

Supplemental information

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Supplementary Materials

Notch4 signaling limits regulatory T cell mediated tissue repair and promotes severe lung inflammation in viral infections

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Supplementary Figure Legends.

Fig. S1. Expression of Notch1, Notch2 and Notch3 on circulating Treg cells of control and COVID-19 subjects. Related to Figure 1. **A-C** Flow cytometric analysis and graphical representation of Notch1 (A), Notch2 (B) and Notch3 (C) expression in Treg cells of control and COVID-19 subject groups. Each symbol represents one subject. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett's post hoc analysis.

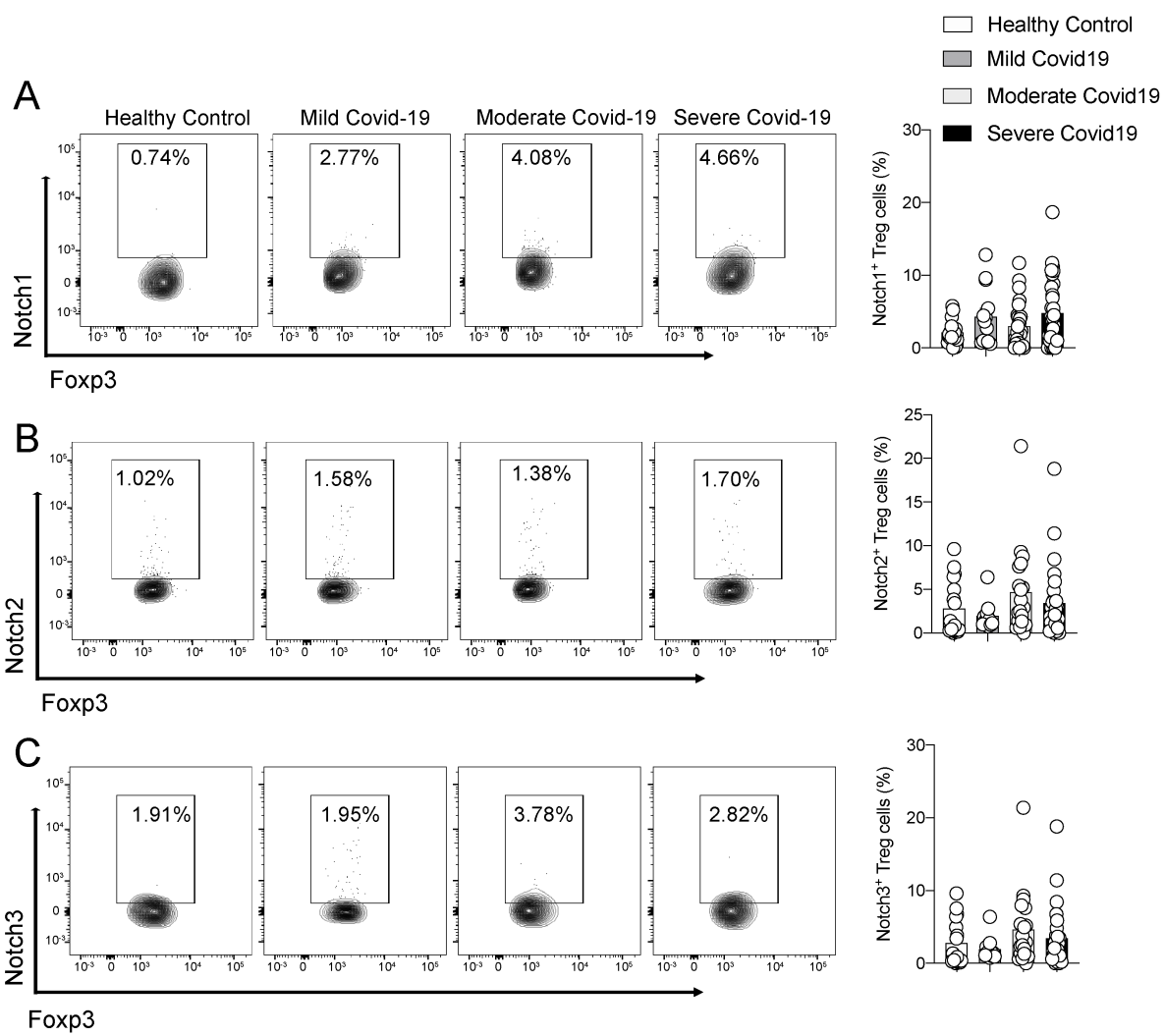


Fig. S2. Impact of Treg cell-specific deletion of different Notch, Hippo and Wnt pathway components on poly I:C-induced lung injury. Related to Figure 2. **A, B,** Weight index (A) and peak weight loss (B) of *Foxp3*^{YFPCre}, *Foxp3*^{YFPCre}*Rbpj*^{Δ/Δ}, *Foxp3*^{YFPCre}*Notch1*^{Δ/Δ}, *Foxp3*^{YFPCre}*Notch2*^{Δ/Δ} and *Foxp3*^{YFPCre}*Notch3*^{Δ/Δ} mice either