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ORIGINAL ARTICLE

Autoimmunity and Clinical Immunology

# Notch4 regulatory T cells and SARS-CoV-2 viremia shape COVID19 survival outcome

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## Abstract

**Background:** Immune dysregulation and SARS-CoV-2 plasma viremia have been implicated in fatal COVID-19 disease. However, how these two factors interact to shape disease outcomes is unclear.

**Methods:** We carried out viral and immunological phenotyping on a prospective cohort of 280 patients with COVID-19 presenting to acute care hospitals in Boston, Massachusetts and Genoa, Italy between June 1, 2020 and February 8, 2022. Disease severity, mortality, plasma viremia, and immune dysregulation were assessed. A mouse model of lethal H1N1 influenza infection was used to analyze the therapeutic potential of Notch4 and pyroptosis inhibition in disease outcome.

**Results:** Stratifying patients based on %Notch4$^+$ Treg cells and/or the presence of plasma viremia identified four subgroups with different clinical trajectories and immune

**Abbreviations:** COVID-19, coronavirus disease 2019; Foxp3, forkhead box protein 3; H1N1, Influenza A virus subtype; LDC7559, gasdermin D inhibitor; Notch4, neurogenic locus notch homolog 4; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Treg cell, regulatory T cell.

Mehdi Benamar and Peggy S. Lai contributed equally.

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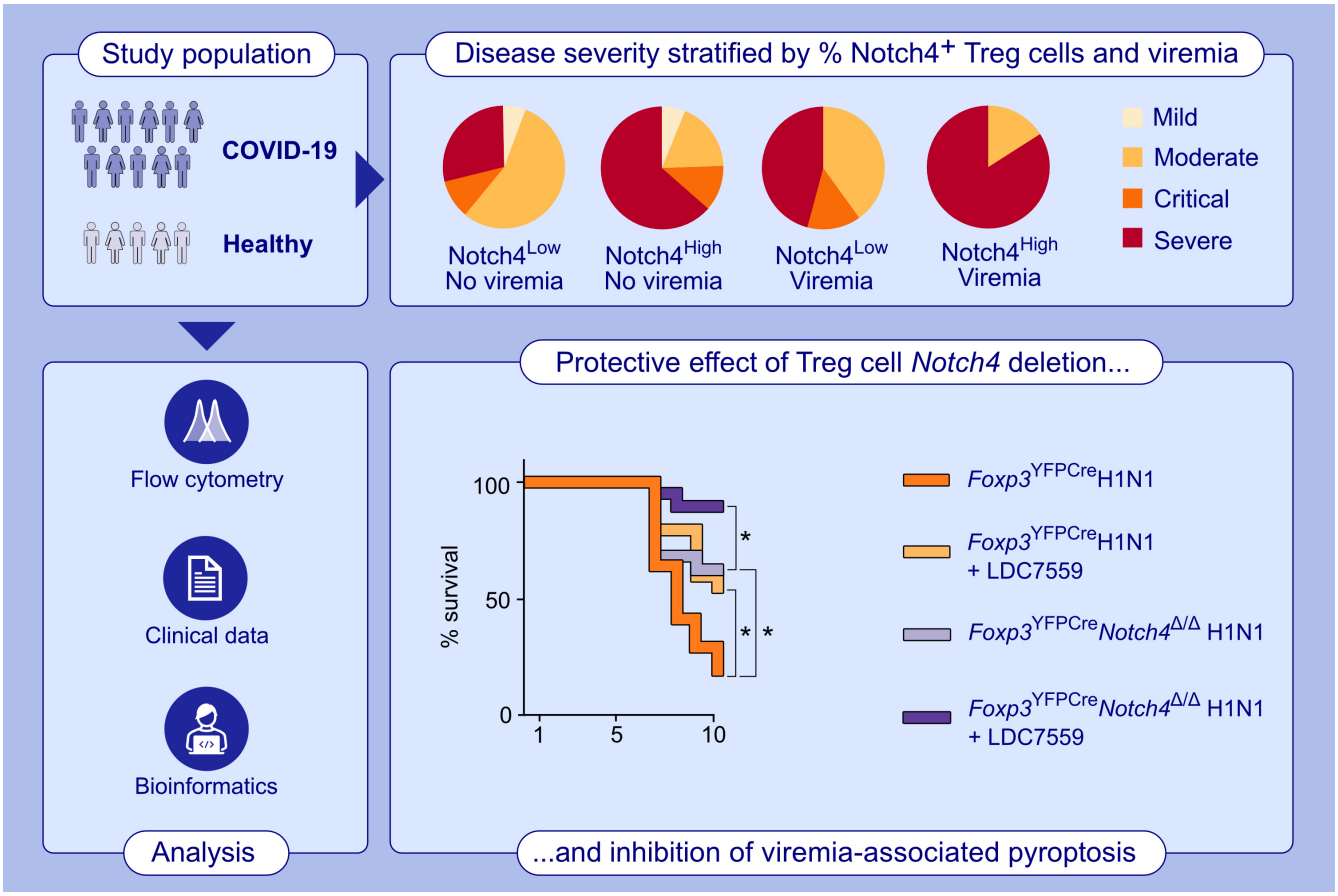
phenotypes. Patients with both high %Notch4$^+$ Treg cells and viremia suffered the most disease severity and 90-day mortality compared to the other groups even after adjusting for baseline comorbidities. Increased Notch4 and plasma viremia impacted different arms of the immune response in SARS-CoV-2 infection. Increased Notch4 was associated with decreased Treg cell amphiregulin expression and suppressive function whereas plasma viremia was associated with increased monocyte cell pyroptosis. Combinatorial therapies using Notch4 blockade and pyroptosis inhibition induced stepwise protection against mortality in a mouse model of lethal H1N1 influenza infection.

**Conclusions:** The clinical trajectory and survival outcome in hospitalized patients with COVID-19 is predicated on two cardinal factors in disease pathogenesis: viremia and Notch4$^+$ Treg cells. Intervention strategies aimed at resetting the immune dysregulation in COVID-19 by antagonizing Notch4 and pyroptosis may be effective in severe cases of viral lung infection.

**KEYWORDS**
COVID19, Notch4, pyroptosis, regulatory T cells, survival, viremia




**GRAPHICAL ABSTRACT**

Notch4 expression on Treg cells and SARS-CoV-2 plasma viremia are associated with increased COVID-19 severity and mortality both independently and especially in combination. Stratification based on %Notch4 Treg cell and viremia identified patient subgroups with distinct clinical and immunological attributes. In a proxy influenza virus infection model, combinatorial targeting of Treg cell Notch4 and viremia-induced monocytic cell pyroptosis protected mice against mortality.

Abbreviations: COVID-19, coronavirus disease 2019; Foxp3, forkhead box protein 3; H1N1, Influenza A virus subtype; LDC7559, gasdermin D inhibitor; Notch4, neurogenic locus notch homolog 4; SARS-CoV-2; severe acute respiratory syndrome coronavirus 2; Treg, regulatory T cell.

## 1 | INTRODUCTION

As of February 2024, over 774 million people have been infected with SARS-CoV-2, the causative agent of COVID-19, with over 7 million resulting deaths worldwide.[1,2] COVID-19 ranges from asymptomatic disease to fatal respiratory failure.[3] Factors associated with disease severity and mortality include underlying clinical factors such as age and medical comorbidities,[4] extent of viral replication,[5–7] serum levels of the acute phase protein pentraxin-3[8–10] and degree of host immune dysregulation.[11] Progress has been made in developing therapies targeted towards COVID-19 infection. Treatments focused on limiting viral replication have shown to be more effective when administered early and prior to hospitalization,[12–14] whereas therapies focused on immune dysregulation appear to be more effective later in the course of disease.[15–17] Antiviral therapies are often administered in conjunction with immune modulators in the inpatient setting, although their efficacy as a monotherapy has been debatable in more severe illness.[18] Despite the increasing number of therapies available for treatment of COVID-19, case fatality rates in critically ill patients with SARS-CoV-2 have remained unchanged over time,[19] highlighting the need for identifying effective therapies in patients with severe COVID-19 infection.

