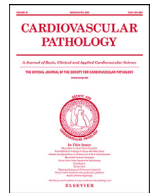




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Original article

COVID-19 myocarditis: quantitative analysis of the inflammatory infiltrate and a proposed mechanism ^{☆,☆☆}

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ABSTRACT

COVID-19 has a significant effect upon the cardiovascular system. While a number of different cardiovascular histopathologies have been described at post-mortem examination, the incidence of typical viral myocarditis in COVID-19 positive patients appears very low [1–3]. In this study, we further characterize and quantify the inflammatory cell infiltrate in a COVID-19 study cohort and compare the findings to both an age and disease matched control cohort and a cohort of patients diagnosed with typical inflammatory myocarditis. All study and control cohorts had 1 or more of the comorbidities most commonly associated with severe disease (hypertension, type II diabetes, obesity, or known cardiovascular disease). The results demonstrate a skewed distribution of the number of CD68+ cells in COVID-19 hearts, with upper quantiles showing a significant increase as compared to both matched control hearts, and those with myocarditis. In contrast, hearts from typical inflammatory myocarditis contained increased numbers of CD4+, and CD8+ cells compared to both COVID-19 and control cohorts. In conclusion, the presence of an increased number of CD68+ cells suggests that COVID-19 may incite a form of myocarditis different from typical viral myocarditis, and associated with diffusely infiltrative cells of monocytes/macrophage lineage.

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1. Background

The SARS-CoV-2 coronavirus has resulted in a global pandemic and the loss of over 3.5 million lives. Initial published reports from China and Europe focused on the pulmonary pathology of COVID-19, yet data to suggest cardiovascular involvement was described early on as a potential source of significant morbidity and mortality [4,5,6].

Early studies of the cardiac morbidity of COVID-19 focused on the presence or absence of myocarditis. Many early descriptions of a typical viral myocarditis in hospitalized patients and even in patients that had recovered from COVID-19 were derived from clinical, radiological, and laboratory measurements, rather than tissue diagnosis [7]. There have been a number of autopsy case series

that have documented several varying histopathologic changes, including what is considered viral myocarditis [8,9]. While there are differences in what is considered myocarditis in the published reports, the largest autopsy series published to date indicates that the overall rate of lymphocytic myocarditis is low (<2%) [2].

Previous coronavirus pandemics (SARS; >8000 infected) and epidemics (MERS; >2000 infected) yielded only one reported case of myocarditis based upon MRI diagnosis [10,11]. Despite the overall low incidence of viral myocarditis in the SARS-CoV-2 pandemic, some have suggested that the inflammatory infiltrate in decedent COVID-19 hearts consists primarily of mononuclear cells of the monocyte and/or macrophage cell line [9]. Recent studies have additionally indicated that following the innate immune response, the systemic cellular immune response differs between mild and severe COVID-19 [12]. Interestingly, it has been suggested that cellular immune dysregulation of innate cells, particularly the monocyte/macrophage cell line, may persist into the recovery phase regardless of the severity of the initial infection [12]. To determine better therapies for acute COVID-19 disease, it will be important to identify the cause of variability in immune response between mild and severely affected patients. Furthermore, given the growing concern regarding the post-acute sequelae of COVID-19, and the reported occurrence of cardiac symptoms in this patient

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Table 1
Demographic and histopathologic findings. Key: ¹Mimimal time from diagnosis/symptoms to death; ²Both type I and type II

	COVID-19	Control	Myocarditis
Study Characteristic			
Gender (M/F)	5/5	5/5	3/2 (one not known)
Age (mean; range)	64 (49-79)	56 (30-79)	49 (30-59)
BMI (mean; range)	33 (22-45)	32 (17-45)	Not recorded
Race (AA/Caucasian/Hispanic)	6/3/1	9/1/0	3/1/?
Known Heart Disease (incl. Afib)	3	0	Not recorded
Hypertension	8	10	Not recorded
Type 2 Diabetes	4	10	Not recorded
Renal disease	2	10	Not recorded
Cancer	1	0	Not recorded
Obesity (BMI>30)	4	4	Not recorded
Coronary stenosis (maximal lesion any vessel) (mean; range)	22% (0-75%)	18% (0-75%)	Not recorded
Laboratory Parameter			
Elevated Troponin I nl<0.04	6/10	2/10	3/4
Elevated BNP (pg/mL) nl<100	6/10	5/8	2/2
Elevated D-dimer (ng/mL DDU) nl<250	8/8	4/4	2/2

population, it is important to determine whether subtle changes in COVID-19 hearts may yield important clues to susceptibility to long term cardiac consequences.

