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Et al.

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Abstract

BACKGROUND

Risk of severe coronavirus disease 2019 (COVID-19) increases with age, is greater in males, and is associated with decreased numbers of blood lymphoid cells. Though the reasons for these robust associations are unclear, effects of age and sex on innate and adaptive lymphoid subsets, including on homeostatic innate lymphoid cells (ILCs) implicated in disease tolerance, may underlie the effects of age and sex on COVID-19 morbidity and mortality.

METHODS

Flow cytometry was used to quantitate subsets of blood lymphoid cells from people infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), comparing those hospitalized with severe COVID-19 (n=40) and those treated as outpatients for less severe disease (n=51). 86 healthy individuals served as controls. The relationship between abundance of specific blood lymphoid cell types, age, sex, hospitalization, duration of hospitalization, and elevation of blood markers for systemic inflammation, was determined using multiple regression.

RESULTS

After accounting for effects of age and sex, hospitalization for COVID-19 was associated with 1.78-fold fewer ILCs (95%CI: 2.34–1.36; $p = 4.55 \times 10^{-5}$) and 2.31-fold fewer $CD16^{+}$

natural killer (NK) cells (95%CI: 3.1–1.71; p = 1.04 x 10^{-7}), when compared to uninfected controls. Among people infected with SARS-CoV-2, the odds ratio for hospitalization, adjusted for age, sex, and duration of symptoms, was 0.413 (95%CI: 0.197–0.724; p = 0.00691) for every 2-fold increase in ILCs. In addition, higher ILC abundance was associated with less time spent in the hospital and lower levels of blood markers associated with COVID-19 severity: each two-fold increase in ILC abundance was associated with a 9.38 day decrease in duration of hospital stay (95% CI: 15.76–3.01; p = 0.0054), and decrease in blood C-reactive protein (CRP) by 46.29 mg/L (95% CI: 71.34–21.24; p = 6.25 x 10^{-4}), erythrocyte sedimentation rate (ESR) by 11.04 mm/h (95% CI: 21.94–0.13; p = 0.047), and the fibrin degradation product D-dimer by 1098.52 ng/mL (95% CI: 1932.84–264.19; p = 0.011).

CONCLUSIONS

Both ILCs and NK cells were depleted in the blood of people hospitalized for severe COVID-19, but, among lymphoid cell subsets, only ILC abundance was independently associated with the need for hospitalization, duration of hospital stay, and severity of inflammation. These results indicate that, by promoting disease tolerance, homeostatic ILCs protect against morbidity and mortality in SARS-CoV-2 infection, and suggest that reduction in the number of ILCs with age and in males accounts for the increased risk of severe COVID-19 in these demographic groups.

Introduction

The risk of disease severity in people infected with SARS-CoV-2 increases with age and is greater for men than for women[1–9]. Similar trends exist in people infected with SARS-CoV[10–12] or MERS-CoV[13], and have been observed in laboratory animals challenged with SARS-CoV or SARS-CoV-2[14,15]. Yet, mechanisms underlying these effects of age and sex on COVID-19 morbidity and mortality remain poorly understood. The composition and function of the human immune system changes with age and exhibits sexual dimorphism[16–19], with consequences for susceptibility to infection, autoimmune disease, and response to vaccination[2,16,17,20–22]. Better understanding of these effects might provide clues as to why the clinical outcome of SARS-CoV-2 infection is so variable.

Survival after infection with a pathogenic virus such as SARS-CoV-2 requires not only that the immune system control and eliminate the pathogen, but that disease tolerance mechanisms limit tissue damage caused by the pathogen or by host inflammatory responses[23–25]. Genetic and environmental factors identified in animal models influence disease tolerance mechanisms that promote host fitness without directly inhibiting replication of the pathogen[23–28]. Particular lymphoid cell types, including $CD4^+$ T cells and innate lymphoid cells (ILCs), are implicated in promoting disease tolerance[29–34]. SARS-CoV-2 viral load does not discriminate symptomatic from asymptomatic infection[35–38], and this discrepancy between SARS-CoV-2 viral load and severity of COVID-19 is especially pronounced in children, who rarely manifest severe COVID-19[39–42], but may have viral loads as high as very sick adults[43,44]. These observations indicate that age-dependent, disease tolerance mechanisms influence the severity of COVID-19.