The profound immune dysregulation associated with severe COVID-19, characterized by the emergence of a cytokine storm that contributes to tissue injury in the lungs and other organs suggests the disruption of immune regulatory mechanisms that would normally restrain such an outcome.[20,21] Such a process may also contribute to the pathogenesis of other diseases including sepsis and acute respiratory distress syndrome due to viral or other causes. Regulatory T (Treg) cells play an important role in limiting an exuberant host response to pathogens that may inadvertently result in collateral immune pathology.[22–24] Beyond their role in immune regulation, Treg cells have been shown to play a critical role in facilitating tissue repair.[25–27] Most studies to date of immune dysregulation in COVID-19 have focused on broad surveys of all immune cell subtypes, as the prominent lymphopenia seen with acute COVID-19 infection and the scarcity of biospecimens have impacted the ability to investigate specific immune cell populations. A few studies have shown perturbations in Treg cell populations that are correlated with COVID-19 severity,[28,29] raising the possibility that Treg cells may serve as a potential therapeutic target.[30] To date, proposed Treg cell-based therapies have focused on adoptive cell transfer, which is however hindered by significant tissue engineering challenges in regards to the stability, specificity, and targeting of delivered cells.[31]

We have previously shown that Notch4 expression on circulating Treg cells is associated with disease severity in a small observational cohort of COVID-19 patients and in asthma.[27,32,33] In mechanistic studies performed in murine models, respiratory H1N1 viral infections were found to increase Notch4 expression on lung Treg cells with subsequent increased lung inflammation and reduced production of the tissue repair cytokine amphiregulin. Treatment with a neutralizing anti-Notch4 monoclonal antibody or deletion of Notch4 reversed lung inflammation in mice inoculated with influenza virus without increasing the respiratory viral load.[27] This raised the possibility that Notch4 expression on Treg cells maybe a biomarker of and a contributor to mortality independent of SARS-CoV-2 plasma viremia.

In this paper, we performed a deeper analysis of COVID-19 patients in a bi-national cohort of 280 patients with COVID-19, and found that the clinical trajectory and survival outcome in hospitalized patients with COVID-19 are based on two cardinal factors involved in disease pathogenesis: plasma viremia and Notch4$^+$ Treg cells. Notch4 expression on circulating Treg cells and SARS-CoV-2 plasma viremia are separately and especially in combination associated with increased 90-day mortality independently of baseline comorbidities. Immune analysis revealed that Notch4 and plasma viremia control different arms of the immune response against SARS-CoV-2 infection. Notch4 controls Treg cell suppressive function and amphiregulin production, whereas plasma viremia controls monocytic pyroptosis, a key mechanism implicated in the emergence of the cytokine storm in COVID-19.[34,35] Notch4 and pyroptosis inhibition alone or in combination induce stepwise protection against mortality in a mouse model of lethal H1N1 influenza infection. These results also identify a novel intervention strategy aimed at resetting the immune dysregulation in COVID-19 by antagonizing Notch4 and pyroptosis in severe cases of viral lung infection.

## 2 | RESULTS

### 2.1 | Treg cell Notch4 and viremia are combinatorically predictive of COVID-19 severity and mortality

To dissect the respective roles of immune dysregulation and the viral load in determining disease severity in individuals with COVID-19, we analyzed a binational cohort of 280 patients hospitalized with COVID-19 in Boston, Massachusetts and Genoa, Italy between June 4, 2020 and February 8, 2022 as well as healthy control individuals from the respective sites (study flow diagram, Figure S1). Characteristics of the participants are described in Tables 1 and 2. For the overall cohort, 44.3% of the participants were recruited from Genoa and 55.7% from Boston, the median age was 68 years, 55.0% were male, 81.4% were White, and 15.6% were of Hispanic ethnicity. The average BMI was 28, and the average Charlson comorbidity index score was 4, indicating a moderate burden of baseline comorbidities. The average duration of symptoms prior to seeking medical care was 5 days, and the median time from hospital admission (or first positive RT-qPCR for SARS-CoV-2 for those not hospitalized) to first research blood draw was 5 [interquartile range (IQR) 3–10] days, see Figure S2. 26.8% of participants required ICU admission, 24.3% ultimately required intubation and mechanical ventilation, and 1.4% requiring venovenous extracorporeal membrane oxygenation (ECMO) for refractory respiratory failure. 54.3% of the cohort received remdesivir, 78.6% received corticosteroids, and 8.2% received monoclonal antibodies to interleukin-6 (IL-6). When

**TABLE 1** Characteristics of study population overall and stratified by %Notch4$^{+}$ Treg cells and plasma viremia.

| | All Subjects (280) | Notch4$^{low}$ No Viremia (158) | Notch4$^{low}$ (+) Viremia (47) | Notch4$^{high}$ No Viremia (44) | Notch4$^{high}$ (+) Viremia (31) | *p*-value[a] |
|---|---|---|---|---|---|---|
| Age (yr) | 68.0 [55.0, 77.0] | 66.0 [54.0, 77.0] | 72.0 [61.5, 82.0] | 67.5 [48.8, 77.3] | 68.0 [59.5, 73.0] | .343 |
| Male | 55.0% | 54.4% | 42.6% | 65.9% | 61.3% | .134 |
| Site | | | | | | .002 |
| Boston | 55.7% | 51.9% | 46.8% | 56.8% | 87.1% | |
| Genoa | 44.3% | 48.1% | 53.2% | 43.2% | 12.9% | |
| White race | 81.4% | 84.2% | 80.9% | 84.1% | 64.5% | .076 |
| Hispanic | 15.6% | 14.8% | 6.4% | 25.0% | 20.0% | .090 |
| BMI[b] | 28.0 [24.4, 33.3] | 27.6 [24.2, 31.7] | 27.3 [24.6, 30.3] | 29.8 [25.3, 34.8] | 31.6 [27.4, 36.1] | .052 |
| Symptom (days) | 5.0 [2.0, 9.0] | 5.0 [2.0, 9.0] | 5.0 [2.5, 8.0] | 6.5 [2.0, 9.0] | 7.0 [2.0, 9.5] | .777 |
| Charlson[b] | 4.0 [2.0, 5.0] | 3.0 [1.0, 5.0] | 4.0 [2.00 6.0] | 3.5 [2.0, 5.0] | 4.0 [3.0, 6.0] | .014 |
| SAPS[b] | 25.0 [18.0, 34.0] | 23.0 [18.0, 28.8] | 25.5 [18.5, 34.0] | 27.0 [21.3, 34.0] | 37.0 [23.5, 45.5] | .001 |
| SOFA[b] | 2.0 [2.0, 4.0] | 2.0 [2.0, 4.0] | 3.0 [2.0, 4.0] | 3.00 [2.0, 5.0] | 6.0 [4.0, 8.0] | <.001 |
| ICU | 26.8% | 11.4% | 21.3% | 47.7% | 83.9% | <.001 |
| Hospital LOS | 17.0 [8.0, 28.0] | 11.0 [6.0, 23.0] | 18.0 [10.0, 25.5] | 22.0 [17.0, 29.0] | 27.0 [18.50, 34.0] | <.001 |
| Severity[c] | | | | | | <.001 |
| Mild | 2.9% | 3.8% | 0.0% | 4.5% | 0.0% | |
| Moderate | 42.9% | 56.3% | 36.2% | 20.5% | 16.1% | |
| Severe | 10.4% | 10.8% | 14.9% | 11.4% | 0.0% | |
| Critical | 43.9% | 29.1% | 48.9% | 63.6% | 83.9% | |
| Treatments[b] | | | | | | |
| Remdesivir | 54.3% | 48.7% | 51.1% | 54.5% | 87.1% | .001 |
| Corticosteroids | 78.6% | 74.7% | 89.4% | 68.2% | 96.8% | .004 |
| Anti IL-6 mAb | 8.2% | 9.5% | 6.4% | 6.8% | 6.5% | .857 |
| Any oxygen | 88.6% | 84.8% | 93.6% | 88.6% | 100% | .063 |
| Oxygen ≥15LPM | 32.9% | 19.6% | 44.7% | 43.2% | 67.7% | <.001 |
| LPM | | | | | | |
| NIPPV | 15.7% | 13.3% | 17.0% | 13.6% | 29.0% | .163 |
| Intubation | 24.3% | 9.5% | 19.1% | 43.2% | 80.6% | <.001 |
| ECMO | 1.4% | 0.6% | 0.0% | 2.3% | 6.5% | .066 |
| Vasopressors | 14.3% | 1.9% | 12.8% | 29.5% | 58.1% | <.001 |
| RRT | 5.0% | 2.5% | 6.4% | 6.8% | 12.9% | .084 |
| Anti-coagulation | 6.4% | 6.3% | 6.4% | 6.8% | 6.5% | .999 |
| Insulin Drip | 6.1% | 2.5% | 2.1% | 9.1% | 25.8% | <.001 |
| Absolute lymphocyte count, cells/uL | 1015 [653, 1528] | 1220 [883, 1715] | 790 [600, 1480] | 985 [670, 1400] | 560 [360, 770] | <.001 |
| Treg cells, cells/uL | 1.5 [0.6, 4.2] | 1.9 [0.9, 4.7] | 1.1 [0.5, 2.4] | 1.2 [0.5, 3.5] | 1.3 [0.6, 2.6] | .144 |
| % Notch4 Treg cells | 16.2 [9.5, 25.7] | 12.2 [7.1, 17.3] | 13.4 [9.7, 19.1] | 32.8 [28.6, 39.1] | 29.7 [27.4, 37.3] | <.001 |
| Plasma viremia[d] | 27.9% | 0.0% | 100.0% | 0.0% | 100.0% | <.001 |
| Mortality | | | | | | |
| 30-day | 11.1% | 4.4% | 21.3% | 15.9% | 22.6% | .001 |
| 90-day | 17.5% | 8.8% | 25.5% | 25.0% | 38.7% | <.001 |

