then markedly increase local tissue injury by further stimulating immune cell infiltration and by excitotoxicity (Collard et al. 2002). Macrophages, one of the more abundant infiltrating immune cells in cytokine storm reactions, also releases high levels of glutamate on stimulation (Piani et al. 1991). With a massive infiltration of neutrophils and macrophages into the injured areas of the lung, one can appreciate the tremendous inflammatory-excitotoxic enhancing effect of further glutamate release from these immune cells.

De Cunha et al. conducted an in vitro study of the effect of blocking NMDA receptors (using MK-801) using a cecal ligation and perforation model (CLP) of sepsis (da Cunha et al. 2010). In the study, they divided male Wistar rats into four groups: group 1 as sham operated controls, group 2. CLP rats treated with saline given immediately after injury and then 12 hours later. Group 3. CLP plus MK-801 in a dose of 0.3mg/kg given 4 hours after injury and group 4. CLP plus MK-801 in the same dose given 7 hours after injury. In addition, they performed a survival study by dividing the animals into three groups. Group 1 was given 50mg of saline subcutaneously. Group 2 was given MK-801 (0.03mg/kg) subcutaneously bid for two days starting 4 hours after the CLP lesion. Group 3 was given MK-801 (0.30mg/kg) bid for two days starting four hours after the CLP lesion. They recorded the animals' mortality for the next five days.

They observed an increase in NR1 and NR2A receptor subunits within the lungs at 6 hours, but not at 12 or 24 hours. There occurred a statistically significant increase in bronchoalveolar lung fluid (BALF) protein level and LDH activation after the CLP lesion, as normally occurs with such lesions. The MK-801 significantly decreased the BALF volume when the receptor blocker was given at four hours but not when given first at 7 hours. Importantly, they found that the CLP lesion resulted in significantly higher lung oxidative injury in the untreated group than the MK-801 treated group. That is, blocking NMDA receptors significantly reduced lung oxidative injury.

Also, of critical importance, the massive increase in total immune cell count within the BALF of the CLP lesioned animals, was significantly decreased by treatment with MK-801 when given at 4 hours, but not when first given at 7 hours.

Histopathological examination of the lungs of CLP lesioned animals without treatment demonstrated typical alveolar disruption, massive inflammatory cell infiltration, with moderate alveolar exudate. When MK-801 NMDA receptor blocker was given at four hours, they observed only mild inflammatory cell infiltration and no alveolar exudates.

The survival study demonstrated that among the CLP-lesioned animals given the higher dose (0.3mg/kg) of MK-801 there was a significantly improved survival when compared to those given a vehicle. The group given the vehicle had a 20% survival rate as compared to a 30% survival in the group given the lower dose MK-801 (0.03mg/kg), and a 45% survival among the group given the higher dose of MK-801 (0.3mg/kg).

In the same study, they also tested the effect of MK-801 against LPS stimulated release of TNF-alpha from spleen cells. The MK-801 NMDA receptor blocker significantly decreased the TNF response following LPS stimulation. In unstimulated spleen cells, neither NMDA, MK-801 or NMDA plus MK-801 had any effect on TNF-alpha release, suggesting that only in primed immune cells are the NMDA receptors active. TNF-alpha, along with IL-1, have been shown to play a major role in the cytokine storm reaction (Tisncik JR et al. 2012).

They concluded from these results that NMDA receptors are upregulated in the lungs during sepsis and that this effect is time dependent, with a reduction in response of the inflammatory cells by MK-801 occurring at 4 hours but not at 7 hours following sepsis onset. In essence, the combined effect of early NMDA receptor activation and increased insertion of NMDA receptor subunits may explain the increased survival observed by reducing the role played by these glutamate receptors in sepsis-induced lung injury. As with excitotoxic injury in the CNS, nitric oxide upregulation plays a major role in the pathogenesis of septic-induced lung injury. Glutamate and NMDA both stimulate the production of nitric oxide via upregulation of iNOS in rat alveolar macrophages. (Shang et al. 2010) Glutamate alone is known to provoke acute lung injury (Shen et al. 2007).

Macrophages, both derived from the blood and intrinsic alveolar macrophages, are considered to be major immune cell types within the lungs in the cytokine storm reaction. In addition, there is compelling evidence that alveolar macrophages are the major source of monocyte chemoattractants, such as MCP-1 (CCL2; Brieland et al. 1992). In the Dickman et al. study using a CLP lesion, the fact that they observed a significant increase in MCP-1 levels 12 hours after the septic lesion was initiated explains why NMDA receptor blockade with MK-801 had no effect if given at 7 and 12 hours (Dickman et al. 2004). A massive influx of macrophages into the lung lesion worsened the injury as at this stage there is already an overwhelming inflammatory response caused by the release of reactive oxygen and nitrogen species from the earlier glutamate stimulated immune cells.