The present study quantitatively examines the immune cells present in heart samples from a cohort of COVID-19 decedents, and demonstrates a significant increase in the number of CD68 positive cells compared to a control group matched for age and comorbidities and to a cohort who were diagnosed with acute viral myocarditis at autopsy. The implications of these findings are discussed in the context of COVID-19 disease.

2. Material and methods

2.1. Cohort

We identified 10 non-consecutive decedents whose death was due to COVID-19 infection. The deaths occurred between March and September of 2020 and all autopsies were performed at University Medical Center-New Orleans (UMC-NO). A control group of 10 decedents (5 male and 5 female) was selected, all of whom had pre-mortem diagnosis of HTN, DM2, and CKD and had died and had an autopsy performed during the same period. The myocarditis control group consisted of 5 patients with a confirmed diagnosis of myocarditis who had an autopsy during the years 2015-2020. Detailed demographics are shown in Table 1 for all 3 groups. The COVID-19 and control groups were compared for age, BMI, percentage of coronary artery stenosis as well as serum troponin, d-dimer, and BNP levels. Some demographic and laboratory data was not available for each included decedent.

2.2. Histologic analysis

For each case, sections of the heart were reviewed by an experienced cardiac pathologist (RVH) and areas were selected for further analysis based upon the hematoxylin and eosin (H&E) appearance. From each heart, 2 sections of the left ventricle and one section of the right ventricle were selected and stained for the following T lymphocyte and monocyte/macrophage markers; CD3, CD4, CD8 and CD68. After staining, each slide was digitized into a whole slide image utilizing a Leica Aperio AT2 slide scanner. An investigator blind to the patient group associated with each slide then chose three regions per slide for analysis within the myocardium, measuring 1.37 mm² each, with a goal of identifying those which contained the largest number of cells within a standard unit area, while excluding tissue outside of the myocardium. The positivity for each cell type was then quantified using a modified version of the Leica ImageScope Cytoplasmic staining algorithm to aver-

age positivity within these regions. Matlab (Mathworks) software was used to perform statistical analysis on the resulting data across groups.

3. Results

3.1. Patient demographics and laboratory data

The demographic and laboratory data are summarized in Table 1. One purpose of this study was to compare a COVID-19 cohort to a control group of similar age demographics and comorbidities. The study was designed to contain an equal number of males and females between the two major comparison groups. The racial and ethnic makeup of our patient population at UMC-NO reflects the makeup of the greater New Orleans area population and therefore, the majority of our patients self-identify as African-American (60%). Racial/ethnic self-identification within the medical record was utilized, with the COVID-19 group consisting of 6 African-Americans, 3 Caucasians, and 1 Hispanic. The control group contained 9 African-Americans and 1 Caucasian. Given the fact that COVID-19 death rates are higher in the African-American and Hispanic communities, our cohort reflects the local population and should be compared to other parts of the country/world with differing populations. The myocarditis group consisted of 3 males (2/3 African-American) and 1 African-American female. In one patient, the information was unspecified. A two-tailed t-test (assuming unequal variances), performed in Excel, was performed on BMI data. There was no significant difference in obesity between study and control groups (BMI mean = 33.2 vs. 31.63; p-NS).

The laboratory data shown in Table 1 reflects the most commonly ordered tests at our institution that correlated with COVID-19 mortality (troponin, D-dimer, and BNP). The levels shown are the highest level recorded for that parameter during the patient's terminal hospitalization. Not all tests were ordered in all patients, especially in the control group where COVID-19 was not suspected or diagnosed. In the COVID-19 group, troponin was measured in 9/10, D-dimer in 8/10, and BNP in 8/10. In the control group, troponin was ordered in 6/10, D-dimer in 5/10, and BNP in 8/10. In the myocarditis group, 4/5 had troponin measured, 2/5 had D-dimer levels, and 3/5 had BNP measured. Values from the decedent from 2015 were not available.

