Rationale and Investigational Study Protocol for Treatment of COVID-19 Severe Viral Pneumonia with Isolated, Placental, Mesenchymal Stem Cell Exosomes

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) has reached pandemic levels worldwide. COVID-19, the respiratory infection caused by this virus, can cause a spectrum of illness ranging from mild upper respiratory symptoms, such as cough and congestion, to severe pneumonia leading to respiratory failure and death. The rapid transmission of the virus, the high mortality rate associated with this disease relative to other respiratory viruses such as influenza, and the lack of any effective treatment have created a panic that has resulted in a profoundly negative impact on the economy and society. Though most people are likely to experience only mild or moderate symptoms after exposure to the virus, and the mortality rate remains below 5%, the healthcare system could easily be overwhelmed by the needs of severely ill patients, who are usually over 65 years of age and/or have other underlying illnesses.

Because healthcare resources are inadequate to meet the potential demands associated with rapid transmission of the virus, governments have implemented social countermeasures to prevent the spread of the virus. In an effort to contain the virus, behavioral modifications have been recommended, or in some cases mandated. These behavioral and business restrictions will remain in effect until one of the following occurs: healthcare resources can be developed to meet the demand; the virus reaches an equilibrium as the population develops natural immunity; or until an effective treatment for the disease is found. These preventive measures such as frequent hand washing, disinfecting surfaces, social distancing, crowd avoidance and self-quarantine are likely to serve only to slow the progression of infection in the absence of effective treatment.

Ongoing efforts to develop medical countermeasures for COVID-19 have focused on three main areas: diagnostic testing, vaccination, and treatment. Unfortunately, diagnostic testing does not serve much purpose in the absence of effective treatment. If the consequence of confirmation of this diagnosis is either self-quarantine or hospital admission without adequate treatment, testing does not provide great utility in overcoming this pandemic. Immunization may be another reasonable approach, but this measure will have no impact on the thousands of people who are already infected with the virus, and by the time that an effective vaccine has been developed and production scaled up to adequate levels, many more people will have contracted the virus and the vaccine will serve no purpose for them. Identifying effective treatments for COVID-19 seems to be the most rational approach to managing the SARS-Cov-2 pandemic.

Cell-based therapy strives to treat or prevent injury and disease by naturally repairing, restoring and regenerating damaged or diseased organs and tissues. This field has exploded in recent years to meet the needs of patients with both complex and common medical problems. Some cell-based therapies aim to slow or stop degenerative or pathophysiologic processes that ultimately present themselves as symptomatic conditions. Other regenerative therapies activate the body's endogenous repair system by influencing the behavior of somatic and progenitor cells to stop degenerating and start regenerating. In the case of pneumonia, acute lung injury (ALI), acute respiratory distress syndrome (ARDS) and sepsis, studies investigating therapy using mesenchymal stem cells have demonstrated safety and some positive effects on these conditions. A very recent study using adipose-derived stromal cells has shown a positive impact specifically on a small group of patients in China with COVID-19. The most recent scientific evidence indicates that the mechanism of action of this type of cell therapy lies in exosomes.

Exosomes are sophisticated, nano-scale, biologic signals that are contained within extracellular vesicles. These membrane-enclosed capsules, ranging in size from about 20 to 200 nanometers, are filled with cellular signaling information. Virtually every type of cell produces exosomes as a means of intercellular

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communication, and exosomes are the essence of the message of the parent cell. Mesenchymal stem cell-derived exosomes (MSC exosomes) have demonstrated a remarkable capacity for immunomodulation and tissue regeneration in preclinical studies. In vitro and in vivo studies using cellular, animal and ex vivo human organ models have provided impressive evidence to suggest potential efficacy as a therapy for ALI/ARDS, including those from bacterial and viral infectious etiologies, which is the cause of death for patients who have contracted COVID-19.

The rationale for the use of MSC exosomes stems from the comprehensive scientific and clinical evidence of safety and efficacy of this type of treatment for the severe, life-threatening, respiratory illness caused by SARS-Cov-2 and other infectious and non-infectious etiologies. Preclinical studies have investigated the use of various different types of MSC exosomes, and the optimal candidate for exosome-based therapy for COVID-19 appears to be isolated, placental, mesenchymal stem cell-derived exosomes. A rationale for the use of this type of MSC exosomes follows.

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Concise Summary of Rationale

Virus & Disease

- Severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) has reached a pandemic level.
- SARS-Cov-2 causes COVID-19, an acute respiratory illness that can be life-threatening.
- SARS-Cov-2 is highly contagious and transmitted through respiratory droplets.
- SARS-Cov-2 remains contagious on surfaces from a few hours to a few days.
- Overall mortality rate is less than 3%; above 60 mortality rate goes up, and over 80, is 25%

Crisis Response

- Existing health care resources are not sufficient to handle rapid disease transmission.
- Social countermeasures have been implemented to slow the spread of the virus.
- Behavioral and business restrictions have had a profoundly negative socioeconomic impact.
- Development of medical countermeasures is directed at testing, vaccination and treatment.
- Testing only serves a purpose if there is effective treatment for the disease.
- Vaccine development will take a long time to complete clinical trials and scale production.
- Effective treatment for COVID-19 holds the greatest promise for ending this pandemic

Mechanism of Disease

- SARS-Cov-2 targets type II alveolar epithelial cells in the lungs
- SARS-Cov-2 triggers a massive immune response in an attempt to reduce viral titers
- Release of very high levels of inflammatory mediators causes cytokine storm
- Cytokine storm damages the delicate barrier between the air spaces and the blood vessels
- Disruption of the alveolar-capillary barrier allows fluid to leak into airspaces
- Fluid in air spaces interferes with gas exchange and oxygenation of the blood
- COVID-19 can progresses from acute lung injury (ALI) to acute respiratory distress syndrome (ARDS)

Stem Cell Therapy

- Stem cells have the capacity to repair, restore or regenerate diseased or damaged tissues
- Stem cell therapy can slow or stop or many pathologic disease processes
- Stem cells can activate the body's endogenous repair system to reverse damage
- A small study in China successfully treated COVID-19 with a form of stem cell therapy
- Stem cells work through a paracrine mechanism known as exosomes.

Exosomes

- Exosomes are complex, biologic messages contained within extracellular nanovesicles
- Exosomes are naturally occurring and form a network of intercellular communication
- Exosomes produced by stem cells (MSC exosomes) mimic their effects and can be equally effective
- MSC exosomes exhibit antiviral, immunomodulatory and tissue-regenerative properties
- MSC exosomes have shown efficacy in models of infectious pneumonia, ALI, ARDS, & sepsis
- Neonatal MSC exosomes derived from chorionic plate MSCs have greatest therapeutic potential
- Neonatal MSC exosomes have shown no adverse effects in IRB approved human pilot studies
- Neonatal MSC exosomes are more scalable and easier to store and distribute than live MSCs

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Mechanisms of Exosomes

- Downregulate inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-12p40, IFN-γ, NF-κB and HMGB1, NLRP3, Ferritin, Kynurenine
- Upregulate of anti-inflammatory cytokines such as TGF-β3 and IL-10
- Promote anti-inflammatory & highly phagocytic M2 macrophage phenotype
- Transfer regenerative factors such as KGF and Ang-1 to damaged cells and progenitor cells
- Promote regeneration of junctions between both epithelial cells and endothelial cells
- Restore the normal permeability of the alveolar-capillary barrier
- Restore normal alveolar fluid clearance by type II alveolar epithelial cells
- Promote proliferation of type II alveolar epithelial cells and bronchioalveolar stem cells
- Replenish alveolar epithelial cells lost to damage caused by inflammatory cytokines & virus
- Direct and indirect antiviral effects through transfer of miRNA

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SARS-Cov-2 (Coronavirus)

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) originated in the Wuhan province of China. This virus, known by several other names including Wuhan coronavirus and 2019 novel coronavirus, is a positive-sense, single-strand 30 kb RNA virus of the Coronaviridae family. The virus is approximately 50-200 nm is diameter. This virus exhibits some similarities to other coronaviruses such as SARS (2003) and MERS (2006). The SARS-Cov-2 RNA genome codes for 10 proteins including orf1ab polyprotein, surface glycoprotein, ORF3a protein, envelope protein, membrane glycoprotein, ORF6 protein, ORF7a protein, ORF8 protein, nucleocapsid phosphoprotein, and ORF10 protein. Each of these is a potential target for siRNA and/or exosomal miRNA. SARS-Cov-2 has some similarity to bat coronaviruses and causes a human illness known as COVID-19. Affinity of SARS-Cov-2 to the human angiotensin converting enzyme 2 (ACE2) receptor and a polybasic cleavage site may contribute to the transmission and pathogenicity of the virus.

COVID-19

COVID-19 is the infectious respiratory illness caused by SARS-Cov-2. This disease is characterized by upper and lower respiratory symptoms, as well as other systemic symptoms. Fever (50-100%), cough (75%) and shortness of breath (20-50%) are characteristic symptoms of the infection, but other symptoms include sputum production (25-50%), myalgia (10-15%), headache (10%), nausea (5-10%), diarrhea (5%), rhinorrhea (5%), pharyngitis (2%) and hemoptysis (1-5%). The causes of death in fatal cases are related to respiratory failure, hypoxemia and cardiovascular complications. The vast majority of cases result in only mild symptoms, but infection in patients over 60 years of age or those with underlying medical conditions can develop severe viral pneumonia, which can progress to ARDS and multi-organ system failure.

Epidemiology of COVID-19

SARS-Cov-2 can be spread from person to person via respiratory droplets released by coughing or sneezing and remains infectious in aerosolized airborne form. The virus may also be transmitted from contaminated surfaces, and depending on the type of surface, the virus may remain contagious from less than 24 hours to several days. After exposure to the virus, onset of symptoms may occur from between 2 to 14 days with a mean incubation period of 4-5 days. Under 50 years of age, the mortality rate in all age groups is about 0.1%. Over 80 years of age, the mortality rate may be as high as 25%. The overall mortality rate of the virus across all age groups appears to be between 2-3%. Compared to the Spanish flu of 1918, which had a mortality rate of 1%, and the more common influenza virus, which has a mortality rate of less than 1%, COVID-19 presents a significant therapeutic challenge. Two interesting observations are that vertical transmission from mother to newborn seems to be very low and that there have been no fatal cases in patients less than age 10.

In the absence of an effective treatment, there are three possible outcomes: avoidance of infection, acquired immunity and death. The highly contagious nature of the virus has contributed to its rapid transmission and a prevalence of infection that has reached a pandemic level. Like other infectious diseases, the virus is likely to reach an equilibrium, in which the rate of infection equals the rate of immunity, however It is likely that many more cases of infection will occur prior to reaching this natural equilibrium. If the mortality rate remains near 2% throughout the global population, that would equate to 150 million deaths worldwide.