sham-treated or treated with poly I:C, as indicated. **C,** AHR in the respective mouse groups in response to methacholine. **D-F,** Graphical representation of lung tissue neutrophils (D) and M1 (E) and M2 macrophages (F). **G, H,** Flow cytometric analysis (G) and graphical representation (H) of Yap and β-Catenin in lung tissue Treg cells of *Foxp3*^{YFPCre} mice that were either sham-treated or treated with poly I:C. **I, J,** Weight index (I) and peak weight loss (J) of *Foxp3*^{YFPCre}, *Foxp3*^{YFPCre}*Yap1*^{Δ/Δ}*Wwtr1*^{Δ/Δ}, *Foxp3*^{YFPCre}*Ctnnb1*^{Δ/Δ} and *Foxp3*^{YFPCre}*Yap1*^{Δ/Δ}*Wwtr1*^{Δ/Δ} *Ctnnb1*^{Δ/Δ} mice either sham-treated or treated with Poly I:C, as indicated. **K,** AHR in the respective mouse groups in response to methacholine. **L-N,** Graphical representation of lung tissue neutrophils (L) and M1 (M) and M2 (N) macrophages. Each symbol represents one mouse (n=5 per group). Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: Two-way ANOVA with Sidak's post hoc analysis (**A,C,I,K**); One-way ANOVA with Dunnett's post hoc analysis (**B,D,E,F,J,L,M,N**);. **P<0.01, ***P<0.001, ****P<0.0001.