Acute lung injury by glutamate is dose dependent and directly related to activation of glutamate receptors as shown by the complete protection from lung injury afforded by NMDA blocking agents (AP-5, AP-7 and MK-801; Said, Berisha & Pakbaz 1996). Magnesium, a blocker of NMDA receptors, was also shown to be protective when added to the lung perfusate.

Glutamate Receptors and Immune Cells

Lymphocytes

Several studies demonstrated lymphocyte activity being influenced by glutamate (Droge et al. 1988; Eck, Drings & Droge, 1989). Kostanyan et al. demonstrated that human lymphocytes express quisqualate-sensitive (AMPA) binding on specific sites of the lymphocyte outer membrane (Kostanyan , Merkubva, Navolotskaya & Nurieva, 1997). Others have also shown lymphocyte activation by AMPA receptor stimulation, which is intimately involved in lymphocyte adhesion and chemotaxis (Ganor et al. 2003).

Metabotropic glutamate receptors have also been demonstrated on lymphocytes. Pacheco et al. demonstrated group I and group III metabotropic glutamate receptors on human lymphocytes (R. Pacheco, Ciruela et al. 2004). They also demonstrated that mGluR5 was expressed constitutionally in T-cells whereas mGluR1 was expressed only after a T-cell receptor-CD3 complex was formed. Kostanyan et al. demonstrated that human lymphocytes express quisqualate-sensitive (AMPA) binding on specific sites of the lymphocyte outer membrane (Kostanyan, Merkubva, Navolotskaya & Nurieva, 1997).

Lombardi et al. in a study using healthy young volunteers found that glutamate in a concentration between 0.001 and 1mM could significantly potentiate lymphocyte response but lymphocyte reactivity progressively declined when exposing PHA-activated lymphocytes to higher concentrations of glutamate (3mM and 100mM; Lombardi et al. 2001). This observed suppression of lymphocyte activity was shown not to be due to glutamate injury to lymphocytes, but rather is acting through functional glutamate receptor mechanisms.

One of the characteristics of SARS-CoV-2 infections is a lymphocytopenia, which could be explained by the high levels of glutamate occurring secondary to widespread inflammation (Xu et al. 2020). The Lombardi study found that NMDA, AMPA and kainite (KA) all produced a significant potentiation of calcium response in activated lymphocytes, similar to the glutamate response described by others (Lombardi et, 2001).

A number of diseases can raise glutamate levels sufficiently to impair lymphocyte immune functions, such as AIDS, neoplastic disease, hepatic encephalopathy, intense inflammation, traumatic injury and autoimmune disorders (Aliprandi et al. 2005).

Glutamate (NMDA) stimulated lymphocytes also produce high levels of reactive oxygen and nitrogen species, which is prevented by exposure to the NMDA blocker MK-801 (Tuneva, Bychkova & Boldyrev, 2003). The normal range of blood glutamate in human circulation is between 70 and 100mM but can reach levels considerably higher in these conditions. Stimulation of NMDA receptors on lymphocytes has also been shown to increase the production of proinflammatory cytokines, such as IL-1ß, IL-6 and TNF-alpha (Valdychenskaya et al. 2011). Both free radicals and pro-inflammatory cytokines inhibit glutamate uptake, leading to higher levels of extracellular glutamate, which acts in an autocrine manner to worsen the pathology in the lungs. Blocking NMDA receptors on lymphocytes has been shown to inhibit T helper cell differentiation, thereby reducing the secretion of proinflammatory cytokines (Gao et al. 2011). Several researchers concluded that glutamate should be considered an immunotransmitter (Valdychenskaya et al. 2011).

Neutrophils

Circulating neutrophils during inflammatory states are captured by pulmonary vessel endothelial cells, followed by leukocyte tethering and rolling activation, and subsequent tight adhesion to endothelial cells. Transendothelial diapedesis results in invasion of these immune cells into the alveoli (Ley, Laudanna, Cybulsky & Nourshargh, 2007). Gupta et al. demonstrated that glutamate could stimulate neutrophil migration by acting through class I metabotropic glutamate receptors (Gupta , Palchaudhuri & Chattopadhyay, 2013; Gupta & Chattopadhyay, 2009).

