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Multisystem Inflammatory Syndrome in Children is driven by zonulin-dependent loss of gut mucosal barrier

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Abstract:

Background: Weeks after SARS-CoV-2 infection or exposure, some children develop a severe, life-threatening illness called Multisystem Inflammatory Syndrome in Children (MIS-C). Gastrointestinal symptoms are common in MIS-C patients and severe hyperinflammatory response ensues with potential for cardiac complications. The cause of MIS-C has not previously been identified.

Methods: Here, we analyzed biospecimens from 100 children: 19 children with MIS-C, 26 with acute COVID-19, and 55 controls. Stool was assessed for SARS-CoV-2 by RT-PCR and plasma was assessed for markers of breakdown of mucosal barrier integrity, including zonulin. Ultrasensitive antigen detection was used to probe for SARS-CoV-2 antigenemia in plasma, and immune responses were characterized. As proof of concept, we treated a MIS-C patient with larazotide, a zonulin antagonist, and monitored impact on antigenemia and clinical response.

Results: We showed that in MIS-C, prolonged presence of SARS-CoV-2 in the GI tract leads to release of zonulin, a biomarker of intestinal permeability, with subsequent trafficking of SARS-CoV-2 antigens into the bloodstream, leading to hyperinflammation. The MIS-C patient treated with larazotide displayed a coinciding decrease in plasma SARS-CoV-2 Spike antigen levels, inflammatory markers, and a resultant clinical improvement above that achieved with currently available treatments.

Conclusion: These mechanistic data of MIS-C pathogenesis provide insight into targets for diagnosing, treating, and preventing MIS-C, which are urgently needed for this increasingly common severe COVID-19-related disease in children.
**Brief Summary:**
In MIS-C, increased gastrointestinal mucosal permeability allows SARS-CoV-2 antigens in the GI tract to leak into the bloodstream, triggering cytokine storm and hyperinflammatory responses.

**Keywords:**
Introduction:

Most children who are acutely infected with SARS-CoV-2 develop mild upper respiratory symptoms or experience asymptomatic infection. Several days to weeks after resolution of the initial infection, some of these children will develop a severe life-threatening illness, termed Multisystem Inflammatory Syndrome in Children (MIS-C), which is an immune activation syndrome associated with prior SARS-CoV-2 infection or exposure. MIS-C patients present with persistent fever, marked gastrointestinal symptoms, cytokine storm, myocardial dysfunction, and cardiogenic shock with ventricular dysfunction in the setting of multisystem inflammation, reminiscent of, yet distinct from, toxic shock syndrome or Kawasaki Disease(1). Eighty percent of these hospitalized children develop cardiac pathology(2), including coronary artery dilations, myocardial dysfunction, or ventricular failure with hypotensive shock(3). The cause of this late-phase severe illness in children has not previously been identified, leaving treatment options and prevention strategies nebulous. As the prevalence of SARS-CoV-2 infections among children and adolescents is increasing(4), MIS-C cases are increasing as well. A better understanding of this life-threatening illness is urgently needed.

Children with MIS-C display increased monocyte recruitment and hyperphagocytosis(5) with cytokine storm(6), T-cell activation(7), and inflammation driven by expansion of immunoglobulins(5, 8). A recently discovered superantigen-like motif near the S1/S2 cleavage site on the SARS-CoV-2 Spike protein(9, 10) is hypothesized to drive this hyperinflammatory response in MIS-C. However, in most MIS-C cases, SARS-CoV-2 is undetectable by reverse transcription- polymerase chain reaction (RT-PCR) of the nasopharyngeal swab, leaving the etiology and timing of this hyperinflammatory response yet to be elucidated(2, 11). In adults, there is increased recognition that the gut serves as a nidus for SARS-CoV-2(12) and that in severe COVID-19, dysbiosis and disruption of the gastrointestinal (GI) barrier drive inflammatory
activation(13, 14). Although GI symptoms predominate MIS-C symptomatology(2), the role of the GI tract in the pathogenesis of MIS-C has not been evaluated.

In this study, we provide evidence that GI sources of SARS-CoV-2 viral antigenemia may instigate and drive MIS-C. We demonstrate that weeks after the initial infection, SARS-CoV-2 RNA remains in the GI tract, and zonulin-instigated hyperpermeability of the mucosal barrier coincides with SARS-CoV-2 antigenemia. Current treatment strategies are targeted at dampening the inflammatory response but do not address mucosal permeability or antigenemia. Here, we provide insight into the mechanism of disease pathogenesis and present evidence related to the trigger of MIS-C, thereby offering biomarkers for early detection of disease and avenues for prevention and treatment of MIS-C.

Results:
This study included biospecimens from 100 children composed of 19 children who were clinically diagnosed with MIS-C as defined by Center of Disease Control (CDC) criteria (Supplemental Table 1), 26 children with COVID-19 confirmed by RT-PCR, and 55 non-COVID controls (32 pre-pandemic). The average age of children with MIS-C (8 years old) was younger than those who presented with COVID-19 (14 years old), consistent with national averages(2, 15). MIS-C patients presented with a median of three days of acute symptoms associated with MIS-C (range 1-28 days), after a previous COVID-19 exposure or SARS-CoV-2 infection 26 days prior to presentation for MIS-C (range 13-62 days). Notably, GI symptoms were predominant in the MIS-C cohort, affecting 89% of these patients, compared to 27% of children with acute COVID-19 (Fisher’s exact test, P<0.0001) (Table 1). Figure 1 outlines the specimens collected for analysis and timing of specimen collection from children with MIS-C or acute COVID-19.
**SARS-CoV-2 in the gastrointestinal tract of children with MIS-C coincides with loss of intestinal epithelial barrier function.**

