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Full Length Article
Basic Research

Mechanism for the attenuation of neutrophil and complement hyperactivity by MSC exosomes

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ABSTRACT

Complements and neutrophils are two key players of the innate immune system that are widely implicated as drivers of severe COVID-19 pathogenesis, as evident by the direct correlation of respiratory failure and mortality with elevated levels of terminal complement complex C5b-9 and neutrophils. In this study, we identified a feed-forward loop between complements and neutrophils that could amplify and perpetuate the cytokine storm seen in severe SARS-CoV-2-infected patients. We observed for the first time that the terminal complement activation complex C5b-9 directly triggered neutrophil extracellular trap (NET) release and interleukin (IL)-17 production by neutrophils. This is also the first report that the production of NETs and IL-17 induced by C5b-9 assembly on neutrophils could be abrogated by mesenchymal stem cell (MSC) exosomes. Neutralizing anti-CD59 antibodies abolished this abrogation. Based on our findings, we hypothesize that MSC exosomes could alleviate the immune dysregulation in acute respiratory failure, such as that observed in severe COVID-19 patients, by inhibiting complement activation through exosomal CD59, thereby disrupting the feed-forward loop between complements and neutrophils to inhibit the amplification and perpetuation of inflammation during SARS-CoV-2 infection.

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Introduction

The COVID-19 pandemic is a global public health emergency caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), affecting ~200 million individuals to date, with a significant mortality rate of ~2% (WHO, covid19.who.int). The unprecedented rapid development and rollout of vaccines have signiﬁcantly reduced mortality. However, a significant number of people remain unvaccinated and are at risk of developing severe disease. Severe COVID-19 disease is essentially a manifestation of dysregulated immune responses in the lungs that could lead to multi-organ pathologies. Current COVID-19 interventions are generally symptomatic and palliative treatments, and there is an urgent unmet need for effective drugs to alleviate immune dysfunctions in the lungs of severely ill COVID-19 patients to reduce mortality and improve recovery.

Mesenchymal stromal cell (MSC)-derived exosomes, which have documented immunomodulatory and regenerative properties, have been proposed as a promising therapeutic to alleviate immune dysfunction and reduce disease severity in fatally ill COVID-19 patients [1]. Although several preclinical and clinical studies have demonstrated their safety and efficacy in mitigating the symptoms associated with severe COVID-19 [2,3], the precise mechanism by which MSC exosomes exert their effects to benefit COVID-19 patients remains elusive.

Our innate immune system serves as the first line of defense against viral invasion. However, an augmented or dysregulated
innate immune response could exacerbate the pathogenesis of a viral infection such as the development of acute respiratory distress syndrome (ARDS) and cytokine storm syndrome in severe COVID-19 [4–7]. Complement is an integral component of the innate immune system that eliminates viral pathogens directly and indirectly through opsonization, formation of the membrane attack complex (MAC) C5b-9 and recruitment of other leukocytes to promote an anti-viral inflammatory response [8,9]. However, clinical data have revealed that increased activation of the complement system is a distinct immunologic feature correlating with worse COVID-19 outcomes [10–17]. Elevated levels of circulating complement markers were present in patients with COVID-19 compared with other etiologies of acute respiratory failure, and these markers can distinguish those with worse outcomes in the setting of SARS-CoV-2 infection [10]. Indeed, complement activation has been known to play a central role in endothelial injury and hypercoagulability, leading to multi-organ failure and pathogenesis of COVID-19. As such, complement inhibitors to combat SARS-CoV-2 infection are currently being explored in multiple phase II and III clinical trials [18–21].