Finally, we recorded the maximal extent of coronary artery stenosis estimated at autopsy for each decedent with available data. As expected, there was considerable variability between both the individuals and the three groups (Table 1). However, some interesting observations can be made. In the COVID-19 group, only 3 had narrowing greater than 50%, none had acute coronary

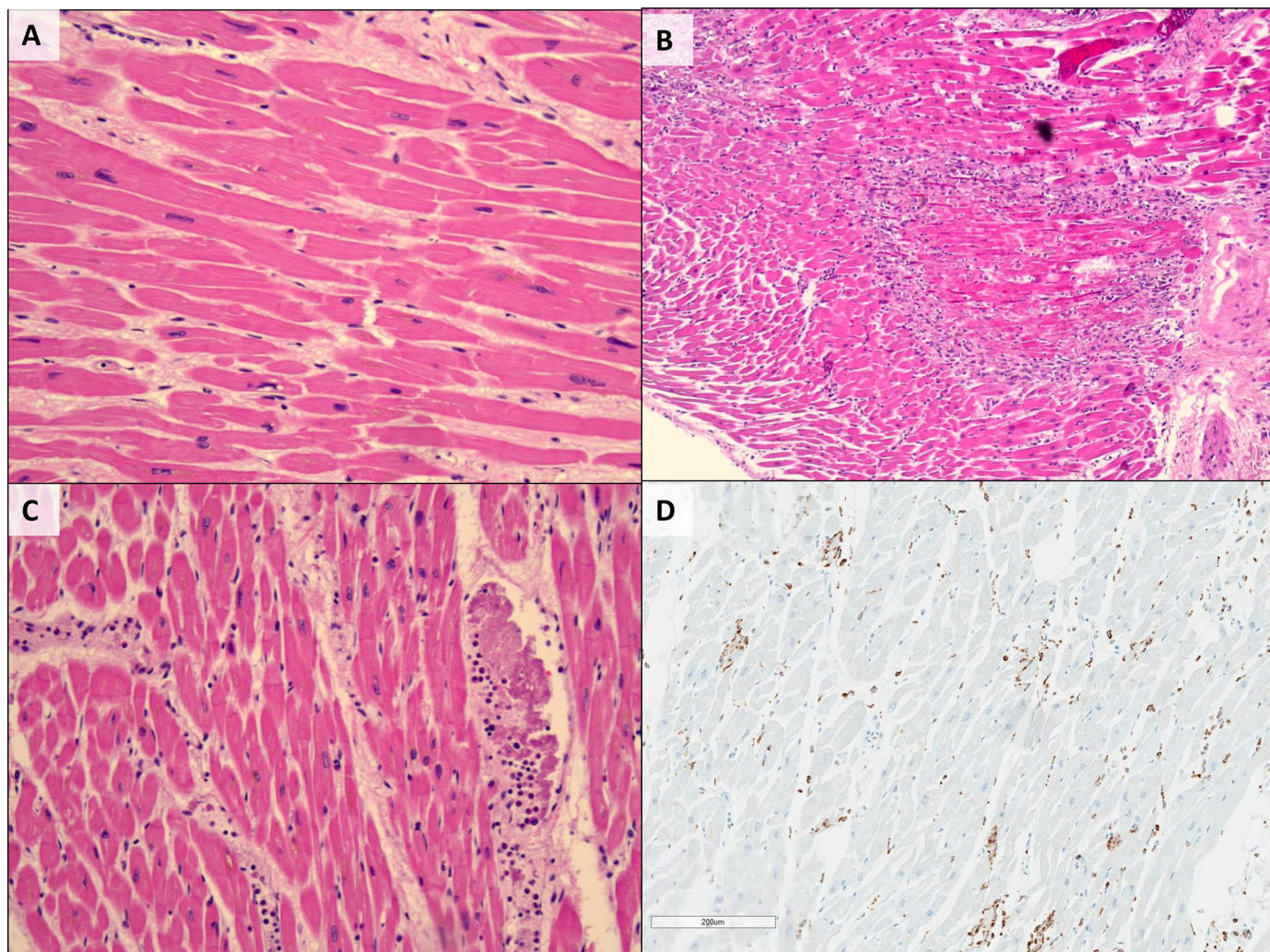


Fig. 1. (A) Cardiac myocytes from control patient (H&E). (B) Myocarditis, characterized by patchy, dense inflammation within the myocardium (H&E). (C) Endotheliitis and diffuse, perivascular distribution of inflammation in COVID-19. (D) CD68 immunostaining highlighting the presence of CD68+ cells in a mild, diffuse intravascular and perivascular distribution in a case of COVID-19.

events (grossly evident thrombosis/plaque erosion/plaque hemorrhage) and 3 had no measurable narrowing. The COVID-19 decedent with the highest measured troponin level (37.4 ng/ml) only had a 25% maximal stenosis and no grossly identifiable acute coronary event. In the control group, 2 decedents had stenosis greater than 60% while 6 had no recorded coronary artery disease. Only one decedent in the myocarditis group had any coronary narrowing and it was estimated at only 20%.