MIS-C develops several weeks after a SARS-CoV-2 infection/exposure (Table 1) and viral load in respiratory secretions is known to decrease over the course of 7-10 days after infection(11, 16, 17). As most children with MIS-C have negative nasopharyngeal viral swabs(11), MIS-C is unlikely to be related to this initial infection of the respiratory tract. To assess the presence of SARS-CoV-2 in the GI tract, we measured SARS-CoV-2 RNA from MIS-C stool samples collected weeks after initial SARS-CoV-2 infection/exposure. Indeed, a majority of patients showed detectable viral loads in stool ranging from $1.5 \times 10^2$ - $2.5 \times 10^7$ RNA copies/mL, suggesting an on-going nidus of infection in MIS-C (Supplemental Table 1).

An intact, functional intestinal mucosal barrier should prevent the passage of large antigens from the gut lumen into the bloodstream, including viral antigens derived from SARS-CoV-2 present in the GI tract(12). Zonulin is a family of structurally and functionally related proteins that reversibly regulate intestinal permeability by modulating intercellular tight junctions(18-20).

Increased circulating zonulin levels resulting in increased intestinal permeability have been reported in several diseases(21) including auto-immune and hyper-inflammatory diseases such as celiac disease(22), inflammatory bowel disease(23), and Kawasaki disease(24). Zonulin release from epithelial cells can result in permissibility of paracellular trafficking of large inflammatory antigens from the gut lumen into the bloodstream. In this study, children with MIS-C had significantly increased release of zonulin into circulation compared to controls ($P = 0.003$) as detected by mass spectrometry analysis (Figure 2A), which can result in a breakdown of mucosal barrier function. Correspondingly, children with MIS-C also had increased lipopolysaccharide binding protein (LBP) compared to controls ($P = 0.007$) (Figure 2B) signaling increased microbial translocation. Soluble CD14, another marker of microbial translocation, also showed an increased trend in MIS-C ($P = 0.1$) (Figure 2C), highlighting a
loss of gastrointestinal mucosal barrier integrity. None of these markers of gastrointestinal barrier integrity, zonulin, LBP nor CD14, were significantly increased in acute COVID-19-infected children. Identification of SARS-CoV-2 within the stool, coupled with the loss of tight junctions’ competency seen in MIS-C but not acute COVID-19, suggests that GI sources of SARS-CoV-2 viral components could breach the mucosal barrier and enter circulation.

**Children with MIS-C have SARS-CoV-2 antigenemia.**

Although viremia and antigenemia have been shown to correlate with severe acute COVID-19 in adults(17, 25), viremia has not been detected in MIS-C(11); and antigenemia has not previously been assessed in children. Using Single Molecule Array (Simoa) assays(25), SARS-CoV-2 Spike, S1, and Nucleocapsid antigens were detected in the plasma of children with MIS-C despite being weeks past the initial SARS-CoV-2 infection or exposure. The SARS-CoV-2 Spike protein was significantly elevated in MIS-C patients compared to healthy controls (P < 0.0001) and to children with acute COVID-19 (P < 0.001) (**Figure 2D**). The SARS-CoV-2 S1 protein was also detected at significantly elevated levels in MIS-C patients compared to healthy controls (P = 0.004) and to children with acute COVID-19 (P = 0.02) (**Figure 2E**). The SARS-CoV-2 Nucleocapsid protein was increased in MIS-C patients, although the levels did not reach significance (**Figure 2F**). There was no significant increase in SARS-CoV-2 antigenemia in children with acute COVID-19 as compared to healthy controls. SARS-CoV-2 antigens showed no correlation with age (**Supplemental Figure S1**). Circulating SARS-CoV-2 antigen indicates that viral components leak from infected tissues. We attribute circulating antigen in MIS-C patients to leakage from the GI system, as corroborated by the increased zonulin levels and microbial translocation markers we detected in MIS-C patients.

The SARS-CoV-2 Spike protein, specifically the S1 component(9, 10), has been hypothesized to have superantigen-like properties that are similar to those seen in the bacterial superantigen
mediated toxic shock syndrome. Superantigens bind to specific β-chains of T cell receptors (TCR) at the variable domain in a complementary-determining region 3 (CDR3)-independent manner(26), thereby bypassing TCR specificity and resulting in skewing and overrepresentation of specific T cell receptors. Recent reports have identified TCR Beta Variable (TRBV) gene skewing in MIS-C, with a profound expansion of TRBV11-2(10, 27). Our data reveal a strong correlation between S1 antigenemia and the previously reported TRBV11-2 expression in MIS-C(10) (Pearson correlation, r = 0.89, P = 0.0005) (Supplemental Figure 2). There is no correlation between the Spike or Nucleocapsid antigens and TRBV11-2 expression (Supplemental Figure 2).

Consistent with hyperinflammatory responses, children with MIS-C displayed cytokine storm with significantly elevated levels of IL-1β, IL-6, IL-10, and TNFα (Supplemental Figure 3A-D). Notably, the antiviral cytokine, IFNγ, is also significantly increased in MIS-C patients compared to healthy controls and children with acute COVID-19 (Supplemental Figure 3E). This increase in IFNγ is typical of a viral exposure, however, it can be paradoxically suppressed in severe acute COVID-19 in adults(28). IL-12p70, IL-8, IL-5, and IL-22 were also assessed but were not altered in MIS-C or acute pediatric COVID-19 (Supplemental Figure 3F-I).

