Sex-Specific Systemic Inflammatory Responses in Mice Infected with a SARS-CoV-2-like Virus and Femur Fracture

[1]Matthew Patrick, [2]Austin Foster, [2]Arun Aneja and [1]Ramkumar T. Annamalai

[1]Department of Biomedical Engineering, University at Buffalo, Buffalo, NY 14260

[2]Department of Orthopaedic Surgery and Sports Medicine, University of Kentucky, Lexington, KY 40536

Author Correspondence:

Ramkumar T. Annamalai
760 Press Avenue
138 Healthy Kentucky Research Building
University of Kentucky
Lexington, KY 40536
Email: ram.kumar@uky.edu

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Abstract

This study investigates the systemic inflammatory response in mice infected with a murine coronavirus (MHV), which shares a common genus with SARS-CoV-2, and sustaining a fracture. The study reveals that the combined inflammatory incidents of MHV infection and fracture disrupt the systemic immune response in both female and male mice, likely leading to immune dysregulation, altered cell recruitment, and disruption of the typical inflammatory cascade. Notably, the study uncovers sex-specific responses that modulate circulating immune factors. Females exhibit elevated levels of inflammatory factors, whereas males demonstrate a diminished response. This divergence is mirrored in cell populations, suggesting that the quantity of immune factors released may contribute to these discrepancies. The findings suggest that an overproduction of proinflammatory cytokines may induce a dysregulated immune response, contributing to the observed poorer prognosis in comorbid cases. These insights could pave the way for therapeutic advancements and treatment strategies aimed at reducing mortality rates in COVID-19 patients with fractures.

1. Introduction

The global health crisis, COVID-19, instigated by the novel SARS-CoV-2 virus, has been declared an international public health emergency. As of October 23, 2023, the disease has affected over 650 million individuals globally and claimed the lives of more than 1 million people in the US alone[1]. Despite the plethora of studies published in the last few years on COVID-19, a clear understanding of the disease is still elusive.

COVID-19 presents two distinct yet overlapping pathological phases. The initial phase is driven by the virus itself, while the subsequent phase is a result of the host's immune response[2]. The severe clinical deterioration observed in patients is often attributed to an unregulated escalation of the host's immune response, akin to sepsis-induced cytokine release syndrome (CRS)[3]. This leads to tissue damage and respiratory failure (acute respiratory distress syndrome, ARDS), which is the primary cause of mortality in COVID-19 patients[4].

Comorbid conditions such as diabetes[5], hypertension[6], and fractures[7] are often worsened by CRS induced by COVID-19, leading to a more severe disease course and complicating recovery. Among these comorbidities, fractures represent a particularly dangerous and poorly understood condition. Limited clinical studies have been conducted on COVID-19 patients with fractures and the impact of COVID-19 on the musculoskeletal system. From this limited literature, patients with a proximal femoral fracture experience an increase in mortality rate from 10.3% to 30.4% when infected with COVID-19[8]. This increased mortality rate may be attributed to the fracture's role in initiating inflammation for healing[9]. Therefore, a comprehensive understanding of how COVID-19 and fractures influence systemic inflammation is crucial for developing targeted strategies to reduce mortality in COVID-19 patients with bone fractures.

In our study, we employ a murine coronavirus (MHV) that belongs to the same genus as SARS-CoV-2 and exhibits similar inflammatory symptoms. This model allows us to investigate the comorbidities associated with COVID-19 without significant infection risk. Our findings provide insights into how COVID-19 infection and fractures affect systemic immune responses in both female and male mice.