Another immunologic indicator of poor disease outcome in COVID-19 is the increased number of circulating neutrophils. In critically ill patients, extensive neutrophil extracellular traps (NETs), a prominent signature of neutrophil activation, are detected in sera and lungs, thereby predisposing the patients to thrombosis, organ damage and, eventually, death [22–28]. This is further exacerbated by the hyperactivation of complements in the lungs of severely ill COVID-19 patients [2], as activated complements are potent chemoattractants for neutrophils [29,30]. Together, the clinical data suggest that the cardinal roles of complements and neutrophils in the pathogenesis of severe COVID-19 intersect. Therefore, elucidating and modulating this intersection represents a potential strategy to prevent the devastating complications in SARS-CoV-2–infected patients [31,32].

In this study, we show that the terminal complement activation complex C5b-9 can directly release the NETs and interleukin (IL)–17 by neutrophils. MSC exosomes can suppress this complement-mediated neutrophil activation via a CD59-dependent mechanism. Together, our data revealed a novel cross-talk between two innate immune components, complement and neutrophil, and further highlighted the potency of MSC exosomes in suppressing the activation of this innate immune axis. Our data also provide a compelling rationale for the use of MSC exosome to treat severe COVID-19.

Results

Terminal complement complex triggers NET formation and IL-17 release by neutrophils

To characterize the effects of complement activation on neutrophil responses, we purified neutrophils from the blood of healthy human donors and initiated complement activation using a standard protocol of adding complements C5b-6 and C7 followed by C8 and C9. The assembly of terminal complement complex C5b-9 on neutrophil surfaces was confirmed by flow cytometry (Figure 1A). Complement activation triggered the release of NETs in vitro, as visualized microscopically via the colocalization of DAPI-positive extracellular DNA fibers with citrullinated histone H3. In particular, >60% of neutrophils underwent NETosis upon activation of the complement cascade, as determined by DNA size, and the area of released NETs was significantly larger than that from the control group (Figure 1B, C). As the severity of COVID-19 also correlates positively with the level of neutrophil-associated inflammatory mediators such as IL-17 and calprotectin S100A8 and S100A9 [33–38], we determined whether these inflammatory mediators were induced in the neutrophil response to complement activation. Quantitative reverse-transcription real-time polymerase chain reaction (PCR) and ELISA analysis revealed a significant induction of IL-17 transcription and secretion upon complement activation (Figure 1D, E), while levels of S100A8 and S100A9 transcripts remained unchanged (Figure 1F). Together, these data showed that complement activation can specifically induce NETs and IL-17 production by neutrophils. These data also provided a possible mechanistic link between complements and neutrophils to induce hyperinflammatory immune responses in severely ill COVID-19 patients.

Exosomes inhibit complement-induced neutrophil activation

MSC exosomes, which possess immunomodulatory properties, have been proposed as a therapeutic option for COVID-19 to alleviate complications such as acute respiratory distress syndrome [23,39]. However, the mechanism by which exosomes exert their functions remains unclear. To test whether MSC exosomes can intervene in complement-induced neutrophilic responses, we incubated neutrophils with exosomes and complements C5b-6 and C7 followed by C8 and C9. Activation of neutrophils was attenuated upon addition of exosomes. Specifically, exosomes abrogated the release of NETs by neutrophils at concentration as low as 1 µg/mL (Figure 2A, B). Exosomes also inhibited IL-17 production at the transcript and protein levels by C5b-9–assembled neutrophils at 10 and 50 µg/mL (Figure 2C, D). The inhibition was statistically significant at 10 but not 1 µg/mL exosome, indicating a dose-dependent response. However, the level of inhibition at 10 and 50 µg/mL was not significantly different, suggesting that maximum inhibition was reached at 10 µg/mL. Therefore, MSC exosomes can attenuate the complement-mediated NET formation and neutrophil IL-17 production.