COVID-19 severity correlates with lymphopenia[45–50] and with reduction in specific blood lymphoid cell populations[47,51–54]. Nonetheless, assessment of lymphoid cell abundance, in the context of a disease for which age and sex are risk factors for severity, is complicated by differences in lymphocyte abundance with age and sex[16,17]. The goal of this observational study was to determine whether abundance of any blood lymphoid cell population correlates independently with clinical outcome of SARS-CoV-2 infection.

Methods

PERIPHERAL BLOOD MONONUCLEAR CELLS

As part of a COVID-19 observational study, peripheral blood samples were collected from 91 individuals with SARS-CoV-2 infection at the Massachusetts General Hospital and affiliated outpatient clinics. Request for access to coded patient samples was reviewed by the Massachusetts Consortium for Pathogen Readiness and approved by the University of Massachusetts Medical School IRB (protocol #H00020836). Some demographic, laboratory, and clinical outcome data were included with the coded samples. Samples from 86 blood donors, either collected prior to the SARS-CoV-2 outbreak or from healthy individuals screened at a blood bank, were included as controls.

FLOW CYTOMETRY

Peripheral blood mononuclear cells (PBMCs) were stained with panels of fluorescent antibodies (Table S1) and detected on a BD Celesta flow cytometer using previously established gating strategies[55]. Cell subsets were identified using FlowJo™ software (Becton, Dickson and Company). Representative gating strategies are shown in Figure S1.

STATISTICAL ANALYSIS AND DATA VISUALIZATION

Data were prepared for analysis with the *tidyverse*[56] package, and visualized using the *ggplot2*[57] and *ggpubr*[58] packages, within the R computer software environment (version 4.0.2)[59]. Multiple linear regression analysis tested for independent effects of age, sex,

and COVID-19 status on log2 transformed cell counts measured by flow cytometry, using the *lm* function in R. Pairwise group comparisons were performed on estimated marginal means generated from multiple linear regression using the *emmeans* package[60], with multiple comparison correction using the Tukey adjustment. Association between log2 transformed cell counts and hospitalization, in SARS-CoV-2-infected individuals, was determined with multiple logistic regression including age, sex, and duration of symptoms at time of blood collection, as additional independent variables using the *glm* function in R. Group differences in age and sex were determined with pairwise, two-sided, Wilcoxon rank-sum tests, or Fisher's exact test, as indicated, with Bonferroni correction for multiple comparisons.

Results

THREE GROUPS OF BLOOD DONORS

The first group of blood donors included SARS-CoV-2-infected people hospitalized for severe COVID-19 (N = 40), among whom 33 (82.5%) were admitted to the ICU, 32 (80%) required intubation with mechanical ventilation, and 7 (17.5%) died (Table 1). This group had a mean age of 57.6 (range 24 to 83) and was 60% male. The second group consisted of people infected with SARS-CoV-2 but who did not require hospitalization for COVID-19 (N=51). This group had a mean age of 36.8 years (range 23-77) and was 25.5% male (Table 1). Differences between the two SARS-CoV-2-infected groups, in terms of median age ($p = 5.22 \times 10^{-8}$) and sex ratio ($p = 3.66 \times 10^{-3}$) (Figure S2), were consistent with the established greater risk of severe COVID-19 in older individuals and in males[1–6,8,61]. The third group included 86 SARS-CoV-2-negative controls. The age of this group spanned the range of the two SARS-CoV-2-infected groups (mean age 50.9; range 23 to 79) and the percentage of males (56%) was similar to that of the hospitalized group (Table 1 and Figure S2). Available information concerning ethnicity and race of the blood donors was insufficient for statistical comparisons among the groups (Table S2).

SARS-CoV-2 INFECTION AND LYMPHOID CELL ABUNDANCE

The effect of COVID-19 severity on lymphocyte abundance was assessed by multiple linear regression, with age, sex, and group (hospitalized, outpatient, and uninfected), as independent variables. After accounting for effects of age and sex, total lymphocytes among PBMCs were decreased 1.33-fold (95%CI: 1.49–1.19; $p = 1.22 \times 10^{-6}$) in