2. Methods

2.1. Animals and studies

All animal procedures were performed in compliance with the protocol approved by IACUC at the University of Kentucky. 12-week-old, wild type C5BL/6 mice were randomly assigned to either control (n = 5 male, 5 female), fracture (n = 5 male, 5 female), MHV (n = 5 male, 5 female), or MHV + fracture (n = 5 male, 5 female) groups. MHV mice were infected intranasally with 10^4 PFU murine hepatitis virus (MHV-VR764, ATCC), known to show similar physiological symptoms as COVID-19, two days before fracture. A well-established transverse fracture model was used on mice in the fracture and MHV + fracture groups, as described[10]. Briefly, mice were anesthetized (1.5 mg of ketamine/kg + 1.5 mg of xylazine/kg body mass of mice), a bland ophthalmic ointment was applied, and the surgical site was shaved and wiped with 70% ethanol, followed by 10% povidone-iodine solution. A small incision was made at the patella, and it was laterally dislocated to expose the knee join. Using a 0.5 mm centering bit, a hole was made in the intercondylar notch, and an intermedullary guide tube (0.2 mm) was inserted into the femur. With the tube in place, the right leg was positioned under the guillotine of a murine fracture device (RIsystems) which was then released to create the fracture. The guide tube was then replaced with a 16 mm titanium mouse screw (RISystems). The skin at the patella was sutured with 6-0 vicryl suture (Ethicon). Then, 1 mL of saline was administered

intraperitoneally for hydration followed by subcutaneous administration of buprenorphine SR (1.5 mg/kg body mass) for analgesia. The animals were then placed into a recovery cage and monitored as per the established post-operative animal care protocols. Animals were monitored daily for 7 days and weekly thereafter to ensure post-operative recovery.

Blood was collected longitudinally in K2 EDTA Microvette tubes (Sarstedt) from the right submental vein at 5 days prior to surgery for a baseline and at days 2 and 7 post-surgery. Extracted blood was kept on ice until being centrifuged at 900 g for 5 minutes to separate plasma, which was then collected in low temperature freezer vials (VWR) and kept at -80°C until needed. The remaining blood was immediately stained and analyzed through flow cytometry (See section 2.3 for details).

2.2. MicroCT imaging and morphometric analysis

Representative images of fractures were captured using microcomputed tomography (microCT), performed using a high-resolution small animal imaging system SkyScan1276 microCT (Bruker, Billerica, MA) with an Al 1mm filter with the voltage set to 70 kV and image pixel size 20.32 µm. Images were then reconstructed (NRecon) with smoothing set to 3, ring artifact correction set to 4, and beam hardening correction set to 30%. Implants were carefully removed, and femurs with surrounding musculature were placed in a new dry tube before scanning.

2.3. Flow cytometry and multiplex

Once plasma was separated, red blood cells (RBC) were lysed from the remaining blood by following the manufacturer's instructions using 1 X RBC lysis buffer (eBioscience). Lysis buffer was then diluted with 1 X Dulbecco's phosphate-buffered saline (PBS, Gibco) and removed through 400G centrifugation for 5 minutes. Remaining cells were then fixed with 4% buffered formalin for 15 minutes. Cells were then washed with 1 mL PBS and formalin removed by centrifugation for 5 minutes and aspiration of the supernatant. cells were then resuspended in anti-mouse CD16/32 (eBioscience) for 10 minutes at 4°C to block Fc receptors. Cells were then stained for 45 minutes in the dark at 4°C with various fluorescently labeled anti-mouse antibodies to identify specific immune cell populations. Including lymphoid ($CD45^+/CD11b^-$), myeloid ($CD45^+/CD11b^+$), M1 and M2 monocytes ($Ly6G^-/Ly6C^{hi/low}$), macrophages ($Ly6G^-/Ly6C^+/F4/80^+$), M2 macrophages ($Ly6G^-/Ly6C^+/F4/80^+/CD206^+$), dendritic cells ($CD11c^+$), T cells ($CD3^+$), T helper cells ($CD3^+/CD4^+$), cytotoxic T cells ($CD3^+/CD8^+$), T regulatory cells ($CD3^+/CD4^+/CD25^+$), and B cells ($CD19^+$). All staining and blocking solutions were prepared in FACS buffer (a PBS solution with 5% fetal bovine serum (Gibco) and 0.1% sodium azide). A table of conjugates, clones, dilution factors, and suppliers for antibodies is in supplementary material. Finally, staining solution was washed with FACS buffer, removed through 400G centrifugation for 5 minutes, and resuspended in FACS buffer. Data was collected immediately using a BD FACSymphony A3 and analyzed using FlowJo software. Fluorescence minus one controls were used to set negative gates with <1% noise.