Pathology of COVID-19

The primary pathology of COVID-19 is pulmonary because the virus has an affinity to the ACE2 receptor, which is found in high concentrations in the type II alveolar epithelial cells in the lungs. After

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internalization of the virus into these cells, viral replication occurs over several days during the replicative phase of this disease, during which only mild symptoms may be present. When the innate immune response fails to contain this virus, the adaptive immune response begins to effectively reduce viral titers by inducing a substantial release of inflammatory cytokines, signaling mediators that cause inflammation and increased activity of immune cells.

This massive cytokine storm disrupts the epithelial-endothelial cell barrier between the alveoli and the pulmonary microvasculature causing diffuse alveolar damage and allowing proteinaceous fluid to accumulate in the alveoli. These inflammatory cytokines also damage the cells responsible for clearance of this fluid from the alveoli. The pattern of damage to the cells also seems to indicate a direct viral effect in addition to the hyperinflammatory injury. ALI can progress to ARDS as accumulation of fluid in the lungs obviously interferes with normal oxygenation of the blood causing hypoxemia and hypercapnia. The increased intrapulmonary pressure also increases the mean pulmonary arterial pressure causing acute cor pulmonale.

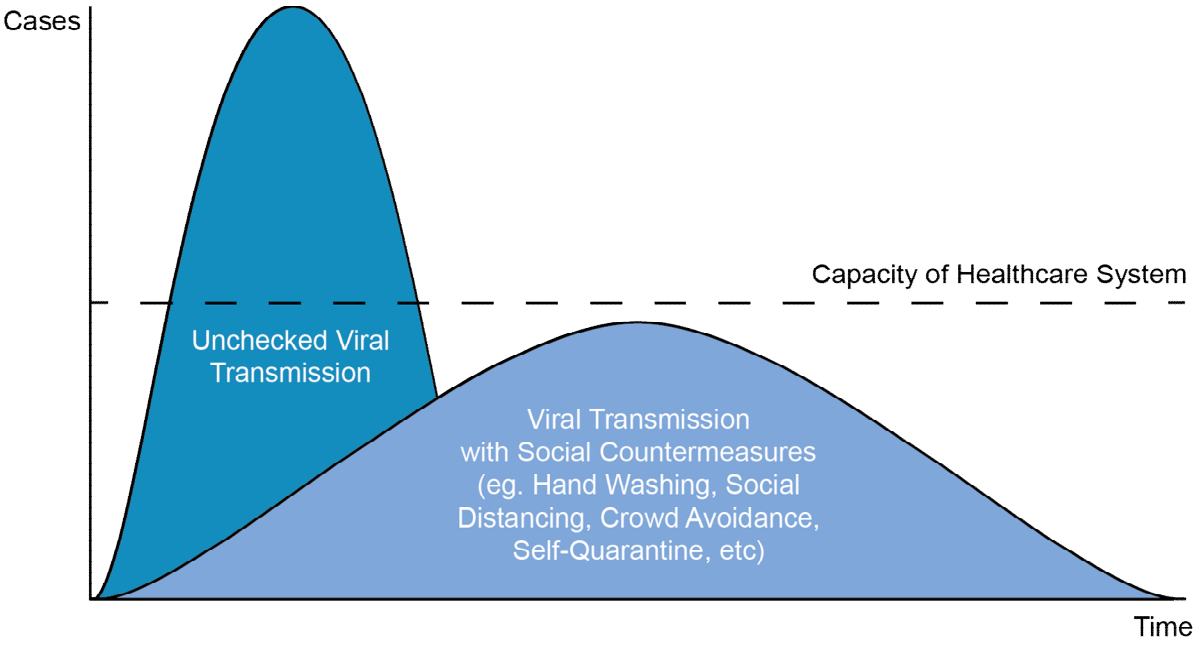

Figure 1. Number of cases of COVID-19 relative to the capacity of the healthcare system, with and without implementation of social countermeasures, and the corresponding socioeconomic impact.

Social Countermeasures for SARS-Cov-2

The lack of effective, clinically proven therapy for COVID-19, which causes a respiratory illness that can lead to respiratory failure and has a comparatively high mortality rate for a viral infection, has forced the population to rely on disease prevention measures. Efforts to reduce the rate of disease transmission are essentially behavioral modifications, both self-imposed and government mandated. These social countermeasures include such behaviors as frequent hand-washing, more vigilant disinfection of surfaces, social distancing, and crowd avoidance and self-quarantine. While these measures may be effective in slowing the rate of transmission of this virus to allow healthcare resources time to scale up resources to meet the new demands, this is not a definitive resolution for this pandemic. Behavioral modifications such as these will never be completely effective, because humans are incapable of perfect execution of these measures, whether inadvertently or by lack of intent. Anything less than absolute compliance with perfect execution of these measures will still allow transmission of the virus from person to person or by contact with contaminated surfaces. The consequence of rigorous self- or government-

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
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imposed deployment of these social countermeasures has been socio-economic collapse, which we are witnessing now as events are cancelled, gatherings are prohibited, education is suspended, businesses are closed, and people are confined to their homes. By intention, these restrictions are likely to slow disease transmission, which will also prolong the need for these restrictions and their impact on the economy and society.

Medical Countermeasures for COVID-19

Confirmation of the diagnosis of COVID-19 was the primary focus of initial clinical research and development aimed at ending the SARS-Cov-2 pandemic. At the outset of COVID-19, no diagnostic tests were available for this novel coronavirus. Government health organizations were unable to respond to the immediate need for diagnostic modalities, while the virus rapidly spread throughout the world. At this point, the viral RNA genome and protein composition are widely known, and diagnostic tests have become available. Unfortunately, without an effective treatment for the disease, positive diagnosis only affords the opportunity to try to prevent transmission via quarantine of affected patients.

A safe, effective vaccine that can provide immunity to SARS-Cov-2 is much more likely than social countermeasures to stop transmission and to effectively prevent COVID-19. This novel coronavirus has some similarities to previous coronaviruses such as those that caused SARS and MERS, however the virus has distinct differences that complicate development of a vaccine. Notwithstanding, clinical trials of vaccines are underway. Unfortunately, these efforts will take time to demonstrate the safety and efficacy of these new drugs with de novo clinical trials. Scaling vaccine production adequately to meet worldwide demand will also take time after regulatory approval for widespread use. During this time, many more people will develop COVID-19 and vaccination after the fact will be ineffective for them.

Development of an effective therapy for COVID-19 seems to hold the most promise in terms of dealing with this pandemic. Existing treatments for moderate to severe cases of pneumonia and ARDS have limited efficacy. As COVID-19 is a viral community acquired pneumonia, it makes sense that antiviral drugs, such as oseltamivir, used as post-exposure prophylaxis may prevent viral infection and reduce shedding of the virus to reduce transmission.[1] Unfortunately, antiviral medications are only likely to have an effect early in the disease course, because the pathogenesis of COVID-19 involves viral replication only in the early stages of the disease, and antiviral medications are not likely to have any effect on the cytokine storm that causes the advanced life-threatening disease.

Hydroxycholoroquine (HCQ), an anti-malarial drug also used to treat autoimmune conditions has shown some antiviral activity against SARS-Cov-2 in vitro.[2] In addition to the antiviral properties, this drug also has some immunomodulatory properties making it useful for treatment of rheumatic diseases such as rheumatoid arthritis and systemic lupus erythematosus. While this drug has been reported to inhibit SARS-Cov-2 in vitro, and has some immunomodulatory activity that could theoretically reduce the inflammatory-mediated pulmonary damage caused by the cytokine storm of COVID-19, a controlled study comparing the use of HCQ and placebo showed now significant difference in outcomes.[3] This finding may be due to the mechanism of action of hydroxychloroquine, which increases the pH of macrophage

[1] Effectiveness of oseltamivir in preventing influenza in household contacts: a randomized controlled trial. JAMA. 2001; 285: 748-754

[2] In vitro antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Yao et al. Clin Infect Dis. 2020; (published online March 9.) DOI:10.1093/cid/ciaa237

[3] Chen et al. A pilot study of hydroxychloroquine in treatment of patients with common coronavirus disease-19 (COVID-19). JOURNAL OF ZHEJIANG UNIVERSITY, March 2020. DOI : 10.3785/j.issn.1008-9292.2020.03.03

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
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lysosomes, interfering with the ability of macrophages to digest antigen proteins and present them to CD 4+ T cells.[4] HCQ also does not have the capacity to directly promote regeneration of damaged tissues.

Acute Lung Injury (ALI) in COVID-19

The cytokine storm induced by the adaptive immune response to SARS-Cov-2 and other viruses, such as influenza causes diffuse alveolar damage that results in ALI, which can progress to acute respiratory distress syndrome (ARDS). This pulmonary disease is a rapidly progressive form of acute respiratory failure characterized by severe hypoxemia and non-hydrostatic pulmonary edema. ARDS is most commonly caused by infection, but it may also result from other pulmonary insults such as trauma and other inflammatory disease, causing a total of about 200,000 cases annually in the US. The mortality rate associated with ARDS varies depending on the severity of the injury and the specific classification criteria applied, but an estimated mortality rate may be 45% for ARDS, 20% for ALI and 5-10% for acute respiratory failure without ALI. Amongst cases of ARDS, the mortality rate for mild, moderate, and severe cases is about 35%, 40%, and 45%, respectively. Survivors of ARDS may experience brain dysfunction, cognitive impairment, anxiety, physical limitations, and increased risk of hospital readmission.

Pathogenesis of ALI/ARDS

Acute lung injury is characterized by injury to the lung epithelium that leads to impaired resolution of pulmonary edema and also facilitates accumulation of protein-rich edema fluid and inflammatory cells in the distal airspaces of the lung. Inflammatory mediators produced by neutrophils and macrophages as well as viruses, such as influenza, damage the tight junctions between alveolar epithelial cells allowing pathologic flow of proteinaceous fluid into alveoli. Normal alveolar fluid clearance from the alveoli to the interstitium adequately removes any fluid accumulation, but the rate of fluid clearance is impaired by infection, inflammatory cytokines and the mechanical ventilation frequently employed in ARDS. Cytokine storm decreases the number of α-epithelial sodium channel (α-ENaC) subunits in the apical membrane of alveolar epithelial cells, which contributes to increased accumulation and impaired clearance of fluid from the alveoli. Exposure of cultured type II alveolar epithelial cells to IL-1β, TNF-α, and IFN-γ increases the protein permeability of alveoli by 5-fold over 24 hours.[5] Impaired pulmonary function due to pulmonary capillary endothelial and alveolar epithelial cell dysfunction is exacerbated by damage to type II alveolar cells (pulmonary progenitor cells), which also interferes with normal surfactant function.