**MIS-C immunoprofiles reflect ongoing mucosal exposure to SARS-CoV-2.**

Immunoglobulin IgM, IgG, and IgA subsets targeting SARS-CoV-2 antigens in MIS-C patients were most highly elevated against Spike and S1 proteins (Figure 3A-C; Supplemental Figure 4), corresponding to the antigens most highly detected in the bloodstream of these children. As expected, anti-Spike-IgM is highest in acute COVID-19 reflecting early adaptive immune responses (Figure 3A). However, anti-Spike-IgM remains higher than would be expected given that MIS-C presents weeks after the original infection/exposure to SARS-CoV-2 but is on a
downward trend over time (Figure 3D). As expected, anti-Spike-IgG, anti-S1-IgG, and anti-RBD-IgG are highest in the delayed-onset MIS-C (Figure 3B; Supplemental Figure 4) and remain plateaued over time (Figure 3E). IgA is the immunoglobulin most reflective of mucosal immunity and typically wanes following viral clearance (29). Here, anti-Spike-IgA, anti-S1-IgA, and anti-RBD-IgA are all significantly increased in MIS-C (Figure 3C; Supplemental Figure 4), and anti-Spike-IgA remains unexpectedly elevated for months after the initial SARS-CoV-2 infection (Figure 3F). The persistence of elevated anti-SARS-CoV-2 IgA and IgM in MIS-C patients supports the hypothesis of ongoing viral antigenic exposure and inflammation in the GI mucosal surfaces of children with MIS-C.

Plasma neutralization capacity against SARS-CoV-2 was compared between children with MIS-C and children with acute COVID-19. Interestingly, although children with MIS-C have significantly elevated IgG levels against SARS-CoV-2 in MIS-C relative to children with acute COVID-19, neutralization titers were comparable between children from both groups (Figure 3G). This suggests that in children with MIS-C, increasing quantities of IgG antibodies against the SARS-CoV-2 Spike protein do not result in a gain in neutralizing capacity over time. Ineffective neutralization and poor antigen clearance, combined with on-going viral antigen leakage from GI sources, could partially explain the high levels of antigen detected in MIS-C subjects.

**Temporal kinetics reveal SARS-CoV-2 antigenemia is inadequately contained by humoral responses in MIS-C.**

Both SARS-CoV-2 antigen and immunoglobulins are detectable in MIS-C patients at levels significantly higher than healthy controls. Antigenemia in adults occurs early in the COVID-19 disease course and is associated with pulmonary symptoms (25), whereas in MIS-C, antigenemia is found in the setting of prominent GI symptoms, weeks to months after resolution.
of a SARS-CoV-2 upper respiratory tract infection or asymptomatic infection. To understand the relationship between viral antigenemia and the humoral response, we investigated longitudinal samples from MIS-C patients. First, we assessed antigen over time since MIS-C symptom onset. Previous reports show that in adults with acute COVID-19, SARS-CoV-2 antigens are rapidly cleared as the patient reaches seroconversion(25, 30). In contrast, our studies show that Spike antigen rises over the first few days of MIS-C symptoms and persists for greater than 10 days, occasionally through six months (Supplemental Figure 5), despite seroconversion in anti-Spike-IgG and -IgA antibodies. The high presence of Spike protein in seroconverted patients was not observed in any adult COVID-19 cases(25). We also found that SARS-CoV-2 antigen levels did not significantly decrease following initiation of steroid and/or immunoglobulin replacement (IVIG) therapy in our cohort (Figure 4A), which are the only currently recommended treatments for MIS-C(31). This suggests that current therapies are targeted towards the downstream consequences of MIS-C, namely the inflammatory responses, but fail to address the on-going antigenemia instigating ongoing inflammation. Figure 4B provides a detailed overview of Spike antigenemia and the humoral inflammatory responses in a representative hospitalized child with MIS-C, relative to SARS-CoV-2 exposure, symptom onset, and treatment course.

**Zonulin antagonism reduces Spike antigenemia and cytokine storm, with subsequent improvement of clinical outcome in MIS-C**

While steroids and IVIG do not block leakage of SARS-CoV-2 antigen across the mucosal barrier, therapies targeting GI mucosal permeability could potentially reduce or prevent antigenemia. Larazotide, a zonulin antagonist, is an investigational therapy that has been well characterized in pre-clinical trials(32, 33) with an excellent safety profile(34) and is currently in phase 3 trials for treatment of refractory celiac disease(35). Given this theoretical benefit, we obtained United States Food and Drug Administration (FDA) approval for compassionate use of
larazotide 10mcg/kg every 6 hours to treat a critically-ill 17-month-old boy with MIS-C after he failed to improve with anti-inflammatory therapies. The toddler, who had a complex past medical history including partial duplication of chromosome 14, biliary atresia status post Kasai procedure and gastrostomy tube placement, with frequent episodes of ascending cholangitis, required hospitalization for severe COVID-19, complicated by respiratory failure and cardiac resuscitation. One month after he was diagnosed with COVID-19, he developed abdominal compartment syndrome with a CRP of 286 mg/L, ferritin of 51,223 µg/L, and an NT-proBNP of 16,462 pg/mL without evidence of cardiac injury on echocardiogram. He was treated with IVIG, steroids, and anakinra with transient improvement of inflammatory markers but rapid recrudescence of symptoms. We detected 1,020 copies of SARS-CoV-2 RNA in his stool, as quantified by RT-PCR, and a plasma Spike antigen level of 566 pg/mL over two weeks after initiation of IVIG and steroid treatment for MIS-C. After the initiation of Larazotide, his CRP, which had quickly rebounded to 173 mg/L following withdrawal of anakinra, dropped by 85% from peak values, and Spike antigen dropped by 90% to 59 pg/mL (Figure 5A). The SARS-CoV-2 Nucleocapsid protein also dropped by 98% from 77 pg/mL to 1.45 pg/mL (limit of detection). His fever curve, which also rebounded after discontinuation of anakinra, improved, as did ferritin and d-dimer levels (Figure 5A). He also experienced an improvement in cytokine levels and inflammatory mediators, including IL-1β, IFNγ, IL-2R, and IL-17, representing improvement in inflammasome activation, viral-induced interferon release, and immune cell activation (Figure 5B). These cytokine and inflammatory mediators initially spiked around the development of MIS-C then improved following IVIG but plateaued or rebounded despite being on steroids. An improvement in these inflammatory mediators was seen following the precipitous drop in SARS-CoV-2 antigens, which occurred as a result of or coincident with the initiation of larazotide therapy. Importantly, he achieved his longest stretches without fever since admission, his GI symptoms improved and he was able to resume full feeds, and his ventilatory status improved.
**Discussion:**