Glutamate alone has been shown to reduce endothelial barrier function, leading to pulmonary edema (Collard et al. 2002; Nassar et al. 2011). As the neutrophils accumulate in massive numbers within the pulmonary tissues, they release high levels of glutamate, further driving neutrophil migration and leakage of fluid through the vascular barriers. Studies using a mouse model for traumatic brain injury have also shown mGluR5 of group I metabotropic receptors play a significant role in neutrophil migration into the site of injury (Yang et al. 2017). In this study, they found that deficiency of mGluR5 inhibits neutrophil infiltration into the injury site as well as inhibiting the release of inflammatory cytokines and chemokines.

The interaction between CD11b/ CD18 on neutrophils, as well as ICAM-1 on endothelial cells, is critical for neutrophil adherence to endothelial cells in the process of transmigration. Li et al. (2015) demonstrated that NMDA increases CD11b on neutrophils.

These studies clearly demonstrate that glutamate receptors, both ionotropic and metabotropic, play a critical role in neutrophil migration, pulmonary edema, release of high concentrations of free radicals, lipid peroxidation products, release of pro-inflammatory cytokines and chemokines, and ultimate destruction of alveoli structures and prevention of gas exchange.

Macrophages

As with the other immune cells thus far discussed, monocytes/macrophages also release high levels of glutamate upon stimulation (Piani D et al. 1991). Macrophages are a heterogenous population of immune cells in which the microenvironment determines the functional immune status, which includes either a classical activated state (M1) or an alternatively activated state (M2; Montovani, Sica & Locati, 2007).

Extracellular glutamate increases macrophage migration by activating class I and 5 mGluRs (Chiocchetti et al. 2006). Stimulation of mGluR5 appears to be mainly immunosuppressive, by reducing nitric oxide production and increasing production and release of IL-10 (Byrnes et al. 2009; Werry et al. 2011). Alveolar macrophages also express functional NMDA receptors, and release glutamate into the alveolar space during infections (Reijerkerk et al. 2010; Shang et al. 2010).

While under physiological conditions macrophages reduce extracellular glutamate levels and are therefore protective, in the face of intense inflammation the opposite can be true. This is based on how macrophages regulate extracellular glutamate levels and the effect of inflammation on this transport.

Human macrophages have two transport system for glutamate, a sodium dependent Excitatory Amino Acid Transport system (EAATs or X_{AG} system), consisting of 5 subtypes and a sodium-

independent glutamate/cystine antiporter system (X_C^-; Rimaniol et al. 2000). It has been shown that EAAT function is developmentally restricted in cultured astrocytes and macrophages (Stanimirovic et al. 1999; Rimaniol et al. 2000). These studies suggest that under inflammatory conditions circulating monocytes/macrophages may rapidly acquire EAAT transporters in a dose dependent manner (Klegeris, Walker & McGeer, 1997). The same was found for alveolar macrophages, in that they possessed no EAATs until stimulated. Under inflammatory conditions circulating macrophages may acquire EAATs by stimulation from TNF-alpha or other inflammatory mediators (Piani et al. 1991; Zerangue, Arriza, Amara & Kavanaugh, 1995).

Elevation of extracellular glutamate levels from macrophages occurs principally by the X_C^- system and not by EAATs (X_{AG}; Rimaniol et al. 2000; Watanabe & Bannais, 1987). Elevation of extracellular glutamate, as would occur during inflammation, inhibits cystine entry into the cell, thereby reducing the production of glutathione, a major cell antioxidant and protectant (Eck & Droge, 1989). EAATs would also be inhibited by elevated levels of reactive oxygen and nitrogen species and inflammatory cytokines, especially TNF-alpha (Trotti, Danbolt & Volterra, 1998; Zou & Crews, 2005). Under inflammatory conditions reverse glutamate transport can occur, dramatically raising extracellular glutamate levels (Grewer et al. 2008; Mandolesi et al. 2015). Not only would this suppression of glutathione synthesis increase damage and death to lung tissues directly, but also large number of macrophages, depleted of their protective glutathione, would release their stores of glutamate into the extracellular space upon cell destruction, further enhancing excitotoxic damage to the lungs. In combination, macrophages contribute to the extensive lung pathology by releasing massive concentrations of inflammatory cytokines, glutamate, reactive oxygen and nitrogen species and lipid peroxidation products into the alveolar fluid (BALF) and along the major airways deep in the lungs. In essence, the cytokine storm appears to be an immunoexcitotoxic storm.