An Immune Monitoring 48-Plex Mouse ProcartaPlex™ Panel (Thermo Fisher, EPX480-20834-901) was used on collected plasma, processed following the manufactures instructions. Briefly, the capture bead mix was introduced into each well of the plate. Subsequently, the plate was washed using a Hand-Held Magnetic Plate Washer (EPX-55555-000). Following this, samples, standards, and both low and high controls were dispensed into the wells in duplicate. The plate was then sealed with the provided Plate Seals and Microplate Lid, and agitated at a speed of 600 rpm for a duration of 2 hours at room temperature. After this, the plate was subjected to a series of three washes with wash buffer. The detection antibody mix was then

added to each well, the plate was resealed, and agitated for 30 minutes at room temperature. The plate was then washed thrice again, followed by the addition of Streptavidin-PE to each well. The plate was then resealed and agitated for 30 minutes at ambient temperature, followed by another series of three washes. Reading buffer was then added to each well and allowed to incubate for 5 minutes. Finally, the plate was processed on a xMAP INTELLIFLEX system with a 50 µL acquisition volume, a DD Gate ranging from 4000 to 13000, and a low PMT reporter gain setting.

2.4. Statistics

All measurements were performed with at least 4 replicates. Data are plotted as means with error bars representing the standard deviation. For statistical analysis of multiple groups, a two-way ANOVA was used followed by a Holm-Sidak test. All statistics tests were done using GraphPad Prism. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1 MHV infection with fracture increases the mortality rate of female and male mice.

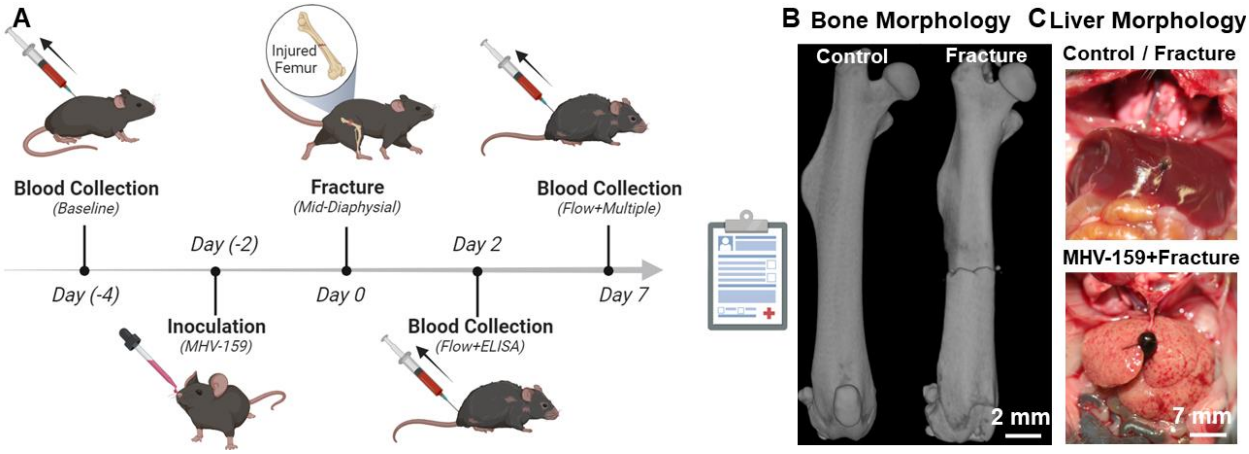

Figure 1. Experimental Timeline, Fracture, and Organ Analysis. A) Blood collection, inoculation, fracture, and endpoint timeline for the study. **B)** Representative micro-CT images of a control and fractured mouse femur on day 7. **C)** Representative images of liver in control and MHV infected mice.

All mice, except those in the control and fracture groups, were treated strictly according to the timeline (**Fig.1A**), with inoculation omitted for the exceptions. All mice in the control group were visually healthy, remaining active and alert over the experimental time course. All mice in the fracture group had slowed movement for a few days post fracture but recovered quickly and as expected. Within 1-2 days post inoculation, all infected mice began displaying signs of illness such as hunched posture, wincing, shivering, and general lethargy, indicating a successful infection. All mice in the MHV group continued to show symptoms throughout the experiment. Toward the endpoint mice seemed to become more active and the physical signs of illness had diminished. Some mice in the MHV + Fracture group seemed to decline in health while others improved. Only mice in the MHV + Fracture group died before the experimental endpoint, a male mouse at day 5 and a female mouse at day 6 post fracture. Therefore, there was an 20% mortality rate in the MHV + Fracture groups and 0% mortality rate in all other groups. All mice that survived were sacrificed 7 days post fracture. At this time, or at the time of death, the mice were opened for a visual inspection of organ health. 1 of 10 mice in the MHV group showed signs of moderate surface level liver damage while 2 of 10 mice in the MHV + fracture group

