



Innate Recognition and Signaling during Times of Pandemic and Beyond from Intensive Care Perspective

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ABSTRACT

The systemic inflammatory response (SIRS) underlies the majority of intensive care-related conditions. Depending on the origin it may become a governing force of organ dysfunctions. The immune response therefore may be a contemptuous reaction. While necessary for viral, or bacterial elimination, clearance of debris, and regeneration, when dysregulated, overpowering, or chronically ongoing, it may lead to significant collateral damage, organ failure, and autoimmunity. Understanding the immune response in specific complex situations, monitoring, and targeted influencing may become a future step in intensive care management. Toll-like receptor four (TLR4) is a representative innate immune receptor with authoritative downstream signaling and regulatory functions. The following review aims to bridge the logics of innate immune recognition, signaling, and influence on intensive care-related acute conditions by TLR4. We demonstrate that overwhelming innate immune response can be blunted, skewed, and consequently, adaptive immunity positively influenced, but such an approach must be careful and targeted for specific situations optimally under comprehensive immune monitoring. The unanswered questions of the field, as well as possible caveats of such novel approaches, are mapped through discussing in vitro and animal models, human trials.

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ABBREVIATIONS

ICU; SIRS; TLR4; LPS; LBP; CD14; MD-2; MHC; DAMPs; NETs; IL, TNF; IFN, ROS; HMGB1; HSP; NADPH; cGAS/STING; MyD88; TRIF; TRAM; MAL; IRF3; FADD; NF- κ B; MAPK; p38; JNK; ERK; CD11b; Bcl-2; CARS; MDR; AB; KP; MRSA; ROSC; ACEII; OSA; SARS-Covid2; RSV; CFU; ARDS; BAL; IRI; ATI; ATII; p53.

1. INTRODUCTION

Due to its proximity and early engagement innate immune signaling has an important function in many Intensive Care (ICU) related conditions. The ICU patient often suffers severe organ dysfunctions, the management of which is largely supportive. Maintenance of oxygenation, gas exchange, avoidance of energy exhaustion, sheltering from opportunistic pathogens, thromboembolic complications, providing energy and fluids, preserving cardiovascular stability, mechanical waste clearance until the indigenous healing process reestablishes organ functions is the centerpiece of our work. One of the least understood and targeted „organs” in intensive care is the immune system for its complexity, ubiquitous expression, and complicated network of communication. The application of biological treatment is yet awaiting and may become more substantial in severe hyperinflammatory conditions, like severe sepsis, acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS), hypoxic and ischaemia-reperfusion injury, immune exhaustion. Such effort requires enhanced and deep understanding of immunological processes by ICU clinicians, as well as clinical monitoring. It is important to recognize that immune function and modulation is often a matter of degree, timing, and context.

2. THE ORIGIN OF THE TLR4 MOLECULE, LIGANDS, AND SIGNALING PATHWAYS

The first sentinel cells of the immune system are equipped to sense danger, either external or pathologically modified self. One of the crucial, proximal sensors is TLR4 [1]: one among ten toll-like receptors in humans, the cell surface molecule originally discovered in drosophila, the fruit fly. The innate immune system components display a high degree of conservation through species and cell components are selected during

evolution to fit the purpose of the whole organism the best.

TLR4 remained present in both immune and non-immune human cells, providing physiological functions of defense and healing. Significant levels are present on all innate immune cells and many tissue-resident cells. The archetypic external ligand for TLR4 is lipopolysaccharide (LPS) [2], present on the outer membrane of gram-negative bacteria. Upon encounter between host and microbe, LPS is released from the outer membrane of gram-negative bacteria, binding to a lipopolysaccharide-binding protein (LBP), and is driven and presented to TLR4/MD-2/CD14 complex on the cell surface. When the cluster of differentiation 14 (CD14) or TLR4 is lacking, mice are resistant to LPS or live gram-negative bacteria-induced septic shock, this is how crucial TLR4 appears to be in mediating septic immune response. Upon LPS binding to a myeloid differentiation factor-2 (MD-2) molecule, homodimerization takes place and intracellular signaling pathways become activated. There are several TLR4 antagonists in development and research utilization. Synthetic LPS derivatives of nonpathogenic photosynthetic *Rhodobacter sphaeroides* (LPS-RS) are potent antagonists of toxic LPS in both human and murine cells. LPS-RS is a pentaacylated LPS as oppose to the hexaacylated toxic LPS of pathogenic gram-negative bacteria. The mechanism of acting is twofold, one is the competitive antagonism of pathogenic LPS by binding to the same site on MD-2, the second mechanism is the inhibition of 6LPS/MD-2 signaling via 5LPS/MD-2 complex. To overdrive LPS-RS inhibition of TLR4 activation, a hundredfold increase in 6LPS dose is necessary. One of the commercially available preparations, Eritoran has been tested in preclinical and clinical settings of several disease models. TAK-242 on other hand binds to the intracellular portion of TLR4 and inhibits LPS induced cytokine production. Nowadays computer-based receptor

and ligand targeted new drugs are designed and tested [3].