*Note*: Continuous data shown as median [IQR]. Categorical data are shown as %.

[a]2-tailed p-value based on Pearson chi-square test for categorical data and Kruskal-Wallis test for continuous data.

[b]Abbreviations. BMI = Body Mass Index. CCI = Charlson Comorbidity Index. SAPS = Simplified Acute Physiology Score II, on hospital admission. SOFA = Sequential Organ Failure Assessment Score, on hospital admission. Anti-IL-6 = anti-interleukin-6 monoclonal antibody, such as tocilizumab. NIPPV = non-invasive positive pressure ventilation. ECMO = Extra-corporeal Membrane Oxygenation. RRT = renal replacement therapy.

[c]Severity defined based on the standardized WHO classification of patients for mild, moderate, severe, and critical. Mild = patients who did not require hospitalization. Moderate = patients who required hospitalization but without significant hypoxia or respiratory distress. Severe = patients who required hospitalization and respiratory rate >30 breathes/min or severe respiratory distress or SpO2 <90% on room air. Critical = patients who required ICU admission for acute respiratory distress syndrome or sepsis.

[d]Proportion of patients with detected SARS-CoV-2 RNA in plasma.

**TABLE 2** Additional laboratory tests and immune cell characterization of study population overall and stratified by %Notch4$^{+}$ Treg cells and plasma viremia.

| | All Subjects (280) | Notch4$^{low}$ No Viremia (158) | Notch4$^{low}$ (+) Viremia (47) | Notch4$^{high}$ No Viremia (44) | Notch4$^{high}$ (+) Viremia (31) | *p*-value[a] |
|---|---|---|---|---|---|---|
| Platelets (x10$^{3}$/μl) | 207.0 [159.0, 255.00] | 211.00 [164.50, 273.75] | 194.00 [141.75, 251.75] | 202.00 [163.00, 250.00] | 203.00 [154.50, 232.50] | .337 |
| D-Dimer (ng/ml) | 1075.00 [693.00, 1721.00] | 958.50 [656.25, 1580.25] | 1176.00 [741.00, 1625.25] | 986.00 [667.50, 1790.50] | 1227.00 [788.00, 2492.00] | .464 |
| LDH (units/L) | 327.50 [251.50, 431.00] | 307.00 [236.00, 376.00] | 312.50 [244.75, 427.25] | 386.00 [288.50, 496.50] | 527.50 [386.75, 689.75] | <.001 |
| Ferritin (ng/ml) | 515.00 [273.00, 993.00] | 439.00 [248.00, 817.00] | 373.00 [259.00, 1107.00] | 709.00 [299.50, 1299.75] | 896.00 [609.00, 1523.00] | .001 |
| CRP (mg/L) | 74.75 [40.85, 131.80] | 70.95 [38.25, 121.25] | 77.20 [40.70, 133.50] | 73.15 [45.75, 149.10] | 120.40 [67.55, 212.85] | .020 |
| ESR (mm/hr) | 43.00 [27.50, 59.00] | 34.00 [22.00, 52.25] | 52.00 [38.00, 82.00] | 50.50 [32.25, 64.75] | 49.00 [32.25, 69.00] | .008 |
| CK (U/L) | 115.50 [60.00, 230.25] | 97.00 [58.50, 211.50] | 100.00 [52.00, 178.00] | 135.00 [67.00, 221.75] | 243.00 [163.00, 444.75] | .001 |
| Bilirubin (mg/dL) | 0.50 [0.40, 0.70] | 0.50 [0.39, 0.70] | 0.52 [0.33, 0.70] | 0.70 [0.51, 0.98] | 0.50 [0.40, 0.60] | .001 |
| % Teff Cells, Activated[b] | 48.10 [36.70, 57.05] | 49.30 [37.10, 59.80] | 47.70 [37.80, 55.10] | 48.80 [36.70, 53.90] | 44.30 [35.55, 53.75] | .405 |
| % Teff Cells, Naïve | 34.15 [23.95, 47.35] | 32.90 [23.10, 46.85] | 38.40 [28.10, 47.15] | 37.20 [27.55, 53.25] | 31.20 [25.08, 42.77] | .281 |
| % Teff cells, Follicular Helper | 0.70 [0.29, 1.71] | 0.56 [0.23, 1.33] | 0.77 [0.30, 1.90] | 0.93 [0.44, 1.78] | 0.88 [0.43, 2.17] | .271 |
| % Treg cells[c] | 3.90 [2.28, 6.92] | 3.80 [2.05, 5.71] | 3.36 [2.20, 5.51] | 3.78 [3.03, 5.97] | 7.45 [3.76, 11.60] | .006 |
| % Treg cells, Naïve | 1.63 [0.46, 5.72] | 1.91 [0.45, 5.83] | 2.83 [0.96, 7.07] | 1.84 [0.83, 4.74] | 0.80 [0.00, 1.18] | .009 |
| % Treg cells, Activated | 83.90 [73.12, 94.10] | 83.10 [72.40, 92.40] | 83.50 [72.40, 89.10] | 86.00 [81.80, 94.90] | 92.40 [67.80, 96.60] | .191 |
| % Treg cells, Follicular | 1.77 [0.41, 5.58] | 1.46 [0.00, 4.29] | 1.82 [0.70, 6.00] | 2.70 [0.66, 7.35] | 4.10 [1.58, 7.43] | .008 |
| % Treg cells, Helios+ | 31.65 [0.71, 50.90] | 36.50 [1.17, 53.40] | 37.50 [11.00, 54.90] | 21.10 [0.49, 40.90] | 21.00 [0.41, 27.40] | .034 |
| % Treg cells, Central Memory | 28.75 [13.93, 43.72] | 28.20 [8.96, 43.80] | 31.70 [17.60, 42.40] | 20.00 [15.80, 41.30] | 38.70 [23.05, 51.60] | .168 |
| % Treg cells, TEMRA | 1.37 [0.33, 8.32] | 1.48 [0.22, 8.31] | 1.75 [0.45, 7.09] | 2.56 [0.65, 10.80] | 0.45 [0.00, 4.17] | .174 |
| % Treg cells, Effector Memory | 61.80 [41.85, 78.65] | 62.75 [40.95, 80.65] | 48.65 [41.55, 74.53] | 66.10 [57.20, 76.10] | 58.50 [51.23, 70.53] | .473 |
| % Teff cells, Central Memory | 23.00 [9.80, 37.25] | 23.10 [6.86, 36.50] | 19.30 [12.80, 31.30] | 16.80 [5.13, 36.30] | 34.10 [22.25, 50.25] | .009 |
| % Teff cells,TEMRA | 5.02 [2.66, 14.80] | 5.50 [2.80, 20.35] | 4.26 [2.32, 8.37] | 7.45 [3.41, 19.20] | 4.71 [2.63, 5.89] | .122 |
| % Teff cells, Effector Memory | 31.40 [21.80, 48.92] | 33.80 [21.60, 52.80] | 30.80 [22.70, 44.80] | 29.40 [23.00, 47.30] | 28.80 [16.85, 43.20] | .615 |
| % Treg cells, CD307c+ | 12.20 [5.67, 23.90] | 11.70 [5.89, 21.60] | 13.20 [3.15, 19.80] | 18.20 [8.71, 33.30] | 9.04 [4.85, 22.40] | .030 |
| % Treg cells, Notch1+ | 5.65 [1.83, 22.20] | 5.62 [1.45, 15.20] | 5.43 [1.88, 33.20] | 9.09 [2.68, 28.00] | 4.23 [2.26, 10.32] | .329 |
| % Treg cells, Notch2+ | 5.04 [2.42, 19.23] | 4.82 [2.48, 14.30] | 6.65 [1.73, 34.40] | 8.57 [2.82, 45.50] | 3.43 [2.30, 5.22] | .022 |
| % Treg cells, Notch3+ | 3.68 [2.23, 6.60] | 3.50 [2.42, 6.59] | 3.56 [1.92, 7.97] | 5.86 [3.58, 14.80] | 3.04 [1.94, 5.06] | .008 |
| % Teff cells, CD307c+ | 2.11 [1.19, 4.67] | 1.83 [1.17, 3.65] | 2.17 [1.10, 3.28] | 5.23 [1.99, 8.81] | 2.09 [1.38, 5.47] | .008 |
| % Teff cells, Notch1+ | 1.61 [0.57, 3.54] | 1.11 [0.46, 3.19] | 1.92 [0.52, 3.46] | 2.45 [1.00, 3.75] | 2.38 [1.23, 4.34] | .006 |
| % Teff cells, Notch2+ | 1.98 [0.95, 4.88] | 1.69 [0.86, 4.18] | 2.06 [0.89, 5.21] | 4.39 [1.37, 9.37] | 1.96 [1.00, 4.14] | .012 |
| % Teff Notch3+ | 0.69 [0.42, 1.17] | 0.66 [0.42, 1.07] | 0.68 [0.40, 1.48] | 0.82 [0.53, 3.32] | 0.72 [0.38, 1.04] | .165 |
| % Teff cells, Notch4+ | 1.54 [0.68, 3.41] | 1.32 [0.55, 3.13] | 1.57 [0.57, 1.85] | 4.78 [1.38, 7.91] | 1.09 [0.88, 3.84] | .001 |