comparison to uninfected controls (Table S3). The abundance of specific lymphoid cell populations, as a fraction of total lymphocytes, was then assessed. Holding age and sex constant, individuals hospitalized with COVID-19 had 1.78-fold fewer ILCs (95%CI: 2.34–1.36; $p = 4.55 \times 10^{-5}$) and 2.31-fold fewer $CD16^+$ natural killer (NK) cells (95%CI: 3.1–1.71; $p = 1.04 \times 10^{-7}$), than did uninfected controls (Table S4 and Figure 1). After consideration of age and sex, neither $CD4^+$ T cells nor $CD8^+$ T cells were depleted (Table S4 and Figure 1). SARS-CoV-2-infected people who were treated as outpatients had no reduction in ILCs, but 1.44-fold fewer $CD16^+$ NK cells (95%CI: 1.93–1.07; $p = 1.68 \times 10^{-2}$), and 1.26-fold more $CD4^+$ T cells (95%CI: 1.06–1.5; $p = 0.01$), than did uninfected controls (Table S4 and Figure 1). Estimated marginal means for uninfected, hospitalized, and outpatient groups, adjusted for age and sex, are shown in Figure 1. After accounting for the effect of group, the effects of age and sex on lymphoid cell abundance was consistent with previous reports[16–19], with $CD4^+$ T cells, $CD8^+$ T cells, and ILCs decreasing with age, $CD16^+$ NK cells increasing with age, and both $CD4^+$ T cells and ILCs less abundant in males (Table S4 and Figure S3).

ASSOCIATION OF LYMPHOID CELL ABUNDANCE WITH HOSPITALIZATION

Association between lymphoid cell abundance and hospitalization in individuals infected with SARS-CoV-2 was determined with multiple logistic regression, including age, sex, and duration of symptoms at the time of blood draw, as additional independent variables. Abundance of ILCs, but not of $CD16^+$ NK cells, $CD4^+$ T cells, or $CD8^+$ T cells was associated with odds of hospitalization: the odds ratio for hospitalization, adjusted for age,

sex, and symptom duration, was 0.413 (95%CI: 0.197–0.724; p = 0.00691), or a decrease of 58.7%, for each 2-fold increase in ILC abundance (Table 2).

ASSOCIATION OF LYMPHOID CELL ABUNDANCE WITH DURATION OF HOSPITALIZATION AND MARKERS OF INFLAMMATION

The relationship between lymphoid cell abundance and duration of hospital stay, or with peak blood values for markers of inflammation, was assessed with multiple linear regression, including age, sex, and cell abundance as independent variables. Holding age and sex constant, abundance of ILCs, but not of $CD16^+$ NK cells, $CD4^+$ T cells, or $CD8^+$ T cells, was associated with length of time in the hospital, and with blood levels of markers associated with systemic inflammation (Table 3). Each two-fold increase in ILC abundance was associated with a 9.38 day decrease in duration of hospital stay (95% CI: 15.76–3.01; p = 0.0054), 46.29 mg/L decrease in blood C-reactive protein (CRP) (95% CI: 71.34–21.24; $p = 6.25 \times 10^{-4}$), and 11.04 mm/h decrease in erythrocyte sedimentation rate (ESR) (95% CI: 21.94–0.13; p = 0.047). Abundance of both ILCs and $CD4^+$ T cells was associated with blood levels of the fibrin degradation product D-dimer, with each two-fold increase in cell abundance associated with a decrease in D-dimer by 1098.52 ng/mL (95% CI: 1932.84–264.19; p = 0.011) and 1868.85 ng/mL (95% CI: 3375.63–362.06; p = 0.016), respectively.

DISCUSSION

This study demonstrated that, after accounting for effects of age and sex, ILCs and CD16$^+$ NK cells, but not CD4$^+$ or CD8$^+$ T cells, were decreased in individuals hospitalized with COVID-19 (Table S4 and Figure 1). Among people infected with SARS-CoV-2, higher abundance of ILCs, but not of NK cells or T cells, was associated with decreased odds of hospitalization, shorter duration of hospitalization, and lower blood level of factors associated with systemic inflammation (Table 3). Considering the known homeostatic function of ILCs[55,62–65] and the host responses necessary to survive pathogenic infection[23–25], these findings support the hypothesis that loss of disease tolerance mechanisms increase the risk of morbidity and mortality with SARS-CoV-2 infection[66].