There is emerging apprehension of certain infective, ischaemic and metabolic processes cumulating in cell damage, exposing, misfolding, and incorrectly compartmentalizing DNA and other self-proteins. These molecules can be recognized by TLR4 and other pattern recognition receptors (PRRs), leading to a self-aggravating process of SIRS often with tissue and organ severance. Intracellular organisms, when targeted by the immune system, inescapably cause the death of the host cell too. Often, particularly in an underlying inflamed or infectious environment, the cells do not undergo a programmed form of death, rather necroptosis or pyroptosis takes place, leading to a vicious circle of inflammation.

Among the first immune sentinels are neutrophils, processing pathogens by phagocytosis or NETosis. Neutrophils are short-lived, and upon fulfillment of killing function, not only die via silent apoptosis but swell, burst, create NETs (neutrophil extracellular traps). Even though NETs participate in host clearance, they also become an inflammatory trigger if produced in large quantities or not cleared properly. *Streptococcus pneumoniae*, *Haemophilus influenzae* for example, activate NET formation, partially via the participation of TLR4 and reactive oxygen species (ROS). LPS may lead to TLR4 and /or ROS-dependent or independent NET formation depending on the bacterial source. Neutrophils release the content of chromatin and DNA, further perpetuating autoinflammation. Hereditary insufficiency of neutrophils is one of the most severe immune deficiencies, hence the role of neutrophils in pathogen clearance should not be underestimated. While they are essential to clearing pathogens from the lungs, NETs also contribute to worsening chronic obstructive pulmonary disease (COPD) pathology and reduction of pulmonary function. NET formation upon pathogen encounter or opsonization by antibodies leads to type I interferon production from plasmacytoid dendritic cells and inflammasome activation. The precariousness of the staggering activation lies in the exhaustion of the host, and irreversible and severe organ damage. The issue of collateral damage triggers the task to identify the appropriate target and modify the immune response so that the host function would be preserved and accessory damage minimized while maintaining antiviral, antibacterial activity.

There is a spectacular range of damage- and self-associated TLR4 ligands [4]: high mobility group box 1 protein (HMGB1), heat shock proteins (HSP60,70,90, etc), self DNA, RNA, S100proteins, chaperone proteins such as gp96, fibronectin, surfactant protein A, CD138, defensins. These molecules are released from the damaged or dead cells. Damage-associated molecular patterns (DAMPs) do not require coreceptors to be recognized by TLR4. HMGB1 is a chromatin protein which functions in stabilizing DNA and in gene transcription. Upon TLR4 engagement on neutrophils, HMGB1 participates in the release of reactive oxygen species by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. HSPs are intracellular proteins, released in response to cell stress such as infection, toxins, or hypoxia. Similar to HMGB1, they can be secreted from cells by noncanonical leaderless secretory pathways, or released during necrosis. DNA can be recognized besides TLR4 by the cGAS/STING pathway. The stimulator of interferon genes (STING) molecule participates in the production of type I interferons (α, β , etc), crucial antiviral cytokines, which however lead to cell apoptosis, aggravating, for example, myocardial necrosis [5].

Membrane-bound TLR engagement by exogenous or endogenous ligands is followed through adaptor molecules by the activation of the wide range of genes. The intracellular portion of TLR4, upon homodimerization and activation, binds to the TIR adaptor molecule. The four TIR adaptor proteins: Myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (MAL), TIR-domain-containing adaptor inducing interferon- β (TRIF), Trif related adaptor molecule (TRAM) mediate all the signaling events by TLRs in general [6]. In the absence of MyD88 and TRIF, no TLR signaling takes place [7]. The downstream TLR4 signaling separates into MyD88 dependent and TRIF dependent pathways. MyD88 serves as an adaptor not only for TLRs but for the interleukin-1 (IL-1) family as well (IL-1 β , IL-8, IL-33), while TRIF is the TLR related adaptor molecule capable of apoptosis activation, via Fas-associated protein with death domain (FADD)-caspase 8 axis. Cardiomyocyte-specific caspase 8 transgenic mice develop apoptosis and severe dilated cardiomyopathy because cardiomyocytes have very limited ability to regenerate [8]. The TRIF adaptor is very own to TLR3 and 4, but while TLR3 can signal via TRIF exclusively, in the case of TLR4, both TRAM and TRIF are required for IFN regulatory

factor3 (IRF3) activation and type I interferon production. MyD88 dependent engagement leads to early activation of the NF- κ B pathway and after internalization to endosomes, TLR4 recruits the TRIF adaptor, leading to the activation of the IRF3 pathway, production of type I interferons, and the second phase of NF- κ B, mitogen-activated protein kinase (MAPK) and IL-1 β activation. The MAPK family has 3 members: p38, c-Jun N-terminal kinase (JNK), and extracellular-signal-regulated kinase(ERK). Importantly, p38 activation is important for antigen presentation via major histocompatibility complex(MHC)I and MHCII molecules to CD8+ and CD4+ lymphocytes and costimulatory molecule expression, even though the effect of TLR stimulation can under certain conditions be inhibitory as well [9].