Damage to the delicate alveolar-capillary barrier causes fluid accumulation in the air spaces of the lungs, significantly interfering with gas exchange in the alveoli and the clearance of the fluid. Normally, pulmonary capillary endothelial cells form a tight barrier that separates the pulmonary capillaries and the alveoli, which prevents the passage of proteinaceous fluid and inflammatory cells between these cells. Adherence junctions, formed by the association of VE-cadherin proteins on the membrane of adjacent endothelial cells, create the alveolar-capillary barrier. Inflammatory cytokines and other signaling proteins present in cytokine storm disrupt these adherence junctions between endothelial cells allowing leakage of the capillaries. Neutrophils, activated platelets, M1 macrophages, NK effectors, and bacterial products, such as endotoxin, can also damage or destroy the endothelial cells themselves, further increasing the permeability of this barrier.

[4] Fox et al. Mechanism of action of hydroxychloroquine as an antirheumatic drug. Semin Arthritis Rheum. 1993 Oct;23(2 Suppl 1):82-91.

[5] Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin- 1. Fang et al. The Journal of biological chemistry 2010;285:26211–26222

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ARDS is characterized by diffuse alveolar damage that causes interstitial and alveolar edema, which progresses through exudative, proliferative, and fibrotic phases to pulmonary fibrosis. Accumulation and impaired clearance of proteinaceous fluid in the alveoli, and the cardiac sequelae result in acute onset of tachypnea, hypoxemia, and loss of compliance of the lungs. Severity of ARDS can be characterized using various measures, such as AECC and Berlin criteria or Lung Injury Scores, based on PaO2/FiO2 and other clinical data. Biomarkers such as soluble intercellular adhesion molecule-1, von Willebrand factor antigen, IL-6, IL-8, SP-D, PAI-1 and sTNFR1 can provide additional information about the progression of the disease. In almost 50% of cases, hypoxemia can progress to become more severe, despite increasing ventilatory support and supplemental oxygen, eventually leading to complete respiratory failure.

The cytokine storm that precipitates ARDS also affects the pulmonary circulation causing diffuse pulmonary endothelial injury and thrombus formation. Fibrosis of the pulmonary endothelium can occlude these alveolar capillaries and other small vessels and contribute to pulmonary hypertension. Hypoxemia and hypercapnia, which alter vasomotor tone, increased intrathoracic pressure due to ventilator support and thrombosis all exacerbate this pulmonary hypertension leading to secondary cardiac effects such as cor pulmonale.

Endogenous Pulmonary Repair Systems

Natural systems for endogenous repair of the pulmonary damage associated with ALI/ARDS exist and begin operating even during the inflammatory phase of this disease. Increased production of anti-inflammatory cytokines, such as IL-10 and IL-1ra, by macrophages, dendritic cells and CD4+ T cells helps to reduce the release of inflammatory cytokines and increase the activity of phagocytes. Regulatory T-cells (Tregs), present in the air spaces in ARDS, produce a significant amount of IL-10, which is important in the resolution of ALI through downstream upregulation of TGF-β. Tregs require upstream signaling with TGF-β to suppress Th17 cells and modulate the immune response. Phagocytosis of apoptotic neutrophils and bacteria also stimulates additional anti-inflammatory signaling and supports clearance of mucosal leukocytes. Modulating the hyperinflammatory immune response to the offending agent or pathogen is essential for recovery from ARDS.

Cellular regeneration is another important part of endogenous repair in ALI/ARDS. After epithelial damage, basal cells in the large airways and Clara cells in the smaller airways auto-regenerate to produce ciliated and secretory cells. Bronchioalveolar stem cells (BASCs), progenitor cells at the bronchioalveolar duct junction that produce surfactant protein C (SPC), have greater auto-regenerative capacity and proliferate even after injury with naphthalene and bleomycin, which kill Clara cells and alveolar epithelial cells, respectively. BASCs can contribute to the production of alveolar epithelial cells. Type II alveolar epithelial cells that express SPC auto-regenerate to produce types I and II alveolar epithelial cells.

Endothelial signaling, which involves VEGF, EGF and Tsp1, promotes epithelial progenitor cell proliferation and supports differentiation of BASCs to become airway and alveolar epithelial cells. Interestingly, Tsp1 knockout studies indicate that conditioned media of lung epithelial cells restores alveolar repair.[6] Other molecules, such as sphingolipid sphingosine-1-phosphate (S1P), Angiopoeitin-1 (Ang-1), Adrenomedullin and Slit, act to restore the alveolar-capillary barrier by altering the cytoskeleton

[6] Lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-Thrombospondin-1 axis. Lee, Joo-Hyeon et al. “Lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-thrombospondin-1 axis.” Cell vol. 156,3 (2014): 440-55. doi:10.1016/j.cell.2013.12.039

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to increase cell overlap and inducing adherence and tight junction assembly to promote inter-cellular adherence and reduce vascular leakage.

Treatment of ARDS

Although clinical researchers have made efforts to develop pharmacologic interventions to treat ARDS, there is no effective pharmacotherapy for this syndrome. Efforts to deploy surfactant and corticosteroids as treatments have not improved outcomes. Neuromuscular blockade has demonstrated some marginal benefit for patients, who cannot be adequately sedated and resist assisted ventilation. The use of inhaled vasodilators has not provided any significant clinical benefit with the exception of nitric oxide, which may improve oxygenation and reduce pulmonary artery pressure in some patients with ARDS.

Because pharmacotherapy has been largely ineffective for ARDS, treatment efforts have been focused on respiratory support. One part of these efforts consists of ventilator management. Optimizing tidal volume at or near 6 ml/kg body weight may improve outcomes. Positive end-expiratory pressure (PEEP) has positive effects on damaged areas of the lung by maintaining aeration and reducing cyclic inflation and collapse, but has negative effects on normal areas of the lung due to hyperinflation. The net effect of PEEP seems to be beneficial with reduced refractory hypoxemia, reduced death due to refractory hypoxemia, and reduced requirement for rescue therapy due to intractable hypoxemia, barotrauma, or acidosis.[7] Recruitment maneuvers with intermittent increases in transpulmonary pressure above those used for tidal inflation, high-frequency oscillation ventilation, and partial liquid ventilation have also been employed with variable improvements. Clinicians have also applied other measures unrelated to ventilator support such as fluid management, hemodynamic monitoring, prone positioning and extracorporeal membrane oxygenation (ECMO).

Stem Cell Therapy

Failure to identify effective pharmacotherapy for ARDS may be due to the multifactorial etiology of the pulmonary dysfunction consisting of multiple concurrent cellular, biochemical and pathophysiologic processes. Cell-based therapy may be uniquely suited to managing clinical challenges with multifactorial etiologies, because of the numerous components of this type of therapy and the multiple cellular pathways on which they act. In a murine model, autologous endothelial progenitor cells (EPCs) improved survival, reduced lung edema, and increased IL-10, after endotoxin-induced lung injury.[8] MSCs have been found to have remarkable therapeutic effects in multiple murine models of acute lung injury, including bleomycin,[9] intratracheal[10,11,12] or intraperitoneal[13] endotoxin, cecal ligation and puncture,[14,15]

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[12] Islam MN, Das SR, Emin MT, et al. Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. Nat Med. 2012;18(5):759–765.

[13] Xu J, Woods CR, Mora AL, et al. Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. Am J Physiol Lung Cell Mol Physiol. 2007;293(1):L131–L141.

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Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

pseudomonal abdominal sepsis,[16] and *E-coli* pneumonia.[17] Human bone marrow-derived stromal progenitor cells reduced inflammation and improved alveolar fluid clearance in *ex vivo* human lungs injured with live E-coli.[18] Intravenous or intratracheal mesenchymal stem cells (MSCs) normalized lung capillary and alveolar growth in mouse and rat BPD models, without engraftment.[19,20] The effect of MSC administration was to increase BASCs in the BPD model by a paracrine mechanism.

Cell-Based Therapy for COVID-19

Optimal therapy for mild, moderate and severe, life-threatening COVID-19 disease would have antiviral activity to inhibit viral replication, modulate the hyperinflammatory immune response and directly promote damaged pulmonary tissue regeneration. The biological activity of mesenchymal stem cells has been a focus of scientific and clinical investigation for many years. In vitro and in vivo studies have demonstrated that these progenitor cells have the capacity for immunomodulation and tissue regeneration, as well as antiviral and antibacterial effects. Mesenchymal stem cells have also demonstrated beneficial effects in preclinical studies of ALI, ARDS, infectious pneumonia (bacterial and viral) and sepsis, all of which are related to the pathogenesis of COVID-19.

Mesenchymal Stem Cells (MSCs)

Mesenchymal Stem Cells (MSCs) are multipotent, progenitor cells that have remarkable, clinically relevant, biological properties. These cells, which can be found in multiple different types of tissue, are present on the first day of life, and their descendant progenitor cells persist until the end of life, as part of an endogenous repair system. The number of MSC-derived, stromal, progenitor cells and the level of function of these progenitor cells declines consistently with age, which means that the biological properties of neonatal MSCs are substantially different than those of older progenitor cells even after as little as two decades of life, meaning that neonatal MSCs have greater therapeutic potential than adult stromal progenitor cells.

Therapeutic Mechanisms of MSCs in ALI/ARDS

The mechanism of action of MSCs, used for treatment of acute lung injury, has been studied in animal models and in humans. Proposed mechanisms of MSC therapy in ALI include: reducing alveolar-capillary barrier permeability,[21,22,23,24] in part by secretion of angiopoietin-1;[25] increasing alveolar fluid clearance, at

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

least in part by secretion of keratinocyte growth factor;[26,27] shifting cytokines and resident macrophages from pro- to anti-inflammatory;[28] improving bacterial clearance by enhancing phagocytosis and secreting antibacterial peptides;[29,30,31] and transferring mitochondria to alveolar epithelial cells, rescuing ATP generation.[32] Preclinical research has identified several proteins, produced by MSCs, that have beneficial effects.