Although children were initially considered to be relatively spared from COVID-19, it is now evident that children can become infected with SARS-CoV-2 but only display minimal or absent symptoms during the acute infection stage (11). The prevalence of COVID-19 in children and adolescents has climbed over the past few months (4, 36). As a result, MIS-C, once reported to be a life-threatening but rare late manifestation, is also increasing in frequency. Identifying children early in the disease course of MIS-C, before cardiac complications ensue is critical; a dire need exists to understand the pathogenesis driving MIS-C to treat and prevent this increasingly prevalent disease in children. The data provided here represent the first evidence of prolonged exposure to SARS-CoV-2 in the GI tract of children with MIS-C coinciding with zonulin release indicating loss of mucosal barrier integrity, and SARS-CoV-2 antigenemia, which may be driving the hyperinflammatory responses defining MIS-C (Figure 6). Further, as proof of concept, we show that inhibition of intestinal permeability in a patient with MIS-C prevents SARS-CoV-2 antigens from trafficking into the bloodstream, conferring clinical benefit by addressing the underlying trigger for MIS-C rather than just the inflammatory consequences.

SARS-CoV-2 antigenemia has not previously been reported in MIS-C. Although epidemiologically linked to prior SARS-CoV-2 infection, previous studies have been unable to attribute MIS-C to viremia (11). In contrast, 77% of adults with severe COVID-19 requiring intensive care unit (ICU) admission for respiratory failure were found to have plasma SARS-CoV-2 antigenemia (25), felt to originate from respiratory sources (25). Nucleocapsid and S1 antigens were the most prominent SARS-CoV-2 antigens detected in the blood of adults with severe COVID-19. In children with MIS-C, the Spike and S1 protein are the predominant antigens. The difference in make-up of SARS-CoV-2 antigenemia warrants further investigation, as this could provide insight into differences between MIS-C and severe adult COVID-19.
The S1 subunit of the Spike protein (S1), which is seen at significantly elevated levels in MIS-C, contains the superantigen-like motif that can interact with T cell receptors (TCRs) and major histocompatibility complexes (MHC) II to trigger superantigenic responses leading to skewed TCR repertoire in MIS-C patients with expansion of the T cell receptor beta variable gene(9, 10). Further, the SARS-CoV-2 superantigen-like motif was found to have remarkable sequence and structural similarity to the *Staphylococcus exotoxin* B (SEB) superantigen motif that interacts with both the TCR and CD28(37) and mediates toxic shock syndrome. Here, we show, for the first time, a direct correlation with S1 antigenemia and specific TRBV11-2 skewing seen in MIS-C.

The source of the antigenemia in MIS-C is likely the gut. MIS-C develops days to weeks after resolution of the initial infection, often when SARS-CoV-2 viral levels are low/undetectable in respiratory secretions(11), making the MIS-C trigger less likely to be from upper respiratory tract sources. In adults with COVID-19, SARS-CoV-2 can be detected in the stool(38) and on intestinal biopsies(39), with anti-SARS-CoV-2 IgA levels remained elevated for over 4 months after the acute infection. These findings suggest that the GI tract may serve as a nidus for SARS-CoV-2 with ongoing immune activation(12). Further, increased severity of acute COVID-19 in adults is associated with increased frequency of GI symptoms(40). In our MIS-C patient cohort, the majority of stool samples from children with MIS-C contained SARS-CoV-2 RNA and most MIS-C patients presented with unusually severe GI symptoms, abdominal pain, vomiting and diarrhea, in addition to severe myocardial dysfunction and cardiac shock. Although extensive tissue studies are necessary to provide direct evidence of SARS-CoV-2 infection in the GI tract, these data, in addition to the predominance of severe GI symptoms in MIS-C patients, point to a viral or viral antigenic source that originates from the GI system at the time of MIS-C presentation. Research is needed to better understand what the impact of the
gastrointestinal environment is on the SARS-CoV-2 virus and superantigen-like viral motifs, and why children are more likely to develop antigenemia weeks after the acute infection than adults.