3.2. Qualitative histology findings

3.2.1. H&E findings

In all ten COVID-19 hearts, there was no evidence of lymphocytic myocarditis. In general, there was no specific pathology outside of interstitial fibrosis, which is likely related to underlying hypertension. The most common finding was individual cell degeneration/necrosis as previously reported, and prominent endothelial cells [1]. The case that exhibited the highest troponin level contained larger areas of myocyte necrosis. This case contained primarily epicardial necrosis, which was associated with epicardial venous occlusion. Additional findings in the COVID-19 cohort included epicardial lymphocytic infiltrates (2 hearts) and prominent collections of neutrophils in small vessels (2 hearts) (Fig 1C). One

heart contained individual myocytes surrounded by mononuclear inflammatory cells that upon further workup were determined to be primarily CD68 positive cells.

In the control hearts, the most common finding was perivascular and interstitial fibrosis again likely associated with the underlying comorbidities present in all decedents. Two hearts exhibited hyperacute (12–24 hours old) subendocardial necrosis likely related to terminal events. No significant inflammatory infiltrates or epicardial infiltrates were noted (Fig 1A). The myocarditis hearts contained areas of inflammatory cell infiltrates associated with significant myocyte necrosis (Fig 1B), present in a patchy distribution. The inflammatory cells were primarily mononuclear but in one case, a significant eosinophilic population was present.

3.2.2. Immunohistochemistry findings

The most striking finding in the COVID-19 group was the presence of increased numbers of CD68+ cells indicating cells of monocyte/macrophage lineage. The CD68+ cells were mostly located in the interstitial space near small blood vessels and inside larger vessels, in a relatively diffuse distribution throughout the sections examined (Fig 1C-D). Interestingly, in some hearts there were a significant number of CD68+ cells seen in the epicardial adipose tissue. Lymphocytes (CD3+, CD4+, and CD8+ cells) were