The aids for TLR4 internalization from the cell surface to the endosomes depend on the immune cells type. In dendritic cells, it is CD11b (complement receptor 3). CD11b in macrophages however disarms the TIRAP-MyD88 complex and therefore has a regulatory function by inhibiting the LPS effect. Importantly LPS can be endocytosed using CD14, even in the absence of TLR4, in the contrary TLR4 is unable to endocytosis without CD14. NF- κ B-dependent cytokine stimulation is hence not entirely dependent on internalization. Upon internalization intracellular killing and antigen processing takes place and antigen-specific adaptive immunity triggered. The downstream signaling pathway of TLR4 leads to the production of cytokines, cell migration, proliferation and /or apoptosis, and antigen presentation The MyD88 pathway activates IL-1 β , TNF α , IL-6, IL-10, IL-12, etc. production, TRIF leads to type I interferon synthesis. While the evolutionary conserved reactive oxygen species(ROS) are important in host defense, they have the potential to cause not only significant collateral tissue damage but also suppress the activation of the adaptive immune T cells response, including that of memory phenotype, perhaps participating in compensatory anti-inflammatory response syndrome (CARS) stage during sepsis. TLR2 and 4 activations in macrophages increased the mitochondrial ROS production and hypoxia-induced ROS production under experimental circumstances generated cell exhaustion (CD8+, PD1+, Tim3+ phenotype) [10]. TLR4 activation propagates into systemic inflammatory response syndrome(SIRS) irrespective of eliciting injurious stimulus. SIRS can be driven by pathogens, but

importantly cell debris, cell metabolites, hypoxia, and trauma activate inflammation in a manner that may become deleterious via energy exhaustion, pathologically increased vascular permeability, swelling, cell death, thrombosis, and fibrosis in the chronic stage(Fig1).

3. TLR4 IN BACTERIAL SEPSIS

TLR4 activation is an important innate defense mechanism for pathogens. It appears to be, however, a double-edged sword, and the balance between pathogen clearance and detrimental hyperinflammation can be tilted towards the latter. For the needs of the clinician, the animal models can become afflictive for the differences in immune response among species, but also because the experiments using mice devoid of TLR4 beginning the conception, without its ability to condition the immune response and participate in the development, and without the potential of responding at any degree at all, can be deceptive. All is done with the best possible intention and bridging the two worlds is demanding. When analyzing bacterial pathogens one has to take into account the severity of sepsis, the virulence of particular pathogens, their ability to evade the immune response, and the baseline condition of the host. It is necessary to realize that our immune system is not yet sophisticated enough to timely elicit a specific response that would eliminate the pathogen with minimal collateral damage. The below will demonstrate the need to account for the invading pathogen in accessing TLR4 function, as they have the differing capacity to stimulate and evade the immune response.

Polymicrobial sepsis-induced upon caecal ligation and puncture consistently demonstrated improved survival upon anti-TLR4 antibody treatment and in knock-out (KO)state, with improved cardiac function [11,12]. The initial phase of sepsis is characterized by a hyperinflammatory response, later immune exhaustion and persistent immune suppression prevail [13]. The immune exhaustion or immune suppression is due to massive T lymphocyte apoptosis and profound long-lasting lymphopenia leads to increased mortality, on the contrary early monocyte apoptosis improves survival. Another hallmark of immune suppression due to acute sepsis is the increase in regulatory T cells, myeloid-derived suppressor cells, and IL-10 producing B cells. During this phase, frequent nosocomial infections, with significant lethality emerge [14]. During the immunosuppressive

phase of sepsis immune stimulation would be desirable. Of such IL-7 appears to be a promising treatment, inducing antiapoptotic programming via B cell lymphoma 2 (Bcl-2) activation in T cells without eliciting cytokine storm. When mice were infected with the influenza virus and treated with Eritoran, subsequent immune suppression was less pronounced and lung pathology in nosocomial methicillin-resistant staphylococcus aureus (MRSA) infection was mitigated [15]. Acinetobacter baumannii(AB) multidrug-resistant (MDR) is responsible for high ICU mortality. The multiresistant highly pathogenic pathogen has evolved into significant danger with the increased utilization of mechanical ventilation and indwelling invasive devices. AB is characterized by perseverance, resistance, and withstanding dry conditions. The multiresistant XDR phenotype is only sensitive to colistin and is responsible for significant mortality due to pneumonia and blood-borne infections. TLR4 deficiency leads to variable outcomes depending on bacterial virulence. The differences demonstrate that while a proinflammatory environment is needed for pathogen clearance, an overwhelming response creates accessory injury, that can lead to death. In hypervirulent strains in susceptible mice, inoculation induces a hyperinflammatory syndrome and TLR4 deficiency rescues 100% of mice from otherwise lethal sepsis. Strikingly, bacterial clearance is not significantly affected, but the cytokine storm is blunted leading to a survival advantage. In situations when less virulent strains are used to infect mice, the response is slower neutrophil recruitment and slower, but ultimately effective bacterial killing and mice having less pulmonary damage. The essential role of reactive oxygen species in killing AB was demonstrated on gp91^{phox}-/- superoxide deficient mice, whose inoculation resulted in a thousandfold increase in bacterial load and death by 48hours [16]. Another important multiresistant pathogen is Klebsiella pneumoniae (KP). KP is a major reservoir of antibiotic resistance genes that can translocate to other bacterial strains. Klebsiella's danger lies in its multitude of munitions it uses to evade the immune response. Even though bacteria in general, use several receptors for entry, in the case of KP, TLR4 appears to be a major receptor recognizing both the capsular polysaccharide and the lipopolysaccharide [17]. Severe KP infections occur primarily in immune-compromised patients with diabetes or malignancies when further immune suppression of any sort could be detrimental, even though

since 1980 hypervirulent strains emerged causing severe diseases and organ abscesses in healthy individuals. TLR4 deficiency in mice in this case leads to an increase in mortality, notably one of KP's evasion techniques is NF- κ B signaling suppression. The KP capsule is so sturdy that it protects against phagocytosis, opsonophagocytosis, or complement-mediated lysis [18].

