COVID-19 Therapy With Mesenchymal Stromal Cells (MSC) and Convalescent Plasma Should Consider Exosome Involvement

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Short title:

**Exosomes from MSC and in Convalescent Plasma may give more optimal
therapy to COVID-19 patients**

Keywords:

Exosomes, Extracellular Vesicles, Mesenchymal Stromal Cells, MSC, miRNA, Cell Therapy, nanoparticles

Abbreviations:

IV, intravenous administration,

MSC, mesenchymal stromal cells.

MSC^{exos}, MSC-derived nano vesicle exosomes,

MHC, major compatibility complex,

GFP, green fluorescent protein,

Ab, Antibody

Ag, antigen

ABSTRACT

Exosome extracellular vesicles as biologic therapy for COVID-19 therapy are discussed for two areas. The first involves growing use of mesenchymal stromal cells (MSC) for the profound clinical cytokine storm and severe pneumonia in Covid-19 patients. Instead, it is recommended to switch to treat instead with their MSC-released exosomes. This is because many reports in the literature of have shown definitively that the release of exosomes from the in vivo administered MSC is actually responsible for their beneficial effects. Further, the exosomes are superior, simpler and clinically more convenient compared to their parental MSC. Additionally, in the context of COVID-19, the known tendency of MSC to aggregate causing lung dysfunction might synergize with the pneumonia aspects, and the tendency of MSC peripheral vascular micro aggregates might synergize with the vascular clots of the COVID-19 disease process, causing significant central or peripheral vascular insufficiency.

The second involves use of COVID-19 convalescent plasma for its content of acquired immune antibodies that must consider the role in this therapy of billions of exosomes in the

plasma. Many of these derive from activated immune modulating cells and likely transfer miRNAs that acting epigenetically to also influence the recipient response to the virus. These immune activated plasma exosomes may either be responsible for positive effects of the plasma beyond the contained immune antibodies, or could be inhibitory. Pre selection of plasma with the best antibodies and the best exosomes would produce the most optimum therapy for very severely affected COVID-19 patients.

COVID 19 THERAPY WITH MESENCHYMAL STROMAL CELLS

Introduction to the wide clinical usefulness of mesenchymal stromal cells (MSC)

MSC have been safely administered to humans in many clinical instances over the past 25 years. Commonly employed harvest sites for in vitro expansion of MSC precursors include bone marrow, adipose tissue, placenta, and umbilical cord. These antecedents of the employed MSC are present in these diverse connective tissue sites at a very low concentration; in the range of one per 10,000 cells (0.001%).¹

Since MSC are self-replenishing, adherent to culture surfaces and do not have specific surface markers of most immune and myeloid cells, they can be grown in vitro to huge numbers; up to 100% for in vivo use. Culture is performed on increasingly large surface areas of sterile flasks under strict aseptic conditions. The MSC and MSC exosome (MSC^{exos}) phenotype is positive for MHC Class I, CD73, CD90, and CD105, and the exosomes also express typical surface marker tetraspanins (CD9, CD63 and CD81). They do not express typical T cell, B cell, macrophage, myeloid or embryonic cell surface marker antigens, and generally also no expression of MHC-II (HLA DR in humans) nor positive costimulatory surface molecules like include CD40, CD40L;¹ overall suggesting immunomodulatory rather than effector properties.

Administering MSC intravenously (IV) to rodents with induced models of clinical diseases at just a million cells, can result in reversal of abnormalities for weeks thereafter. All told, these effects are nutritive, trophic, reparative, anti-inflammatory, and anti-immunologic; as well as healing micro vascular abnormalities.² Thus, regarding very ill patients with COVID-19 infections, MSC may be ideal for decreasing the pathogenesis of severe viral pneumonia and resulting acute respiratory distress syndrome (ARDS). In addition to infection, there are two accompanying potentially severe and life endangering immunologic syndromes that MSC^{exos} may have special properties for beneficial

treatment. The first is cytokine storm induced by the over reacting immune system, occurring in severe viral diseases.^{3,4} The second is emerging Kawasaki-like Multisystem Inflammatory Syndrome (MIS) of Children also resembling toxic shock syndrome.^{5,6,7}

Potential immune mediated Multisystem Inflammatory Syndrome as sequela of COVID-19 infection potentially benefitted by MSC therapy

MIS in children is a seeming sequela of COVID-19 can occur after seeming recovery as a different delayed immune response. Clinically there is with unrelenting fever, skin rash with some of the following: non-purulent conjunctivitis or muco-cutaneous inflammatory edema (oral, hands or feet), generalized extremity pain and GI problems like diarrhea, vomiting, or abdominal pain. Systemically, there can be hypotension progressing to shock, refractory to volume resuscitation and eventually requiring inotropic support due to the dominant myocarditis with severe inflammation causing left ventricular dysfunction pericarditis, valvulitis, or coronary inflammation with aneurisms, eventuating into acute heart failure or cardiac arrest.^{6,7,8} Like toxic shock and cytokine storm there appears to be a diffuse inflammatory process with development of small pleural, pericardial, and ascitic effusions.⁶ Laboratory tests show: elevated Troponin and cardiac enzymes, as well as evidence of coagulopathy (by PT, PTT, elevated d-Dimers) and markers of inflammation (ESR, C-reactive protein, or procalcitonin) without microbial infectious cause. Treatment with immune globulin, the previous approach to Kawasaki syndrome, can be associated with recovery of left ventricular systolic function. However, therapy with MSC or convalescent plasma has not previously been contemplated.