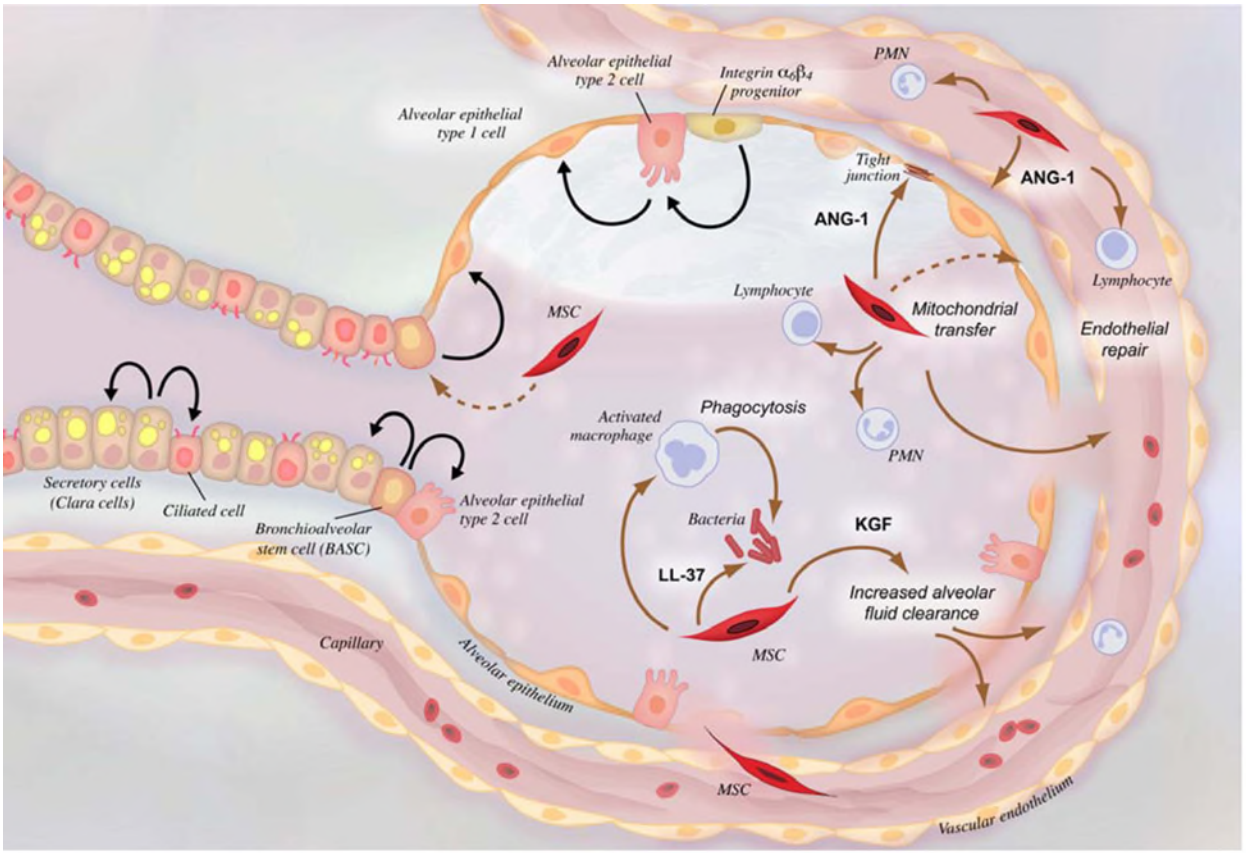

Figure 2. Cellular and biochemical mechanisms of immunomodulation, alveolar-capillary repair and restoration of proteinaceous fluid clearance after acute lung injury. Endogenous and exogenous cell-based pathways for recovery from acute respiratory distress syndrome. Clin Chest Med. 2014 Dec; 35(4): 797–809.

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

Hepatocyte growth factor (HGF) significantly decreased expression of proinflammatory cytokines and reduced levels of profibrotic factors, while enhancing expression of the anti-inflammatory cytokine. IL-10.[33] Angiopoietin-1 (Ang1) is an important regulator of angiogenesis, vascular stabilization, and anti-inflammatory actions by improving vascular permeability and inhibiting leukocyte-endothelium interactions. MSCs over-expressing Ang-1 reduced the recruitment of inflammatory cells into the lung and pulmonary vascular endothelial permeability compared with administration of MSCs alone.[34] Interleukin-33 (IL-33) is produced in response to endothelial and epithelial injury and participates in promotion of immunoregulatory and anti-inflammatory response by acting directly on macrophages through inhibition of the Toll-like receptor 4 expression.[35] MSCs overexpressing its receptor, soluble IL-1 receptor-like-1 (sST2), provided a superior therapeutic effect than MSCs on the detrimental immune-inflammatory response occurring in ALI.[36] Interleukin-10 expression is severely reduced in mice suffering from ALI, and the response of T regulatory cells (Tregs) and dendritic cells (DCs) against transfusion-related ALI is in part mediated by IL-10.[37] MSCs overexpressing IL-10 resulted in a sustainably higher serum concentration of IL-10 compared to direct injection, thereby inducing anti-inflammatory effects facilitating survival in an endotoxin-induced ALI mouse model.[38] Keratinocyte growth factor is significantly upregulated following epithelial damage[39], and can protect lung tissues from oxidative insults and facilitate the regeneration of type II alveolar epithelial cells, thereby maintaining the integrity of the alveolar barrier.[40] MSC-based KGF therapy ameliorated pulmonary microvascular permeability and attenuated proinflammatory responses in a mouse model of LPS-induced ALI.[41]

MSCs in Clinical Trials for ARDS

Human clinical trials have studied intravenous administration of allogeneic, bone-marrow derived MSCs in patients with moderate to severe ARDS, and found no significant adverse events at the time of treatment. In one study, two patients with severe ARDS were treated with MSCs, and both patients demonstrated improved lung function and resolution of multiorgan failure.[42] These early successes prompted further clinical investigation of the safety and efficacy of allogeneic MSCs as a therapy for ARDS.

A pilot study evaluating 12 adult patients, meeting the Berlin definition of ARDS with a PaO2:FiO2 ratio less than 200 mmHg, were randomized to receive allogeneic adipose-derived MSCs or placebo. Patients

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

received one intravenous dose of 1×10^6 cells/kg of body weight. There were no infusion toxicities or serious adverse events related to MSCs administration and there were no significant differences in the overall number of adverse events between the two MSC and control groups. Length of hospital stay, ventilator-free days and ICU-free days at day 28 after treatment were similar. Clinical effect with the doses of MSCs used was weak, and the investigators concluded that further optimization of this strategy was required to reach the goal of reduced alveolar epithelial injury in ARDS.[43]

A phase 1 clinical trial was performed to evaluate the safety of a single dose of allogeneic, bone marrow-derived MSCs in patients with moderate-to-severe ARDS, defined by the acute onset of the need for positive pressure ventilation, a PaO2:FiO2 ratio less than 200 mm Hg with at least 8 cm H2O positive end-expiratory airway pressure (PEEP), and bilateral infiltrates consistent with pulmonary edema on chest radiograph. Each of three groups of 3 patients was treated with either low dose MSCs (1×10^6 cells/kg), intermediate dose MSCs (5×10^6 cells/kg), or high dose MSCs (10×10^6 cells/kg). Investigators reported that a single intravenous infusion of allogeneic, bone marrow-derived human MSCs was well tolerated in all nine patients with moderate to severe ARDS.[44]

A Phase 2a trial to assess safety and efficacy after administration of MSCs to patients with moderate to severe ARDS was conducted. Ventilated patients, admitted to five academic medical centers with moderate to severe ARDS, defined by PaO2:FiO2 ratio less than 200 mm Hg on PEEP ≥8 cm H2O, received either MSCs (10×10^6 cells/kg) or placebo. Investigators concluded that dose of intravenous MSCs was safe in patients with moderate to severe ARDS, but larger trials were needed to assess efficacy, and that the viability of MSCs needed improvement.[45]

In addition to failure to demonstrate efficacy with these doses of allogeneic bone marrow-derived mesenchymal stem cells for ARDS, the major disadvantages of MSCs as a therapeutic agent are the risk of iatrogenic tumor formation and graft-versus-host disease, as well as the prohibitive cost of production, storage, and distribution of cells in tissue transplant facilities, which limits access.[46]

Paracrine Mechanism of MSCs

The most recent studies clearly demonstrate a paracrine mechanism underlying the immunoregulatory and tissue repair functions of MSCs, which are mediated by exosomes. In numerous models of human diseases, exosomes produced by MSCs mimic their beneficial effects, and these exosomes have demonstrated efficacy equivalent to MSCs in models of severe infectious pneumonia, ALI, ARDS, and sepsis. Exosomes are also much easier to store, distribute and increase scale of production. As an acellular product, MSC exosomes also lack any risk of malignant transformation or graft-versus-host disease that may be present with live cells. Knowing that MSC exosomes can provide equivalent benefits to MSCs, and that they lack the major disadvantages of MSCs, it seems reasonable to explore the benefits of MSC exosomes in the case of ALI/ARDS and COVID-19. The antiviral, immunomodulatory and regenerative properties of MSC exosomes, and their safety profile, make this novel therapy an

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

excellent candidate for the treatment of mild, moderate and severe, life-threatening cases of respiratory illness and ARDS caused by SARS-Cov-2 and other respiratory viruses such as influenza.

Exosomes

Exosomes are naturally occurring, biologic messengers that contain complex, cell-signaling information within extracellular nanovesicles produced by virtually all living cells. This subtype of extracellular vesicles, 20-200 nm in size and of endosomal origin, is released from parent cells by fusion of the multivesicular body with the cell membrane. Exosomes contain hundreds of proteins, mRNA and microRNA (miRNA) that evoke different behaviors in different cell types and under different conditions. Exosomal proteins, such as growth factors, growth factor receptors, anti-inflammatory cytokines, immunomodulators, enzymes, binding proteins and tumor suppressors, are responsible for some of the effects of exosomes on cell behavior. Transfer of RNA to target cells that internalize exosomes directly affects cell behavior by influencing protein production, in part, through direct translation of exosomal mRNA in these cells. Exosomal microRNA transferred into target cells acts as an inhibitor of specific protein production in these cells by blocking ribosome binding to specific, complementary mRNA and by accelerating the degradation of these mRNA strands. These powerful cell-signaling mediators are contained within a phospholipid membrane similar to the parent cell that protects the contents of the exosomes from degradation by circulating proteases or RNAses and facilitates internalization of the exosomes into the target cells. Exosomes are the messengers in a sophisticated network of intercellular communication that mediates the paracrine mechanisms of the parent cells.