In the mouse model of E.coli induced peritonitis with 10^4 colony forming unit (CFU) of E.coli, TLR4 KO mice had similar mortality dynamics to wild type, but MyD88 KO and TLR2/4 double KO had worse outcomes. TLR4 KO had a more gradual septic immune response that potentially rescued them from deleterious effects of immune system overactivation. In a model of the lethal dose of intraperitoneal E.coli (10^8 CFU), anti-TLR4 antibody rescued mice from death if it was given up to 7 hours after lethal E.coli challenge [19]. The effectiveness of monoclonal antibody therapy depends however on the type of antibody, dose, timing, affinity, etc [20]. Certain pathogenic and commensal bacteria evade endocytosis by producing dephosphorylated LPS. The commensal Bacteriodes thetaiotaomicron has an altered LPS structure to evade TLR4 recognition, perhaps a beneficial feature to preserving the immune environment silent. When a randomized clinical trial conducted on severe sepsis patients led to no significant difference in disease outcomes and mortality, the idea of antagonizing TLR4 in septic patients was abandoned. There is however still a lack of understanding, as to why abundant basic research data differed so greatly from clinical results. Is it due to the constitutive lack of TLR4 in knockout and mutant mice, versus short-lived low dose inhibition of TLR4 when already after LPS stimulation via pathogenic microbes has already taken place? Is it due to internalization of TLR4 upon 6LPS encounter or that TLR4 blockade has been counterbalanced and complemented by other immune activators, or that bacterial clearance was influenced negatively in a way that has ultimately led to the loss of survival benefit?

4. TLR4 ACTIVATION DURING HYPOXIA AND ISCHAEMIA-REPERFUSION INJURY

Acute ischaemia in either local or global form emerges upon occlusion of arterial blood supply or disequilibrium between cell oxygen supply and

demand [21]. The unfortunate side effect of reperfusion is further exasperation of the inflammatory response causing deterioration of organ function. Ischaemia-reperfusion injury (IRI) promotes cell death, transcriptional reprogramming, SIRS upon release of DAMPs, TLR4 and complement activation. TLR4 engagement has been demonstrated in all experimental models of IRI and TLR4 inhibition led to partial restoration of organ function in analyzed experimental and clinical models. Upon global cerebral hypoxia, TLR4 activation contributes to neuronal cell death in hippocampus, which is partially prevented in TLR4 KO mice [22]. Acute myocardial ischaemia type I is managed by recanalization of coronary arteries, and while imperative, recanalization contributes to reperfusion injury. Targeting IRI remains in that account a conspicuous aspect of management [23]. While acute ischaemia for a short period of 5 minutes has shown to be protective via preconditioning, ischaemia lasting beyond 20 minutes deprives the myocardium of oxygen and nutrients and acuates myocardial cell death starting from subendocardium extending gradually transmurally. Impaired oxidative phosphorylation in mitochondria causes ATP depletion and attenuated contractility. Upon reperfusion arrhythmias may arise, while stunning continues, and microvascular obstruction due to capillary damage aggravates cell death. Clinical studies suggest, that reperfusion injury worsens myocardial infarction by increasing infarct size. The contributors of myocardial reperfusion injury are oxidative stress, the opening of mitochondrial permeability transition pores, calcium overload, SIRS [24]. Several groups demonstrated that constitutive TLR4 deficiency spared the heart tissue from necrosis to a certain extent. Eritoran diminished infarct size significantly in mice pretreated before coronary artery occlusion of the left anterior coronary descending artery. In a similar study, Yorkshire pigs were used, coronary artery obstruction was applied for 45 min, and 10 minutes later the animals were treated with ApTOLL, a small TLR4 inhibiting RNA, pig hearts were rescued from injury, had improved left ventricular ejection fraction day 7, inflammatory cytokines were dampened [25]. Cardiopulmonary resuscitation upon cardiac arrest both outpatient and inpatient has a low survival rate, with frequently poor quality of cerebral functions. Periods of cerebral hypoxia and subsequent reperfusion injury upon return of spontaneous circulation (ROSC) propagate into cerebral swelling and neuronal cell death. TLR4 has been