Cytokine storm in severely ill patients infected with COVID-19 virus

The cytokine storm occurs during primary active COVID-19 infection. It is due to rapid release of a variety of inflammatory cytokines from viral-activated immune system cells that are overreacting to the initial acute persistent viral infection. There are high fevers, edema, great muscle aches, extreme fatigue and nausea with multiple organ damage., Clinically there is rapid developing patchy bilobar pneumonia with pulmonary edema, dysfunctional air-exchange indicated by dangerously low pO₂; all often progressing to (ARDS) induced by infection of alveolar cells. The pneumonia is visible as inflammatory lesions with ground-glass opacity on CT scan, with acute cardiac injury; in some leading to death.^{8a} Considerable vascular abnormalities due to vascular injury and inflammation are induced by infection of the endothelium.⁹

This severe COVID-19 cytokine storm syndrome is primarily a virally triggered macrophage activation syndrome.¹⁰ Accordingly, there is preferential production of a variety of macrophage activating factors such as: IFN- γ , its associated IP10 chemokine, monocyte chemoattractant protein 1 (MCP1) and monocyte chemoattractant protein 1 (MIP10); together with granulocyte-colony stimulating factor (GSCF), as well as IL-2, IL-6, IL-7, and TNF α .^{8a,9,11} The rises in macrophage directed cytokines are accompanied by severe lymphopenia; especially reduced CD4⁺ and CD8⁺ T cell counts. They induce pulmonary edema, dysfunctional of air-exchange with severe fall in pO₂, acute respiratory distress syndrome, acute cardiac injury, and often secondary infection, leading to death. The severe CD3⁺ T cell depletion with bursts of IL-6 and IL-8 cytokines are together particularly bad prognostic signs.¹²

Numerous observations that MSC used for healing therapy are not found at their apparent site of action

A crucial point is that when labeled MSC are given they seldom are found in the organ systems they are repairing. Numerous studies prove this is because they are primarily in the lungs, where they are eliminated by 24-48 hours after administration,^{12a} and during which they release their MSC-derived exosomes (MSC^{exos}) that actually are responsible for the healing processes they mediate.

MSC can be harvested from precursors in a variety of connective tissues greatly enriched by simple cultures for use as trophic and anti-inflammatory natural healing cells. It has been shown over the last several years that there is no evidence that MSC go to their targeted organs that were prospering.¹³

Therefore, it is now incontrovertible, that healing, trophic and anti-inflammatory actions of administered MSC are due to their released exosomes (MSC^{exos})

Indeed, the therapeutic effects of systemic MSC can be replicated by systemic transfer of exosomes produced and secreted by the MSCs (MSC^{exos}) in a variety of experimental injury models like myocardial infarction,^{14,15} with arrhythmia.¹⁶ Similarly in: CNS ischemic stroke,^{17,18} traumatic brain injury,^{19,20} spinal cord injury,²¹ ischemia-reperfusion renal injury and dysfunction,^{22,23} liver drug injury, ischemia and fibrosis,^{24,25} lethal irradiation enteropathy,²⁶ and hematopoiesis restoration,²⁷ and finally bronchopulmonary dysplasia²⁸ and bleomycin pulmonary fibrosis.^{29,30} In these various experimental diseases, few, if any, of the systemically administered MSCs were

detected at lesion sites that were healing and further, their derived exosomes were found to deliver almost identical healing.

Additionally, MSC^{exos} replacing their parental MSC modulate immune functions,^{31,32} and consequently there is similar data as the above in models of autoimmune diseases; such as: Systemic Lupus,^{33,34,35} and the CIA model of rheumatoid arthritis.^{36,37} Type I Diabetes Mellitus^{38,39} Inflammatory bowel disease,⁴⁰ and the EAE model of multiple sclerosis.^{41,42,43}

Finally, there are beginning studies of rodent models of MSC^{exos} therapy in Alzheimer's disease,⁴⁴ Parkinson's' disease⁴⁵ status epilepticus⁴⁶ and even autism.⁴⁷ Therefore, it can be seen clearly that so called "cell therapy" with MSC is giving way to administration of their derived MSC^{exos}. Further, it is also readily apparent that a very wide number of clinical tissue and organ injury diseases, as well as a variety of inflammatory, fibrotic and degenerative diseases have been found potentially susceptible to the unusual and wide healing properties of MSC^{exos} instead of treatment with their much more complicated parental MSC. It is our contention that this also applies to the use MSC^{exos} rather than the MSC in the treatment of patients with severe COVID-19 clinical syndromes.