ILCs and NK cells are innate immune lymphoid cells that lack clonotypic antigen receptors but share many developmental and functional characteristics with T cells[30,62,63,67,68]. Like CD8$^+$ T cells, NK cells kill virus-infected cells using perforin and granzyme[30,67]. FcγRIII (CD16)-positive NK cells link innate and acquired immunity by binding virus-specific immunoglobulins that target virus-infected cells for antibody-dependent cellular cytotoxicity[69]. The lack of association in this study between the abundance of these CD16$^+$ NK cells and odds of hospitalization, duration of hospitalization, or blood markers of inflammation, is consistent with the paradoxical finding that individuals with severe COVID-19 have more robust SARS-CoV-2-neutralizing antibody responses[70,71]. Findings such as these indicate that stronger adaptive antiviral immune responses do not necessarily result in decreased morbidity and improved survival.

ILCs represent innate counterparts of $CD4^+$ T cells[30,67] that promote tissue homeostasis and repair[55,62–65], maintaining the integrity of epithelial barriers in the lung and intestine[29–32]. ILCs with a similar marker profile to those detected in the blood are found in human lung and bronchoalveolar fluid[29], and experiments with animal models demonstrate that these ILCs promote airway epithelial integrity and restore tissue homeostasis in the lung after challenge with influenza virus[29]. Reduction in blood ILCs in people with HIV-1 infection correlates with decrease in colon lamina propria ILCs[55], suggesting that the reduction in blood ILCs reported here with SARS-CoV-2 reflects tissue abundance of these cells. Decreased abundance of ILCs with increasing age and male sex (Table S4 and Figure S3) may therefore explain why morbidity and mortality associated with SARS-CoV-2 infection is worse in males and more severe with older age[1–6,8,61]. This conclusion is also supported by the association reported here between ILC abundance, odds of hospitalization, length of stay in the hospital, and levels of the markers of COVID-19 severity, CRP, ESR, and D-dimer[9,72–74] (Table 3).

In conclusion, these results support the idea that the clinical outcome of SARS-CoV-2 infection is at least in part dependent upon disease tolerance mechanisms, reflected in peripheral blood counts of homeostatic ILCs, and, additionally offer potential explanation for the effects of age and sex on risk of morbidity and mortality. The findings of this observational study warrant establishment of prospective cohort studies to determine whether abundance of ILCs, or of other lymphoid cell subsets associated with disease tolerance[29–34], predict clinical outcome for infection with SARS-CoV-2 or other lethal pathogens.

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Table 1: Demographic and Clinical Characteristics of Blood Donor Groups

Characteristic	Uninfected N=86	Hospitalized N=40	Outpatient N=51
Mean age (range) - years	50.9 (23-79)	57.6 (24-83)	36.8 (23-77)
Sex – number (%)			
Male	48 (55.8)	24 (60)	13 (25.5)
Female	38 (44.2)	16 (40)	38 (74.5)
Mean symptom duration at sample collection (range) – days		21.8 (5-66)	26.9 (1-61)
ICU admission – number (%)		33 (82.5)	
Intubation with mechanical ventilation – number (%)		32 (80)	
Deaths – number (%)		7 (17.5)	
Max lab value – mean (range)			
CRP – mg/L		228.6 (6.5-539.5)	
ESR – mm/h		89.0 (15-146)	
D-dimer – (ng/mL)		5700 (351-11923)	

Table 2: Odds of Hospitalization*

Cell count†	Odds Ratio‡	95% Confidence Interval	P-Value
$CD4^+$ T	0.501	0.184–1.07	0.106
ILC	0.413	0.197–0.724	0.007
$CD8^+$ T	1.22	0.635–2.64	0.579
$CD16^+$ NK	0.814	0.53–1.21	0.309

*Adjusted for age, sex, and symptom duration at time of sample collection
† per 10^6 lymphocytes
‡ per 2-fold increase in cell population abundance

Table 3: Association of cell type abundance with time in hospital and laboratory values†

Cell count‡	Days hospitalized	CRP§	ESR§	D-dimer§
$CD4^+$ T	-10.843	-3.335	-2.674	-1868.847*
	[-22.511, 0.825]	[-56.162, 49.492]	[-23.840, 18.492]	[-3375.630, -362.063]
ILC	-9.381**	-46.288***	-11.035*	-1098.515*
	[-15.755, -3.008]	[-71.337, -21.238]	[-21.936, -0.134]	[-1932.842, -264.188]
$CD8^+$ T	3.366	32.247	15.317	486.192
	[-8.992, 15.724]	[-16.509, 81.003]	[-4.127, 34.761]	[-1049.836, 2022.221]
$CD16^+$ NK	-4.775	-14.619	-5.159	-404.873
	[-11.251, 1.701]	[-44.011, 14.774]	[-16.809, 6.491]	[-1316.261, 506.516]