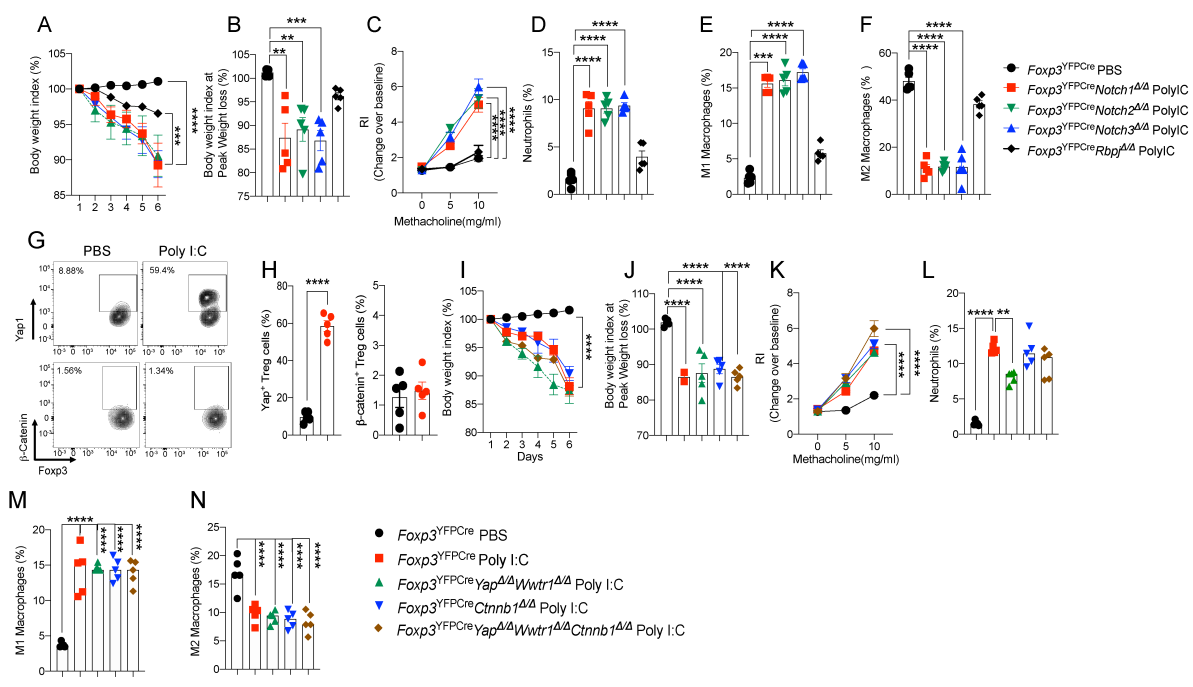


Fig. S3. Treg cell-specific deletion of *Il6ra* partially protects against Poly I:C-induced lung injury. Related to Figure 2. **A**, BAL concentrations of IFN γ , TNF α , IL-12p70, IL-1 β , IFN β , IL-10, IL-1 α , MCP-1, IL-27, GM-CSF, IL-17, OSM, LIF and CXCL5 in PBS or Poly I:C-treated *Foxp3*^{YFPCre} and *Foxp3*^{YFPCre}*Notch4* ^{Δ/Δ} mice, the former also co-treated with either isotype control or anti-Notch4 mAb. **B**, Weight index (A) and peak weight loss of *Foxp3*^{YFPCre} and *Foxp3*^{YFPCre}*Il6ra* ^{Δ/Δ} mice either sham-treated or treated with Poly I:C, as indicated. **C**, AHR in response to methacholine. **D, E**, Flow cytometric analysis (D) and graphical