In MSC treatment of spinal cord injury (SCI), results show that MSC^{exos} mediate the tissue repair and functional recovery

We confirmed the failure to find IV delivered MSCs at their apparent site of action in our studies of MSC-induced healing of spinal cord injury (SCI) in rats. I.V. injected fluorescent-labeled MSCs were not detected at lesion sites in the spinal cord, that none-the-less improved.²¹ I.V. infused green fluorescent protein (GFP)-expressing MSCs trafficked transiently to the lungs, spleen and liver, where they survived for only a few days.^{12a} and none were detected within the contused spinal cord that was healing.²¹

This argued that the therapeutic effects are not mediated by MSC local replacement of damaged cells at the SCI site, nor by MSC paracrine effects at the site nor unlikely long distant SCI-directed effects on local tissue repair at the site of injury. Instead, it indicates that the exosomes alone secreted by the MSC acted in an endocrine manner while stationed in SCI-distant organs like the lung and lymphoid organs. There, over a couple of days the MSC release MSC^{exos} into the general circulation, to then target and affect the distant damaged SCI tissues, to actually be responsible for the SCI healing and trophic effects. The proof was that the in vitro culture-derived MSC^{exos} administered IV mediated all of

the actions of the MSC in vivo and when they were labeled could be localized as preferentially targeting healing M2-type macrophages at the site of SCI.²¹

The value of MSC and MSC-derived exosome clinical therapy in humans with a variety of diseases

The above discussion dealt with properties of MSC and MSC^{exos} in a large variety of disease models of experimental animals; a necessary prerequisite to human use. The natural initial dominance of clinical trials with MSC currently is reflected in the ten human disease trials listed at the NIH clinical trials.com site that include: diabetic nephropathy, multiple sclerosis, non-healing wounds and burns, lupus nephritis, allogenic transplantation, acute renal injury, refractory thrombocytopenia and liver failure. The more pertinent use of MSC^{exos} instead is the we are considering here important we are considering here for therapy in appropriate severe COVID-19 patients considering here rather than MSC.

At the clinical trials.com site there also are ten projects listed using MSC^{exos} therapy; most in various cancers but one very interestingly testing MSC^{exos} aerosol treatment of normal volunteers. This certainly could be an antecedent to such therapy in patients with COVID-19 pneumonia that is not possible with cells like the MSC. It follows on phase I/II trials with human bone marrow-derived MSCs safely administered to patients with acute respiratory distress syndrome (ARDS) and septic shock,⁴⁸ and pre clinical studies of Therapeutic Use of MSC^{exos} in acute lung injury.⁴⁹ Also, and very pertinent here, is study of therapy in ARDS in experimental influenza pneumonia, with aerosol application of exosomes directly into the COVID-19 affected lung.^{49a} This is an application of the well known clinical fact that applying topical therapy at the disease site offers the best therapeutic index, as is done routinely with aerosol corticosteroids applied in allergy patients to atopic noses and asthmatic airways. Indeed, the airway exosomes that are released during influenza virus infection serve as a key component for stimulating the antiviral innate immune response.⁵⁰ Thus, there is already is a considerable literature on the beneficial use of MSC in patients with a variety of diseases. Focusing on severe acute pulmonary disease as most pertinent here, patients with ARDS benefitted from their administration^{51,52,53} However, note that clinical trials of ARDS with MSC^{exos} has shown greater and more consistent benefit in part due to problems of variability of viability and effects among the MSC when compared, while the MSC^{exos} have been much more consistent. Still there currently are many more worldwide clinical trials with the MSC, rather than with their seemingly superior derived MSC^{exos}. **54**

Use of MSC^{exos} therapy in relevant human pulmonary diseases

Thus, despite the numerous and growing data favoring replacement of MSC cell therapy with their produced MSC^{exos}, that have been documented to actually be mediating their effects, some of the cognoscenti core of Mafia-like leaders of the MSC field, still in 2020, extol and focus on and invest in treatment employing the MSC. This results in numerous current companies still gearing up to deliver to patients raw MSC from various sources. This now seems to be a distinctly uninformed and inferior approach. It ignores the greater simplicity and most importantly the far less dangerous alternative use of the MSC-derived equally effective secreted MSC^{exos}. However, the superior MSC^{exos} alternative is now beginning to attract more research and commercial considerations with attempts to develop new methods for generation and isolation of MSC^{exos} that will simplify commercial use. Still, even in 2020, many studies persist that ignore the fact of exosome mediation of MSC effects, even when they consider the source of non-cellular trophic actions of MSC “secretome” factors.

Use of MSC and MSC^{exos} therapy in humans with COVID-19 pulmonary diseases.