showed visual surface level signs of severe liver damage (**Fig.1B**). However, a more in-depth histology study would be warranted to fully evaluate the liver health. Ultimately, the mouses overall health seemed worse when recovering from both infection and fracture. Looking at the systemic inflammatory response in each group will help us explain this discrepancy.

3.2 Circulating cell populations are most influenced under MHV infection and fracture

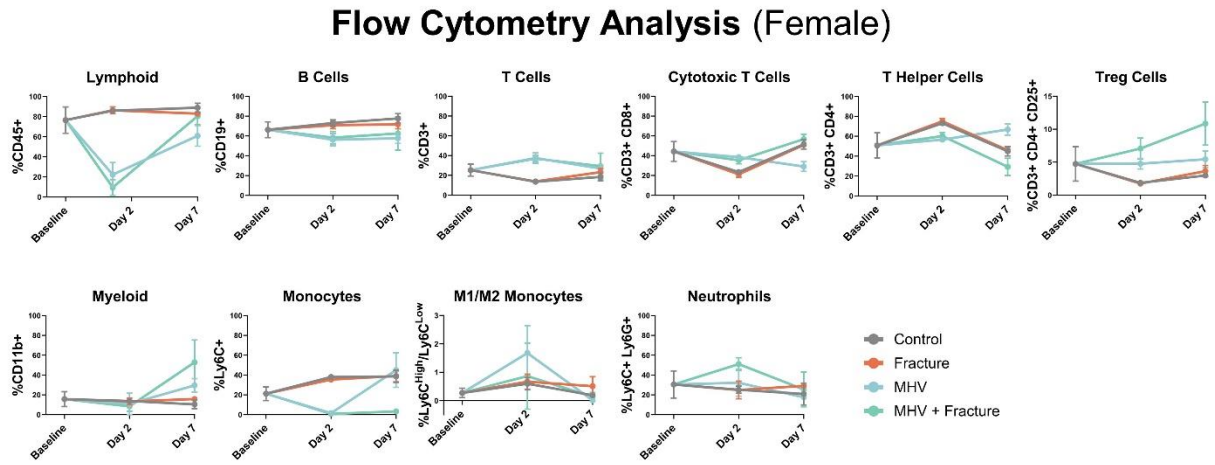

Figure 2. Immune Cell Populations of Female Mice Over Time. The myeloid and lymphoid populations and sub populations of all experimental groups in female mice at baseline, day 2 and day 7.

Circulating immune cell population changes were comparable between female and male mice. At day 2, several cell populations (lymphoid, B cells, T cells, cytotoxic T cells, T helper cells, T regulatory cells, monocytes, and neutrophils) exhibited significant differences in female mice when comparing conditions within the time point. Data for macrophage, M2 macrophage, and dendritic cell populations were not obtained for female mice. In the MHV and MHV + Fracture groups, lymphoid, B cells, T helper cells, and monocytes demonstrated a significant decrease, while T cells, cytotoxic T cells, and T regulatory cells showed a significant increase compared to the Control and Fracture groups. No significant change was observed when comparing the Control vs. Fracture groups and the MHV vs. MHV + Fracture groups. At day 7 in the MHV group, lymphoid and B cells populations were significantly reduced compared to the Control and Fracture groups. Conversely, T cells, T helper cells, and myeloid cells in the MHV group showed a significant increase compared to Control and Fracture groups. However, the cytotoxic T cells population in the MHV group was significantly lower than all other groups. In the MHV + Fracture group at day 7, the monocyte population was significantly reduced compared to all other groups. When comparing time points within each condition, many significant variations occurred. Most notably, in the MHV group, the M1/M2 population had a significant increase compared to baseline while all other group did not. Additionally, the T regulatory population in the MHV + Fracture group was significantly increased compared to baseline at days 2 and 7 while all other groups showed decreased or unchanged levels. A detailed list of all Holm-Sidak comparison p-values is available in the **Supplementary material**.