*Note*: Continuous data shown as median [IQR].

[a]2-tailed *p*-value based on Kruskal-Wallis test for continuous data.

[b]CD4 Teff cell subpopulations including T follicular helper cells are calculated as percent of CD4$^{+}$ T cells.

[c]Treg cells are calculated as percent of CD4$^{+}$ T cells. Treg cell subpopulations including T follicular regulatory cells are calculated as percent of total Treg cells.

accounting for the subset of patients with repeated blood draws for immunologic analysis, the median time to the highest %Notch4$^+$ Treg cells was 6 [IQR 3–12] days.

SARS-CoV-2 viremia was detected in 27.9% of patients. In the overall cohort, the average %Notch4$^+$ Treg cell was 16.2%. When we stratified patients based on %Notch4$^+$ Treg cells into high vs. low groups using a cutoff of 24.5%, as determined by an univariate receiver operating characteristic curve analysis for mortality prediction (see Methods), the average %Notch4$^+$ Treg cell value was 13.0% in the low %Notch4$^+$ group (Notch4$^{low}$) whereas it was 31.0% in the high %Notch4$^+$ group (Notch4$^{high}$).

We then asked whether Notch4$^+$ Treg cell frequency or plasma viremia predicted disease severity. Disease severity was assessed using the standardized WHO classification of patients into mild (did not require hospitalization), moderate (requiring hospitalization but without significant hypoxia or respiratory distress), severe (requiring hospitalization and respiratory rate > 30 breathes/min or severe respiratory distress or SpO2 < 90% on room air) and critical (required intensive care unit (ICU) level of care for acute respiratory distress syndrome or sepsis) disease (Table 1). Notch4$^+$ Treg cell frequencies and the plasma viral load were increased in more severe diseases (Figure 1A,B). When compared with healthy controls, %Notch4$^+$ Treg cells were higher in patients with mild COVID-19 ($p < .05$), and even more so in patients with more severe disease (Figure 1A, Kruskal-Wallis $p < .001$, Cuzick trend $p < .001$). Similarly, plasma viral load was higher in patients with more severe COVID-19 disease (Figure 1B, Kruskal-Wallis $p < .001$, Cuzick trend $p < .001$). After stratifying patients by %Notch4$^+$ Treg cell values (Notch4$^{high}$ versus Notch4$^{low}$) and by SARS-CoV-2 viremia ((+) viremia versus no viremia), participants with Notch4$^{high}$ Treg cells and plasma viremia manifested the highest severity of illness and the largest number of complications, whereas participants with Notch4$^{low}$ Treg cells and -no plasma viremia had the lowest severity of illness and the fewest number of organ failures (Table 1, Figure 2A). When comparing patients in the Notch4$^{High}$ (+) viremia group to those in the Notch4$^{low}$/no viremia group, the sequential organ failure assessment (SOFA) score was higher (6 vs. 2), with a higher proportion requiring mechanical ventilation (80.6% vs. 9.5%), ECMO (6.5% vs. 0.6%), vasopressors (58.1% vs. 1.9%), renal replacement therapy (12.9% vs. 2.5%), ICU level of care (83.9% vs. 11.4%), and higher 90-day mortality (38.7% vs. 8.8%). While lymphopenia was noted in all four subgroups, absolute numbers of Treg cells did not vary significantly across groups (Table 1). Plasma viral load decreased in all groups over time when assessed longitudinally throughout the hospital stay, whereas the frequencies of Notch4$^+$ Treg cells increased with prolonged hospital stay in the Notch4$^{low}$ (+) viremia group (Figures S2 and S3).

Analysis of patient mortality revealed 49 deaths occurring within 90 days of hospital admission, resulting in an overall 90-day mortality rate of 17.4%. Exploratory analysis showed that clinical variables such as the Charlson comorbidity index, age, D dimer levels, severity of illness scores such as the severity of disease classification system (SAPS II) and SOFA scores and need for ICU admission or therapies for organ failure (such as high flow oxygen, vasopressors, or renal replacement therapy) were highly correlated with 90-day mortality, as were the proportion of Notch4$^+$ Treg cells and the presence of SARS-CoV-2 viremia (Figure S4). The average % Notch4 + Treg cells was higher in those who died vs. those who survived at 90 days (22.3% vs. 15.8%, $p = 0.002$, Kruskal-Wallis's rank sum test).

Kaplan–Meier and adjusted survival estimates for 90-day mortality are depicted in Figure 1D–G. High %Notch4$^+$ (Figure 1D) and (+) plasma viremia (Figure 1E) were both associated with decreased survival. 90-day mortality was the highest in the Notch4$^{high}$ (+) viremia group at 38.7%, compared to 8.8% in the Notch4$^{low}$ /no viremia group (Figure 1F). In crude unadjusted analyses using Cox proportional hazards model, higher proportions of Notch4$^+$ Treg cells were associated with higher 90-day mortality, as were plasma viremia, age, and the Charlson comorbidity index, and IL-6 (Table 3). The association between %Notch4$^+$ Treg cell expression and 90-day mortality remained statistically significant in Cox proportional hazards model after adjusting for age, gender, time to blood draw, serum level of IL-6, and the Charlson comorbidity index (Figure 1G). Each 10% increase in %Notch4$^+$ Treg cells was associated with an HR of 1.37 (95% CI 1.12–1.67) for death, while the presence vs. absence of plasma viremia was associated with an HR of 2.38 (95% CI 1.29–4.40) (Table 3). The estimates for the association between %Notch4$^+$ Treg cells and 90-day mortality were unchanged when adjusting for lymphopenia (absolute lymphocyte count) in a sensitivity analysis (Table S1). Predicted adjusted 90-day survival probability was lowest for patients in the Notch4$^{high}$/ (+) viremia group at 75.4%, followed by the Notch4$^{high}$/no viremia and the Notch4$^{low}$/(+) viremia groups (84.5 and 86.6%, respectively), and highest for the Notch4$^{low}$/no viremia group (95.5%) (Figure 1G).