It is early days in the treatment of COVID-19 patients with MSC. In recent studies from China preliminarily tested MSC treatment of COVID-19 pneumonia.^{54a,54b,54c} There was a remarkable reversal of disease and laboratory abnormalities within days of administering infusions of about 5×10^7 MSC from allogeneic umbilical cord, with no toxicities. Also, there were accompanying decreases of inflammatory cytokine levels and increased anti-inflammatory factors and suppressive IL-10. MSC recovered from the patients showed high levels of anti-inflammatory and trophic factors including TGF β . These results and other facts about MSC, as reviewed here, have led to plans for MSC trials in COVID-19 patients.^{54d,54e}, as already begun in several countries and as well for MSC^{exos}.^{54f}

There are a great number of advantages for the use of MSC^{exos} compared to therapy with their parental MSC.

MSC^{exos} mitigate many safety concerns compared to use of viable replicating MSC. The cells may never really disappear in recipients, compared to the non-immortal exosomes. Further in the context of COVID-19, the known side effect that MSC IV administration may result in a coagulopathy⁵⁵ with aggregating or clumping in the viral injured microcirculation,⁹ perhaps due to a complement related immune reaction to the abnormal endothelium.⁵⁶ This can cause lung dysfunction that might synergize with the COVID-19 pneumonia effects. Similarly, the tendency toward similar MSC-associated peripheral vascular micro aggregates might synergize with the vascular clots of the COVID-19 disease process of causing central or peripheral vascular insufficiency leading to heart

attack with cardiac arrest, stroke, or pulmonary embolism.**57,58** In this time of hyper media coverage of COVID-19 virus, a few such cases would dissuade thousands from considering the MSC approach. As noted MSC^{exos} have significantly less stimulatory HLA-complex molecules and surface co-stimulators.¹ Thus, they are less innate immune stimulatory, nor acquired immunogenic, so that even allogeneic sources of MSC^{exos} have intrinsic tissue and cell preferential targeting/binding ability via their surface signature bar codes, compared to the MSC for which the peripheral tissues have no known receptivity.

MSC^{exos} are durable in culture for large scale manufacture, so that many can be harvested by repeated collections of conditioned media from the same adhering MSC donor cells in bioreactors with huge surface areas. Thus, MSC^{exos} have reduced time and cost for producing therapeutic patient material. Further, biogenesis and secretion of the MSC^{exos} can be boosted several fold by incubation in neuro bioamines.**59** They are non-viable but instead are subcellular particle organelles without functional genomic DNA, so they pose no risk of transferred abnormal chromosome numbers (aneuploidy), nor development of cancer promoting mutations and consequential oncogenicity.

The comparative safety of exosomes is emphasized by the fact that there are billions in blood plasma and patients have been receiving blood and plasma for many decades without problems,**59a** whereas MSC as cells are difficult to standardize or predict their actions in vivo. On a practical level MSC^{exos} can be freeze-thawed at least once without toxic cryo-preserved, with no lost activity for months to years, and it has been reported that they can be freeze-dried,**60** for superior storage allowing on site administration without refrigeration. This can result in uncomplicated use on hospital wards or in poorer countries with low technology medical facilities. Thus, the MSC^{exos} are available for immediate use post thawing without washing compared to cells for easier routine hospital use; they are an 'off-the-shelf' therapeutic with less manufacturing and storage costs.

MSC^{exos} represent a thousand-fold purification as the actually effective component of MSC, via reduction in size and volume and have very different proteome and transcriptome compared to parental cells; obviously important considering that they are responsible for the biologic effects of the MSC that is most often med by the miRNAs that they deliver. As non-cells that are non-proliferating and not subject to differences in viability, MSC^{exos} can be more precisely standardized per dose and duration of biologic action, compared to the difficult variability of live MSC. Therefore, several reviews highly recommended that MSC^{exos} hold great potential for targeted tissue repair.**61,62,63,64,65** Over all, exosomes are simpler, safer, with lower cost, and more easy storage, transportation and use at

the bedside, and of course they are the essential ingredient of the MSC.

COVID-19 CONVALESCENT PLASMA LIKELY CONTAINS IMPORTANT EXOSOMES

As noted, exosomes are nano vesicles made by all cells and are present in all body fluids. They contain greatly varying RNAs, proteins and lipid mediators that can alter the function of targeted cells near and far away via transit in the blood plasma. Especially important is that exosome miRNAs transferred to targeted acceptor cells can epigenetically alter functional properties since exosomes are the chief mechanism for regulation of the behaviors of different and distant cells genetically by such RNA transfers. Exosomes often act epigenetically by their transferred miRNA inactivating cytoplasmic mRNA encoding proteins that act to alter nuclear DNA by methylation and other means to change gene expression.

Exosomes in transit in the plasma have been studied in a variety of conditions because they can be markers of diseases, severity of disease and selection of therapies. Blood plasma exosomes are especially useful in cancer for “liquid biopsies” not only for diagnosis and prognosis, but also as mediating tumor induced resistance to routine radiation and drug therapies. As example here in COVID-19 ARDS patients, there can be a puzzle about the beneficial use and dose of corticosteroids and NSAIDs,⁶⁶ in whom convalescent plasma exosomes might play a significant role. In fact, one study compared convalescent plasma to 1.5gm pulsed methylprednisolone in 19 vs. 21 SARS patients with progressive disease and found the plasma gave significantly better results.^{66a} Further the exosomes are undoubtedly an indicator of intercellular communications among the cells of the immune system to importantly serve towards choices for patient therapy.