Other Immune Cells Damaged by Glutamate

Functional glutamate receptors have also been demonstrated on thymocytes, dendritic cells (DCs) and natural killer cells (NK cells; Rezzanni et al. 2003; Pacheco et al. 2006; Kuo et al. 2001). Each of these immune cells play critical roles in both a normal response to respiratory viral and bacterial infections and in the pathological cytokine storm reaction. Elevated glutamate levels, acting through glutamate-linked cell signaling, could affect each of the cells in terms of effective immune responses, either resulting in a hyperimmune response or impairing viral and/or bacterial clearance in the lungs. DCs are the most potent of the APCs, in part by direct activation and differentiation of naïve T-lymphocytes (Banchereau & Steinman, 1998).

Pacheco et al. (2006) demonstrated that dendritic cells release glutamate upon stimulation, which was dependent on the X_C^- system. Exposure of DCs to lipopolysaccharide increased their basal glutamate release, and glutamate was shown to modulate cytokine production. For example, in this study after 48 hours following T-cell-DC cell contact, the released glutamate acted via mGluR1 on the T-cells to increase secretion of IL-6 as well as TNF-alpha, IL-2, INF-gamma and IL10. Stimulation of mGluR1 on the T-cells increased TNF-alpha production with a positive feedback upon dendritic cells. Glutamate, when acting through the mGluR5 was shown to inhibit T-cell proliferation, again possibly contributing the lymphopenia seen with the cytokine storm associated with SARS-CoV-2 infections. They also demonstrated that DCs and macrophages both release the same concentration of glutamate upon stimulation.

Controlling Immunoexcitotoxic Reactions in the Cytokine Storm Reaction

Experimental in vivo studies using various models have shown the effectiveness of blocking excitotoxicity in preventing and reducing the pathological damage caused by the cytokine storm. Li et al. used bleomycin to induce acute lung injury and fibrosis in mice, which produced similar lung injury as seen with highly pathogenic influenza and SARS-CoV-2 (Li et al. 2015). Their previous study demonstrated that glutamate alone could cause acute lung injury (Shen et al. 2007).

In their bleomycin study they demonstrated that the drug selectively induced the release of very high concentrations of endogenous glutamate into the lungs, resulting in acute lung injury (Li et al. 2015). They used memantine, a glutamate NMDA receptor blocker in the study. Animals given the memantine demonstrated significantly less histological lung damage, pulmonary permeability and lung wet/dry ratio than the untreated animals. The meantine appeared to work by suppressing neutrophil accumulation and by decreasing IL-1ß and TNF-alpha, while increasing the anti-inflammatory cytokine IL-10. They also demonstrated that activation of the NMDA receptors increased expression of the adhesion molecule CD11b on neutrophils, thus increasing transmigration of neutrophils into the alveolar space from surrounding vessels.

The increase wet/dry weight of the animals' lungs, along with protein leakage into the alveolar spaces, is consistent with the high incidence of pulmonary edema seen with pathogenic lung infections. Glutamate is known to induced pulmonary edema by increasing vascular permeability, which can be inhibited by MK-801 (Nassar et al. 2011).

Glutamate has been shown to mediate lung injury secondary to both hyperoxia and sepsis, suggesting that glutamate receptor activation is a common event with acute lung injury from various causes (Wang et al. 2009; Da Cunha et al. 2010). Studies in which other glutamate receptor inhibitors reduced lung pathology add further evidence of the importance of the excitotoxic arm of immunoexcitotoxicity in infectious lung injury.

Curcumin has been shown to provide powerful protection against the cytokine storm reaction in at least two cytokine storm animal models. In one such model of a pulmonary infection-induced cytokine storm utilizing mice infected with reovirus serotype I, strain Lang (reovirus I/L), researchers were able to reproduce the acute exudative phase, which included hyaline membrane formation as seen in human cases of virally induced cytokine storm. The untreated survivors also developed intra-alveolar and interstitial fibrosis as seen in human cases (Avasarala et al. 2013).

The researchers first established that curcumin had no effect on viral clearance or viral replication. They used three staining methods, haematoxylin and eosin (H&E), Sirus Red and Mason's trichrome to determine histological damage to the lungs, paying particular attention to diffuse alveolar damage (DAD), which is characterized by edema, capillary dilation and hemorrhage with hyaline membrane formation. Mice surviving the acute phase of the disease developed significant pulmonary fibrosis, just as seen in human cases.