Exosomes do not affect PMA-induced NETosis and IL-17 release

Several stimuli are known to trigger NET formation and IL-17 production by neutrophils. To determine whether inhibition of NETosis and IL-17 release by exosomes is specific to complement-mediated activation of neutrophils, we treated neutrophils with another known neutrophil activator, phorbol myristate acetate (PMA), which activates neutrophils through the signal transduction enzyme protein kinase C [40]. PMA induced NETosis and IL-17 production at the transcript and protein levels in neutrophils, but MSC exosomes did not have any effect on these processes (Figure 3A–D). Therefore, exosomes suppressed complement but not PMA-mediated activation of neutrophils. This suggested that exosomes exert their inhibition of complement-mediated neutrophil activation through the complement cascade and not through the neutrophil per se.

MSC exosomes inhibit complement-induced neutrophil activation through a CD59-dependent mechanism

MSC exosomes have been reported to inhibit the formation of the terminal complement complex, C5b-9, through their expression of CD59 [41]. To investigate whether exosomal CD59 is required for exosome-mediated suppression of complement-induced neutrophil activation, a neutralizing antibody against CD59 was added to the mixture of activated complements and neutrophils. The anti-CD59 antibody abrogated the inhibitory effects of exosomes on complement-mediated NETosis. Notably, the NETs released by neutrophils in the presence of exosomes and the anti-CD59 antibody were of comparable size to those found in the control group without exosome treatment (Figure 4A, B). Consistent with this, the inhibition of IL-17 release by exosomes was also abolished by anti-CD59 antibody treatment (Figure 4C, D). Of note, anti-CD59 antibody alone does not trigger the release of NETs or IL-17, ruling out nonspecific effects of anti-CD59 antibody on neutrophil activation (Figure 4A–D). Together, these results indicate that inhibition of C5b-9 formation by CD59 on the MSC exosome abolished complement-induced neutrophil NETosis and IL-17.
Hyperactivation of the complement system and neutrophilic immune responses have been associated with worse outcomes in COVID-19 patients [10–12,16,23,28,35,42]. It remains unclear, however, whether these are two independent events or are interrelated components of a broader immune cascade. In this study, we demonstrated that initiation of the complement activation cascade induced NET release and IL-17 production by neutrophils. These neutrophilic responses were suppressed by MSC exosomes via a CD59-dependent mechanism (Figure 5). Because CD59 inhibits only the terminal step of the complement activation cascade, i.e., C5b-9 complex, our data also demonstrated that C5b-9, and not the other complement complexes, was the main effector of neutrophil activation. Collectively, our results provide a mechanistic basis for how exosomes can inhibit complement-induced neutrophil activation, potentially mitigating the vicious cycle that perpetuates inflammation in the lungs of severely ill COVID-19 patients.

Clinical observations strongly indicate that patients with severe COVID-19 are predisposed to complement activation and dysregulated neutrophilic responses [24,25,43]. Interestingly, it has been reported that the sera from COVID-19 patients can trigger the release of NETs by healthy control neutrophils [24,44], consistent with a possibility that complements present in the sera can activate neutrophils [45]. In this study, we present novel findings on the cross-talk between the terminal complement complex and neutrophils. We showed that the assembly of complement complexes can directly trigger NET formation and IL-17 release by neutrophils. NETs are known to fuel pro-inflammatory immune responses, and their aggregation culminates in vascular occlusion and organ damage, whereas IL-17 has been reported to exacerbate inflammation and destruction of the lung parenchyma [37,46,47]. Hence, it is likely that the complement system and neutrophils form a feed-forward loop to escalate inflammation, tissue injury and thrombosis that, if left unchecked, would lead to coagulopathy, respiratory failure and other devastating complications observed in COVID-19.

MSC exosomes are an attractive cell-free therapeutic option to treat and manage symptoms associated with COVID-19, and they have demonstrated promising results toward alleviation of major COVID-19 complications such as cytokine storm and ARDS in a small but growing number of clinical trials [2,3]. The rationale for such a treatment approach was predicated on the superior regenerative and immunomodulatory capacity of MSC exosomes [3]. Exosomes have been shown to secrete anti-inflammatory cytokines that interact with immune cells such as T cells, B cells and macrophages, thereby preventing overactivation of the immune system. Furthermore, they can deliver various growth factors that help initiate tissue repair and regeneration during lung injury [48]. Together, these studies support...
the potential utility of exosomes as a treatment option for COVID-19 patients with severe complications. However, the precise therapeutic effect of exosomes on COVID-19 patients, and the exosome attribute responsible for mediating such an effect, have not been elucidated.