Exosomes may have been involved but unmeasured in favorable history of convalescent plasma therapy of viral diseases

Prior positive findings concerning exosomes in convalescent plasma include serious RNA viral infections.^{66b} Mostly beneficial effects were mostly poorly controlled recent studies of viral diseases antecedent to COVID-19; such as infections with related coronaviruses like SARS^{67,68} and MERS,^{69,70} as well as Ebola,^{71,72} avian influenza⁷³ and influenza pneumonia^{74,75,76}, with generally over all 60-80 % improvement in severity or death rates.

Three influenza studies deserve further mention. In one, 93 patients with severe pandemic influenza A (H1N1) 2009 virus infection, in a prospective cohort study were treated with convalescent plasma having a neutralizing antibody titer of $\geq 1:160$, vs controls matched by age, sex, and disease severity.^{76a} Mortality in the treatment group was significantly lower than in the nontreatment group (20.0% vs 54.8%; $P = .01$) a 64% reduction. Subgroup demonstrated that plasma treatment was associated with significantly lower viral load, IL-6, IL-10 and TNFa vs. the control group ($P < .05$).

Another study published recently (2019) examined anti-influenza immune plasma in patients with severe influenza A in a prospective randomized, double-blind, phase 3 trial, comparing clinical efficacy of convalescent plasma with high titer anti-influenza A antibodies to convalescent plasma with low titers.⁷⁷ There was overall benefit but none in high titer vs. low titer plasma was found as in the prior phase 2 trial. This raises questions about the amount or class of Ab needed or other factors in the plasma such as perhaps strongly involved immune exosomes in both lots. Further, 34% of those treated experienced serious adverse events including ARDS and allergic transfusion reactions; highlighting the potential hazards of convalescent plasma.

The third study was a systematic review and meta-analysis of the effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology in several related studies published between 1918 and 1925 showing a 75%% reversal of case-fatality rate in the treated group vs the nontreated controls.⁷⁸ Thus, this meta-analysis of the use of convalescent plasma in the pooled influenza and other virus studies sustained the positive conclusions of the individual past studies. Note also that in the previous early twentieth century convalescent sera and plasma were used to seemingly be able to stem outbreaks of viral diseases such as poliomyelitis,⁷⁹ measles,^{80,81} mumps ⁸², and a meta-analysis of studies using convalescent blood products to treat the Spanish influenza pneumonia of 1918-19.⁸³

Unusual properties of exosomes possibly contributing to the positive effects of convalescent plasma

Convalescent exosomes in general can inhibit pathogen proliferation and transmission directly. They can allow the host to mount effective immune responses against pathogens, that includes activating antiviral mechanisms and transferring antiviral miRNAs derived from a variety of responding cells.^{66b} In influenza virus infection, induced miRNAs in the exosomes regulate viral replication and have favorable influence on the host acquired immune response.⁸⁴

The structure of exosome membranes often changes as a consequence of infections, including alterations in the quantity of structural proteins and particularly and qualitative changes of constituent lipids influencing spatial configurations, resulting in particularly high viscosity that enables certain of these nano vesicles to withstand noxious environments.**85,86,86a,87**. As example, there is there is increasing evidence that immune activation of donor cells, as undoubtedly happens in viral illness due directly to the virus and the resulting immune response, alters membrane lipids to give some produced exosome subpopulations unusual features that may have great relevance to their role in the beneficial effects of convalescent plasma. As example, there is there is increasing evidence that immune activation of exosome donor cells, as undoubtedly happens in viral illness, due directly to the virus and to the resulting immune response. This alters membrane lipids of some produced exosome subpopulations unusual features that may have great relevance to their role in the beneficial effects of convalescent plasma.

This activation can include exosomes with unusual resistance that allows them to function in noxious environments like the acid/enzymes of the stomach and in hypoxic environments; both of which likely apply to affected pulmonary tissue in COVID pneumonia and ARDS. Such “activated” exosome subpopulations from immune and perhaps viral activated cells are able to acquire particular surface abilities. These are ability to be systemically active after oral administration.**88,89** Additionally, there is newly acquired ability to bind Ag-specific Ab free light chains.**88,89,90** This can render suppressive T cell-derived exosomes Ag-specific,**90** enabling their surface anti-peptide Ab to bind peptide-Ag/MHC on targeted APC,**89,91** for transferring the specific miRNA-150.**88,89,92** This miRNA leads to inhibited production of cytokines like IFN- γ by finally targeted Th1 T cells.**93** This T cell subset is most important in pathogenicity of COVID-19 infection-associated cytokine storm.**1,10**