implicated in pathogenesis based on the already described general mechanisms of SIRS activation [26] and aquaporin stimulation in the brain. The way the cell dies influences the immune response. Under the conditions of prolonged energy deprivation, apoptosis is inhibited and necroptosis prevails [27]. Apoptosis is a physiological *modus operandi*, but if apoptotic cells are not cleared timely, secondary necrosis is initiated. As oppose to immunologically silent apoptosis, necrosis triggers an immune response and ultimately may lead to the establishment of autoimmunity. Necrotic cell death occurs during injury, viral and bacterial infections, ischaemia-reperfusion, UV radiation, oxidative stress-mediated via $TNF\alpha$, TLR3, and TLR4, interferon signaling, etc. Necroptosis leads to the disintegration of the plasma membrane and the release of DAMPs. Pyroptosis occurs upon the activation of inflammasomes [28]. After the clearance of dead tissue and resolution of inflammation, an anti-inflammatory switch takes place, in macrophages from M1 to M2 phenotype, regulatory T cells rise [29]. Lung regeneration does happen. For example, in rodents after 2 - 3 weeks upon pneumectomy, there is a dramatic increase in alveolar cell number and formation of new septa [30]. A certain degree of immune activation is perhaps beneficial for tissue regeneration, but overt activation via TLR4 leads to fibrosis representing a restrictive environment for lung mechanics. For example, in the systemic sclerosis model, TLR4 is a key driver of tissue fibrosis. Sensing of DAMPs appears to trigger a profibrotic environment via myofibroblast differentiation and fibrotic gene-environment stimulation [31]. Upon LPS stimulation of fibroblasts, primarily genes involved in extracellular matrix remodeling and tissue repair become activated. Temporary, low-grade TLR4 is necessary for normal wound healing, oligodendrocyte differentiation in the spinal cord [32], and osteoblast formation. The uncontrolled, excessive or persistent, and unresolved TLR4 activation, among other signaling pathways, is the factor that tilts the balance towards fibrosis. Acute inflammation, ongoing or recurring hypoxia can ultimately lead to pulmonary fibrosis with chronically impaired lung mechanics and oxygenation. Similarly, the chronic phase of acute myocarditis [33], and acute kidney injury [34] can resume in fibrosis with permanently impaired organ functions. If the inflammatory response is regulated during the early stages, such irreversible consequences may be effectively blunted [35]. Pulmonary fibrosis is

particularly prominent if the etiology is viral pneumonitis [36]. In the pathogenesis of heart failure, angiotensin-converting enzyme (ACE) inhibitors bear a compelling antifibrotic effect. Hypoxia response involves chiefly the activation of hypoxia-inducible factor (HIF) and NF- κ B transcription factors. HIF-1 and HIF-2 participate in the regulation of macrophage functions during hypoxia. Hypoxia aggravates reactive oxygen species activity and activated HIF-1 elicits negative feedback on ROS production. It appears that during hypoxia innate immune cells gain a survival advantage over adaptive immune cells. Hypoxia-activated macrophages generate increased phagocytic activity and cytokine levels. There are direct hypoxia-responsive elements in the genes of TLR1,2 and 6, with significant TLR4 engagement present in acute and chronic hypoxia, too.

RAW264.7 macrophages upon 8 h exposure to hypoxia and subsequently to LPS, demonstrate a pronounced COX-2, IL-6, RANTES, and IP-1 expression. The HIF1- TLR4 crosstalk is bidirectional. This has been demonstrated in vitro in SIHA cells, using the experimental cervical cancer model. Superfluous activation of HIF1 α has been implicated in cervical cancer cell growth and invasiveness in vitro via TLR4 activation and ROS production. Silencing TLR4 via siTLR4 led to abolishing such activity [37]. The DAMPs engagement has been demonstrated in kidneys in SD rats exposed to hypoxic conditions, by placement to hypoxic chamber daily for 8 hours during 2 weeks to mimic obstructive sleep apnea (OSA). In peripheral blood increased TLR2,4, IL-6, TNF α levels were present and kidney damage with HMGB1 deposition was demonstrated [38]. Acute induction of HIF-1 was necessary in experiments to achieve cardioprotection in cardiac ischaemic preconditioning [39].

5. TLR4, THE VIRUS, AND ARDS

The first viral pandemic in modern history was the „Spanish“ flu between 1918 and 1920, caused by the H1N1 influenza A virus, claiming a toll of up to 100 million human lives worldwide, with a fulminant course, patients dying within few days most likely from cytokine storm and consequences of acute ARDS. Since then, SARS in 2003, H5N1 in 2006, H1N1 in 2009 [40] emerged and experts anticipate new and new viral pandemics to egress periodically in the future. Before mutual adaptation of the host and pathogens shapes new mutants to a level of