Increased zonulin levels indicate breakdown of intestinal epithelial tight junctions, which may allow the leak of SARS-CoV-2 antigen into the blood stream. Zonulin is a modulator of intercellular epithelial tight junctions; various stimuli, including dysbiosis(41) activate MyD88-dependent zonulin release, allowing zonulin to bind to its target protease activated receptor (PAR)2 and transactivate the epidermal growth factor receptor (EGFR)(42). This triggers downstream signaling that leads to phosphorylation of tight junction proteins, including zonula occludens (ZO)1 and myosin 1c, ultimately causing disassembly of tight junctions and increased paracellular permeability to macromolecules(43). This mechanism has already been described for a variety of other chronic inflammatory diseases(44). In a murine model of Kawasaki disease vasculitis, increased intestinal permeability has been associated with higher levels of circulating IgA and its deposition in cardiovascular lesions(24). Blocking intestinal permeability in this murine model of Kawasaki Disease with the zonulin inhibitor AT1001 (larazotide acetate, currently in phase 3 clinical trial for celiac disease) significantly reduced the development of cardiovascular lesions, suggesting a possible involvement of zonulin in this process(24). A similar mechanistic link between zonulin-dependent Spike protein trafficking from the GI tract into the bloodstream in MIS-C pathogenesis is further supported by our successful use of larazotide acetate in a toddler with MIS-C, described here. Following the initiation of larazotide, blood Spike protein levels decreased by 90%, ameliorating inflammation and improving MIS-C-related symptoms. Similarly, in Kawasaki disease, patients presenting with prominent GI symptoms at the onset of illness are less likely to respond to IVIG therapy alone and thus more likely to develop coronary artery aneurysms(45). Here, we show elevated levels of zonulin in MIS-C indicates increased permeability in the setting of detecting SARS-CoV-2 in the stool.
We also show an increase of translocation of microbial factors in MIS-C, not seen in acute COVID-19 in children, signifying increased breakdown of gastrointestinal barrier integrity in MIS-C. Although it’s possible that these microbial factors contribute to the general inflammatory state of MIS-C, other diseases associated with increased translocation of microbial factors (46, 47), lack the distinct clinical symptomatology and pathology of MIS-C. This suggests the intestinal leakage of the SARS-CoV-2 antigens themselves are responsible for triggering the profound cytokine release and hyperinflammatory response seen in MIS-C.

As zonulin-dependent loss of gut integrity develops in MIS-C but not COVID-19-infected children, this suggests that a chronicity of SARS-CoV-2 dysbiosis in the gut results in a cumulative increase in breakdown of mucosal barrier integrity. Additionally, this suggests that intervening and preventing mucosal epithelial damage early in the course could prevent the development of MIS-C. Larger, prospective studies are needed to better define the development of dysbiosis in MIS-C.

Analysis of the kinetic profile of Spike in MIS-C patients show that antigen leakage increases over time after the onset of MIS-C symptoms. Humoral responses alone are ineffective at controlling elevated levels of antigen in MIS-C, and in fact contribute to the inflammatory profile(5). We show that in MIS-C, antibodies, especially anti-Spike IgM and IgA, continue to rise in response to antigen, suggesting ineffective adaptive immunity plus ongoing antigen release from increased intestinal permeability. This data support previous reports of an expansion of inflammatory antibodies that are generated(5) for multiple non-COVID-19 pathogens, including common coronaviruses, influenza and RSV(11), plus numerous self-antigens(8, 48), which in turn activate monocytes and elicit hyperphagocytosis(5), macrophage activation, and cytokine storm(6, 7). Of note, the assays used in our experiments detect the Spike protein S1-S2 extracellular subunits but cannot not distinguish between live or dead virus. However, viral
antigen can drive hyperinflammatory responses and live virus is not required to induce an immune response.

Importantly, antigen levels persist for days to weeks after hospitalization and treatment for MIS-C in some cases, revealing that current treatments (i.e. steroids and/or IVIG) target inflammatory responses but may not adequately mitigate intestinal permeability and SARS-CoV-2 antigen leak into the blood. This prolonged antigen leak may explain the recrudescence of symptoms in some children and justify the rationale for a prolonged taper of immunomodulatory therapies in MIS-C(49). Additionally, detection of bloodborne antigen and zonulin within the first few days of MIS-C symptom onset could identify those at risk for developing MIS-C. Conversely, increased levels of zonulin in children recently infected with SARS-CoV-2 may identify individuals at increased risk for developing MIS-C. Here, we show the proof of concept that zonulin antagonism directly reduces SARS-CoV-2 antigenemia with abatement of the cytokine storm and subsequent clinical improvement in a single patient. Although clinical trials will be needed to test whether larazotide is effective at treating or preventing MIS-C, this study highlights the proof of concept that zonulin antagonism reduces SARS-CoV-2 antigenemia, inflammatory markers improved, and the patient defervesced and clinically stabilized following initiation of treatment. Diagnostic and therapeutic strategies aimed at targeting intestinal permeability could offer a new avenue for identifying, treating, or preventing MIS-C.

**Conclusion:**

Here, we report compelling evidence that in MIS-C, zonulin-dependent loss of gastrointestinal tight junctions results in SARS-CoV-2 antigenemia, driving a hyperinflammatory immune activation. These data suggest that in MIS-C, the presence of SARS-CoV-2 in the GI tract may lead to local mucosal inflammation, increased zonulin release, and subsequent increased gut permeability allowing SARS-CoV-2 antigens, including the superantigen-like motif of the Spike
protein, to traffic across mucosal barriers and into the bloodstream. The data shown here advances our understanding of MIS-C pathogenesis, revealing potential biomarkers for MIS-C diagnosis, and prospective pathways for treating and preventing MIS-C, all of which are urgently needed for this new and increasingly common life-threatening disease in children.