representation (E) of Notch4 expression in lung tissue Treg cells of *Foxp3*^{YFPCre} and *Foxp3*^{YFPCre}*Il6ra* ^{Δ/Δ} mice that were either sham-treated or treated with Poly I:C. **F-H**, Flow cytometric analysis and graphical representation of lung tissue neutrophils (F) and M1 (G) and M2 macrophages (H). Each symbol represents one mouse (n=5 per group). Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: Two-way ANOVA with Sidak's post hoc analysis (**A,C**); One-way ANOVA with Dunnett's post hoc analysis (**B,E,F,G,H**); **P<0.01, ****P<0.0001.

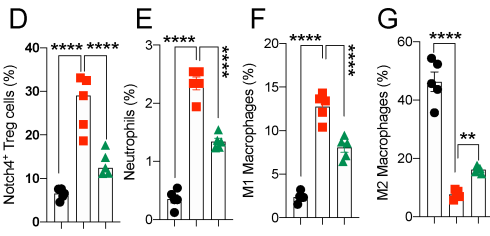
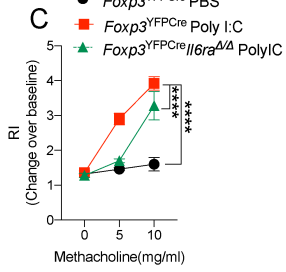
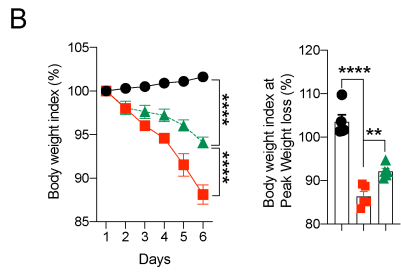
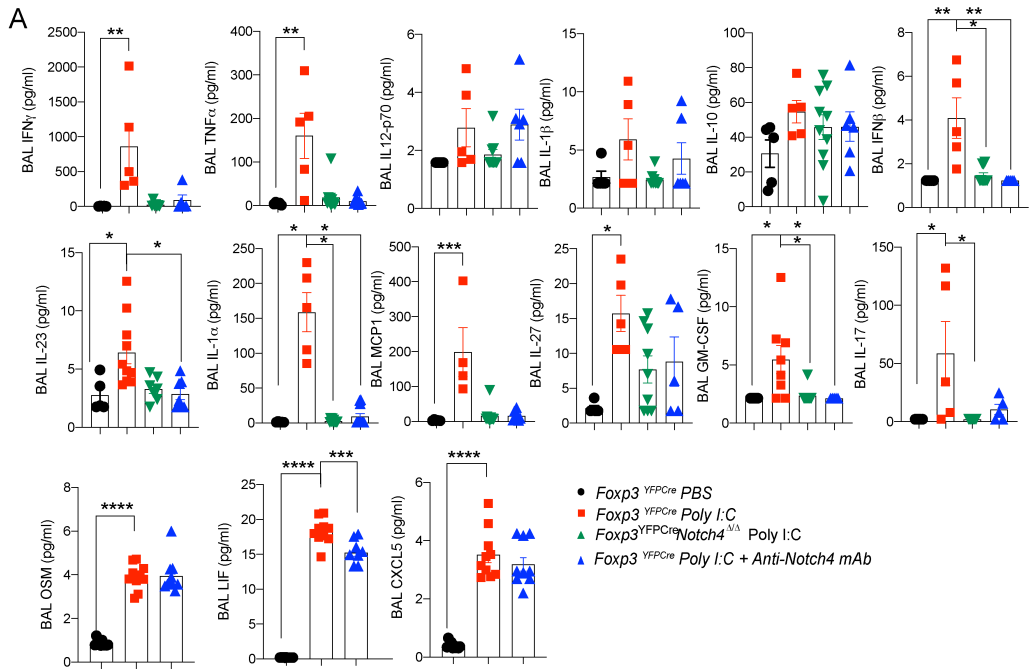