Here, we demonstrate for the first time that exosomes suppress complement-mediated neutrophil activation and inhibit the release of NETs and IL-17 by neutrophils (Figure 5). We identify CD59 as the MSC exosome attribute that mediates this suppression. We
previously showed that MSC exosomes inhibit the assembly of terminal C5b-9 complement complex through CD59, a known inhibitor of the final stage in the complement activation cascade and an abundant MSC exosomal protein [41]. Consistent with the role of CD59 in mediating the inhibitory effect of MSC exosomes on neutrophil activation, this inhibitory effect was attenuated by a CD59-neutralizing antibody.

Figure 3. Exosomes do not affect PMA-induced NETs and IL-17 release by neutrophils. (A) Representative images of NET formation by neutrophils PMA stimulation, in the presence or absence of different concentrations of exosomes. Cells were stained for nuclei (DAPI, blue) and H3 Citrullinate R2/8/17 (green). Scale bar, 25 μm. (B) Quantification of NET release by neutrophils pooled from three independent experiments performed using neutrophils from three unique donors. At least 100 cells were quantified from each experiment. The neutrophils used in these experiments were from different donors than those in Figures 1, 2 and 4. Data shown as mean ± SEM (n = 3). ***P < 0.0001, two-way ANOVA. (C) mRNA expression levels of Il17a relative to hprt1 in neutrophils upon PMA stimulation, in the presence or absence of different concentrations of exosomes (n = 5). Five independent experiments were performed using neutrophils from five unique donors. The neutrophils used in these experiments were from different donors than those in Figures 1, 2 and 4. *P = 0.03, unpaired two-tailed Student’s t test. (D) Neutrophils were stimulated with PMA in the presence or absence of different concentrations of exosomes, and culture supernatants were assayed for IL-17 by ELISA (n = 3). Three independent experiments were performed using neutrophils from three unique donors. **P < 0.01, unpaired two-tailed Student’s t test. (Color version of figure is available online.)
By inhibiting the formation of the terminal complement complex on neutrophils through CD59, MSC exosomes attenuated the activation of neutrophils and the manifestations of neutrophil activation, namely NETosis and IL-17 production. Significantly, we did not observe any inhibitory effect of exosomes on PMA-induced NETs and IL-17 release, indicating that MSC exosomes act specifically

Figure 4. α-CD59 abolishes inhibitory effects of exosomes on C5b-9-induced NETosis and IL-17 production. (A) Representative images of NETs formation by neutrophils upon assembly of C5b-9, in the presence or absence of exosomes and α-CD59. Cells were stained for nuclei (DAPI, blue) and H3 Citrullinate R2/8/17 (green). Scale bar, 25 μm. (B) Quantification of NET release by neutrophils pooled from three independent experiments performed using neutrophils from four unique donors. At least 100 cells were quantified from each experiment. The neutrophils used in these experiments were from the same donors as those in Figures 1 and 2. Data shown as mean ± SEM (n = 4). *P = 0.02, two-way ANOVA. (C) mRNA expression levels of IL17a relative to hprt1 in neutrophils upon assembly of C5b-9, in the presence or absence of exosomes and α-CD59 (n = 3). Three independent experiments were performed using neutrophils from three unique donors. The neutrophils used in these experiments were from the same donors as those in Figures 1 and 2. *P = 0.02, unpaired two-tailed Student’s t test. ***P = 0.0003, one-way ANOVA. (D) C5b-9 were assembled on neutrophils in the presence or absence of exosomes and α-CD59, and culture supernatants were assayed for IL-17 by ELISA (n = 3). Three independent experiments were performed using neutrophils from three unique donors. The neutrophils used in these experiments were from the same donors as those in Figures 1 and 2. *P = 0.02, unpaired two-tailed Student’s t test. **P = 0.004, one-way ANOVA. (Color version of figure is available online.)
on complement-mediated activation of neutrophils. Together, these results also revealed for the first time that C5b-9, and not the other complement complex upstream of C5b-9, is the major complement complex that activates neutrophils.