Methods:

Antigen Simoa assays

SARS-CoV-2 antigen Simoa assays were prepared and performed using commercial antibodies for: spike assays (Sino Biological 40590-T62), S1 assays (Sino biological 40150-D001), and nucleocapsid assays (Sino Biological 40143-R004, 40143-R040), and a custom antibody for S1 as previously described(25). Plasma samples were diluted 8-fold in Homebrew Detector/Sample Diluent (Quanterix Corp.) with Halt Protease Inhibitor Cocktail (ThermoFisher Scientific) and EDTA. Detector antibodies were diluted in Homebrew Detector/Sample Diluent to 0.3 µg/mL, and streptavidin-β-galactosidase (SβG) concentrate (Quanterix) was diluted to 150 pM in SβG Diluent (Quanterix). Antibody-conjugated capture beads were diluted in Bead Diluent, with a total of 500,000 beads per reaction (125,000 S1 beads, 125,000 S2 beads, and 250,000 647 nm dye-encoded helper beads for the S1/S2 multiplex assay, and 125,000 nucleocapsid beads and 375,000 647 nm dye-encoded helper beads for the nucleocapsid assay). All reagents were diluted in plastic bottles that were loaded into the HD-X Analyzer (Quanterix). The assays were performed on an HD-X Analyzer in an automated three-step assay format according to the manufacturer's instructions and as previously described(50). In each assay, capture beads were incubated with the sample for 15 minutes, detector antibody for 5 minutes, and SβG for 5 minutes, with washing steps in between. The beads were then resuspended in 25 µL resorufin-β-galactopyranoside and loaded into the microwell array for imaging. Average enzyme per bead (AEB) and sample concentration values were calculated by the HD-X Analyzer software. All samples were measured in duplicates.
Multiplexed quantitative plasma proteomics

Protein concentration in plasma samples was determined using a BCA assay and 100 µg of plasma per sample was denatured in 6 M guanidinium chloride for 5 min at 95 °C. Disulfide bonds were reduced and free thiols alkylated as previously described(51). Proteins were purified using solid-phase-enhanced sample-preparation (SP3) technology with a 1:1 mixture of hydrophobic and hydrophilic Sera-Mag Speed Beads (GE Life Sciences) essentially as previously described(52) including sequential digestion with Lys-C and trypsin. For multiplexed quantification, peptides were labeled with TMT16 reagents (Thermo Scientific)(53, 54). Samples were then combined to pools of fifteen samples and one bridge sample generated by combining plasma proteome digests prior to TMT labeling and used for quantitative comparison across TMT sets(54). The combined sample pools were fractionated using high pH reversed-phase (HPRP) chromatography as previously described(51). Twelve fractions per samples were analyzed in 3-hour runs via reversed phase LC-M2/MS3 on either an Orbitrap Fusion Lumos or an Orbitrap Eclipse mass spectrometer using the Simultaneous Precursor Selection (SPS) supported MS3 method(55, 56) essentially as described previously(57) and using Real-Time Search when using the Orbitrap Eclipse(58). MS2 spectra were assigned using a SEQUEST-based(59) in-house built proteomics analysis platform(60) using a target-decoy database-based search strategy to assist filtering for a false-discovery rate (FDR) of protein identifications of less than 10 % (61). Searches were done using the Uniprot human protein sequence database (UP000005640)(60). Only MS3 with an average signal-to-noise value of larger than 10 per reporter ion as well as with an isolation specificity(55) of larger than 0.75 were considered for quantification. Zonulin (pre-haptoglobin, HOY300), lipopolysaccharide binding protein and soluble CD14 were quantified. Protein concentration data were normalized as previously described(54). A one-way ANOVA was performed to compare relative protein concentrations between COVID-19, MIS-C and healthy control samples. If the ANOVA showed significance with a p-value
<0.05, a two-sided T-test was performed between paired sample groups to determine which proteins are significantly varied between cohorts.

**TCR immunosequencing:**

TCR immunosequencing and analysis was originally performed at Cedars-Sinai Medical Center, on the MIS-C patients included here, as recently reported (10). Briefly, RNA was isolated from peripheral blood of controls and MIS-C patients and assessed for quality with a bioanalyzer (Agilent). Sequencing of the TCR genes was performed using the QIAseq Immune Repertoire RNA Library Kit (Qiagen) and the NovaSeq6000 system (2 x 250bp, 11M average reads per sample) as reported earlier. For analysis, the MiXCR framework (3.0.8) was used for annotation of TCR rearrangements and clone construction, whereby the default MiXCR library served as reference for sequence alignment and each unique complementarity-determining region 3 (CDR3) nucleotide sequence was defined as one clone. Only productive sequences with a read count of ≥2 were considered for further analysis as detailed (10).

**Immunoglobulin Simoa assays**

SARs-CoV-2 serological Simoa assays for IgA, IgM and IgG against four viral antigen S1, Spike, Nucleocapsid, and RBD were prepared and performed using commercial antibodies: IgG assays (Bethyl Laboratories A80-148B), IgM assays (Thermo Fisher MII0401), IgA assays (Abcam ab214003) as previously described (30). Plasma samples were diluted 4000-fold in Homebrew Detector/Sample Diluent (Quanterix Corp.). Four antigen-conjugated capture beads were mixed and diluted in Bead Diluent, with a total of 500,000 beads per reaction (125,000 of each bead type). Biotinylated anti-human immunoglobulin antibodies were diluted in Homebrew Detector/Sample Diluent to final concentrations of: IgG (Bethyl Laboratories A80-148B): 7.73ng/mL, IgM (Thermo Fisher MII0401): 216ng/mL, IgA (Abcam ab214003): 150ng/mL. Streptavidin-β-galactosidase (SβG) concentrate (Quanterix) was diluted to 30 pM in SβG
Diluent (Quanterix). The serology assay was performed on an HD-X Analyzer (Quanterix) in an automated three-step assay. Average Enzyme per Bead (AEB) values were calculated by the HD-X Analyzer software. AEB values were converted to normalized antibody titers using four calibrators that were included in each HDX run.