Fig. S4. Protective effect of Notch4 inhibition in influenza A H1N1 virus infection.

Related to Figure 3. **A**, Flow cytometric analysis (A) and graphical representation of lung neutrophils in the indicated mouse groups. **B**, Flow cytometric analysis and graphical representation of lung M2 macrophages in the indicated mouse groups. **C**, Flow cytometric analysis and graphical representation and graphical representation of lung M1 macrophages in the indicated mouse groups. **D**, BAL concentrations of IFN γ , TNF α , IL-12p70, IL-1 β , IFN β , IL-10, IL-1 α , MCP-1, IL-27, GM-CSF, IL-17, OSM, LIF and CXCL5 in PBS or H1N1-infected *Foxp3*^{YFPCre} and *Foxp3*^{YFPCre}*Notch4* ^{Δ/Δ} mice, the former also co-treated with either isotype control or anti-Notch4 mAb. Each symbol represents one mouse (n=5 per group). Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett's post hoc analysis (**A-D**). **P<0.01, ***P<0.001, ****P<0.0001.

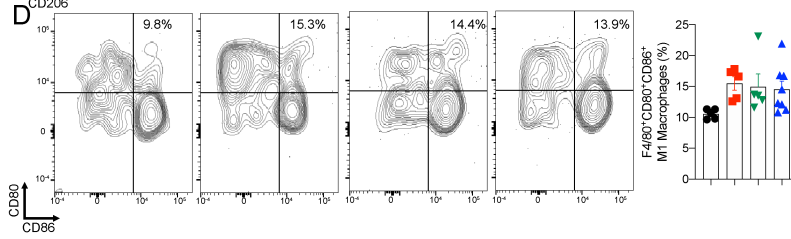
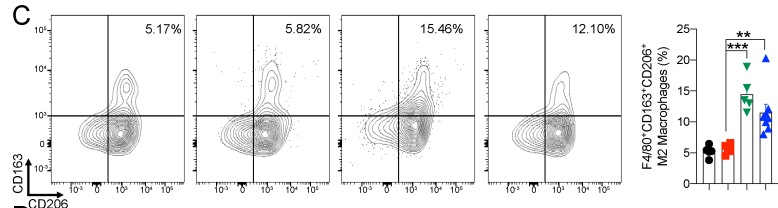
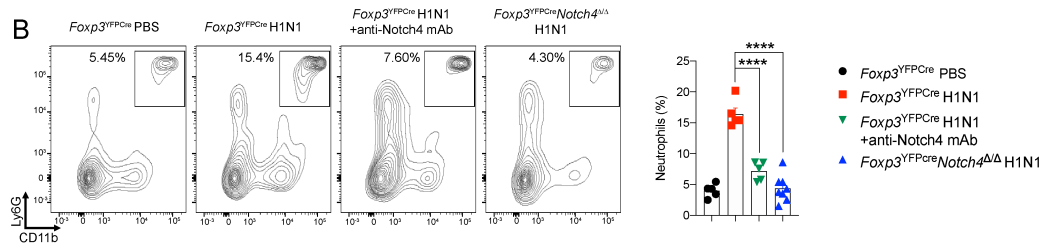
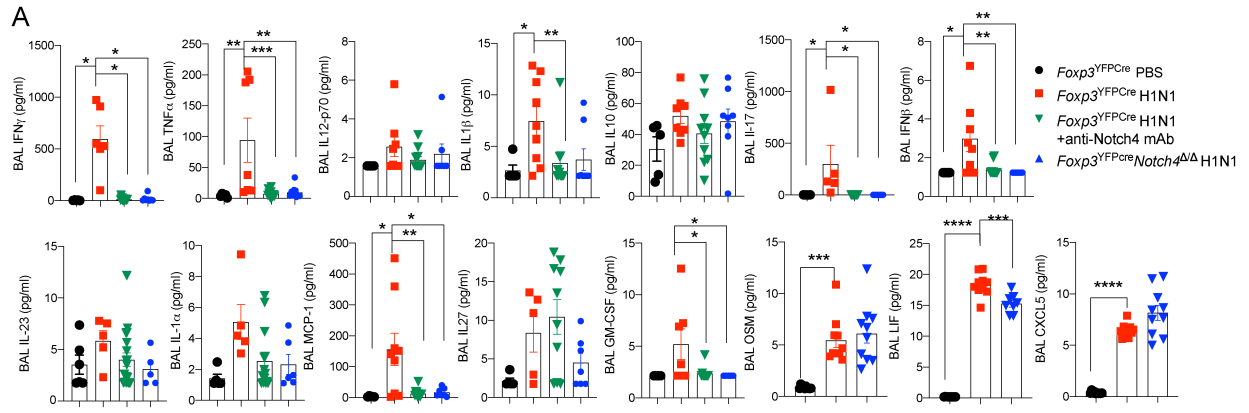


Fig. S5. Characterization of lung T cell infiltrates in H1N1 infected mice. Related to Figure 3. **A,B**, Graphical representation of CD8⁺ T cell frequencies (A) and IFN γ ⁺CD8⁺ T cell frequencies and numbers (B) in the lungs of *Foxp3*^{YFPCre} and *Foxp3*^{YFPCre}*Notch4* Δ/Δ mice that were either sham-treated or infected with H1N1 virus, in the case of the *Foxp3*^{YFPCre} mice either alone or together with an anti-Notch4 mAb, as indicated. **C,D**, Graphical representation of CD4⁺ T cell frequencies (C) and IFN γ ⁺CD4⁺ Teff cell frequencies and numbers (D) in the lungs. **E**, Graphical representation of IFN γ ⁺ Macrophage frequencies and numbers. **F**, Graphical representation of IFN γ ⁺ILC1 frequencies and numbers. **G**, Graphical representation of IL-17⁺CD8⁺ T cell frequencies and numbers in the lungs. **H**, Graphical representation of IL-17⁺CD4⁺ T cell frequencies and numbers in the lungs. Each symbol represents one mouse (n=5 per group). Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett's post hoc analysis (**A-H**). *P<0.05, ***P<0.001, ****P<0.0001.

• *Foxp3^{YFPcre}PBS* • *Foxp3^{YFPcre}H1N1* • *Foxp3^{YFPcre}H1N1 +anti-Notch4mAB* • *Foxp3^{YFPcre} Notch4^{Δ/Δ}H1N1*