While inhibiting infection and suppressing deleterious aspects of the immune response of the cytokine storm, exosomes in convalescent plasma, particularly those from DC and B cells can also serve to augment the antiviral immune response by acting as miniature nano APC,**94** (Rodrigues M, Fan J 2018). These can induce healing M2-type macrophages,**95** and promote generation of the particular subset among Th2 T cells that produce immunosuppressive IL-10 and TGF- β . **1,10** A pertinent example is that exosomes used as vaccines containing the S protein of the coronavirus pathogen of SARS pneumonia induce high levels of neutralizing antibodies.**96**

Many things in biology and medicine acting in viral infections can function to favor the host or the pathogen. Exosomes can be tailored for function in either way by producing cells to alter target cell activities in many ways to then mediate their essential central function of delivering specific mediators. This applies especially to delivery of gene expression altering small RNAs that can function in both positive or negative directions to modulate the infection or the immune response depending on circumstances. They can promote immune resistance to viral infections in several ways; such as: inhibiting pathogen infection, proliferation and transmission directly, and indirectly Inhibiting Infection by Stimulating Immune resistance Responses by Improving functions of Macrophages, NK cells, T cells, and B cells.

Therefore, there are important functions that could be revealed in the study of patient plasma exosomes in COVID-19 infection that would bear consideration in selection of particular plasmas for therapy according to their potential positive effects on the infection and on the infection associated deleterious immune responses like ARDS and the coagulopathy, as well as the anti-viral Ab titers.

Potential beneficial functions of the exosomes in convalescent plasma of patients with COVID-19 infections

Up to now there have been few observations of exosomes in the plasma of patients with the COVID-19 infections and its syndromes. In adults, plasma blood tests early in COVID-19 infections for studying exosome phenotype, proteome and transcriptome might be useful as “a liquid biopsy” in diagnosing those patients that will go on to severe sudden pneumonia and the need for artificial ventilation, as well as predicting the likelihood and extent of the cytokine storm syndrome. Similar analysis of plasma exosomes might be useful in distinguishing the pediatric patients that will go on to have the severe Kawasaki Disease-like MIS affecting the heart and great vessels.

Per treatment, firstly COVID-19 convalescent plasma analysis of contained anti-viral antibodies is thought useful for selecting particularly strongly therapeutic lots for treatment of the infections and/or induced associated syndromes. This would be based on in vitro assay of the acquired infection-associated viral antigen-specific IgG antibody titers against the virus that if used for treatment can help block or clear the infectious agent early in the illness, and is hoped to be useful additionally for treating the viral and host response mediated pathology.

Secondly, the use of COVID-19 convalescent plasma for its content of acquired immune antibodies must also consider the role in this therapy of the billions per milliliter of exosomes contained in the

plasma. These likely mediate functions of viral activated immune modulating cells due transferred miRNAs acting epigenetically. These immune activated plasma exosomes may be importantly involved in the positive effects of the plasma beyond the contained immune antibodies, or could possibly be inhibitory. Pre selection of plasma with the best antibodies and the best exosomes would produce the most optimum convalescent plasma therapy for very severely affected COVID-19 patients.

Before the development of antibiotics in the 1930s and 1940s convalescent patient plasma or serum of intentionally immunized animals were the only organism-specific mode for treatment of infections like pneumonias.**97,98** Moreover, now for the first time in the use of convalescent plasma, it is realized that in addition to antibodies against the infectious agent, it also contains biologically active exosomes in billions per milliliter. Experimental work in other pandemics suggests that these nano vesicles could also be powerful inhibitors of destructive pneumonias and the excessive immune response that is driving the over blown cytokine storm of severe in severe infections like with COVID-19.

It will be at the least several months or more until really effective drug treatment of active infection will be available to replace convalescent plasma. Additionally, even longer time must elapse until effective vaccines are available to prevent infection. Therefore, for now and many months ahead, given the large continuous number of new patients and the substantial severity and death rate, use of convalescent plasma for its antibody content, will be the only viral-specific treatment available. Appropriately, there are developing individual and inter connected consortium programs with active participation of professional statisticians and experts on designing controls, and for organizing and directing important large scale trials. These will be aimed hopefully at reaching routine use of convalescent plasma for treating hospitalized active cases of COVID-19 infections with severe complications. In the current rush to find effective treatments for such severe current COVID-19 patients, studies are on the way to use convalescent plasma for treatment in which the entire focus is on the contained immune antibodies; with no planned consideration of the important and perhaps crucial exosome aspect.

In contrast, relevant drugs being used today and contemplated are non-specific blockers of limited steps among many constituting the innate and acquired host immune response producing the cytokine storm. These are focused on blocking the IL-6 receptor and signaling, as well as other cytokines,**99,100** or required viral signaling pathways infected host cell. **101,102,103**

As programs for administering COVID-19 convalescent plasma are being comprehensively and hurriedly generated to meet the masses of potential patient recipients, investigators say it is some time before they can even think about the role of the exosome content of the plasma. In 2020 this is a wrong decision about an important element of the plasma therapies never before contemplated since the presence and likely large effects of plasma exosomes was not known in all prior studies.