The mice treated with the curcumin had significantly less inflammatory cellular infiltrate and significantly reduce fibrosis in the survivors. Untreated animals demonstrated significant accumulation of collagen in the lungs, which was greatly reduced by curcumin. They also found a dramatic decrease in recovered cells from the alveolar fluid in the animals treated with the curcumin,

in particular the number of PMNs (GR1+) and CD4+ cells on day five and CD19+ cells on day 9. On day 14 they observed a significant decrease in PMNs, NK cells, CD8+ T-cells and CD19+ B cells in the animals given curcumin. Curcumin was also found to differentially modulate TNF-ß1, NF_kB and p58 signaling, as well as significantly reducing phosphorylated p65 NF_kB as compared to untreated virally infected animals. Overall, curcumin was shown to significantly reduce inflammation and fibrotic lung damage in animals with a viral-induced cytokine storm reaction. The greatest lung damage in the virus infected animals without treatment occurred on day 14, with high levels of IL-6, IFN-gamma and MCP-1. Infected animals treated with curcumin demonstrated downregulation of all these pro-inflammatory factors.

Curcumin has been shown to effectively suppress NF_kB activation, a transcription factor linked to elevation in IL-1, IL-8, IL-6 and TNF-alpha upon immune cell activation (Singh & Aggarwal, 1995; Literat et al. 2001; Oh et al. 2011). Activation of NF_kB is associated with sepsis-induced acute lung injury (Bohrer et al. 1997; Arnalich et al. 2000). Suppression of TGF-ß by curcumin is important as its elevation has been associated with a poorer prognosis in ARDS (Fahy et al. 2003; Fahey, Robins & Constantinescu, 2007). Curcumin has been shown to suppress fibrosis under a number of conditions, such as that induced by cyclophosphamide, whole-body radiation and bleomycin (Venkatesan & Chandrakasan, 1995).

Later stages of sepsis are often associated with systemic organ damage, especially liver injury. In this study, Venkatesan and Chandrakasan demonstrated elevated liver enzymes at day 14 in the untreated virally infected mice. Treatment with curcumin reduced these liver enzymes back to control levels.

Curcumin is known to suppress the release of numerous cytokines and chemokines including IL-1ß, IL-2, IL-6, IL-8, IL-12, INF-gamma, TNF-alpha, MCP-1 and MIP-1alpha from monocytes and macrophages (Abe, Hashimoto & Horie T, 1999; Gao et al. 2004; Fahey, Robins, Constantinescu, 2007). Curcumin has also been shown to lower IL-6, IL-8 and MCP-1 secretion in cultured monocytes treated with high glucose concentrations as well as lowering blood TNF-alpha, IL-6 and MCP-1 levels in diabetic rats (Jain et al. 2009). Zhou et al. found that curcumin stimulated the production of SOCS proteins (Suppressor of Cytokine Signaling) a factor that fine tunes cytokine networks and helps prevent hyperimmune states, such as the cytokine storm (Kedzierski et al. 2014, Zhou et al. 2016).

While a significant portion of the protective effect of curcumin against lung damage by infections appear to be due to its ability to reduce pro-inflammatory mediators, curcumin also inhibits several glutamate receptor subunits and reduces excitotoxicity. Khalil RM and Khedr found that curcumin could protect against monosodium glutamate neurotoxicity in rats by decreasing NMDA2B and mGluR5 in hippocampal neurons (Khalil & Khedr, 2016). Curcumin was found to lower TNF-alpha levels in the hippocampus elevated by MSG, and may also lower glutamate levels. Lowering of extracellular glutamate levels occurs by several mechanisms, such as increased uptake of glutamate into glial cells and macrophages and by conversion of glutamate to glutamine by upregulation of glutamine synthease and glutamate decarboxylase (Zhou & Danbolt, 2014).

Chen et al. demonstrated an anti-inflammatory effect by dextromethorphan (DXM), an NMDA receptor blocker, using a murine model of collagen-induced arthritis and in humans with rheumatoid arthritis (Chen et al. 2017). IL-17A plays an important role in arthritis by enhancing the production

of TNF-alpha, IFN-gamma and IL17A (Mills, 2008). DXM suppresses IL-17A. Reduction of the inflammation is partially due to a blocking effect by DXM on dendritic cell function, suppression of the TH1 response, the TH17 response, or both. In a previous study, Chen et al. demonstrated dextromethorphan could inhibit activation of dendritic cells and dendritic cell functions (Chen et al. 2013) Future studies will have to determine if suppression of NMDA receptors on dendritic cells is responsible for the immune effects observed in these studies.