To decipher the respective role of Notch4 expression in Treg cells and plasma viremia in the clinical trajectory and immunological features of hospitalized patients with COVID19, we analyzed several clinical and immunological parameters. The Notch4$^{high}$/(+) viremia group had the highest acuity of illness with the highest average SOFA and SAPS II scores and required the highest resource utilization for organ failure including ICU admission, intubation and mechanical ventilation, renal replacement therapy, and vasopressor use (Figure 2A; Table 2). Clinical laboratory markers associated with inflammation and poor outcomes such as CRP, ferritin, and LDH[36] were also highest in this group. Furthermore, the Notch4$^{High}$/(+) viremia group also displayed elevated levels of plasma inflammatory cytokines including IL-6, IP-10/CXCL-10, TNF-α, as well as an exuberant antiviral response including elevated IFN-β and IFN-λ1 (Figure 2A). In comparison, the Notch4$^{low}$ (+) viremia group had lower levels of CRP and less organ failure, with high levels of the anti-inflammatory cytokine IL-10 and lower levels of IL-6, IP-10/CXCL-10, and TNF-α. The Notch4$^{high}$/no viremia group also showed high levels of inflammatory cytokines including IL-1β, whereas the Notch4$^{low}$/no viremia group had the lowest levels of inflammatory cytokines (Figure 2A).

To further decipher the immune dysregulation associated with SARS-CoV-2 infection, we performed a multi-dimensional flow cytometric analysis, which was visualized by T-Distributed Stochastic Neighbor Embedding (tSNE). Results showed that each of the four

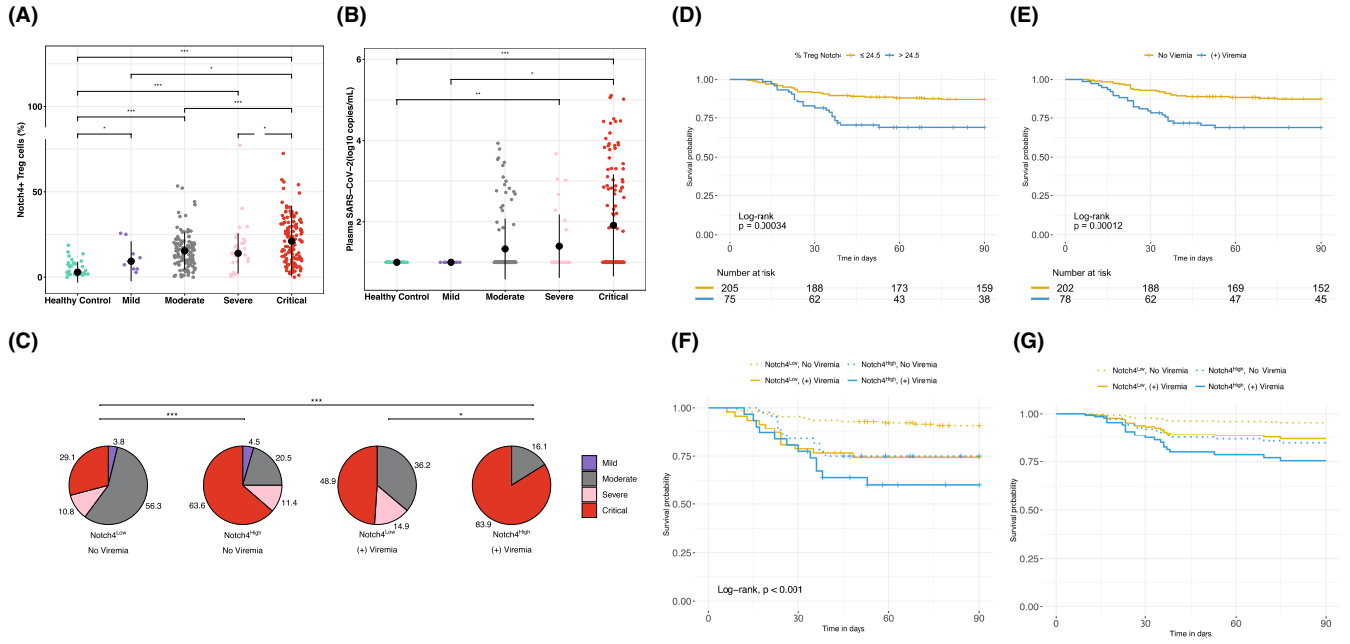



**FIGURE 1** Notch4$^+$ Treg cells and plasma viremia are independently and combinatorially associated with COVID-19 disease severity and mortality. (A, B) %Notch4$^+$ Treg cells among CD4$^+$ T cells and plasma viremia in healthy controls, COVID-19 patients with mild, moderate, severe, and critical illness using the WHO definition for COVID-19 severity. The overall comparison of the Kruskal-Wallis's tests for both A and B were statistically significant ($p < .001$). Only significant pairwise comparisons are included in the figure for ease of interpretation. (C) Pie chart stratified by %Notch4 Treg cells (using a cutoff of 24.5%) and presence of plasma viremia groups. Colors indicate proportion of patients with mild, moderate, severe, and critical disease. The overall Fisher's Exact Test $p < .001$, indicating significant differences between four groups of %Notch4 Treg cells and plasma viremia and COVID-19 severity. Pairwise comparison of Fisher's exact test conducted with a multiple adjustment using Bonferroni correction. Only significant pairwise comparisons are included in the figure for ease of interpretation. (D–F) Kaplan–Meier curves for 90 day survival stratified by discretized %Notch4 Treg (E) by presence or absence of SARS-CoV-2 detected in plasma (F) or by both %Notch4 Treg and plasma viremia. (G) predicted 90-day survival curves based on Cox proportional hazards model adjusting for age, gender, Charlson comorbidity index, IL-6 and time to blood collection. Significance value indicators. NS $p > .05$, $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

groups identified by the combination of Notch4$^+$ Treg cell frequencies and plasma viremia status exhibited a distinct T cell activation profile (Figure 2B,C). Thus, the transition from the Notch4$^{low}$/ no viremia group to the Notch4$^{high}$/(+) viremia group was associated with diminution of the naïve CD4$^+$ Teff cell population and the expansion of the Treg cell central memory compartments (Figure 2B,D). Aside from increased Notch4 expression on Treg cells of Notch4$^{high}$/no viremia and Notch4$^{high}$/(+) viremia groups, expression of other Notch family members, including Notch1, 2 or 3, was either unaffected (Notch1 and Notch3) or minimally changed (Notch2) (Figure 2C,D). tSNE analysis of CD4$^-$ T cell populations showed similar results in terms of decreased naïve and increased central memory cells (Figure S5). Finally, we performed a global analysis integrating all these parameters. To this end, we calculated the Gower's distance between each individual using all clinical, cytokine, and T-cell subpopulations assessed for each patient. Categorizing patients based on high vs. low %Notch4$^+$ Treg cells and presence vs. absence of plasma viremia was a significant explanatory factor for differences in clinical and immunological features among participants (PERMANOVA $R^2$ 0.113, $p < 0.001$, Figure 2E). Together, these results pointed to a step-wise immune dysregulation governed by the contribution of Notch4 and plasma viremia.

Our previous studies have shown that Notch4 inhibits Treg cell suppressive function and amphiregulin production in lung viral infection.[27,33] Moreover, SARS-CoV2 infection has been associated with peripheral monocyte pyroptosis as a function of disease severity.[34] To decipher the combined role of Notch4 and plasma viremia on these parameters, we first analyzed by flow cytometry the amphiregulin production and the Treg cell suppressive capacity in patients from the four respective groups. Both amphiregulin expression and Treg cell suppressive capacity declined as a function of Notch4 expression but not of viremia (Figure 3A–C; see Figure S6A for amphiregulin gating strategy). In contrast, monocyte pyroptosis increased as a function of viremia, as measured by the expression of the pyroptosis effector Gasdermin D,[37,38] but not of Treg cell Notch4 expression (Figure 3D,E; see Figure S6B for Notch4 gating strategy), suggesting that increased Treg cell Notch4 expression and plasma viremia acted to increase disease severity by distinct mechanisms.