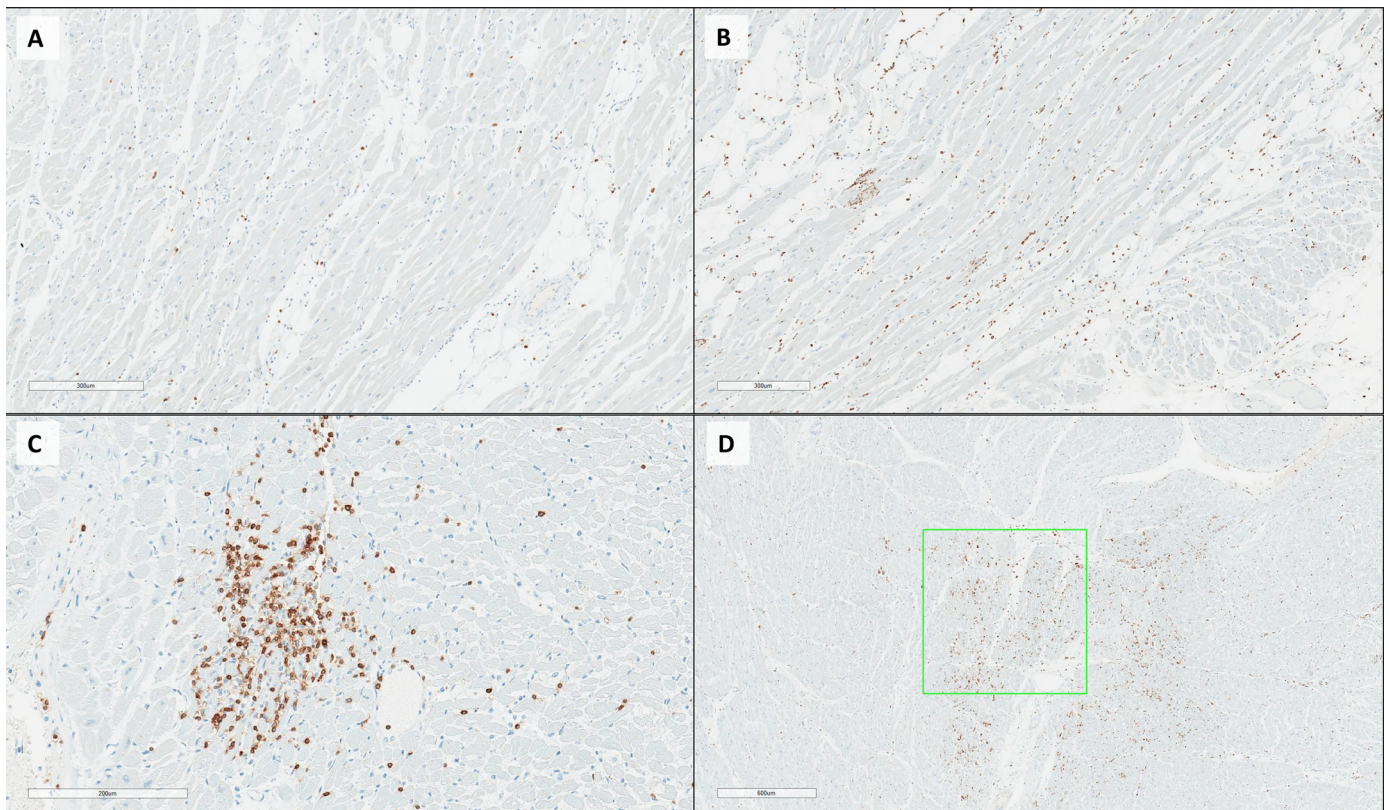


Fig. 2. (A) CD3 immunostaining, demonstrating a paucity of CD3+ lymphocytes in COVID-19. (B) CD68 immunostaining in COVID-19, highlighting a mild, diffuse distribution of CD68+ cells. (C) CD3 immunostaining in a case of myocarditis, demonstrating intense staining for CD3+ lymphocytes in a patchy distribution. (D) CD68 immunostaining in myocarditis, in a similar patchy distribution to that of lymphocytes, with example of region selection box for analysis.

present in the hearts, but not in large numbers, and did not exhibit any specific pattern of distribution. In control hearts, the CD68+ CD4+, and CD8+ cell numbers did not appear different from the COVID-19 group on visual inspection. As expected, the myocarditis group appeared to contain increased numbers of CD3+, CD4+, and CD8+ cells as compared both to control and to COVID-19 groups. The CD68+ cell population was increased compared to control hearts but the distribution was centered on the areas of lymphocytic infiltration and myocyte necrosis (Fig 2).

3.3. Quantitative histology findings

A nested ANOVA performed in Matlab identified Patient Group (COVID-19, control, or myocarditis) as the greatest source of variability among inflammatory marker positivity ($F = 12.6, P < .0001$), with right versus left side of the heart sampling having no significant effect. Patient Group was a significant factor in CD68 positivity ($F = 6.49, P = .0024$), with patients in the COVID-19 group having significantly greater numbers of CD68 positive cells within the myocardium as compared to the myocarditis and control groups (Fig 3A-B). By contrast, a greater number of CD4 and CD8 positive cells was seen in the myocardium of patients with myocarditis as compared to either COVID-19 or controls (CD4: $F = 6.46, P = .0025$; CD8: $F = 6.06, P = .0034$). A significant effect of group was not seen for CD3 staining ($F = 1.85, P = 0.1628$), in part due to large variability of staining within the COVID-19 and myocarditis samples. The effect size of group may also be limited by the number of samples of true myocarditis available for inclusion in the study. Examination of the distribution of CD68+ cells among quantiles of the COVID-19, control, and myocarditis groups was revealing (Fig 3A, C). At lower quantiles, CD68 positivity was similar between COVID-19 and control hearts; however, at upper quantiles, the COVID-19

group showed a distribution skewed towards much higher numbers of CD68 positive cells. The same was true, though to a lesser extent, in a comparison between COVID-19 and myocarditis heart samples, with myocarditis sample size being a potential limitation. The findings of a non-linear Q-Q plot of CD68+ cells within the COVID-19 samples as compared to the control distribution is consistent with the theory that while not all cases differ in positivity from control samples, there are a significant number of COVID-19 samples in upper quantiles with greater CD68 positivity (Fig 3A, C).