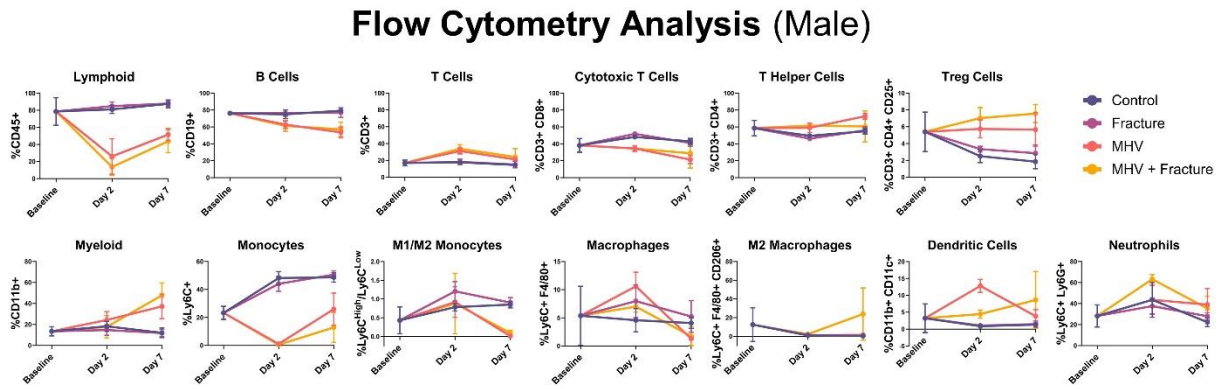

Figure 3. Immune Cell Populations of Male Mice Over Time. The myeloid and lymphoid populations and sub populations of all experimental groups in male mice at baseline, day 2 and day 7.

Male mice exhibited an immune response to each condition that was largely analogous to that of female mice, albeit with a few notable exceptions. On days 2 and 7, male mice displayed an inverse response in cytotoxic T cells and T helper cells compared to females. Specifically, the MHV and MHV + Fracture groups in males demonstrated a decrease in cytotoxic T cells and an increase in T helper cells. The MHV monocyte population in female mice returned to Control and Fracture levels by day 7, while it remained decreased in the MHV + Fracture group in males. Furthermore, the M1/M2 monocyte ratio was significantly reduced at day 7 in both the MHV and MHV + Fracture groups. The dendritic cell population at day 2 was significantly elevated in the MHV and MHV + Fracture groups compared to Control and Fracture groups, with the MHV group also showing a significant increase relative to the MHV + Fracture group. When comparing time points within each condition, male responses largely mirrored those of females and again there were many significant variations. Most notably, in the MHV + Fracture group, neutrophils had a significant increase compared to baseline and day 7 while all other groups remained unchanged or decreased comparatively. These findings suggest that both MHV and MHV + Fracture independently influence systemic cell populations in female mice relative to both Control and Fracture groups. Additionally, subtle sex-specific differences were observed in the immune response.