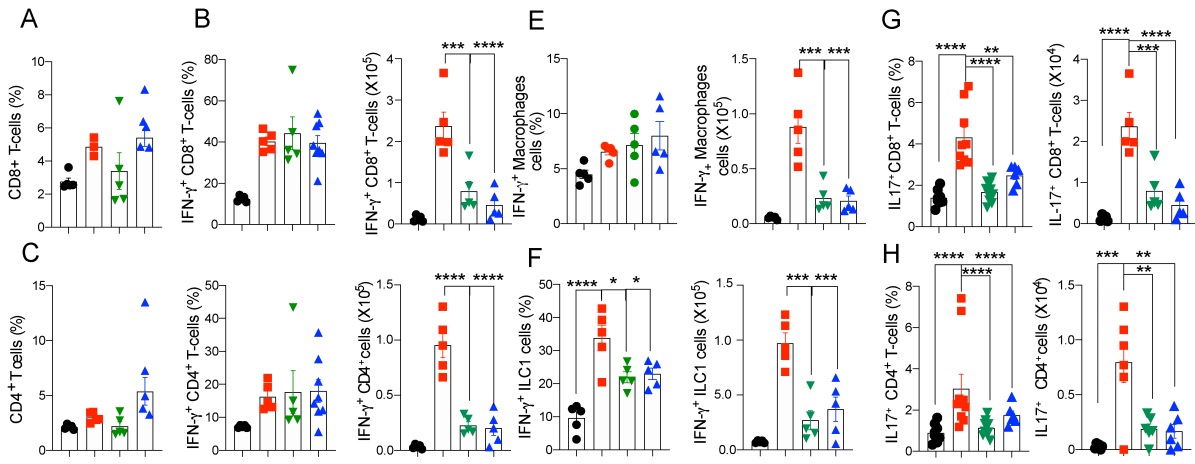


Fig. S6. Validation of neutralizing anti-human Notch4 mAbs and amphiregulin₉₁₋₁₄₀ blocking peptide. Related to Figures 4 and 7. **A.** Reactivity of different Notch4 mAbs to Notch1, Notch2, Notch3 and Notch4 as measured by ELISA. **B, Left,** Flow cytometric analysis Jagged1-Notch4^{GFP} interaction in Jurkat cells that were either sham transfected (control) or transfected with a construct encoding Notch4^{GFP} then incubated with Jagged1 in the absence or presence of the indicated mAb. **B right,** Flow cytometric analysis and graphical representation of Jagged1-PE staining of Jurkat cells that were transfected with a construct encoding Notch4^{GFP} in the absence or presence of the indicated mAb. **C,** Flow cytometric analysis and graphical representation of EGFR phosphorylation at tyrosine 1068 (pEGFR) in HEK293 cells treated with mouse amphiregulin in the presence increased concentrations of amphiregulin₉₁₋₁₄₀ blocking peptide (bp) or a neutralizing anti-amphiregulin mAb, as indicated. **D,** Weight index and peak weight loss of *Foxp3*^{YFPCre} and *Foxp3*^{YFPCre}*Notch4*^{Δ/Δ} mice treated with Poly I:C, either alone or together with anti-amphiregulin neutralizing mAb. **E,** Frequencies of neutrophils and M1 and M2 macrophages in lung tissues. **F,** Serum tetramethylrhodamine isothiocyanate (TRITC) dextran, measured as relative fluorescent units (RFU), in Poly I:C + anti-Amphiregulin mAb-treated *Foxp3*^{YFPCre} or *Foxp3*^{YFPCre}*Notch4*^{Δ/Δ} mice at 1-hour post intra-tracheal instillation. **G,** Frequencies of neutrophils and M1 and M2 macrophages in lung tissues from *Foxp3*^{YFPCre} mice either sham treated or treated with Poly I:C, either alone or together with amphiregulin bp. **H,** frequencies of M1 and M2 macrophages in co-cultures of Poly I:C-treated lung macrophages incubated either alone, with amphiregulin, Treg cells from Poly I:C-treated *Foxp3*^{YFPCre} mice or the combination thereof. Each symbol represents one mouse (n=5-10 per group). Numbers in flow plots indicate percentages.

Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett's post hoc analysis (**D-F**); two-way ANOVA with Sidak's post hoc analysis (**D**) ; Student's two tailed *t*-test (**G**). ** $P < 0.01$, **** $P < 0.0001$).