4. Discussion

Since the onset of the global pandemic, the incidence and significance of myocardial involvement, especially the presence of myocarditis, has been controversial. Early studies indicated that myocarditis was prevalent, and cases of fulminant myocarditis were reported from China [7,13,14]. However, in those studies the diagnosis of myocarditis was based on clinical and radiologic data and when autopsy-based studies appeared, the incidence of typical viral myocarditis became more accurately diagnosed. The largest reported study to date showed that in 277 postmortem examinations, the true incidence of myocarditis was much lower; likely less than 2% [2]. Some early studies reported that COVID-19 hearts contained increased numbers of monocytes, but the significance of the finding was not clear [9]. The observations of Basso et al. were from COVID-19 autopsy cases alone, and were not compared to control hearts with similar age, BMI, and co-morbidities. This study was designed to both control for the most common comorbidities reported in COVID-19 decedents, and to quantify and statistically test for differences in inflammatory cell subsets.

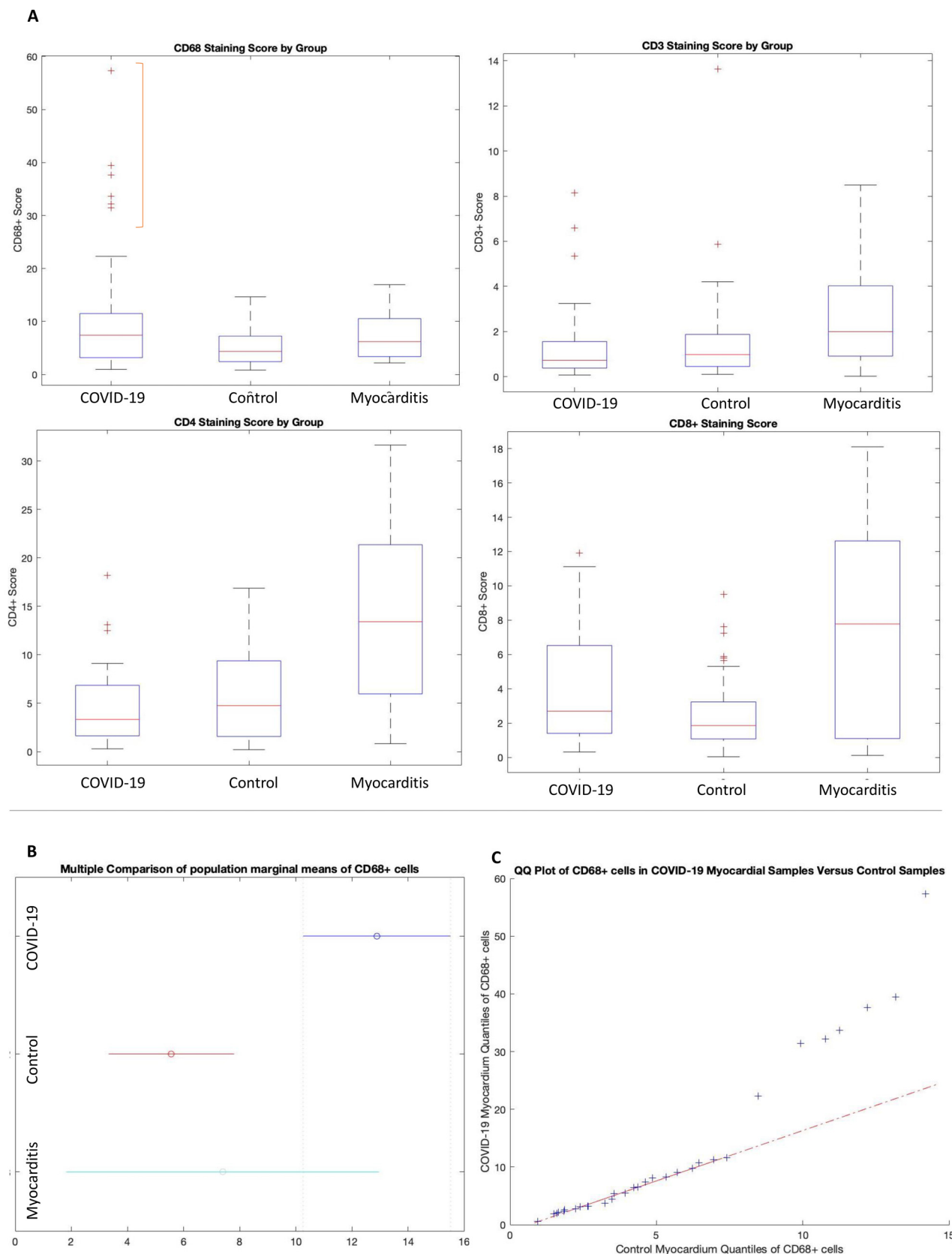


Fig. 3. (A) Boxplots showing median and range of CD68, CD3, CD4, and CD8 staining cells for each Patient Group. A red bracket highlights the numerous outliers of CD68+ cells in the upper quantile of the COVID-19 group, which may represent a subset of COVID-19 patients with greater myocardial inflammation. (B) Population marginal means after correction for multiple comparisons within the nested ANOVA performed on CD68+ cells. The population marginal mean of CD68+ cells is significantly higher for the COVID-19 group as compared to controls. (C) Q-Q plot of CD68+ cell quantiles in the COVID-19 myocardium samples as compared to control quantiles. The relationship is non-linear at higher quantiles, indicating a difference in distribution of CD68 positivity among the COVID-19 group, with higher values at the upper quantiles.

The results confirmed that decedents that died of severe COVID-19 did not exhibit typical viral myocarditis. A significant subset of hearts from COVID-19 patients contained an increase in CD68+ cells when compared to both a matched control group, and to a group with known viral myocarditis. The CD68+ cells were primarily localized in vascular and interstitial spaces and were not seen forming large aggregates or associated with multicellular myocyte necrosis. In one COVID-19 case, CD68+ staining cells were seen