In conclusion, our study elucidates a feed-forward loop between two key immunologic indicators of severe COVID-19, complements and neutrophils, and provides an overarching mechanistic context for the association of elevated complement activation, neutrophils, NETosis and IL-17 with adverse COVID-19 outcomes. This study also determines that MSC exosomes can specifically inhibit this complement/neutrophil axis through their expression of CD59 and provides a scientific rationale for use of MSC exosomes in alleviating the severe manifestations of critically ill COVID-19 patients.

Materials and Methods

Preparation of MSC exosomes

The exosome preparation used in this study was batch AC109 from an ongoing production process that has been in operation for >10 years and has manufactured >100 batches to date. This process was first described in 2010 [1]. The cell source was replaced with the immortalized cell line in 2013 [5], and the process was simplified as described below. Immortalized E1–MYC 16.3 human ESC-derived mesenchymal stem cells were cultivated in DMEM with 10% fetal calf serum as previously described [49]. For MSC exosome preparation, the conditioned medium was prepared by growing 80% confluent cells in a chemically defined medium for 3 days as previously described [50–52]. The defined medium was prepared as follows: 480 mL DMEM (31053; Thermo Fisher), 5 mL NEAA (11140-050; Thermo Fisher), 5 mL L-glutamine (25030-081; Thermo Fisher), 5 mL sodium pyruvate (11360; Thermo Fisher), 5 mL ITS-X (51500-056; Thermo Fisher) and 0.5 mL 2-mercaptoethanol (21985-02; Thermo Fisher). This was supplemented with 0.1 mL basic fibroblast growth factor (bFGF; 0.5 ng/mL, 0.2% BSA in PBS(+) and 0.005 mL platelet-derived growth factor (PDGF; 100 ng/mL PBS(+)}. The latter components were obtained as follows: bovine serum albumin (BSA; A9647; Sigma-Aldrich), PDGF (100-00; CYTOLAB), bFGF (13256-029; Thermo Fisher) and PBS(+) (14040-133; Thermo Fisher). The conditioned medium (CM) was size-fractionated by tangential flow filtration and then concentrated 50× using a membrane with a molecular weight cutoff (MWCO) of 100 kDa (Sartorius, Gottingen, Germany). The MSC exosome preparation was assayed for protein concentration using a Coomassie Plus (Bradford) Assay Kit (Thermo Fisher). The properties of these exosome preparations have been extensively assayed in accordance with Minimum Information for the Study of Extracellular Vesicles 2018 [6] and reported in our earlier papers. These preparations carry exosome-associated markers CD81, ALIX and TSG101 [50,53], and the presence of CD81 on bi-lipid membrane vesicles was confirmed using transmission electron microscopy [54]. For this study, AC109 was assayed for the key identity and potency parameters for MSC-SEV preparations as recommended [7,8]. Specifically, the preparation had a protein concentration of 1.419 ± 0.034 mg/mL, particle concentration of $2.13 \times 10^{11}$ ± 5 × 10^6 particles/mL, particles with a modal size of 125.1 ± 60.0 nm, cholesterol of 8.16 ± 0.32 ng cholesterol/μg protein, CD73/ecto-5'-nucleotidase activity of 27.04 ± 0.79 μl/mg protein and CD59 concentration of 3.15 ± 0.43 ng CD59/ mg protein. The exosome preparation was filtered with a 0.22-μm filter (Merck Millipore, Billerica, MA) and stored in a −80°C freezer until use or lyophilized.