virulence that enables the pathogens to survive without eliminating the host, -since this is the most optimal scenario for viral survival and spreading-, the pathogen can take up hypervirulent forms, and the respiratory route is the easiest way to attack the vulnerable host. ARDS, the most severe form of pulmonary pathology, characterised by bilateral infiltrates on x-ray and progressive hypoxemia leading to respiratory insufficiency, is affected by mortality that currently fluctuates between 27-50% [41]. Significant mortality reduction has been achieved to great extent due to protective mechanical ventilation techniques with proning, opening the lungs, and keeping them open, low tidal volumes (with decreased epithelial injury and cytokine production), non-depolarising muscle relaxant administration, yet the mortality for hyperinflammatory category of ARDS remains high. When phenotypic categorisation was done on two large ARDS cohorts (using data from ALVEOLI trial targeting low versus high PEEP application), and ARMA trial (evaluating low tidal volume ventilation) two distinct phenotypes were established on day 3, with survival benefit of hypoinflammatory over hyperinflammatory phenotype, the latter associated with increased levels of bronchoalveolar lavage (BAL) cytokines: IL-1 β , IL-6, IL-8, TNF- α , reminiscent of NF- κ B and inflammasome activation [42]. The recognition of relatively stable subphenotypes at day 3 has led to further underscoring the ambivalent nature of overt inflammatory reaction in ARDS and to articulating the need to group patients based on dominant relevant disease-modifying parameters to enable valid statistical analysis. A small proportion of patients changed course by swapping phenotype and the outcome of the patients was determined by the nature of the phenotypic change [43]. Many clinical treatment trials in ARDS have failed, in several instances the complexity and heterogeneity of ARDS phenotypes were not accounted for, hence the trial design was incriminated. The disease severity categorisation in ARDS is based on oxygenation index, that is the ratio of arterial oxygen tension to the oxygen fraction administered upon respiration/ventilation. Consequent to subphenotype categorisation, a previously renounced simvastatin treatment led to a 28-day mortality reduction in hyperinflammatory group of patients [44]. Simvastatin has multiple immune downregulatory effects, including depreciation of TLR4 expression demonstrated in rat myocardial infarction, aortic valve stenosis [45], cerebral haemorrhage, sepsis-induced lung injury, etc.

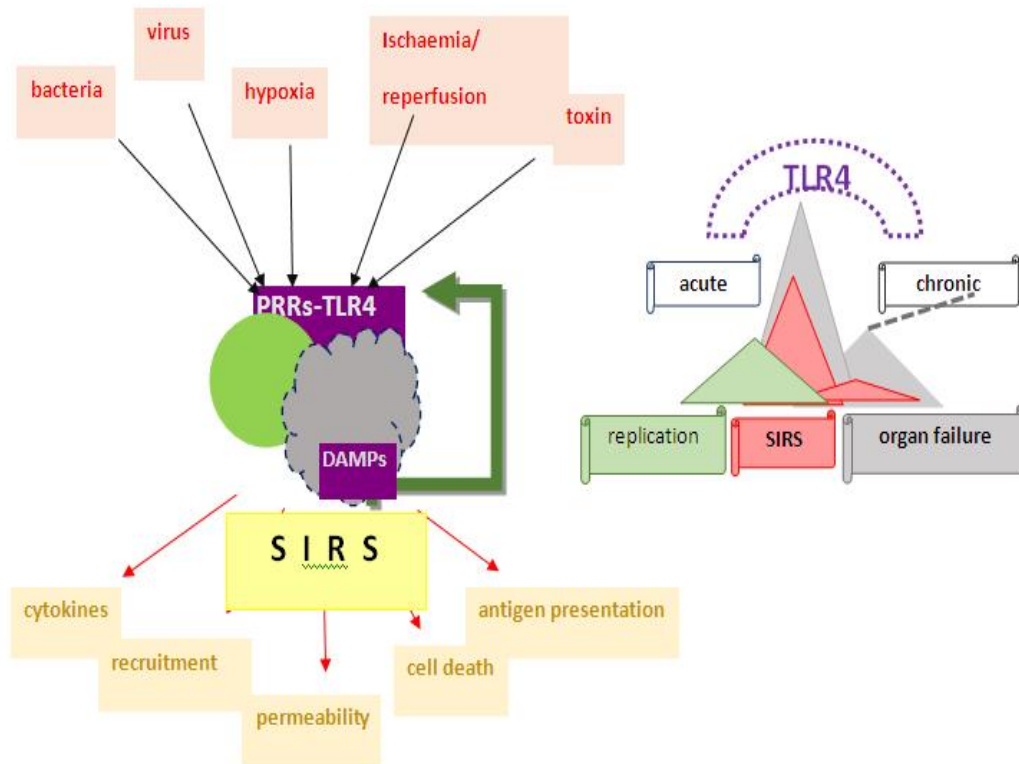


Fig. 1. Self-aggravating tissue injury due to SIRS

Among the important features of ARDS is increased epithelial and endothelial permeability, insufficient fluid clearance leading to non-cardiogenic, protein-rich pulmonary oedema. The management consists of fluid restriction and positive end-expiratory pressure application. In the healthy lungs, active ion transport-created osmotic gradient drives the alveolar fluid clearance from the lungs, with alveolar type I (ATI) and type II (ATII) cells participating in the process. During ARDS the fluid accumulation in alveoli and interstitial tissue - further compromising oxygen uptake by lungs- is due to several contributors. Cell damage, cytokine effect, the important role of complement 5a anaphylatoxin, hypoxia, and hypercapnia contribute to impairment of sodium pumps and disintegration of tight cell junctions between alveolar epithelial and endothelial cells. Elevated dead (functionally not active) pulmonary space, and decreased lung compliance are associated with increased mortality in ARDS [46]. Another hallmark is apoptotic and necroptotic alveolar epithelial cell death induced by neutrophil ROS

and NETs, macrophage TNF-related apoptosis-inducing ligand (TRAIL), as well as loss of surface tension due to defective surfactant production leading to alveolar collapse.

The identification of viral-induced ARDS is not always straightforward, not all viruses are tested for, and the incidence is accordingly reported between 13.4% to 49%. As opposed to TLR4, TLR3, the prototypic PRR for viral double-stranded RNA is protective in influenza or coronavirus induced disease and ARDS via TRIF pathway consistent with the finding, that interferon(IFN) I and III production is mitigated by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [47,48].