MSC Exosome Properties

Numerous preclinical studies have demonstrated that MSC exosomes contain signaling information that induces cellular behavior that could have profound clinical applications. MSC exosomes reduce inflammation, which is understood to be a core mechanism of many diseases and traumatic processes, by downregulation of inflammatory cytokines such as TNF-α, IL-1β, IL-12p40, IFN-γ, NF-κB, HMGB1 and NLRP3, and upregulation of anti-inflammatory cytokines such as TGF-β3 and IL-10.[47,48] Excessive activity of immune cells also plays a role in many autoimmune, infectious and degenerative conditions, and MSC exosomes have demonstrated efficacy in modulating the activity of immune cells.[49,50,51,52,53,54,55,56,57,58]

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

These exosomes also promote the development of connective tissues and blood vessels.[59,60] MSC exosomes have also demonstrated efficacy in maintaining the viability of injured cells.[61] These exosomes also promote the proliferation and migration of endogenous progenitor cells to regenerate damaged tissues that have lost cells due to apoptosis and necrosis.[62,63] In severely damaged tissues that do not survive, these exosomes reduce the extent of fibrosis and scarring. Some MSC exosomes have also shown evidence of suppression of cancer cells and tumor growth in preclinical models.[64,65]

The type of tissue, in which MSCs reside, also influences their function even at the same age, meaning that the biological properties of the exosomes they produce will be different. MSCs originating from the chorionic plate of the placenta show much higher levels of anti-inflammatory and regenerative growth factors, such as Ang-1 and HGF, than do MSCs derived from umbilical cord and amniotic membrane.[66] This observation would account for the differences observed between exosomes produced by MSCs residing in different tissues. Though the ability to differentiate into adipogenic, chondrogenic and osteogenic cells is characteristic of MSCs, the observed effects of MSCs in vitro, in vivo and in clinical trials are not related to their ability to differentiate in vivo, as very few transplanted MSCs show any signs of long-term engraftment. Instead, MSCs function via a paracrine mechanism by releasing exosomes, which influence the behavior of endogenous immune, somatic and progenitor cells.

When considering the biologic properties of neonatal MSC exosomes, it is important to note that their effects are not the result of any single protein, mRNA or microRNA contained within the exosomes. Instead, the biologic properties of these exosomes result from the combined effects of hundreds of proteins, mRNAs and microRNAs acting in concert on multiple signaling pathways in the specific cell that internalized the exosome. The alterations in cellular behavior induced by the composite effect of all these exosomal signaling factors also depends on the biochemical processes occurring in the target cell prior to endocytosis of the exosomal contents. The target cell type-specific effect induced by internalization of MSC exosomes may explain some seemingly paradoxical effects of exosomes, such as the pro-angiogenic effect usually observed in somatic cells and the anti-angiogenic effects observed in neoplastic cells.

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[62] Collino et al. AKI recovery induced by mesenchymal stromal cell-derived extracellular vesicles carrying MicroRNAs. J Am Soc Nephrol 26:2349–2360

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

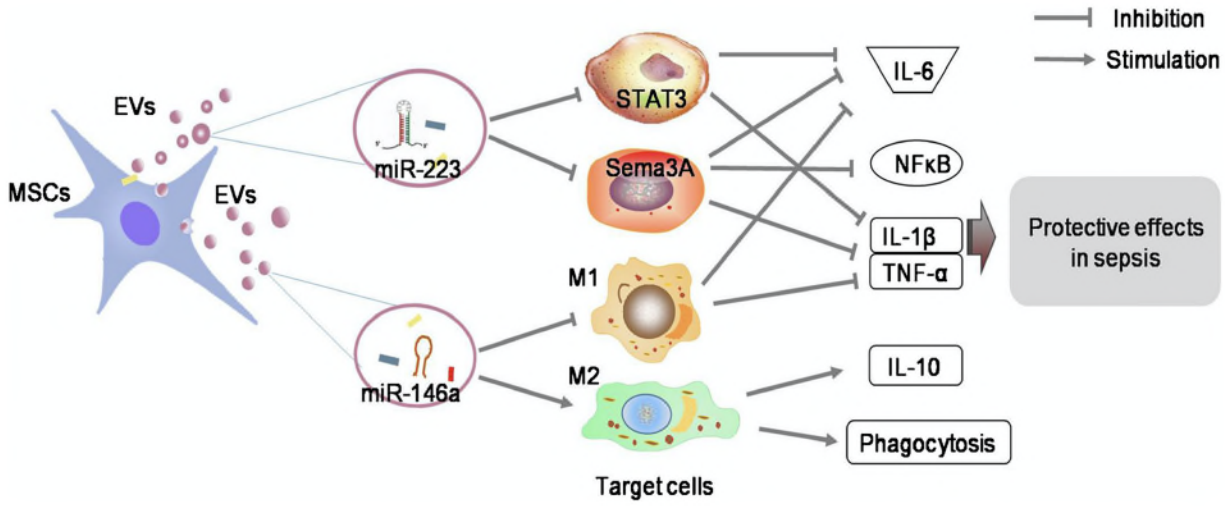

Figure 3. Mechanisms of immunomodulation by MSC exosomes that exert protective effects in sepsis.[67]

Neonatal MSC Exosomes

Exosomes produced by MSCs at different ages contain different levels of specific proteins and RNA, which influence their effects on cellular behavior. The biological properties of neonatal MSC exosomes differ from those of adult stromal progenitor cell exosomes. MSCs of neonatal origin exhibit superior proliferation ability, lower immunogenicity, and lower incorporated mutation than MSCs classically derived from adult bone marrow.[68] Since exosomes reflect the parental cells and mimic their function, their beneficial biological properties of neonatal MSCs can be transferred through the proteins and RNA contained within the exosomes they produce. These observations suggest that neonatal MSC exosomes have greater potential for a broad range of clinical applications.

Adult Stromal Progenitor Cell Exosomes

The closest analog to multipotent, mesenchymal stem cells in an adult are stromal progenitor cells, sometimes referred to as mesenchymal stromal cells. The exosomes produced by these stromal progenitor cells commonly isolated from adipose or bone marrow have different contents than perinatal MSCs, because of the changes in gene expression and protein translation associated with normal aging and the risk of exposure to environmental mutagens and spontaneous mutation. Senescent progenitor cells express different proteins and miRNA than neonatal MSCs. One familiar example of the loss of protein production with age can be illustrated by development of lactose intolerance by many adults, due to the reduced expression of lactase in adults. Cataloguing of microRNA present in circulating extracellular vesicles also indicates that the levels of specific microRNAs change with aging, or in specific disease states. The differences in proteins and RNA contained within exosomes produced by adult stromal progenitor cells and those produced by neonatal MSC exosomes may explain why bone marrow-derived MSC exosomes have been shown evidence of promotion of osteosarcoma, leukemia, metastasis

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

and other tumorigenesis,[69,70,71] while neonatal MSC exosomes appear to have tumor suppressive properties.[72]

Role of Endogenous Exosomes in ARDS

As is the case with many diseases, endogenous exosomes play a role in the pathogenesis of ARDS. LPS administered intratracheally or intravenously in mice stimulates endothelial cells and leukocytes to produce elevated levels of extracellular vesicles, which cause a profound inflammatory response, increasing myeloperoxidase, TNF-α, IL-1β, and IL-10 production in bronchoalveolar lavage fluid and plasma. As previously described, elevated levels of inflammatory cytokines damage the structure of alveoli and the surrounding capillaries, causing severe perivascular and intra-alveolar hemorrhage and formation of a hyaline membrane in the alveoli.[73]

In moderate-to-severe ARDS, MSCs produce extracellular vesicles that reach a level almost 5 times that, which is present in normal patients. These extracellular vesicles contain TGF-β receptor I (TβRI)/Alk5 and the Runx1 transcription factor. The clinical outcome of the ARDS depends heavily on the ratio of two isoforms of Runx1: p52, which is expressed continuously, and p66, whose expression is short lived. A high ratio of p66:p52 isoforms of Runx1 provides a survival advantage, regardless of age, sex, disease severity or length of stay in the intensive care unit. Notably, the Runx1 p66 isoform is expressed by cultured human bone marrow-derived MSCs and is released in exosomes, which can be isolated from the conditioned media and stimulate the proliferation of LPS-treated endothelial cells. Exosomal Runx1-p66 enhances the junctional integrity of LPS-injured endothelial cells and decreases the severity of histologic damage in the lungs of LPS-treated mice.[74] This repair system for ALI mediated by endogenous MSC exosomes suggests the potential of exogenous MSC exosomes as a potential therapeutic agent for ARDS.

Therapeutic Mechanisms of MSC Exosomes in ALI/ARDS

Acellular MSC-conditioned medium (MSC-CM), a mixture of exosomes and soluble factors produced by MSCs, decreased the alveolar influx of inflammatory cells and prevents pulmonary edema formation in part by promoting an alternate anti-inflammatory and highly phagocytic M2 macrophage phenotype, which is much more effective than M1 macrophages in efferocytosis.[75,76,77] Unlike hydroxychloroquine which inhibits macrophage activity, MSC exosomes change the phenotype of macrophages to reduce the release of inflammatory mediators and enhance phagocytosis of cellular debris. As demonstrated by

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[74] Alk5/Runx1 signaling mediated by extracellular vesicles promotes vascular repair in acute respiratory distress syndrome. Shah et al. Clin Transl Med 2018;7:19 doi: 101186/s40169-018-0197-2

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

knockout studies, MSC-CM has ability to reduce alveolar inflammation, restore alveolar epithelial permeability to normal, and improve impaired alveolar fluid clearance in ALI, caused by inflammatory mediators such as IL-1β, TNF-α, and IFN-γ, mediated at least in part by KGF.[78] MSC exosomes containing Ang-1 mRNA restored endothelial cell permeability to human lung microvascular endothelial cells injured by inflammatory mediators. Administration of these exosomes to mice with endotoxin-induced ALI reduced lung inflammation, protein permeability, and pulmonary edema.[79,80] Exosomal miR-126 transferred from endothelial progenitor cells to target endothelial cells in ALI models resulted in improvement in endothelial cell function via downregulation of SPRED1 and promotion of RAF/ERK signaling pathways.[81] Also, miR146a produced by alveolar macrophages significantly suppressed LPS-mediated TNF-α, IL-6, and IL-1β induction by repressing expression of IRAK-1 and TRAF-6.[82]

MSC Exosome Effects in Severe Bacterial Pneumonia

MSCs have been reported to increase bacterial clearance and reduce bacteremia in preclinical models of ARDS and sepsis in part through KGF.[83] Paracrine factors produced by MSCs increase alveolar macrophage phagocytosis and production of directly antimicrobial peptides, such as cathelicidin LL-37, human β-defensin-2, hepcidin, lipocalin-2, indoleamine 2,3-dioxygenase (IDO) and interleukin (IL)-17.[84] Administration of human MSC exosomes improved survival in mice injured with bacterial pneumonia, through keratinocyte growth factor secretion, and decreased the influx of inflammatory cells, cytokines, protein, and bacteria. MSC exosomes enhanced monocyte phagocytosis of bacteria, while decreasing inflammatory cytokine secretion, and increased intracellular ATP levels in injured alveolar epithelial type 2 cells.[85] MSC exosomes have been reported to be just as effective as MSCs in E. coli LPS and live bacteria-induced ALI in mice. In an ex vivo perfused human model of bacterial pneumonia, MSC exosomes administered systemically 1 hour after intrabronchial administration of E. coli bacteria, significantly increased alveolar fluid clearance, reduced lung protein permeability, and numerically lowered the bacterial load in the injured alveoli.[86]