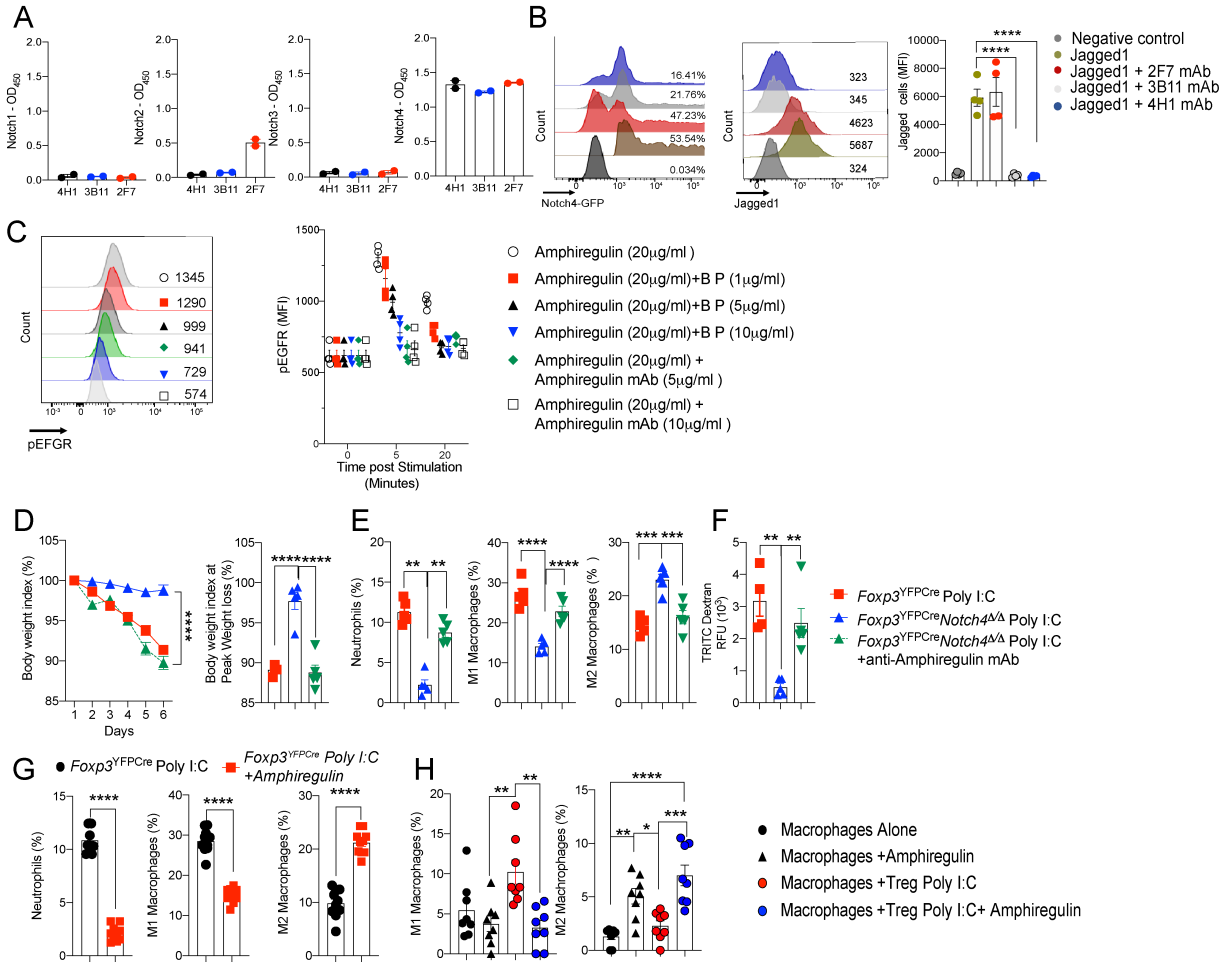


Table S1. Demographics of COVID-19 patients stratified by disease severity and predictors of mortality. Related to Figure 1

Table S1. Demographics of COVID-19 patients stratified by disease severity and predictors of mortality.