Potential roles of exosomes in Covid-19 convalescent plasma derived from viral and immune induced host cells

To my view considering the effects of the exosomes in the convalescent plasma needs to be done sooner rather than later. Looking over prior knowledge of exosomes and especially their role in immune regulation, it seems that plasma during COVID-19 infection, and especially use in those with the cytokine storm and ARDS may be augmentative as likely derived from the over blown innate immune cell response of the macrophage family per Mac 1-type subsets stimulated by $\text{TNF-}\alpha$ of the over blown innate immune response and subsequently during still active infection by the viral antigen activated Th1 cell and Th17 T cell-derived cytokines like $\text{INF-}\gamma$

With disease progression towards resolution and beyond, biology of the exosome response likely converts to reflect the healing positive aspect also in convalescent responses with more generation from the healing and trophic M2-type macrophages making IL-4, IL-10 and $\text{TGF-}\beta$, and perhaps later regulatory Th2 cells also making IL-10 and $\text{TGF-}\beta$, with IL-4, IL-13 and IL-25. Thus, convalescent plasma exosomes should be healing. Therefore, it is postulated that activated exosomes from immune stimulated regulatory and suppressor T cells and M2-type macrophages may make a very significant contribution to the beneficial effects of convalescent plasma. This would be beside and beyond the effects of remaining IgM antibodies of the primary immune response and the later developed crucial higher affinity IgG anti-COVID 19 antibodies of the acquired T cell mediated secondary B cell response. In fact, there may be effective Ag-specific Ab actually on the surface of the exosomes. **88,89,90.**

A preclinical model with antigen-specific antibody coated suppressive exosomes in the convalescent immune plasma

In experiments in mice, repeated Ag high dose induced immune tolerance may be similar to experiencing the great and multiple viral Ag load in COVID-19 infection. In fact, tolerogenic suppression of the immune system is a regular part of many viral infections. **104,105,106**

In the preclinical high Ag exposure system, there is generated suppressive CD8^{pos} T cells that act by producing inhibitory anti-inflammatory and anti-immunological suppressive exosomes.**107**

These exosomes are Ag-specific due to a coating of specific Ab free light chains from neighboring activated B cells,**89,89.90** and are found in body fluids and importantly, also in the plasma.**90** Like the separate protective plasma whole antibodies, these exosome-coating antibody light chains can be quite Ag-specific and by employing their specific binding to Ag-affinity chromatography are easy to isolate and strongly enrich.**88,89,90**.

This can provide a route to determining antibody variable-region gene sequences for development of biotechnology engineered monoclonal COVID-19 Ag-specific high affinity IgG antibodies for more definitive treatments that could be significantly developed before emergence of effective treating drugs or preventing vaccines. Further, determining the crucial suppressor exosome carried miRNAs responsible for the healing by the convalescent plasma exosomes via RNA sequence cloning may isolate crucial inhibitory miRNAs also useful in treating the patients. This convalescent-associated miRNA may lead to the cytosolic mRNA of targeted cells that they affect and subsequently on to the upstream DNA genetic sequence for possible development of molecular therapy for COVID-19 diseases as well. The exquisitely sophisticated currently available molecular methodologies may mean that these steps can be accomplished in a very short time, compared to the necessary much longer time to develop a safe, useful and effective vaccine or appropriate drug therapy.

Finally, in planning convalescent plasma therapy of COVID-19 patients, there will be a focus on determining the plasma samples with the most effective antibodies and titers using pre-testing with assays like in vitro viral neutralization. The argument here is that additional determination of the beneficial biologic effects of contained exosomes in the convalescent plasma should also be considered for constructing more optimal biologic therapy; adding to more precise selection of the most optimal plasma samples to use for the most effective treatment of very sick patients. Also, for some there will be a natural tendency to isolate the antibodies in the convalescent plasma to express potential therapeutic titers in ug/ml. There additionally will be refinements using sophisticated technologies aimed at isolating especially effective elite sub populations of the most effective broadly neutralizing antibodies as seemingly achieved for HIV,**108** and the genes of the very few cells that produce them. However, this wholly antibody focus would mean blindly dispensing with the value of the similarly evolutionary optimized exosomes and thus potentially “throwing out the baby with the bath water.”

Exosomes in convalescent plasma could be mediating negative therapeutic effects overall opposing the antibody beneficial effects

Although there is much data suggesting that the exosome component of convalescent plasma likely contributes to the undoubted positive effects, like all things in biology there can be negative properties in some instances that may be detrimental when determined so that removing exosomes from the plasma may result in a better therapeutic agent for COVID-19 patients. Exosomes definitely can go in both directions dependent on the phase of illness. At the height of the cytokine storm and ARDS there is good reason to think that plasma exosomes mediate pro-disease effects. In convalescent plasma from later in the process, exosomes should have evolved properties to favor suppression of disease as reviewed here and thus be a useful aspect of convalescent plasma. However, the opposite may be true and thus to proceed we need to isolate and then then separate the exosomes from the Ab and remove them if this is so. Use of serum instead of plasma does not help since it contains most of the exosomes found in the plasma.