Innate interferon production is induced upon engagement of certain surface and intracellular pattern recognition molecules, and it is considered an elemental antiviral effector, and the downstream interferon responsive elements: IFN- stimulated genes (ISG)s can alter the majority of viral replication steps [49]. During

influenza virus infection Interferon I and III are complementing, and the deficiency of each is mutually compensatory. While TLR4 signaling can engage innate interferons via TRIF/IRF3 upon internalisation, significant redundancy is expected with other interferon eliciting elements.

Viral evasion of the interferon response is a widespread attribute of infectivity [50]. To further perplex the matter, interferons can cause epithelial cell disruption. During the repair phase, around the 11th day of influenza virus infection IFN I(β) and III(λ) treatment reduced the proliferative activity of ATII cells via the activation of the p53 tumor suppressor pathway [51]. In IFN I or III receptor-deficient mice proliferative activity of ATII cells was restored and viral clearance was not affected, perhaps due to the compensatory nature of the interferon network, and *Str. pneumoniae* bacterial superinfection was also improved in the absence of IFN-III receptor. The main culprit of the compromised lung regeneration was IFNIII(λ).

The pattern recognition profile of viruses and bacteria is very diverse, during virus recognition these are complement receptors (CD21, CD46, CD55), TLR2,4 on the cell surface, TLR 3,7,8 on the endosomal membrane, NOD2, RIG1, MDA5, NLRP3 in the cytoplasm, and importantly receptors that have physiological functions other than immune regulations, CAR (group B coxsackievirus, adenovirus 2), nucleolin (RSV), sialic acid (influenza, adeno-, rotavirus), etc participate in recognition and entry. Animals lacking the preponderant receptor necessitated for viral-host engagement are less susceptible to the virus-induced disease/pathology. The mouse ACE-2 receptor, the main receptor for SARS-CoV-2 virus entry, for example, has a low affinity towards the virus, leading to effete binding and entry, hence the animal gets only mild illness. TLR4 polymorphism in population studies also influences symptom severity in many disease conditions [52]. Recently it has been suggested, that TLR4 may be a co-receptor for the SARS-CoV-2 virus [53]. It is not unusual for viruses to exploit several receptors at the same time for entry. TLR4 has been shown to act as a co-receptor for the respiratory syncytial virus (RSV) [54], Ebola, etc. [55]. *In vitro* studies demonstrated TLR4 activation by S1 subunit of SARS-CoV-2 spike protein in murine and human peritoneal macrophages [56], TLR2 activation upon engagement by the envelope protein, and proinflammatory cytokine production. The disequilibrium between *in vitro* studies of murine

macrophages and the disease severity in mice upon actual viral inoculation further emphasizes the need to validate our data in real-life situations and the disease driving force is the overall cumulative, additive, synergistic, antagonising or neutralising effect of all components in a model with multiple variables [57]. Habitually, at the stage when respiratory function deteriorates to an extent requiring noninvasive or invasive mechanical ventilation, immune pathology and tissue demolition prevail. ARDS, either of direct pulmonary etiology or indirect, either induced by the virus, bacterial sepsis, major trauma, acid aspiration, or chemicals has TLR4 engagement in its repertoire via previously described damage-associated molecular patterns and pathogens. In the acid aspiration induced mouse model, TLR4 mutant mice were partially protected from ARDS, measured by elastance, oxygenation, and survival [58]. Oxidative stress, the extensive activation of ROS also had a profound effect on ARDS severity together with complement C3a, C5a, and pro-inflammatory cytokines IL-6, IL- β , TNF α , and IL-8, increasing vascular permeability and aggravating noncardiac pulmonary edema [59]. Asthmatics [60] and chronic obstructive pulmonary disease (COPD) patients are exceedingly endangered with pulmonary infective and inflammatory pathology, as TLR4 is upregulated in their bronchial epithelial cells and TLR4 signaling is responsible for airway inflammation in COPD.

Downstream mediators of TLR4 signaling such as p38 MAPK are accroached by several viruses and their inhibition has been shown to halt virus replication. In the study by D Marchant et al, viruses expressing green fluorescent protein(GFP) were used to infect pulmonary fibroblasts in the presence of p38MAPK inhibitor and under conditions of MyD88 deficiency or pretreatment with anti-TLR4 antibody. H1N1 and RSV were both dependent on p38MAPK for cell entry and in the absence of p38 MAPK activity virus trafficking towards nucleus was slower, while CVB3 infection was not significantly influenced by p38 MAPK presence. TLR4 inhibition with antibody antecedently to virus inoculation has led to p38 MAPK inhibition and dampening viral infection of the cells [61]. The signaling molecule p38 MAPK is implicated in SARS-Covid 2 infection as well. Mass spectrometry phosphorylation studies showed heavily activated p38 MAPK and dependent transcriptional factors by the virus in Vero cells (African monkey kidney), pulmonary cancer cell lines, and bronchial epithelial cells [62]. The cells

were treated with the p38 inhibitor and decreased viral replication and pro-inflammatory cytokine productions were observed similarly to previous viral experiments using GFP.