Isolation of neutrophils

Venous blood of healthy human donors was collected and diluted 1:1 in PBS before overlaying on Ficoll-Paque (GE Healthcare). After density gradient centrifugation, the polymorphonuclear and erythrocyte-rich pellet was collected, and cells were treated with red blood cell lysis buffer for 15 min at room temperature. Cells were subsequently washed with PBS, and neutrophils obtained were resuspended in PBS and rested at 37°C for ≥1 h.

Assembly of C5b-9

Neutrophils were washed and then resuspended in PBS with C5b-6 (0.125 μg/mL) and C7 (0.5 μg/mL) in the presence or absence of exosomes. The mixture was incubated at 37°C for 15 min before C8 (0.5 μg/mL) and C9 (0.5 μg/mL) were added with or without a CD59 blocking antibody (YTH53.1; Invitrogen) at 37°C for an additional 30-min incubation. As a negative control, C9 was omitted.
Flow cytometry staining for C5b-9

Neutrophils were stained with anti-C5b-9 (aE11; Novus Biologicals), followed by anti-mouse FITC (Sigma-Aldrich), in staining buffer (PBS containing 1% BSA) at 4°C for 1 h. Data were acquired using LSR II (BD Biosciences) and analyzed using FlowJo software (TreeStar).

PMA stimulation

Neutrophils were stimulated with 10 ng/mL PMA, in the presence or absence of exosomes, for 30 min at 37°C.

NETosis assay

Neutrophils were seeded onto poly-l-lysine–coated 48-well plates and allowed to adhere for 30 min at 37°C. The cells were then stimulated with complement components or PMA as described above. Cells were then fixed in 2% paraformaldehyde at room temperature for 15 min and stained with anti-histone H3 (citrulline R2 +R8+R17) antibody (ab5103; Abcam) and DAPI before visualization with a microscope (EVOS). DNA area was measured using DAPI staining in ImageJ software. Image acquisition and analysis were performed by a blinded investigator. At least 100 cells in each experimental group were analyzed.

IL-17 ELISA

2 × 10⁶ neutrophils were stimulated with C5b-9 or PMA as described above. Culture supernatants were collected after 4 h, and IL-17 was detected by human IL-17A ELISA kit (ab216167; Abcam) according to the manufacturer’s protocol.

Quantitative PCR

Purified neutrophils were lysed with TRIzol (Gibco, Thermo Fisher), and RNA was purified using phenol/chloroform extraction. Complementary DNA was reversed transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). The following primers were used for real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems):

\[
\begin{align*}
\text{Il17a} & : \text{CGATGACTTGCAAAATGTCGCAG} \\
\text{s100a9} & : \text{GCCACTGTGGTCTTAGGGGT} \\
\text{hprt1} & : \text{AGTCAAGGGCATATCCTACAAAC}
\end{align*}
\]

The mRNA expression of il17a, s100a8 and s100a9 were normalized to hprt1 using the 2⁻ΔΔCt method.

Statistics

Figures and statistical analyses were generated using GraphPad Prism software. For NETosis analyses, two-way ANOVA was performed. For other analyses, 1-way ANOVA or unpaired two-tailed Student’s t test was performed. A P value of <0.05 was considered significant.

Study approval

Human blood was obtained for research with approval from the Centralised Institutional Research Board of the Singapore Health Services in Singapore.

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Author Contributions

JTL, BZ, KPL and SKL were involved in the conceptualization and design of study. JTL and JKHT performed the experiments. ABHC produced the MSC exosomes, which were characterized by RCL. JTL, BZ, KPL and SKL analyzed and interpreted the data. The manuscript was drafted by JTL and revised by BZ, KPL and SKL. All authors have approved the final article.

Declaration of Competing Interests

SKL holds founding shares in Paracrine Therapeutics.

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