To demonstrate the respective roles of Treg cell Notch4 expression and plasma viremia in disease progression and mortality in severe respiratory viral infections, we employed a mouse model of lethal influenza A H1N1 viral infection. Mice with Notch4-sufficient (*Foxp3*$^{YFPCre}$) or deficient (*Foxp3*$^{YFPCre}$*Notch4*$^{\Delta/\Delta}$) Treg cells were infected with a lethal dose of H1N1 while either being sham-treated

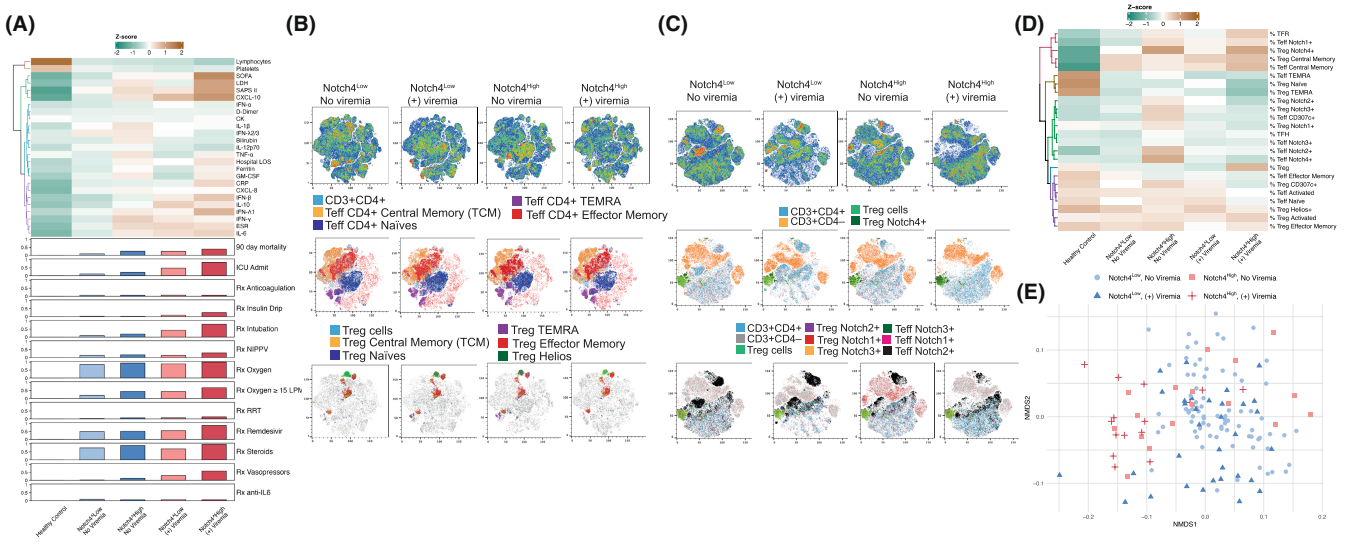



FIGURE 2 Notch4$^+$ Treg cells and plasma viremia define patient subgroups with different Clinical trajectories and immunological features. (A) Clinical variables and plasma cytokine levels in healthy controls and COVID-19 patients stratified by %Notch4 Treg cells and plasma viremia. Colors in heatmap indicate average Z-score values for continuous variables while height of barplot indicates proportion of that group with that outcome or receiving treatment for binary variables. (B) Flow-tSNE plot of CD4$^+$ Teff and Treg TCM (CCR7$^+$CD45RA$^-$), naïve (CCR7$^+$CD45RA$^+$), effector memory (CCR7$^-$CD45RA$^-$) and TEMRA (CCR7$^-$CD45RA$^+$) cells in representative samples. (C) Flow-tSNE plot of Teff and Treg cells, from anti-CD3, CD4, CD25, CD127, Notch1, Notch2, Notch3, Notch4, and FOXP3 staining in representative samples. (D) Heatmap of CD4+ T cell populations in healthy controls and COVID-19 patients stratified by %Notch4 Treg cells and plasma viremia. Colors indicate average Z-score values for that group. (E) Non-metric Multi-dimensional Scaling (NMDS) plot of Gower's distance using clinical variables, plasma cytokine levels, and flow cytometry of CD4+ T cells. Categorizing patients by Treg %Notch4 and plasma viremia explains differences in clinical and immunological features between patients (PERMANOVA $R^2$ 0.113, $p$ <.001). Each symbol represents one patient.

TABLE 3 %Notch4 and plasma viremia are independently associated with 90-day mortality.

| | Univariate[a] | | | Multivariate[b] | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *p*-value[c] | HR | 95% CI | *p*-value[c] |
| % Notch4 Treg cells[d] | 1.36 | (1.14, 1.62) | .001 | 1.37 | (1.12, 1.67) | .002 |
| Plasma viremia[e] | 2.85 | (1.62, 5.00) | <.001 | 2.38 | (1.29, 4.40) | .005 |
| Age (year) | 1.06 | (1.04, 1.09) | <.001 | 1.03 | (1.00, 1.07) | .046 |
| Charlson[f] | 1.39 | (1.25, 1.55) | <.001 | 1.23 | (1.05, 1.45) | .010 |
| Male gender | 0.99 | (0.57, 1.74) | .978 | 0.90 | (0.49, 1.63) | .726 |
| IL-6[g] | 1.00 | (1.00, 1.00) | <.001 | 1.00 | (1.00, 1.00) | <.001 |
| Time to blood collection | 1.01 | (0.97, 1.06) | .502 | 1.02 | (0.98, 1.07) | .372 |

[a]Results from univariate Cox proportional hazards model assessing one variable at a time.

[b]Results from best fitting Cox proportional hazards regression model.

[c]Two-tailed *p*-value for hazard ratio (HR).

[d]HR associated with each 10% increase in % Notch4 expression on Treg cells. Average difference between Notch4$^{high}$ and Notch4$^{low}$ groups is 20%. % Notch4 Treg modeled as a continuous variable.

[e]HR associated with presence versus absence of viremia. Plasma viremia modeled as a binary variable denoting presence or absence of SARS-CoV-2 RNA detected in plasma.

[f]Abbreviation: Charlson, Charlson comorbidity index.

[g]HR associated with each 10% increase in IL-6.

or treated with a pyroptosis inhibitor LDC7559.[39,40] Results revealed that either Treg cell-specific *Notch4* deletion or pyroptosis inhibition provided intermediate protection against H1N1 mortality (Figure 4A). Importantly, the combination of Treg cell *Notch4* deletion and pyroptosis inhibition provided near complete protection against disease lethality (Figure 4A). To enable concurrent immunological

analyses of the respective groups, we employed a mouse model of sublethal H1N1 infection. Results revealed that cumulative treatment with the pyroptosis inhibitor LDC7559 and Treg cell-specific Notch4 inhibition induces a step-wise decrease in disease severity as shown by reduced weight loss (Figure 4B). This protection is associated with a reduction of cleaved Gasdermin D, the pyroptosis effector protein, in monocytic cells (Figure 4C; see Figure S6C for Gasdermin D gating strategy).[37,38] Expression of the inflammasome protein NLR family pyrin domain containing 3 (NLRP3), which is normally upregulated by inflammatory cytokines, was also decreased upon pyroptosis inhibition, a reflection of decreased inflammation in the pyroptosis inhibitor-treated mice (Figure 4D).[41–44] While both Notch4 deletion and pyroptosis inhibition decreased IFNγ production by $CD4^+$ and $CD8^+$ Teff cells and by innate lymphoid cells type 1 (ILC1), superior IFNγ inhibition was obtained with combinatorial therapy (Figure 4E–H and Figure S7). Similarly, combinatorial therapy was more effective in suppressing IL-17 production by $CD4^+$ and $CD8^+$ Teff cells, while LDC7559 treatment was sufficient to reduce IL-17 production by ILC3 (Figure 4E–H and Figure S7). Several studies have shown that IL-17 and IFNγ are involved in COVID-19 severity and poor outcomes.[45–49] These results indicated that combined Notch4 and pyroptosis antagonism increased disease tolerance by protecting against mortality at a given viral inoculum.[50]

## 3 | DISCUSSION

In a large prospective binational study of adult patients with COVID-19, we demonstrate that disease severity and outcome are determined by two key factors, namely immune dysregulation reflected by Notch4 expression on Treg cells and plasma viremia. Both factors were associated with increased disease severity and 90-day mortality independent of each other and also of other comorbidities, while their combination predicted the worst outcomes in terms of disease severity and survival. Furthermore, the four subgroups of patients defined by their stratification based on their %Notch4 Treg cell expression and presence or absence of SARS-CoV-2 plasma viremia differed significantly in their clinical trajectories and immune responses. By identifying different patient subgroups at risk for severe disease and fatal outcomes based on these two factors, our results point to potential interventions that may positively impact host tolerance without impairing viral clearance. In particular, the demonstration that combinatorial targeting of Treg cell Notch4 and the viremia-associated monocytic cell pyroptosis led to near complete protection against mortality in an otherwise lethal H1N1 influenza infection model provides a potentially novel approach to the management of immune dysregulation in hospitalized patients with severe respiratory viral infections including COVID-19.