3.3 Sex-specific changes are prevalent in circulating inflammatory proteins under MHV infection and fracture

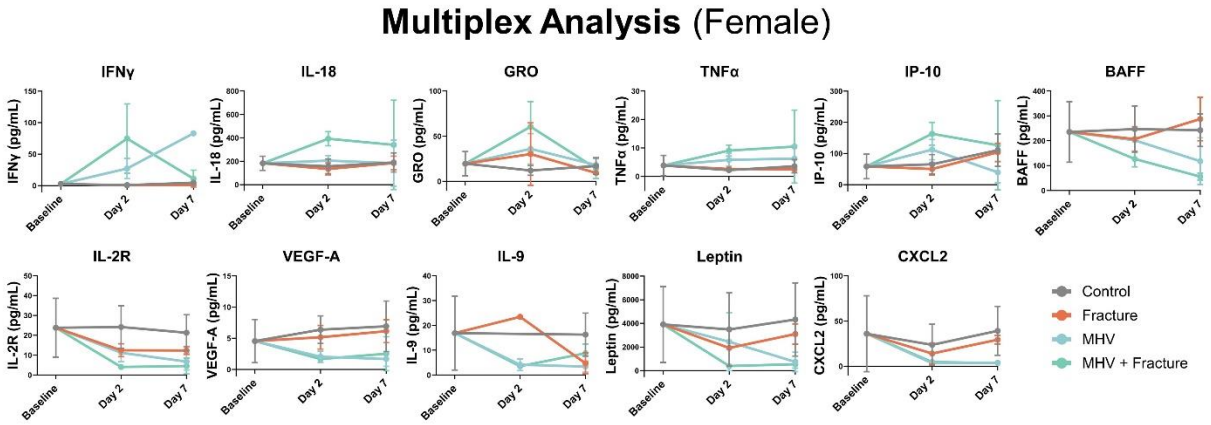

Figure 4. Multiplex Analysis of Female Mice Over Time. The most notable immune factors of female mice at baseline, day 2, and day 7.

Circulating inflammatory cytokines, chemokines, and growth factors, akin to circulating cell populations, provided valuable insights into the mouse response to fracture and infection. Notably, in female mice IFNγ was detected exclusively in samples from virally infected mice at day 2, confirming successful inoculation and infection. Out of the 48 immune factors tested in female mice, only those represented in **Figure 4** were notable (complete results available in the supplementary material). At day 2, IL-18 exhibited a significant 2-fold increase in the MHV + Fracture group compared to all other groups. Similarly, TNFα and IP-10 in the MHV + Fracture group significantly increased compared to Control and Fracture groups but not the MHV group. Also, VEGF-A significantly decreased in MHV and MHV + Fracture groups compared to Control. At day 7, BAFF significantly decreased in the MHV + Fracture group compared to Control and Fracture groups. VEGF-A was only significantly decreased in the MHV group compared to the Fracture group. Lastly, Leptin was significantly decreased in the MHV and MHV + Fracture groups. Interestingly, only one significant difference was seen when comparing the timepoints within each condition. Il-2R in the MHV group was significantly decreased at day 7 vs. day 2.

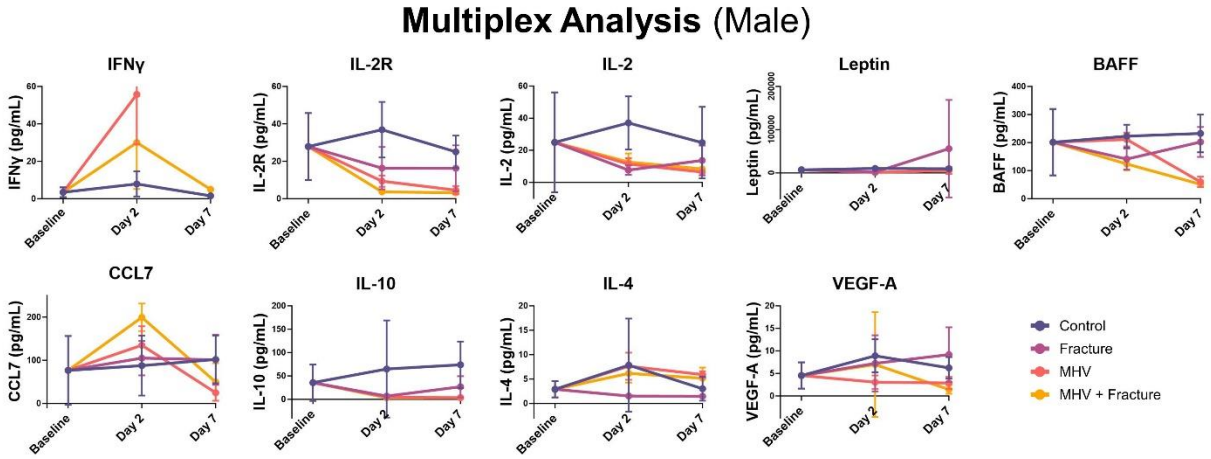

Figure 5. Multiplex Analysis of Male Mice Over Time. The most notable immune factors of male mice at baseline, day 2 and day 7.