surrounding individual myocytes undergoing cell death, but lymphocytes were essentially absent, and the affected myocytes were scattered throughout the wall of the ventricles. In this case, the serum troponin level was 0.38 ng/mL which was just above the panic threshold in our laboratory (<0.02 ng/mL). These findings are consistent with previously reported findings in COVID-19 hearts, which found scattered myocytes undergoing cell death/degeneration [2]. Interestingly, the fact that the cells are of the monocyte/macrophage line suggests that apoptosis may be an important pathway of myocyte death.

Myocarditis is typically defined as the presence of an inflammatory infiltrate intimately associated with adjacent myocyte injury. In cases of myocarditis sufficiently severe to cause cardiac dysfunction, the infiltrate is expected to produce significant myocyte injury. In the myocarditis cohort, inflammatory infiltrate was significant and was associated with obvious myocyte necrosis (Fig 1B). In most cases, lymphocytes were the predominant cell type while in one case eosinophils were the predominant cell type. In these hearts, the number of T lymphocytes (CD4+, and CD8+) was significantly greater than the COVID-19 hearts and the control hearts (Fig 3A). There were marginally fewer macrophages/CD68+ cells as compared to the COVID-19 hearts, with the comparison limited to some extent by the size of the myocarditis cohort (Fig 3B). The CD68+ cells present in the myocarditis samples appeared to be localized in clusters within the myocardium, associated with patches of lymphocytic infiltrate, as opposed to the diffuse distribution pattern seen in COVID-19 hearts. These results suggest that the presence of increased numbers of diffusely distributed CD68+ staining cells in COVID-19 hearts may be a distinct effect of SARS-CoV-2 infection. Importantly, the COVID-19 myocardial samples demonstrated a distribution of CD68 positivity among samples in which numerous outliers were present above the median value (Fig 3A). This was seen in the Q-Q plot of COVID-19 samples as compared to controls, where a relatively linear pattern was observed at lower quantiles, but this change in distribution led to non-linearity, and therefore a difference in distribution as compared to controls, at higher quantiles (Fig 3C). This finding would support the concept that some, but not all, COVID-19 myocardial samples demonstrate an increase in CD68+ inflammatory cells, which is consistent with the clinical picture of variable cardiac effects in COVID-19.