	Overall	Mild	Moderate	Severe	p-value
n	118	20	57	41	
Site, n (%)					<0.001
Boston	38 (32.2)	2 (10.0)	17 (29.8)	19 (46.3)	
Genoa	44 (37.3)	0 (0.0)	28 (49.1)	16 (39.0)	
Istanbul	36 (30.5)	18 (90.0)	12 (21.1)	6 (14.6)	
Age, mean (SD)	63.12 (18.97)	42.60 (15.22)	65.21 (17.39)	70.22 (15.83)	<0.001
Male, n (%)	63 (53.4)	9 (45.0)	25 (43.9)	29 (70.7)	0.022
White, n (%)	108 (91.5)	18 (90.0)	53 (93.0)	37 (90.2)	0.859
BMI, mean (SD)	28.57 (6.88)	26.94 (3.94)	27.98 (6.05)	30.18 (8.69)	0.151
Any medical problems, n (%)	95 (81.2)	8 (40.0)	49 (86.0)	38 (95.0)	<0.001
Immunosuppression	16 (13.6)	1 (5.0)	6 (10.5)	9 (22.0)	0.125
Malignancy	22 (18.6)	1 (5.0)	10 (17.5)	11 (26.8)	0.116
Pulmonary disease	33 (28.0)	2 (10.0)	17 (29.8)	14 (34.1)	0.13
Cardiac disease	70 (59.3)	4 (20.0)	32 (56.1)	34 (82.9)	<0.001
Hypertension	55 (46.6)	4 (20.0)	24 (42.1)	27 (65.9)	0.002
Hyperlipidemia	12 (10.2)	0 (0.0)	6 (10.5)	6 (14.6)	0.205
Endocrine disease	38 (32.5)	2 (10.0)	19 (33.3)	17 (42.5)	0.04
Diabetes	26 (22.0)	1 (5.0)	14 (24.6)	11 (26.8)	0.126
COVID-19 treatments					
Remdesivir	26 (22.0)	0 (0.0)	8 (14.0)	18 (43.9)	<0.001
Hydroxychloroquine	54 (45.8)	15 (75.0)	24 (42.1)	15 (36.6)	0.014
Glucocorticoids	67 (56.8)	0 (0.0)	35 (61.4)	32 (78.0)	<0.001
Anti-IL-6	9 (7.6)	0 (0.0)	2 (3.5)	7 (17.1)	0.016
Supportive care					
Supplemental oxygen	89 (75.4)	9 (45.0)	50 (87.7)	30 (73.2)	0.001
High flow oxygen	23 (19.5)	0 (0.0)	0 (0.0)	23 (56.1)	<0.001
Noninvasive ventilation	8 (6.8)	0 (0.0)	0 (0.0)	8 (19.5)	<0.001
Mechanical ventilation	20 (16.9)	0 (0.0)	0 (0.0)	20 (48.8)	<0.001
ECMO	2 (1.7)	0 (0.0)	0 (0.0)	2 (4.9)	0.148
Dead	19 (16.1)	0 (0.0)	5 (8.8)	14 (34.1)	<0.001
30-day, n (%)	8 (6.8)	0 (0.0)	0 (0.0)	8 (19.5)	<0.001
90-day, n (%)	18 (15.3)	0 (0.0)	4 (7.0)	14 (34.1)	<0.001
Biomarkers, mean (SD)					
Notch4, %	15.00 (14.48)	4.67 (5.57)	10.82 (10.93)	25.84 (15.24)	<0.001
IL-6, pg/mL	89.10 (209.25)	124.22 (172.49)	43.15 (38.47)	137.70 (326.22)	0.071

	Crude OR	95% CI	p-value	Adjusted OR	95% CI	P=
Age, years	1.086	(1.044, 1.140)	<0.001	1.094	(1.036, 1.177)	0.005
Male gender	1.613	(0.598, 4.654)	0.354	3.713	(0.942, 17.922)	0.076
History malignancy	5.954	(2.028, 17.718)	0.001	3.717	(0.950, 15.565)	0.062
Steroid treatment	5.020	(1.552, 22.55)	0.015	5.730	(1.177, 45.520)	0.053
Notch4, %	1.052	(1.020, 1.088)	0.002	1.046	(1.008, 1.090)	0.023
Interleukin-6, pg/mL	0.999	(0.992, 1.001)	0.553	1.000	(0.993, 1.004)	0.883

Disease severity classification. Mild = did not require hospitalization. Moderate = hospitalized but required only supplemental oxygen. Severe = hospitalized and required high flow oxygen, non-invasive ventilation, or mechanical ventilation.

Abbreviations: BMI = body mass index; ICU = intensive care unit; Anti-IL-6 = anti-interleukin-6 monoclonal antibody therapy; ECMO = extracorporeal membrane oxygenation; IL-6 = interleukin-6, OR = Odds Ratio. 95% CI = 95% confidence interval.