Viral-induced ARDS in the influenza model was dampened in TLR4 KO mice, not only that, but bacterial superinfection had a less deleterious effect. When mice were exposed to lethal influenza challenge and beginning day 2 treated with novel TLR4 inhibitor FP7, the animals were rescued with much subtle level of alveolitis, perivasculitis, peribronchiolitis [63]. TLR4 antagonist FP7 and Eritoran both protected against lethal influenza infection in mice [64]. TLR4 inhibition comes with the hope that antiviral response remains overall unaffected. The experimental model of acute myocarditis and chronic dilated cardiomyopathy using heart passaged coxsackievirus B3 infection in mice had shown that mice carrying a disabling point mutation in the TLR4 gene develop less disease [65,66]. The coxsackievirus model mimics an environment where damaged self tissue is already present and memory does not only develop to the host but also modified self.

The degree of tissue damage in severe COVID-19 pneumonia is devastating. ICU patients on mechanical ventilation often show a computed tomography picture of 80- 95% diffuse lung damage and amazingly enough, some of these extremely ill patients survive and recover. The price of survival is immense, recovery is slow, and frequent secondary infections and sepsis, septic shock with opportunistic bacteria further compromise lung function and often terminate our efforts by exhausting body reserves, by inducing multiorgan failure, and by hampering lung functions to unbearable degrees. It would be extremely important to shorten the time of treatment to modify the immune response so that it would last longer, and would be able to prevent and combat superinfections. An overzealous innate immune reaction early on when adaptive immunity has not been established yet, can be damaging and exhaustive.

6. TLR4, SEDATION AND ANALGESIA

Another argument for comprehensive immunological monitoring of ICU patients can be provided by reflecting on variable immune modulative effects of analgesics and sedatives employed in the ICU environment. Propofol and dexmedetomidine exercise protective effects on the brain in ischaemia-reperfusion injury

mediated in some measure by TLR4 inhibition [67], but also chemotaxis and proinflammatory cytokine production is suppressed upon propofol administration. Inhalation agents (sevoflurane and isoflurane) induce apoptosis in normal peripheral lymphocytes in vitro in a time and dose-dependent manner [68].

Propofol exhibits a powerful antioxidant effect by scavenging free radicals in endotoxin-activated macrophages [69]. The problem is not only the matter of anaesthetic and analgesic agents but dose, time, and level of brain inactivation. Inappropriate anaesthesia and hypotension predispose to infection and worsen the outcome of ongoing sepsis. Sedation in general compared to fully awake state improved survival in severely septic Sprague-Dawley rats [70]. Sedation with midazolam and dexmedetomidine both markedly improved survival in polymicrobial septic rats induced by caecal ligation and double intestinal puncture. Similar results were achieved in mice [71]. The mortality was not significantly different in the first 24h if mice were sedated with fentanyl or a combination of fentanyl and midazolam, and survival was improved in mice sedated with midazolam only.

7. CONCLUSION

The immune activation is initiated primarily by organ resident and innate immune cells upon recognition of exogenous and endogenous ligands via pattern recognition molecules. After incitement, an overwhelmingly proinflammatory cascade commences, eliminating the danger. The cascade has several positive feedback mechanisms and it may reach measures representing more harm than benefit for the body. It would be important to modify the primary response by skewing to a chiefly beneficial response. If the proximal contender is modified, canonically, the downstream happenings become positively influenced too. Here we analyze the role of TLR4 pattern recognition receptor, unifying widespread recognition and signaling pathway features in conditions where TLR4 signaling has important implications. Impertinent innate activation may ultimately lead to an overwhelming, aberrant adaptive response and exhaustion. While a suitable and tolerable level of TLR4 activation is desirable, particularly in pathogen clearance, it is the inappropriately and dangerously overwhelming activation that tilts the balance towards a negative outcome. The real challenge is to be able to interfere without negatively affecting pathogen aggressivity and

clearance. It would be outmost beneficial to map the immune response clinically to be able to better determine the time, mode, and level of therapeutic modification, alteration, or fortification. Target cytokine and cell receptor levels are evaluated prior and after treatment application in a portion of clinical studies. I believe it is important to look at the local immune cells and cytokine levels fundamentally, when technically possible, since serum levels may or may not dependably reflect the actual happenings in the case of solitary organ involvement.

TLR4 abolition based on basic research data could be beneficial when appropriately applied in hyperinflammatory conditions and ischaemia-reperfusion injury. The mainstay of therapy is to target the virus and the bacteria, reinstalling oxygenation and perfusion. It would be great to have monoclonal antibodies for viruses and bacteria likewise, to be able to stop entry. Complementing our treatment effort by minimizing collateral damage could further improve survival and quality of life, as well as diminish and shorten morbidity. The ongoing pandemic is not the worst by far in possible scenarios of the outcome, but SARS-Cov-2 related severe ARDS can have high mortality with sweeping lung involvement. The pandemic emphasized the need to recuperate energies towards less invasive, relatively simple, and affordable life-saving procedures. Mass vaccinations will save many, but will likely not eliminate entirely the problem, and the development of new vaccines is time-consuming, altogether with the design of pathogen-specific new target molecules. Modification of exuberant, dysregulated immune response that has a more conserved and collective mechanism of operation optimally under monitored conditions may help save lives and importantly improve the quality of life.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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