Female and male mice shared only four notable immune factors: IFNγ, Leptin, BAFF, and VEGF-A. Of the 48 immune factors tested in male mice, only those represented in **Figure 5**

were notable (full results available in supplementary material). Again, IFNγ was exclusively detected in virally infected male mice at day 2, confirming successful inoculation. In male mice, IL-2R significantly decreased at day 2 in the MHV + Fracture group compared to the Control group. By day 7, both MHV and MHV + Fracture groups showed a significant decrease from the Control group. Unlike females, Leptin significantly decreased at day 2 in the Fracture, MHV, and MHV + Fracture groups compared to the Control group, with no change observed at day 7. Interestingly, BAFF decreased compared to the Control group in the Fracture and MHV groups but not in the MHV + Fracture group. At day 7, consistent with females, BAFF significantly decreased in the MHV + Fracture groups compared to the Control and Fracture groups. At day 2, CCL7 in the Fracture + MHV group significantly increased compared only to the Fracture group. Interestingly, no significant changes were observed in the VEGF-A group in male mice. Finally, at day 7 IL-4 significantly increased only in the MHV group compared to the Fracture group. When comparing time points within each condition, BAFF significantly decreased at day 7 compared to day 2 in both the MHV and MHV + Fracture groups. CCL7 also had a significant decrease at day 7 compared to day 2 but only in the MHV + Fracture group. IL-4 had a significant increase at day 2 when compared to baseline in the MHV group. Similar to the measure of immune cell populations, these findings suggest that both MHV and MHV + Fracture induce distinct immune responses and that sex-specific differences are prevalent.

4. Discussion

It is well known that suffering from a COVID-19 infection while attaining a fracture has a negative impact on patient recovery and increases mortality[11]. COVID-19 is already known to induce a high level of systemic inflammation which is only exacerbated by the inflammatory cascade induced by fracture. Inflammation plays a major role in disrupting the patient's ability to recover. However, inflammation is a complex process that encompasses a plethora of immune cells releasing cytokines, chemokines, and growth factors, all working in tandem to induce a very specific systemic response. Understanding how COVID-19 influences fractures inflammatory response and vice versa will lead to therapeutic advancements, better treatment options, and lower the mortality rate for patients with this common comorbidity. By evaluating the inflammatory response of a murine coronavirus (MHV) that shares a common genus with SARS-CoV-2 and presents with similar inflammatory symptoms, fracture as a comorbidity of COVID-19 can be studied without substantial risk of infection.

As seen in human patients, the MHV + Fracture group had an increased mortality rate of 20%. This indicates that the deadly nature of the comorbidity is still prevalent in the mouse model. This increase in mortality rate could be due to viral and inflammatory induced organ dysfunction. A recent study investigated the effects of SARS-CoV2 drugs on MHV-infected mice and found that MHV without treatment caused severe hepatocellular necrosis[12]. Liver damage is a common and ongoing issue in SARS-CoV2 patients, which is thought to be caused by viral cytopathic damage and immune-mediated hepatitis[13]. In our study, we evaluated the organs at the endpoint and found that both the MHV and MHV + Fracture groups had prevalent organ damage. While the MHV group had some noticeable liver discoloration, the MHV + Fracture group had more severe damage that impacted more mice. A more in-depth study comparing the histology of the livers of the MHV and MHV + Fracture groups, as well as analyzing blood markers of liver health, could be fruitful in understanding the impact of fracture as a comorbidity on organ dysfunction.

Polytropic viruses such as SARS-CoV-2 and MHV can infect multiple cell and tissue types[14]. The respiratory MHV strain initially infects and replicates in the nasal respiratory and olfactory epithelium, then disseminates to the lung, liver, bone marrow, brain, reproductive

organs, and the lymphoid tissue[15]. This broad impact can disrupt tissue function and immune response. Concurrently, a bone fracture triggers the inflammatory and wound healing cascade. This dual activation of the inflammatory system could lead to immune dysregulation and a poorer prognosis. Systemic immune cell populations in both female and male mice were altered by MHV infection, with notable differences between the MHV group and the Control and Fracture groups. Interestingly, fracture alone had minimal influence on the systemic response. However, the combined MHV + Fracture group altered the systemic response most significantly, providing insight into the effect of this comorbidity.