Conclusions

Most practicing physicians, as well as academic physicians, consider the cytokine storm as a purely immunological reaction resulting in progressive damage to the deep lung tissues, leading to a high incidence of death, or severe complication in survivors. Growing evidence indicates that an excitotoxic element is also involved and appears to play a major role in the disorder—perhaps a central role. An interaction between immune/inflammatory mediators, principally pro-inflammatory cytokines and chemokines, and glutamate receptors occurs in the cytokine storm reaction—immunoexcitotoxicity—in the same manner that we see in the central nervous system. In multiple sclerosis, for example, progressive destruction of the axons and neurons of the CNS continue to occur long after the immunological reaction has cooled down (Matute C,2001; Gonsette, 2008). We see a similar relationship in certain viral encephalic infections, where there is a progressive neurodegenerative response after the virus has been cleared by the immune system (Espey, Kustova, Sei & Basile, 2002; Hirai et al. 2017).

In the case of the CNS, it has been determined that there are several pathways of interaction between immunity and excitotoxicity. For example, TNF-alpha, and less so IL-1, stimulate glutaminase production of glutamate by microglia and astrocytes, and these same cytokines can stimulate insertion of certain glutamate receptors that can enhance the neurotoxicity of specific glutamate receptors (GluR2 lacking AMPA receptors; Stellwagen, Beattie, Seo & Malenka, 2005; Beattie, Fergursen & Bresnahan, 2010). Similar interactions are seen with monocytes, macrophages and neutrophils.

As the inflammatory process progresses, elevation in glutamate from the presence of activated macrophages and neutrophils within the alveolar spaces, stimulates an increase in immune cell migration to the lungs, especially neutrophils and macrophages, the principle immune cells found with cytokine storm reactions, which further increases glutamate levels in an autocrine and paracrine manner. With activation of the immune cells, we see a production of high levels of reactive oxygen and nitrogen species and lipid peroxidation products, which inhibit glutamate transport proteins (EAATs) and causes a suppression of the glutamate, cystine antiporter (system X_C^-) function by raising extracellular glutamate levels. This makes the macrophages and neutrophils more susceptible to apoptosis and necrosis, which releases their content of glutamate into the alveolar fluid and bronchial secretions. It would also trigger cell death in the epithelial cells of the airways and alveola. Activation of glutamate receptors on the endothelial cells lining the microvessels within the lungs, increase permeability of the vessels, leading to pulmonary edema, something well demonstrated with primary damage cause by glutamate exposure.

Immunoexcitotoxicity operates in both directions, in that excitotoxicity also triggers inflammation, producing a cyclic cascading effect of destruction. While not conclusively demonstrated, compelling

evidence suggest that activation of immune cells with release of pathogenic levels of pro-inflammatory cytokines always triggers excitotoxicity (immunoexcitotoxicity; Gelbard et al. 1993; Herman et al. 2001; Beattie et al. 2002; Bernardino et al. 2005; Floden,2005; Li & Combs, 2005; Zou & Crews, 2005; Takeuchi et al. 2006; Carmen, Rothstein & Kerr, 2009; Han & Whelan, 2010; Olmos & LLado, 2014). Brison et al. found that excitotoxicity played a major role in paralysis in mice following infection with a human strain of coronavirus with a single mutation in its spike protein (Brison, Jacomy, Desforges & Talbotr, 2011). While this discussion is limited to local inflammatory reaction within the lungs, glutamate receptors in the medial septal area of the brain also regulates immune reactions (Podlacha et al. 2015). NMDA injected into the medial septal nuclei in stressed Wistar rats was shown to significantly increase in NK cell cytotoxicity and cause a rise in large granular lymphocytes in the plasma.

Studies have also shown that neurodegeneration secondary to viral infections may not be secondary to directly infected neurons, rather the damage is secondary to accumulation of glutamate in the extracellular space secondary to interference of glutamate uptake, which is powerfully inhibited by inflammatory cytokines (Darman et al. 2004; Nargi-Aizenman et al. 2004).

In most of these studies the source of the excess glutamate was intrinsic, principally from immune cells. Yet, oral ingestion of glutamate can raise glutamate levels to neurotoxic levels and should be considered. Ironically, many parenteral tube feedings contain high levels of glutamate and other excitotoxic amino acids. This could worsen existing excitotoxicity in seriously ill patients and may play a role in the rapid deterioration of moderately ill patients infected with a respiratory virus, including SARS-CoV-2, especially since so many processed foods contain significant levels of glutamate and other excitotoxin additives.

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