There is a paucity of knowledge about the clinical effects of exosomes in convalescent plasma, and no data are yet available about this aspect of COVID-19 infections. There definitely can be negative effects of exosomes induced during the infection as the virus uses host production of exosomes for its benefit. An important aspect is that virus stimulation of host cell exosome production favorable to thwarting the host is a central mechanism of how the virus controls the host. This might carry over into exosomes in the convalescent plasma and thus call for removal of the exosomes to improve the potential positive effects mediated by the acquired immune system production of beneficial convalescent antibodies. Further, like in suppression of the host effector T cell response in cancers we postulate that negative-acting exosomes in some of the potential COVID-19 therapeutic plasmas may contain suppressive exosomes surface PD-L1,^{109,110} or expressing other co-inhibitory receptors. These would be induced by the virus to thwart the host effector T cell mediated anti-COVID-19 response, and will have to be removed to obtain the best effect in treatment of the ARDS and cytokine storm processes. Finally, note that in EBOLA infections, induced exosomes can express a viral encoded antigen called VP40 that is a suppressor of effector T cells,¹¹¹ and similarly would have to be removed.

Pilot largely uncontrolled treatment studies in 2020 of convalescent plasma for patients with severe COVID-19 syndromes

In a half dozen preliminary reports of convalescent plasma treatment of severe COVID-19 published in early 2020, the patients treated with 2-300ml convalescent plasma, have had no severe adverse effects with clearing of clinical parameters and improvement of cytokine levels in 1-3 days.. One study at heavily loaded Mt Sinai Hospital in New York City, with many available treated patients and disease controls involved 39 patients (the largest number treated), who were compared to carefully matched severe active disease controls, and strongly showed the value of convalescent plasma treatment. Namely, 12.8% of plasma recipients died compared to double that at 24.4% for the matched controls, with respectively 72% percent vs. 67% percent discharged alive.¹¹² Together this and the other recent studies,^{113,114,115,116,117} show for now very strong evidence of the great value of convalescent COVID-19 plasma treatment, but leave open the important question concerning the role of antibodies compared to the contained exosomes.

An uncontrolled similar recent study of convalescent plasma therapy in 10 severe COVID-19 patients in China reported very similar clinical results with reduction of pulmonary chest CT lesions, increase in pO₂ in all patients (indicative of recuperating lung function), and reversal of lymphocytopenia. In some patients there was resolution of COVID-2 viremia and increase in neutralizing antibody titers.¹¹⁶

CONCLUSIONS

These recent publications and many reported ongoing studies in several European countries, will result in more than a thousand patients treated with convalescent plasma by the summer of 2020,¹¹⁸ plus the later available results of large programs involving multiple thousands of patients and now recruiting under the FDA organized Convalescent Plasma Expanded Access Program administered through the Mayo Clinic, involving 40 leading US medical centers and in the UK as the NHS Plasma Program across its 23 main blood centers will provide definitive data to guide recommendations on the general role that convalescent plasma should have in the treatment of COVID-19 patients. For the huge Mayo Clinic Consortium study, as of June 1, 2020; 2400 sites and 7200 physicians were involved with 24,513 patients and 18,543 had been infused.

However, no current or planned studies include evaluating the role and planned participation on the contained exosomes. Certainly, compared to the much ballyhooed Remdesivir and now heavily invested in drug that acts on viral metabolism in which the major favorable result was just four days

shorter hospitalizations, it is clear that convalescent plasma likely is far superior treatment. Surely in such an enormous effort there is room for and great need and it is strongly recommended that a major effort be mounted to determine the role of and relative strengths of contained antibodies vs. carried exosomes; the latter potentially delivering important anti-viral and pro-host epigenetic function altering miRNAs.

SUMMATION

There was a recent editorial by Dr. Sunny Dzik of the MGH, who is the editor of *Transfusion Medical Review*, entitled: “COVID-19 Convalescent Plasma: Now Is the Time for Better Science.”¹¹⁹

I agree completely with his arguments that after almost ten instances of use of convalescent plasma in epidemics and pandemics over more than a century we are now about to use convalescent plasma requiring at long last thoroughly conceived and controlled studies to truly establish efficacy; minimally at least at the level of “first do no harm.” I add bringing attention to the fact that convalescent plasma undoubtedly contains very biologically active exosomes mediating important aspects of the effects of this plasma therapy that were not known of previously, and so far are to be ignored in currently planned better scientifically conceived studies . All told, it is committing medical research malpractice to use MSC instead of the their produced MSC^{exos} that are responsible for their actions. It also can be seen as medical research malpractice to perform studies of convalescent plasma treatment aimed entirely at the antibody content, avoiding the “Elephant in the Room.” These companion immune and viral activated exosomes in billions per milliliter, that undoubtedly play an important role in the effects of the plasma, may be crucial to the outcome of convalescent plasma treatment of very sick patients with COVID-19 syndromes.

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CONFLICT OF INTEREST

None

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