In female mice, major differences were observed in cytotoxic T cells, T helper cells, T regulatory cells, monocytes, M1/M2 monocyte ratio, and neutrophils. The cytotoxic T cells and T helper cells in the MHV + Fracture group returned to the Control groups level while the MHV group remained lower and higher, respectively. Additionally, T regulatory cells in the MHV + Fracture group at day 7 had a significant increase compared to baseline while all other groups remained the same or decreased. While not typically known for their role in wound healing, T cells have been shown to be an important part of fracture healing[16, 17]. While fracture alone was not enough to provoke a change in these populations, the combined inflammatory effect of fracture and infection clearly influences T cell production and recruitment. In the MHV + Fracture group at day 7 the monocyte population remains depleted while the MHV group returned to the Control and Fracture groups level. While at day 2 there is more M1 or inflammatory monocytes in the MHV group compared to all other groups. Additionally, at day 2 there are more neutrophils in the MHV + Fracture group compared to all other groups. Monocytes and neutrophils play many roles in the immune and wound healing responses. These innate immune cells are among the first populations recruited in both response to a viral infection and the fracture inflammatory response[18, 19]. Furthermore, these cells typically direct the immune response in fracture healing, helping to resolve inflammation and transition into the wound healing phase[18].

In contrast, male mice showed fewer changes. The only two circulating cell populations that were influenced by the combined viral and trauma response were dendritic cells and again neutrophils. dendritic cells in the MHV group at day 2 were highly increased, while the MHV + Fracture group increased more than Control and Fracture but was decreased compared to the MHV group. neutrophils in the MHV + Fracture group at day 2 were again increased compared to baseline, while all other groups remained unchanged. dendritic cells and neutrophils play an important role in the innate and adaptive response to viral infection and in the initial stage of fracture healing[20]. Again, the combined inflammatory response seems to influence their infiltration and presents in the circulating blood. Overall, The combined inflammatory incidents appear to disrupt the systemic immune response in both female and male mice, likely leading to immune dysregulation, altered cell recruitment, and disruption of the typical inflammatory cascade[21].

Inflammation is a complex response that includes not only the immune cells but the different cytokines, chemokines, and growth factors that initiate and direct the cellular reactions. In both female and male mice, IFNγ was only seen in the MHV groups. IFNγ is released in response to active viral infections[22], indicating the inoculation was successful. How other inflammatory factors change in response to both MHV infection and bone fracture can help describe why immune cell populations are fluctuating and the presents of immune dysregulation.

In female mice IL-18, TNFα, and IP-10 were all significantly increased compared to the Control group. All these factors play a major role in initiating the inflammatory response by

inducing cell recruitment and activating positive feedback loops to propagate higher levels of inflammation[23-25]. In male mice only BAFF and IL-2R are decreased in the MHV + Fracture group compared to the Control group. Although these factors are known to induce inflammation they also activate B and T cells and are important for the adaptive response[26, 27]. Decreased production of these cytokines could lead to a worse adaptive response and lower overall ability to recover from the infection or subsequent infections.

Our findings suggest a sex-specific response that modulates circulating immune factors. Females exhibit elevated levels of inflammatory factors, whereas males demonstrate a diminished response. This divergence is mirrored in cell populations, suggesting that the quantity of immune factors released may contribute to these discrepancies. Despite the limitations of our study, such as a reduced sample size, the significant responses we measured are likely to profoundly influence the inflammatory response and subsequent recovery in both female and male mice. Our results also point to immune dysregulation, where cytokine overproduction can trigger both pro-inflammatory and immunosuppressive responses.

This study provides critical insights into the systemic inflammatory response influenced by a COVID-19-like virus, both independently and in conjunction with a fracture. Our findings suggest that an overproduction of proinflammatory cytokines may induce a dysregulated immune response, contributing to the observed poorer prognosis in comorbid cases. Notably, the mortality rate for COVID-19 patients who sustain a fracture is threefold. A deeper understanding of this lethal combination could pave the way for therapeutic advancements and treatment strategies aimed at reducing these mortality rates. Moreover, a fundamental comprehension of the inflammatory response to infection alone could prove invaluable when investigating other comorbidities or elucidating why the virus is so deadly in isolation.

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