MSC Exosome Effects in Severe Viral Pneumonia

Influenza viruses cause annual outbreaks of acute respiratory illness that can develop into severe viral pneumonia in patients over 65 years of age, resulting in significant morbidity and mortality through the inflammatory cytokine-mediated mechanisms of ALI and ARDS. In vitro examination of the anti-influenza activity of MSC exosomes in lung epithelial cells demonstrated inhibition of hemagglutination activity of avian, swine, and human influenza viruses at concentrations of 1.25–5 μg/ml**.** MSC exosomes also inhibited influenza virus replication and virus-induced apoptosis in lung epithelial cells. In a pig model of

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

influenza virus, intratracheal administration of MSC exosomes 12 h after influenza virus infection significantly reduced virus shedding in the nasal swabs, influenza virus replication in the lungs, and virus-induced production of proinflammatory cytokines in the lungs. MSC exosomes also alleviated influenza virus-induced lung lesions in pigs, a relevant preclinical large animal model, suggesting that MSC exosomes could be used as cell-free therapy for influenza and viral ALI in humans.[87]

Antiviral Effects of Exosomal miRNA

Elimination of these beneficial effects of MSC exosomes by pre-incubation of exosomes with RNAse, an enzyme which selectively degrades the RNA contained within exosomes, indicates that the anti-influenza activity of MSC exosomes is due to transfer of exosomal RNAs to epithelial cells. Other studies have shown that miR-323, miR-485, miR-491, and miR-654, miR-3145 destabilize and regulate translation of mRNA coding for PB1, an H1N1 influenza viral polymerase complex protein, by targeting the conservative region and inhibit viral replication.[88],[89],[90] let-7c precursor diminishes H1N1 replication by binding to the 3′-UTR of mRNA coding for M1, the most abundant protein in the influenza A virus.[91] miR-33a suppressed the expression of NP and M1 proteins by directly binding to the 3′-UTR of RNA coding for Archain 1 (ARCN1), a component of human coatomer protein complex, which regulates protein transport from the Golgi body to the endoplasmic reticulum and critically modulates influenza virus entry to host cells, viral membrane protein expression, and assembly.[92,93] Also, miR-21 targets mRNA coding for NP, PB1, PB2, PA, NA, and HA protein segments of H1N1 influenza virus.[94] Antiviral activity of exosomal miRNA has also been demonstrated in a study of Hepatitis C viral infection, in which researchers were able to identify four specific miRNA molecules (let-7f, miR-145, miR-199a, and miR-221) that mediated RNA-induced silencing complexes to inhibit viral RNA translation to protein and reduce viral replication.[95]

The miRNAs, which have been identified to target the SARS-CoV2 genome through bioinformatic analysis performed by a group in India, are hsa-let-7a, hsa-miR101, hsa-miR125a-5p, hsa-miR126, hsa-miR222, hsa-miR23b, hsa-miR378, hsa-miR380-5 and hsa-miR98e. These were also reported to target Hepatitis C, Herpes simplex virus 1, Hepatitis B, Influenza A, Enterovirus 71 and Vesicular stomatitis virus. The miRNA viral targets predicted by miRanda includes IFN-B, ATP5B, ERBB2, PB2, PA, NS1, NP, VP1 genes.[96]

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

Rationale and Investigational Study Protocol for Treatment of COVID-19 Severe Viral Pneumonia with Isolated, Placental, Mesenchymal Stem Cell Exosomes

MSC Exosomes and COVID-19

As with other infectious and non-infectious causes of ALI and ARDS, effective treatment of COVID-19 requires immunomodulation of the cytokine storm that causes the vast majority, if not all, of the alveolar-capillary damage that leads to respiratory failure. Also, being that the cause of the cytokine storm in this case is a virus, it follows that any treatment should also exhibit antiviral activity, although this may not be essential, because the cause of severe illness and death is related to the secondary damage to the lungs caused by the inflammatory response to the virus, not the virus itself. Another property of an ideal therapy for COVID-19 would be the capacity to promote and accelerate regeneration of the damaged lung tissue. Cell-based therapy exhibits all three of these properties: immunomodulation, antiviral activity and tissue regeneration.

To this end, one Chinese study has deployed adipose-derived stromal progenitor cells as therapy for COVID-19 patients and seen early clinical success.[97] In this study, these stromal progenitor cells have improved the outcome of 7 enrolled patients without observed adverse effects. Pulmonary function and symptoms of these seven patients were significantly improved in 2 days after cell transplantation. After treatment peripheral lymphocytes were increased and C-reactive protein decreased. Overactivated cytokine-secreting immune cells CXCR3+CD4+ T cells, CXCR3+CD8+ T cells, and CXCR3+ NK cells disappeared in 3-6 days. CD14+CD11c+CD11b mid regulatory DC cell population (syn. DC10 cells) dramatically increased. Levels of TNF-α were significantly decreased, while IL-10 increased. Gene expression profile showed MSCs were ACE2- and TMPRSS2- which indicated MSCs were free from COVID-19 infection. The investigators concluded that intravenous transplantation of MSCs was safe and effective for treatment in patients with COVID-19 pneumonia, especially for the patients in critical condition.

The apparent success of this small pilot study seems promising and should inspire similar efforts to treat COVID-19 with cell-based therapy. Knowing that the mechanism of action of the stromal progenitor cells used in this study depends on the paracrine effect of exosomes produced by these cells, it seems preferable to focus clinical research efforts on the use of exosomes, rather than cells, for treatment COVID-19. MSC exosomes, which have demonstrated the ability to elicit the same effects as MSCs, are more scalable and easier to store and distribute than MSCs, making them easier to deploy rapidly in response to this pandemic. Amongst the different types of MSC exosomes, neonatal MSC exosomes seem to present an optimal therapeutic option because they contain the full complement of proteins, mRNA and microRNA produced by neonatal MSCs. Neonatal MSC exosomes also do not seem to exhibit the same tumorigenic or pro-metastatic properties demonstrated in some preclinical studies by exosomes produced by bone marrow-derived stromal progenitor cells or adipose-derived stromal progenitor cells.

Investigational New Drug Development

As a purified extract of placental mesenchymal stem cells, an injectible suspension of isolated, placental, mesenchymal stem cell-derived extracellular vesicles could be an excellent investigational new drug candidate. These exosomes, produced by mesenchymal stem cells isolated from placental tissue after health Caesarean section deliveries, carry a developmental message which naturally supports healthy fetal development. The natural function of placental MSC exosomes supports the rapid, but controlled, cell division required for fetal development, suppresses inflammation to prevent preterm labor and allow full-term pregnancy, modulates the maternal immune system to prevent rejection of the fetus, promotes angiogenesis to provide adequate blood supply for developing fetal tissues, directs synthesis of

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

connective tissue such as skin, hair, bone, muscle and cartilage, and suppresses oncogenesis through expression of tumor suppressor proteins and microRNA.

The biological properties of these placental MSC exosomes could be used to regenerate tissues damaged by injury and disease like the severe pneumonia and acute lung injury caused by SARS-Cov-2. The ability of MSC exosomes to reduce the inflammatory response and attenuate the acute lung injury of viral pneumonia caused by influenza, and the capacity to promote regeneration of the damaged tissues suggests that this biologic therapy could help to improve the similar condition of critically ill COVID-19 patients, in the same way that cell-based therapy has. If these MSC exosomes also have any direct or indirect antiviral activity against SARS-Cov-2 through transfer of microRNA, as they have demonstrated with influenza, hepatitis C and other RNA viruses, then perhaps MSC exosomes could be used to treat mild or moderate cases of COVID-19 as well, or even act as prophylaxis. This type of unique biopharmaceutical has natural biological properties, which warrant clinical trials to treat many different degenerative conditions, traumatic injuries and disease processes such as COVID-19 caused by the SARS-Cov-2 coronavirus.

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Study Protocol

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Brief Name	Investigational New Drug trial investigating the safety and clinical efficacy of placental, mesenchymal stem cell-derived extracellular vesicles for treatment of COVID-19 severe viral pneumonia in ventilated patients.
Official Title	A randomized, double blind, parallel group, placebo controlled, one center, phase I/IIa study to assess the safety and efficacy of isolated, placental, mesenchymal stem cell-derived extracellular vesicles for the intravenous treatment of adult patients with Severe Viral Pneumonia-Acute Respiratory Distress Syndrome-COVID-19 (sVP-ARDS-COVID-19).
Study Overview	The study is a prospective, randomized, double-blinded, parallel group, placebo controlled study with a primary objective to investigate the safety and efficacy of KLX-100, isolated, placental, mesenchymal stem cell-derived extracellular vesicles for the treatment of COVID-19 severe viral pneumonia in ventilated patients suffering from acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). The novel coronavirus, SARS-Cov-2, is a positive-sense RNA virus that infects type II alveolar epithelial cells and causes COVID-19, an acute respiratory illness characterized by fever, cough and shortness of breath. After infection, COVID-19 progresses through a viral replicative phase of this disease, during which only mild symptoms may be present. By the time the normal adaptive immune response begins, there is a massive release of inflammatory cytokines, signaling mediators that cause inflammation and increased activity of immune cells. This cytokine storm induces recruitment of inflammatory cells such as macrophages to the lungs and disrupts the junctions between both alveolar epithelial cells and pulmonary capillary endothelial cells, which form the alveolar-capillary barrier, allowing influx of proteinaceous fluid into the alveoli. Injury to the alveolar epithelial cells also interferes with their normal ability to clear alveolar fluid from the air spaces. Accumulation of fluid in the alveoli interferes with normal gas exchange, causing impaired oxygenation of the blood, which can lead to hypoxemia. Mesenchymal stem cells (MSCs) are multipotent, progenitor cells, meaning they are able to differentiate into other cell types. These progenitor cells have demonstrated the capacity to modulate the immune system and to regenerate diseased or damaged cells and tissues in vitro, in vivo and in clinical trials. Exosomes are naturally occurring, biologic messengers that contain complex, cell-signaling information within extracellular nanovesicles produced by virtually all living cells. Exosomes produced by MSCs mimic their effects and have demonstrated equivalent efficacy in preclinical models of infectious pneumonia, ALI, ARDS and viral sepsis. Unlike live MSCs, MSC exosomes are acellular, so they carry no risk of malignant transformation or graft-versus-host disease. MSC exosomes are also more scalable and easier to store and distribute. This study is being undertaken to investigate the safety and efficacy of isolated, placental, MSC exosomes for treatment of COVID-19 severe viral pneumonia in ventilated patients. These MSC exosomes show great promise for multiple applications and have demonstrated no adverse effects in previous IRB approved human pilot studies.