Seroconversion classification based upon the early stage classification model trained using an independent panel of 142 positive samples by RT-PCR SARS-CoV-2 and 200 negative pre-pandemic controls. The markers for this model were chosen using a cross-validation step. This cross-validation yielded four markers (IgA S1, IgA Nucleocapsid, IgG Nucleocapsid, and IgG Spike) and exhibited the best performance in the training set. The threshold for a positive test result for the unknown samples was determined based on the cutoff that yielded 100% specificity in the training set(30).

**Cytokine assays**

Cytokines were measured in plasma samples using the CorPlex Cytokine Panel (Quanterix Corp), which included sample diluent buffer. Plasma samples were diluted 4-fold in sample diluent buffer and assays were performed following the CorPlex manufacturer protocols. Each CorPlex cytokine panel kit was analyzed by the SP-X Imaging and Analysis System (Quanterix Corp.).

**Neutralization assay**

The SARS-CoV-2 Spike protein was pseudotyped onto a GFP expression-driving lentivirus reporter vector similar to a previously published protocol(62). In order to improve trafficking to the cell membrane for generation of VLPs, we removed 21 amino acids in the cytoplasmic tail thought to contain a cryptic ER retention signal(63) but left the extracellular domain intact. Plasma samples were heat-inactivated at 58°C for one hour, and a dilution series was created
by performing 5-fold serial dilutions in cell culture medium (DMEM + 10% FBS, 1% P/S) beginning with a 1:50 dilution. Dilutions were then incubated with CoV-2 Spike pseudotyped VLPs for 1 hour at 37°C before seeding modified ACE2-expressing HEK293T cells into the solution. Cells were incubated for 48 hours before trypsinization and fixation (4% paraformaldehyde for 30 minutes) prior to analysis by flow cytometry. ACE2-expressing HEK293Ts were generated by integration of a separate hEf1a-hACE2 lentiviral vector at high MOI prior to FACS for expression of hACE2. hACE2 expression was found to be stable at 95% for over a dozen passages after sorting.

Neutralization capacities for plasma samples were determined through measuring inhibition of GFP production in ACE2 expressing HEK293T cells as a function of plasma dilutions. Relative quantification of GFP+ cells were determined through flow cytometry, and quantities of GFP expressing cells were normalized to the highest dilution of plasma. NT50 was calculated as the dilution corresponding to a normalized signal of 0.5 (i.e. 50% inhibition of GFP expression).

**SARS-CoV-2 Viral RNA detection**

RNA was extracted from 100-200 μL of stool using TRIzol (Fisher Scientific, 15596026) following the manufacturer’s protocol. RNA was isolated from the collected aqueous layer using the RNeasy PowerMicrobiome Kit (Qiagen, 26000-50) and eluted in 50μL of RNase-free water. Viral RNA was quantified as previously reported(64). In brief, Luna Universal Probe One-Step RT-qPCR kit (New England Biolabs) were used, with CDC N1 primers(65) (IDT), targeting the N-gene of SARS-CoV-2: Forward: GACCCAAAATCAGCGAAAT, Reverse: TCTGGTTACTGCCAGTGAATCTG, Probe: FAM-ACCCCGTACGTTGTTGGTGGACC-BHQ1. SARS-CoV-2 synthetic RNA molecules (Twist Bioscience) was included on the same RT-qPCR plate for each run, read out by CFX96 Real-Time Detection System (Bio-Rad). Viral
RNA content in each sample was quantified using the equation RNA Copies = 1,000\times2^{\Delta Ct}, where $\Delta Ct$ is the difference between the cycle threshold of the RNA control and a given sample.

**Statistics**

Multiple comparisons were completed with one way ANOVA with Tukey’s multiple comparisons, dichotomous comparisons used Mann-Whitney t test. Fisher’s exact test and Chi-square were used to analyze categorical differences between groups. P<0.05 was considered significant. All data fittings and statistical tests were performed in Graphpad Prism 9. All figures were plotted in Graphpad Prism 9 and Adobe Illustrator version 2015.

**Study approval**

Participants, including the patient treated with FDA-approved emergency authorization of larazotide, were enrolled in either the Institutional Review Board (IRB)-approved Pediatric COVID-19 Biorepository (MGB #2020P000955) or the Pediatric Biorepository (MGB #2016P000949) at Massachusetts General Hospital after informed consent, and assent when appropriate, was obtained. Blood, nasopharyngeal swabs, oropharyngeal swabs, and stool were collected from children in the Pediatric COVID-19 Biorepository and processed as previously described(66). Demographics, past medical history, and clinical laboratory results were obtained from the medical record. Serum samples were obtained from children enrolled in the Pediatric Biorepository. All procedures were performed in accordance with IRB guidelines. Hospitalized pediatric patients were defined as having MIS-C if they met CDC criteria(65): fever, laboratory evidence of inflammation, evidence of >2 organs affected, plus evidence of SARS-CoV-2 infection (current or recent nasopharyngeal RT-PCR positive for SARS-CoV-2 or positive SARS-CoV-2 serology) or exposure to confirmed or suspected individual with COVID-19 within
the past four weeks. Patients with positive nasopharyngeal SARS-CoV-2 RT-PCR without signs of MIS-C were defined as having acute COVID-19.