Accumulating evidence has emphasized the role of immune dysregulation in COVID-19 disease pathogenesis. Early surveys of hospitalized patients with COVID-19 identified pronounced abnormalities in immune cell populations with severe depletion of T cells, increased numbers of monocytes and neutrophils, and elevated levels of inflammatory cytokines such as IL-6 and IL-1β.[11] While elevated levels of Type I interferons were observed signifying the importance of an effective antiviral response,[51,52] persistent elevations of cytokines involved in the response to fungi or extracellular bacteria were also detected, suggesting some degree of immune "misfiring" in patients with severe disease.[11] In this context, our results confirm the critical function of $Notch4^+$ Treg cells in immune dysregulation, both in the absence or in the presence of systemic viremia. Increased $\%Notch4^+$ Treg cells in COVID-19 subjects was associated with loss of Treg cell suppressive function and decreased amphiregulin production, consistent

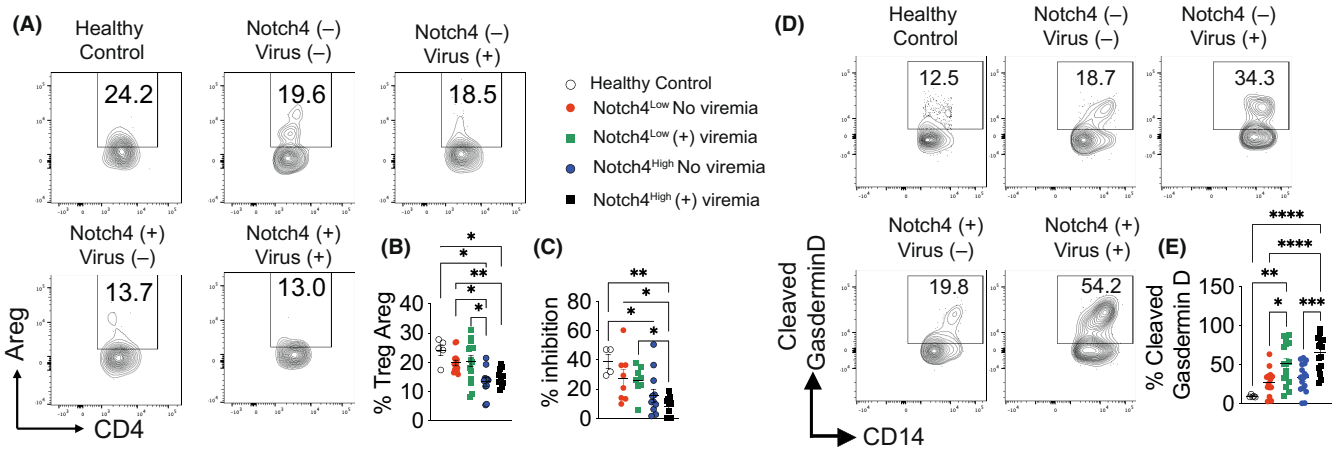



FIGURE 3 $Notch4^+$ Treg cells and plasma viremia associate with distinct immunological responses. (A, B) Flow cytometry analysis (A) and cell Frequencies of Areg+ Treg cells (B) in circulating Treg cells from Healthy control ($n=5$) and COVID-19 patients with $Notch4^{Low}$no viremia ($n=14$), $Notch4^{high}$no viremia ($n=15$), $Notch4^{Low}$(+)viremia ($n=11$) and $Notch4^{high}$(+) viremia ($n=11$), (C) In vitro suppression assay of $CD4^+$ T cells ($T_{eff}$) by $T_{reg}$ cells from COVID-19 patients stratified by %Notch4 Treg cells and plasma viremia. D,E. Flow cytometry analysis, (D) and cell Frequencies of Cleaved Gasdermin D, (E) in circulating monocytes from Healthy control ($n=5$) and COVID-19 patients with $Notch4^{Low}$no viremia ($n=14$), $Notch4^{high}$no viremia ($n=16$), $Notch4^{Low}$(+)viremia ($n=17$) and $Notch4^{high}$(+) viremia ($n=16$). Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett's post hoc analysis (B, C,E) $^*p<.05$, $^{**}p<.01$.