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Study Type	Interventional
Study Phase	Phase I/IIa
Study Design	**Allocation**: Randomized
	Intervention Model: Placebo-controlled, clinical-scoring per protocol
	Masking: Double-blind (subject and investigator)
	Primary Purpose: Investigate safety and efficacy of exosomes for COVID-19
Study Duration	**Patient Endpoints**: 1, ~~2,~~ 3, 4, 5, 6, 7, 8-10, 14, 28, 90 days until release from ICU
	Estimated Study Duration: 6 months

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Condition	Severe Viral Pneumonia-Acute Respiratory Distress Syndrome-COVID-19
Intervention	Treatment Groups: ● Standard of Care ● Standard of Care + Intravenous infusion of isolated, placental, mesenchymal stem cell-derived extracellular vesicles
Study Logistics	**Number of Subjects**: 64 **Indication for Treatment** Severe pneumonia of sufficient severity requiring intensive care, and with at least one of the following major criteria of severity present for less than 18 hours: ● SpO2 < 93% on room air ● RR > 30/min ● HR > 120/min ● Any signs of organ failure ● Requiring invasive mechanical ventilation for respiratory failure due to pneumonia ● Requiring treatment with vasopressors **Screening** Vital Signs: Daily systolic and diastolic blood pressure, heart rate, core temperature [tympanic, rectal or bladder], respiratory rate (in non-ventilated patients) as follows: Screening, Day 1 (at Pre-dose, and at 0.5h (±5 min), 1h (±10 min), 2h (±10 min), 4h (±20 min), 12h (±30 min) and 24 h (±1 h) post each KLX-100 infusion), Day 2 (at least 4 times), Day 3 (at Predose, and at 0.5h (±5 min), 1h (±10 min), 2h (±10 min), 4h (±20 min), 12h (±30 min) and 24 h (±1 h) post each infusion), then at least 4 times daily while in the ICU or, if discharged from ICU at least once on Days 4, 5, 6, 7, 8-10, 14, 29, 90 or study discontinuation. Laboratory studies (hematology, coagulation, clinical chemistry, C-reactive protein, troponin-I and urine analysis): At Screening, Day 0 Pre-dose, and then at least on Days 2, 3 (only hematology and coagulation), 4, 7, 14, 29, 90, 180 and 365 or study discontinuation 12-lead electrocardiogram (ECG): From Screening, Day 1 and Day 3 both 5 hours ± 1h post-study treatment administration. Chest radiographs: Daily PA and lateral chest radiographs Cardiac echocardiogram: At Screening, and Day 3, if patient remains in critical condition Anti-human leukocyte antigen complex (HLA)/donor antibodies (Abs) **Treatment**: One (1) intravenous infusion of isolated, placental, mesenchymal stem cell-derived extracellular vesicles (0.2 mg/kg) will be administered, in addition to the Standard of Care for COVID-19 pneumonia, to patients who meet criteria for initial treatment: ● SpO2 < 93% on room air ● RR > 30/min

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

	● HR > 120/min ● Any signs of organ failure ● Requiring invasive mechanical ventilation for respiratory failure due to pneumonia ● Requiring treatment with vasopressors Subsequent intravenous infusions may be indicated every 48 hours according to criteria indicative of declining pulmonary function: ● PaO2 < 60 without change in respiratory support ● Declining PaO2 on stable respiratory support ● Declining PaO2/FiO2 **Protocol Synopsis:** With both subject and the evaluating physician blinded, subjects will be administered the investigational drug injection and placebo (saline) injection at Visit 1 (Day 0) by a non-evaluating (unblinded) study site staff member and will be followed up and re-evaluated by a blinded evaluating physician at Visit 2 (Day 1), Visit 3 (Day 7), Visit 4 (Day 14), Visit 5 (Day 21), Visit 6 (Day 30), Visit 7 (Day 60), Visit 8 (Day 90), visit 9 (day 120), and visit 10 (day 180). At each visit, participants (and accompanying caregivers) will be informed/reminded about study design, responsibilities, and possible adverse events.

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Primary Endpoints	Safety profile of one (1) or more intravenous infusions of KLX-100, isolated, neonatal, mesenchymal stem cell-derived extracellular vesicles at a dose of 0.2 mg/kg each
Secondary Endpoints	Efficacy 1. Mechanical ventilator and vasopressors treatment-free days (number of days that a patient is alive and free from mechanical ventilation and vasopressors) over 28 days. 2. Percentage of patients alive and free of mechanical ventilation at Day 29. 3. Ventilator free days (VFD) over 28 days. VFD are defined as one point for each day during the measurement period that are both alive and free of mechanical ventilation. 4. Percentage of patients alive and free of vasopressors at Day 29. 5. Vasopressor treatment-free days over 28 days defined as one point for each day during the measurement period that subjects are both alive and free of vasopressors. 6. Time to end of invasive mechanical ventilation. 7. Time to end of invasive and/or non-invasive mechanical ventilation. 8. Time to end of vasopressors treatment. 9. sVP-ARDS-COVID-19 Clinical Response: a. Visit at Day 14±2 assessed as follows: i. Cure: complete resolution of pneumonia signs and symptoms present at baseline, no new symptoms or complications attributable to the pneumonia. ii. Non-response: any of the following: 1. Failure related to pneumonia: a. Persistence/progression of baseline signs/symptoms of pneumonia; or baseline radiographic abnormalities after at least 2 days of treatment; or development of new pulmonary/extra pulmonary findings consistent with active infection, or development of new pulmonary infection or extrapulmonary infection requiring antimicrobial therapy; or persistence/progression of baseline signs/symptoms of severe sepsis; or development of new signs/symptoms of severe sepsis; or death due to sepsis. 2. Failure unrelated to pneumonia: a. Any other cause of clinical response failure than in the investigator's judgement is unrelated to the index pneumonia (myocardial infarction, pulmonary thromboembolism, sepsis of urinary origin, etc.). i. Indeterminate: extenuating circumstances precluding classification to one of the above. b. Visits at Day 8-10 and Day 29 or early discontinuation. i. Time to sVP-ARDS-COVID-19 cure. i. Duration of antiviral treatment. i. Rate of pneumonia recurrence/reinfection after clinical cure. v. Pneumonia recurrence is defined as a new acute clinical episode of pneumonia, after clinical cure of the episode that qualified the patient for the study, based on the presence of two relevant signs (fever,tachypoea, leukocytosis, or hypoxemia) and radiographic findings of new pulmonary infiltrate/s or clinically significant worsening of previous ones. If a pathogen isolated in the recurrent episode is phenotypically different from the one isolated in the previous episode this will be considered as reinfection.

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

10. Time to recurrence/reinfection of pneumonia after clinical cure at sVP-ARDS-COVID-19 clinical response assessments.
11. Survival 28-day all-cause mortality.
12. 28-day sVP-ARDS-COVID-19-associated mortality.
13. Survival at Day 7, 14, 29, and 90 visits.
14. Time to death.
15. Other efficacy endpoints:
    a. Time to discharge from ICU.
    b. Time to discharge from hospital.
    c. Length of stay in ICU and hospital after randomisation.
    d. Number of ICU-free days over 28 days.
    e. Changes in Sepsis-related Organ Failure
    f. Assessment score daily during stay at ICU.
    g. Changes on chest X-ray assessed at Screening, and then as medically required with at least one CXR per sVP-ARDS-COVID-19 clinical response assessment until clinical cure from Day 1 to Day 29 and for pneumonia recurrence/reinfection assessment.
    h. Evolution of partial pressure of oxygen/fraction inspired oxygen (PaO2/FiO2) daily until Day 7.
    i. Need of mechanical ventilation or need for non-invasive ventilation 12 hours after the second KLX-100 infusion.
    j. Use of rescue antibiotics i.e. addition or change of antibiotic treatments due to the occurrence of antibiotic resistance posterior to microbiology results at baseline or insufficient efficacy during the course of the study.
    k. Exploratory Biological Endpoints
        i. Cell responses on Day 0 Pre-dose and Days 7, 14 and 29 or early discontinuation:
        ii. Cell proliferative capacity in the presence and absence of stimulation
        iii. Cell activation status (phenotype pro/anti-inflammatory monocytes, pro/antiinflammatory T cells, HLADR, CD69)
        iv. Secretion assay of peripheral blood mononuclear cells in response to stimulation
        v. Evaluation of RNA expression profiles of blood leukocytes on Screening, Day 0 Post-dose, Day 2, Day 3 Post-dose and Days 7 and 14 or early discontinuation (only if early termination [ET] is before V9 [Day 14]).
        vi. Evaluation of plasma concentrations of biomarkers on Screening, Day 0 Post-dose, Day 2, Day 3 Post-dose, and Days 7 and 14 or early discontinuation (only if ET is before V9 [Day 14]).
            1. Protein biomarkers may include, but are not restricted to: TNF-α, IL-1, IL-6, IL-8, IL-10, IL-17, soluble triggering receptor expressed on myeloid cells 1, C-reactive protein, plasminogen activator inhibitor-1, protein C, sE selectin, angiopoietin-1, and angiopoietin-2, troponin-I, D-dimers, HMGB-1

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Key Publications	1. Administration of microparticles from blood of the lipopolysaccharide-treated rats serves to induce pathologic changes of acute respiratory distress syndrome. Li et al. Exp Biol Med (Maywood) 2015;240:1735–1741 doi: 101177/1535370215591830 2. Alk5/Runx1 signaling mediated by extracellular vesicles promotes vascular repair in acute respiratory distress syndrome. Shah et al. Clin Transl Med 2018;7:19 doi: 101186/s40169-018-0197-2 3. Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin- 1. Fang et al. The Journal of biological chemistry 2010;285:26211–26222 4. Therapeutic Use of Mesenchymal Stem Cell-Derived Extracellular Vesicles in Acute Lung Injury. Lee et al. Transfusion. 2019 Feb; 59(Suppl 1): 876–883 5. Mesenchymal Stromal Cells Modulate Macrophages in Clinically Relevant Lung Injury Models by Extracellular Vesicle Mitochondrial Transfer.. Morrison et al. Am J Respir Crit Care Med. 2017 Nov 15;196(10):1275-1286. 6. Mesenchymal stem cell-derived extracellular vesicles attenuate influenza virus-induced acute lung injury in a pig model. Katri et al. Stem Cell Res Ther. 2018; 9: 17 7. Human mesenchymal stem cell microvesicles for treatment of Escherichia coli endotoxin-induced acute lung injury in mice.. Zhu et al. Stem Cells 32(1): 116-125 8. Conditioned media from mesenchymal stromal cells restore sodium transport and preserve epithelial permeability in an in vitro model of acute alveolar injury. Goolaerts et al. Am J Physiol Lung Cell Mol Physiol 2014;306:L975-85101152/ajplung2422013 9. Exosomes derived from endothelial progenitor cells ameliorate acute lung injury by transferring miR-126. Wu et al. Exp Cell Res 2018;370:13–23 doi: 101016/jyexcr201863 10. Mesenchymal stem cell microvesicles attenuate acute lung injury in mice partly mediated by Ang-1 mRNA. Tang et al. Stem cells 2017;35:1849–1859 doi: 101002/stem2619 11. Mesenchymal stem cell derived secretome and extracellular vesicles for acute lung injury and other inflammatory lung diseases. Monsel et al. Expert Opin Biol Ther 2016;16:859–871 doi: 101517/1471259820161170804 12. Mesenchymal stem cell microvesicles restore protein permeability across primary cultures of injured human lung microvascular endothelial cells. Hu et al. Stem Cells Transl Med 2018;7:615–624 doi: 101002/sctm17-0278 13. Therapeutic effects of human mesenchymal stem cell microvesicles in an ex vivo perfused human lung injured with severe E. coli pneumonia. Park et al. Thorax 2019;74:43–50 doi: 101136/thoraxjnl-2018-211576 14. Therapeutic Effects of Human Mesenchymal Stem Cell-derived Microvesicles in Severe Pneumonia in Mice. Monsel et al. Am J Respir Crit Care Med 2015;192:324-36101164/rccm201410-1765OC 15. Therapeutic effects of human mesenchymal stem cells in ex vivo human lungs injured with live bacteria. Lee et al. Am J Respir Crit Care Med 2013;187:751-60101164/rccm201206-0990OC 16. Treatment of acute respiratory distress syndrome with allogeneic adipose-derived mesenchymal stem cells: a randomized, placebo-controlled pilot study. Zheng et al. Respir Res2014;15:39101186/1465-9921-15-39