Early studies of the incidence and extent of COVID-19 myocarditis described the presence of increased numbers of CD68+ cells [9]. Basso et al. found increased numbers of interstitial macrophages in 86% of the 21 consecutive autopsies they examined. They concluded that the increased numbers of macrophages likely reflected the underlying diseases present in the decedents. This study confirms the presence of CD68+ cells, and we suggest that they may play an important role in COVID-19, especially as they appear commonly in the interstitium and smaller vascular spaces. Others and we have hypothesized that the endothelium is a potential target of the SARS-CoV-2 virus infection [1,15–17]. Some of the co-morbidities identified as independent COVID-19 risk factors, that is, HTN, T2DM, and obesity, are associated with endothelial dysfunction and in light of the conclusion by Basso et al., it is important to control for this potentially confounding effect [18]. The present study shows that COVID-19 hearts uninvolved with any lymphocytic myocarditis contain increased numbers of CD68+

cells as compared to a control group matched for these important comorbid conditions.

4.1 Potential role of macrophages in COVID-19

Early observations of macrophages involving the heart were reported in COVID-19 but the cause of the infiltration was not clear. Macrophages play important roles in several inflammatory and healing related processes. They are involved directly in the detection, phagocytosis, and destruction of infectious organisms but also present cellular antigens to T lymphocytes resulting in the generation of cytokines that activate additional inflammatory reactions. The SARS-CoV-2 virus has been shown to infect epithelial cells in the trachea, lung, ileum, and colon but it is not clear how the virus causes systemic effects involving other organs including the heart and brain [4]. Reports have shown that in severe COVID-19, resident alveolar macrophages are involved with the initial response to viral infection but they die off and monocyte-derived alveolar macrophages are recruited to the site of infection. These cells may contain SARS-CoV-2 viral particles, and may spread antigenic material to other areas of the lung [19,26], and perhaps to the rest of the body.

Macrophages can be broadly divided into two groups; M0 and M1. M0 macrophages are “traditional” macrophages that are involved in infection response and are capable of causing tissue damage as part of that response. Although still not settled, endothelium expresses ACEII receptors and therefore subsequent to viremia, endothelium could be directly infected by the SARS-CoV-2 virus. Since macrophages mediate both local and systemic responses to viral infection, it is possible that they could damage endothelium in an attempt to contain the spread of the virus through infected endothelium. Macrophages are also capable of fixing complement and therefore could cause the direct death of nearby myocytes through the activation of apoptotic attack complexes (C5b-9) [20,21]. Alternatively, damaged endothelium could cause direct activation of the hemostasis pathway resulting in the generation of localized thrombi and subsequent ischemia/reperfusion myocyte injury; both types of injury were identified in this study.

A recent study by Kawakami *et al.* examined the nature of inflammatory cells in COVID-19 hearts. In this study, 5 random hearts were selected from a series of fifteen COVID-19 cases, and the results were compared to a group of 5 controls in which the cause of death was trauma. They showed no differences in the total number of CD3+ and CD68+ cells. Although the total number of CD68+ cells in COVID-19 hearts was greater than controls, and was higher than some would use to diagnose myocarditis, they concluded that criteria for myocarditis was not met. It is not clear, however, how CD68+ cells were localized and quantified in this study. In the present study, we objectively identified representative areas, and used both whole slide digitization and computer-assisted algorithmic quantification to determine differences between heart samples. Furthermore, our control group was matched for comorbidities and obesity; important risk factors for severe COVID-19 disease. Finally, platelet/neutrophil extracellular traps (NETs) have been increasingly recognized as drivers of immunothrombosis [22]. In COVID-19 hearts, we do see neutrophils in increased numbers in small vessels, often associated with or adjacent to mononuclear cells, and therefore the role of NETs in activating local thrombosis and resulting ischemia should be investigated more closely [22–24].

Interestingly, the mononuclear phagocyte system (MPS) is known to be a major contributor to hyper inflammatory and pro-coagulant secretion syndromes, as demonstrated in the previous SARS epidemic, as well as COVID-19 infection [25,26]. Therefore, it is possible that the SARS-CoV-2 virus elicits a unique inflammatory response in infected patients that leads to endothelial in-

jury. In the heart, that injury can lead to clotting at the arteriole, venule and capillary level, initiating thrombosis and resulting ischemia/reperfusion injury. Alternatively, the presence of endothelial injury could attract non-classical monocytes (i.e., M1) to the site resulting in macrophage-induced activation of the complement pathway, and generating apoptotic injury. Whether one of these proposed pathways is more important and/or which local conditions lead to primary activation of one or the other pathway is the subject of our ongoing investigations.

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