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Writing – LY, TG, AFO, AF
Writing – Review & Editing and Supervision: LY, TG, AFO, RL, GC, MA, GA, DRW, AF

Declaration of Interests:
AF is Co-Founder and stockholder of Alba Therapeutics. David Walt has a financial interest in Quanterix Corporation, a company that develops an ultra-sensitive digital immunoassay platform. He is an inventor of the Simoa technology, a founder of the company and also serves on its Board of Directors. Dr. Walt’s and Dr. Fasano’s interests were reviewed and are managed by BWH (DW), MGH (AF) and Partners HealthCare (both) in accordance with their conflict of interest policies.


Table 1: Age and sex were described for all pediatric patients and controls, in addition to clinical features of illness for acute COVID-19 and MIS-C. Significant differences between groups were assessed using one-way ANOVA with Tukey’s multiple comparisons (age); Chi-square (sex); Mann-Whitney t test (days since COVID-19 exposure); Fisher’s exact test (report of respiratory and GI symptoms).

<table>
<thead>
<tr>
<th>Total enrolled (N=100)</th>
<th>MIS-C (n=19)</th>
<th>COVID-19 (n=26)</th>
<th>Controls (n=55)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean years (max, min)</td>
<td>8 (0.17, 21)</td>
<td>14 (2weeks, 22)</td>
<td>12 (2 months, 20)</td>
<td>***</td>
</tr>
<tr>
<td>Sex, number female (%)</td>
<td>6 (32)</td>
<td>13 (50)</td>
<td>31 (56)</td>
<td>ns</td>
</tr>
<tr>
<td>Sex, number male (%)</td>
<td>13 (68)</td>
<td>13 (50)</td>
<td>24 (44)</td>
<td>ns</td>
</tr>
<tr>
<td>Days since COVID-19/exposure, median (max, min)</td>
<td>26 (13, 62)</td>
<td>3 (0, 10)</td>
<td>n/a</td>
<td>****</td>
</tr>
<tr>
<td>Days of MIS-C symptoms, median (max, min)</td>
<td>3 (1, 28)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Reporting respiratory symptoms, number (%)</td>
<td>7 (37)</td>
<td>19 (73)</td>
<td>n/a</td>
<td>*</td>
</tr>
<tr>
<td>Reporting gastrointestinal symptoms, number (%)</td>
<td>17 (89)</td>
<td>7 (27)</td>
<td>n/a</td>
<td>****</td>
</tr>
</tbody>
</table>
Figure 1: Study overview. Timing of sample collection and sample analysis are defined for children with MIS-C or acute COVID-19.
**Figure 2:** A) Plasma zonulin, B) Lipopolysaccharide-binding protein, and C) soluble CD14 quantified by multiplexed mass spectrometry-based proteomics (54, 55), were assessed in children with MIS-C (n=13), COVID-19 (n=21), and non-COVID-19 controls (n=23), compared by ANOVA. D) SARS-CoV-2 Spike, E) S1, and F) Nucleocapsid proteins were quantified from the plasma of children with MIS-C (n=16), children with acute COVID-19 (n=22), and pre-pandemic healthy controls (n=32), compared by one-way ANOVA with multiple comparisons. **** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05. Median values and 95% confidence interval are presented.
Figure 3: Peak values of A) Anti-Spike IgM, B) Anti-Spike IgG, and C) Anti-Spike IgA were quantified from the plasma of children with MIS-C (n=16), children with acute COVID-19 (n=22), and pre-pandemic healthy controls (n=32), compared by one-way ANOVA with multiple comparisons. Time course of D) Anti-Spike IgM, E) Anti-Spike IgG, and F) Anti-Spike IgA were plotted over time following symptom onset for MIS-C. G) The half maximal inhibitory concentrations (IC50) for antibody neutralization for children with MIS-C and children with acute COVID-19 were compared by Mann-Whitney t test. Mean value and standard deviation are presented. **** P<0.0001, ** P<0.01, * P<0.05
Figure 4: A) SARS-CoV-2 Spike, B) S1, and C) Nucleocapsid levels in the plasma of children with MIS-C were quantified before treatment with steroids and/or immunoglobulin replacement therapy, through 14 days following treatment (n=11). Shaded regions signify limit of detection for each specific antigen test. B) Spike levels and Anti-Spike IgM, IgG, and IgA were detailed over the course of illness for a child with MIS-C. Of note, the Spike protein remains above the limit of detection for the Spike antigen test at the 213 day follow-up time point.
Figure 5: Timeline of child treated with larazotide. Hospital course is delineated by hospitalization for acute COVID-19 (Day 0) followed by development of MIS-C (day 39), with treatment courses identified along time course. A) CRP and SARS-CoV-2 Spike antigen levels, median daily temperature curve, d-dimer and ferritin levels throughout hospital course are shown. Light blue box represents normal body temperature. Light grey box highlights days with larazotide treatment. B) Inflammatory cytokines, IL-17F, IL-2R, IFN-γ, and IL-1β, are shown following the development of MIS-C, in relation to treatment courses.
**Figure 6:** Overview of proposed hypothesis driving MIS-C. A) A child is exposed to or infected with SARS-CoV-2. SARS-CoV-2 enters the GI tract. Dysbiosis leads to increased zonulin release and resultant loss of tight junctions. SARS-CoV-2 antigens, especially the Spike protein, breaches the mucosal barrier, entering the blood stream. The superantigen motif of the Spike protein stimulates a pathogenic hyperinflammatory response. B) Overview of cytokine and inflammatory mediator levels in child with COVID-19 followed by the development of MIS-C. Treatments with IVIG, steroids, and larazotide are highlighted.