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

Rationale and Investigational Study Protocol for Treatment of COVID-19 Severe Viral Pneumonia with Isolated, Placental, Mesenchymal Stem Cell Exosomes

Principal Investigator (PI) Contact Information	**First Last.** Bettina Jungwirth, MD **Title**: Prof. Dr. **Institution:** Klinik für Anaesthesiologie University Hospital Ulm, Albert-Einstein-Allee 23, 89081 Ulm, Germany **Direct**: +49(0)731-50060007/60006 **Email**: bettina.jungwirth@uniklinik-ulm.de **Website**: https://www.uniklinik-ulm.de/anaesthesiologie.html
Clinical Contact Information	**First Last.** Manfred Weiss, MD **Title: Prof. Dr.** Klinik für Anaesthesiologie Address Albert-Einstein-Allee 23, Ulm, 89081 **Direct**: +49(0)731-50060226 **Email**: manfred.weiss@uniklinik-ulm.de **Website**: https://www.uniklinik-ulm.de/anaesthesiologie.html
Sponsor Contact Information	Jason Sanders, M.D. President Kimera Labs 2831 Corporate Way, Miramar, Florida 33025 **Direct**: +1 (949) 375-2186 **Email**: jason.sanders@kimeralabs.com **Website**: https://kimeralabs.com

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Inclusion Criteria	● Adult subjects of either gender (aged between 18 years and 80 years old.) ● Body weight between 50 kg and 100 kg. ● Clinical diagnosis of acute (developed within 21 past days) viral pneumonia [COVID-19] based on the presence of two relevant signs: fever, tachypnea, or hypoxemia, radiographic findings of new pulmonary infiltrate, with or without confirmatory test for SARS-Cov-2, while result is pending ● Subjects with severe pneumonia of sufficient severity requiring intensive care, and with at least one of the following major criteria of severity present for less than 18 hours: ○ Non-invasive O2 saturation < 90% on maximum non-ventilated respiratory support ○ Requiring invasive mechanical ventilation for respiratory failure due to pneumonia ○ Requiring treatment with vasopressors (i.e., dopamine >5 g/kg/min or any dose of epinephrine, norepinephrine, phenylephrine or vasopressin) for at least 2 hours to maintain or attempt to maintain systolic blood pressure (SBP) >90 mm Hg (or >70 mm Hg) after adequate fluid resuscitation (i.e. for shock). ● Female subjects of no childbearing potential (e.g. non fertile, pre-menarchic, permanently sterile [e.g. underwent hysterectomy, bilateral salpingectomy or bilateral ovariectomy] or post-menopausal [history of no menses for at least 12 months without an alternative medical cause] or Woman of childbearing potential* with a negative serum or urine pregnancy test (sensitive to 25 IU human chorionic gonadotropin [hCG]) and agree to use an adequate method of contraception for three months after the last dose of the investigational medicinal product according to her preferred and usual life style. Adequate methods of female contraception for this study are: sexual abstinence (refraining from heterosexual intercourse), hormonal contraception (both progesteroen-only or combined estrogen and progesterone; both with inhibition of ovulation or where inhibition of ovulation is not the primary mechanism of action), intra-uterine device, bilateral tubal occlusion, condom use by male sexual partner(s) or medically-assessed successfully vasectomized male sexual partner(s). - A woman of childbearing potential is a woman between menarche and post-menopause (history of no menses for at least 12 months without an alternative medical cause) unless she has undergone hysterectomy, bilateral salpingectomy or bilateral ovariectomy. ● Male subjects agreeing to use one of the following methods of birth control according to his preferred and usual life style for three months after the last dose of the investigational medicinal product: sexual abstinence (refraining from heterosexual intercourse), use of condoms or medically-assessed successful vasectomy, or having a female sexual partner(s) who is using an adequate method of contraception as described above.
Exclusion Criteria	A patient will not be included in the study if he/she meets ANY of the following criteria: ● Subjects with Hospital acquired (HAP)-, Health Care acquired (HCAP)- or Ventilator associated-pneumonia (VAP). ● Subjects with pneumonia exclusively of bacterial or fungal origin*.

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
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	Subjects with bacterial pneumonia co-infected with viruses and/or other microorganisms may be entered into the study. *Due to the short time window (up to 18 hours) between fulfillment of severity criteria (i.e. initiation of invasive mechanical ventilation or vasopressors administration, whichever comes first) and the start of the first dose of study treatment, patients with a pneumonia of suspected viral origin by any established standard diagnostic method routinely applied at the study site (e.g. urinary antigen test, rt-PCR) can be entered into the study (confirmation of viral origin must be obtained afterwards). ● Subjects with known or suspected Pneumocystis jirovecii (formerly known as Pneumocystis carinii) pneumonia. ● Subjects with aspiration pneumonia. ● Subjects with known active tuberculosis. ● Subjects with a history of post-obstructive pneumonia. ● Subjects with cystic fibrosis. ● Subjects with any chronic lung disease requiring oxygen therapy at home. ● Presence of infection in another organ location caused by same pathogen (e.g. ● Pneumococcal meningitis in the context of pneumococcal pneumonia). ● Subjects expected to have rapidly fatal disease within 72 hours after randomization. ● Inability to maintain a mean arterial pressure 50 mmHg prior to Screening despite the presence of vasopressors and intravenous fluids. ● Subjects not expected to survive for 3 months due to other pre-existing medical conditions such as end-stage neoplasm or other diseases. ● Subjects with a history of malignancy in the 5 years prior to screening, except for successfully surgically treated non-melanoma skin malignancies. ● Subjects with known primary immunodeficiency disorder or with HIV infection and acquired immune deficiency syndrome (AIDS) with CD4 count <200 cells/mm3 or not receiving highly active antiretroviral therapy (HAART) for HIV. ● Subjects receiving immunosuppressant therapy (including chronic treatment with anti-TNFa) or on chronic high doses of steroids (single administration of 2 mg/kg body weight or 20 mg/day of prednisone or equivalent for 2 weeks). ● Granulocytopenia, not due to sepsis, as evidenced by leukocyte absolute neutrophil count <500 per µL>21 days prior to onset of pneumonia symptoms. ● Subjects who received stem cell therapy, or allogeneic transplantation (organ or bone marrow transplant) within the past 6 months. ● Subjects receiving treatment with a biological agent (e.g. antibodies, cells), immunotherapy or plasma exchange treatment within the last 8 weeks. ● Subjects with a known liver function impairment associated with liver cirrhosis (Child Pugh C) or known esophageal varices. ● Subjects hospitalized within the previous 15 days ● Conditions resulting in a New York Heart Association or Canadian Cardiovascular Society Class IV functional status.

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

	● End-stage neuromuscular disorders (e.g. motor neuron diseases, myasthenia gravis, etc.) or cerebral disorders that impair weaning. ● Patients with quadriplegia (traumatic or otherwise). ● Patients who have received any other investigational drugs for treatment
ClinicalTrials.gov Identifier	
Internal Study ID Numbers	123/20
Responsible Party	Kimera Labs, Inc
Sponsors / Collaborator(s)	Kimera Labs, Inc
Sponsor Contact	Jason Sanders MD President Kimera Labs 2831 Corporate Way, Miramar, Florida 33025 **Direct**: +1 (949) 375-2186 **Email**: jason.sanders@kimeralabs.com **Website**: https://kimeralabs.com

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de

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Principal Investigator	**First Last.** Bettina Jungwirth, MD **Title**: Prof. Dr., **Institution:** Klinik für Anaesthesiologie **Academic Affiliations:** University Hospital Ulm, Albert-Einstein-Allee 23, 89081 Ulm, Germany **Direct**: +49(0)731-50060007/60006 **Email**: **bettina.jungwirth@uniklinik-ulm.de** **Website**: https://www.uniklinik-ulm.de/anaesthesiologie.html
Location(s)	Institution Klinik für Anaesthesiologie University Hospital Ulm **Direct**: +49(0)731-50060007/60006 **Address**: Albert-Einstein-Allee 23, 89081 Ulm, Germany Ulm, **City**: Ulm, 89081 **Direct**: +49 (0)731 500-55425, Fax: 0731 500-55403 **Email**: janpeter.schroeder@uniklinik-ulm.de; karl.traeger@uniklinik-ulm.de **Website**: https://www.uniklinik-ulm.de/anaesthesiologie/stationen.html
Research Ethics Review	**First Last**: Christian Lenk, MD **Title:** Prof. Dr. Ethikkommission der Universität Ulm **Academic Affiliations**: Inst. für Geschichte, Theorie und Ethik der Medizin **Address:** Parkstrasse 11, 3. OG, **City:** Ulm, 89073, **Direct:** 49 (0)731 500 33720, Fax: +49 (0)731 500 **Email**: christian.lenk@uni-ulm.de

Jason Sanders MD, President, Kimera Labs, jason.sanders@kimeralabs.com
Marion Schneider, PhD, Division of Experimental Anaesthesiology, University Hospital Ulm; marion.schneider@uni-ulm.de
Manfred Weiss, MD, Klinik für Anaesthesiologie, University Hospital Ulm; manfred.weiss@uniklinik-ulm.de