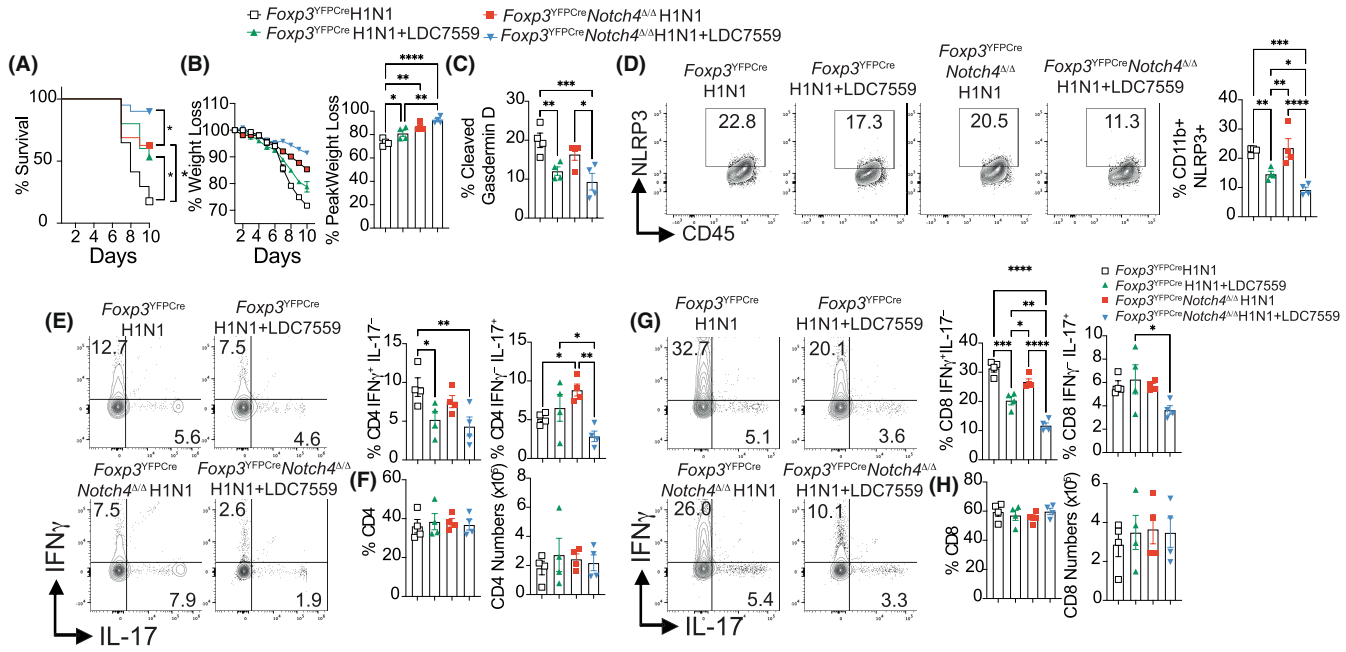



**FIGURE 4** Protective effect of Treg cell Notch4 and pyroptosis inhibition in severe lung virus infection. (A) Survival of *Foxp3*$^{YFPCre}$ or *Foxp3*$^{YFPCre}$*Notch4*$^{\Delta/\Delta}$ mice infected with a lethal dose of H1N1 virus that were either sham-treated ($n=17$) or treated ($n=20$) with LDC7559, as indicated. (B) Weight indices of *Foxp3*$^{YFPCre}$ or *Foxp3*$^{YFPCre}$*Notch4*$^{\Delta/\Delta}$ mice infected with a sublethal dose of H1N1 virus that was either sham-treated ($n=4$) or treated ($n=4$) with LDC7559, as indicated. (C) Cell frequencies of cleaved Gasdermin D expression in lung tissue CD11b$^+$ of the respective mouse groups (n=4). (D) Flow cytometric analysis and cell frequencies of NLRP3$^+$ expression in lung tissue monocytes of respective groups infected with a sublethal dose of H1N1 virus ($n=4$). (E) Flow cytometric analysis and cell frequencies of IFNγ and IL-17 expression in lung tissue CD4$^+$ Teff cells of respective groups infected with a sublethal dose of H1N1 virus ($n=4$).(F) Cell frequencies and numbers of lung tissue CD4$^+$ Teff cells of respective groups infected with a sublethal dose of H1N1 virus ($n=4$). (G) Flow cytometric analysis and cell frequencies of IFNγ and IL-17 expression in lung tissue CD8$^+$ Teff cells of respective groups infected with a sublethal dose of H1N1 virus ($n=4$). (H) Cell frequencies and numbers of lung tissue CD4$^+$ Teff cells of respective groups infected with a sublethal dose of H1N1 virus ($n=4$). Each symbol represents one mouse. Numbers in flow plots indicate percentages. The results represent a pool of 2–4 experiments. Error bars indicate SEM. Statistical tests: Log-rank-test (A), One-way ANOVA with Dunnett's post hoc analysis (B, C, D, E) $*p<.05$, $**p<.01$, $***p<.001$, $****p<.0001$.

with their impaired homeostatic regulatory and tissue repair functions. These functions were spared in patients with isolated viremia, indicative of non-overlapping mechanisms of pathogenesis mobilized by Notch4 and systemic viremia, respectively. Moreover, monocyte pyroptosis increased as a function of viremia but not of Notch4 expression in Treg cells. These findings are particularly relevant to patients who, following the initial viral infection, continue to manifest immune dysregulation either in the absence or in the presence of systemic viremia, as their disease may not be responsive to anti-viral therapy alone. Currently, it remains unclear whether patients with elevated Notch4$^+$ Treg cells, either isolated or in combination with persistent viremia, are at increased risk for long-term complications of COVID-19 as a function of ongoing immune dysregulation. Future studies focused on patients with "Long COVID-19" would help address this issue.

Because hospitalized patients with COVID-19 have features similar to those seen in cytokine storm,[20,53] several clinical trials have focused on immunomodulators as a therapy. Of these, systemic corticosteroids in patients requiring supplemental oxygen have shown the most consistent benefit although a recent meta-analysis of randomized trials showed that mortality rates remain high at 32.7% in critically ill patients despite corticosteroid treatment.[15] Other trials have evaluated more targeted immunosuppressive agents such as IL-6 pathway inhibitors,[16,54] Janus kinase (JAK) inhibitors,[17,55] and IL-1 pathway inhibitors[56,57] with more modest effects. Part of the challenge with the use of immunomodulators is the potential adverse impact on the control of viral replication.[58] Many studies have shown that the detection of SARS-CoV-2 RNA in the blood is associated with increased mortality.[5,59] Early in the pandemic, there was controversy regarding the use of corticosteroids for COVID-19 given prior evidence that early steroid use in severe influenza virus infection leads to increased mortality.[60] Thus, the current paradigm for the treatment of COVID-19 focuses on administration of antivirals shortly after symptom onset when viral replication is thought to peak, with administration of immunomodulators later in the disease course when immune dysregulation is hypothesized to play a larger role in disease progression.[61] However, the time scale and trajectory of each patient is different and impacted by underlying risk factors such as genetics[51] and underlying immunocompromise,[62] making it difficult to determine for any given patient whether viral replication or immune dysregulation is contributing to disease progression at the time of hospital presentation. The ideal immunomodulator should not impair control of viral replication.

Existing studies on Treg cells in COVID-19 have focused on descriptive profiling to discover perturbations compared to either healthy controls, patients with other respiratory infections, or comparisons of COVID-19 patients with different degrees of severity.[28,29,63] These studies were limited by small sample sizes and were not powered to detect associations with mortality. In prior mechanistic studies using murine models, we defined an IL-6-dependent pathway that subverts lung Treg cells to promote tissue inflammation by increasing Treg cell expression of the receptor Notch4; this pathway is activated in response to environmental exposures and respiratory viral infections[27,33] and can be rescued by either Notch4 antagonism with a neutralizing monoclonal antibody, or by the administration of the tissue-protective cytokine amphiregulin.[25,27] It remained unclear how viral replication vs. immune dysregulation individually contributed to disease outcome in a human cohort as we were under-powered to do so and did not assess for plasma viremia. It was also unknown whether Notch4 antagonism would alleviate mortality in a humanized mouse model of respiratory virus-induced immune dysregulation. We now extend our findings using a hypothesis-driven approach to a large observational study of 280 patients with mild, moderate, severe and critical COVID-19 to show that after accounting for the presence of plasma viremia, plasma IL-6 levels, underlying medical comorbidities, age, and sex, Notch4 expression on Treg cells remains a strong predictor of 90-day mortality. These results are novel in that they define the clinical trajectory and disease outcome in hospitalized patients with COVID-19 based on the interplay of two cardinal factors in disease pathogenesis: viremia and immune dysregulation as reflected in Notch4$^+$ Treg cells. They also identify a novel intervention strategy aimed at resetting the immune dysregulation by antagonizing Notch4 and pyroptosis.

There are a few limitations to this study. Although we show the potential role of Notch4 Treg cells as a target of intervention, to demonstrate the additional role of Notch4 Treg as a biomarker for mortality prediction, a much larger sample size with replication cohorts would be needed for such a biomarker study. Due to feasibility issues, blood samples were collected weekly rather than at a finer time scale while patients were hospitalized, which meant that patients with longitudinal samples were those who required longer hospital length of stays. This limitation precluded a more granular description of the temporal dynamics of Notch4 Treg cell expression and viral load on disease outcome. Sequencing was not performed to identify the SARS-CoV-2 variant due to budgetary constraints; our sample size would not allow for stratified analysis based on variant. However, the majority of participants were recruited early in the pandemic (53.6% recruited in 2020, 45.4% in 2021, and 1.0% in 2022) thus the variants present in our study population likely represent those prior to the Omicron and later variants.

In conclusion, elevated circulating Notch4$^+$ Treg cells either alone or in combination with plasma viremia are associated with increased disease severity and 90-day mortality in patients with COVID-19. Treatment with a neutralizing monoclonal antibody against Notch4 in a humanized mouse model of influenza leads to improved survival.[27] Stratification of patients based on Notch4 Treg cell expression and presence of plasma viremia identifies subgroups of patients with different clinical trajectories and immune phenotypes. This study also identifies novel intervention strategies aimed at resetting the immune dysregulation in COVID-19 by antagonizing Notch4 and pyroptosis in severe cases of viral lung infection. Future studies should be aimed at examining the efficacy and safety of an anti-Notch4 monoclonal antibody and pyroptosis inhibition in modulating disease severity and mortality in patients with severe respiratory viral infections.

## AUTHOR CONTRIBUTIONS

M.B., P.S.L., R.D.P and T.A.C. conceived the project and designed experiments. M.B., P.S.L., F.B.O, Q.C, P.C., J.F, M.W., M.N.G, T.M.C.F., H.H., performed experiments R.D.P. and T.A.C. supervised the experimental studies. P.S.L. and R.D.P. supervised patient recruitment and sample collection at the respective centers. D.O., L.M. C.V., S.L., L.M., A.G., M.K, J.V., and H.-Y. S., collected and entered patient samples. L.B. and M.G. provided additional patient samples. E. C. set up the institutional review board protocol at the Boston Children's Hospital and W.P. provided feedback, scientific and funding support. P.S.L, C.A., and C.-Y.H. analyzed clinical data. P.S.L., R.D.P., obtained institutional review board approvals at the respective centers. M.B., and T.A.C. wrote the manuscript.

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## CONFLICT OF INTEREST STATEMENT

T.A.C., H.H. M.B., P.S.L., P.C. and R.D.P. are inventors on provisional patent application US 63/038,186 titled "Methods and Compositions for treating coronavirus infectious disease." H.H. and T.A.C. are co-founders of and hold equity in Alcea Therapeutics.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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