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
† coefficients are for each two-fold increase in cell population abundance, adjusted for age and sex [±95%CI]
‡ per 10^6 lymphoid cells
§ Maximum lab value recorded during course of hospitalization

Figure 1

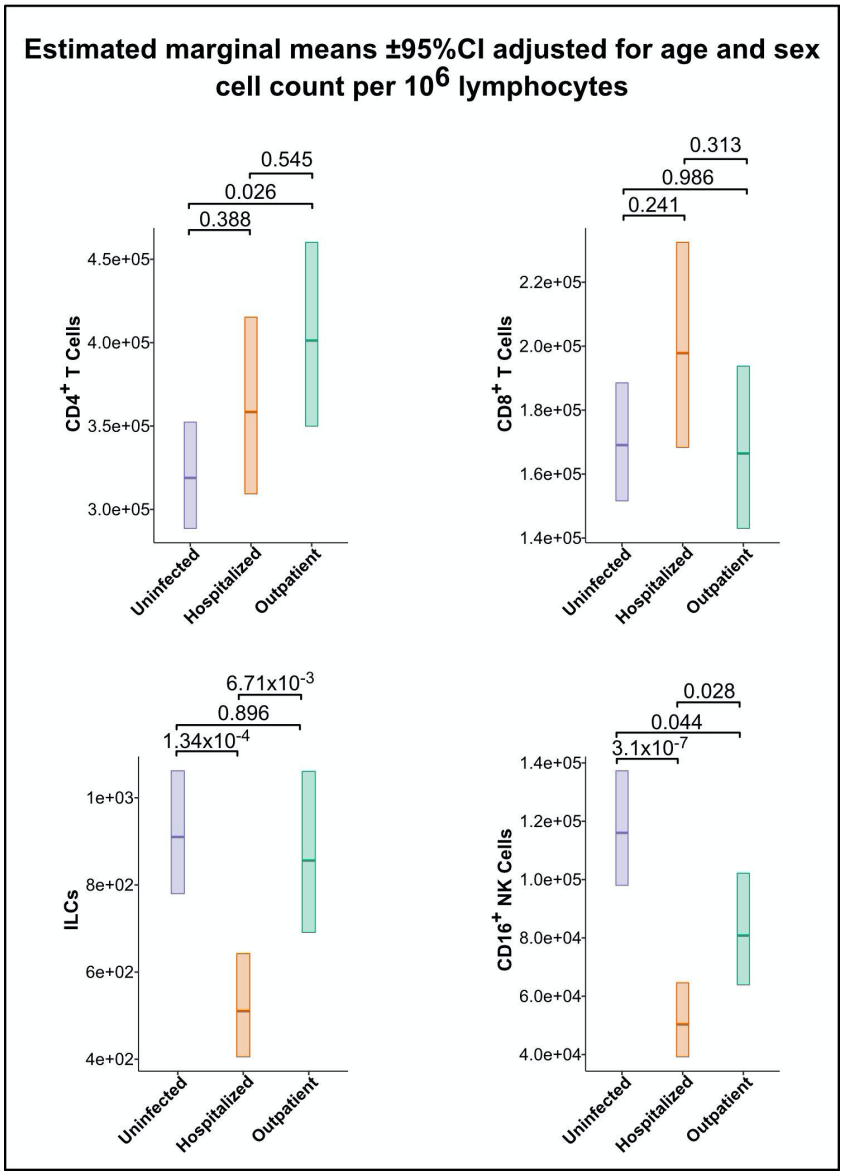

Figure 1: Difference in lymphoid cell abundance by group, shown as estimated marginal means, ±95CI, generated from the multiple linear regressions in Table S4, and averaged across age and sex. Cell counts are per million lymphocytes. P-values represent pairwise comparisons on the estimated marginal means, adjusted for multiple comparisons with the Tukey method.

Table S1: Antibodies Used in Flow Cytometry

Targeting antigen	Company	Catalog Number/Clone/Fluorophore
Anti-Human BDCA1	Biolegend	Cat# 354208 Clone: 201A (FITC) (1:200 dilution)
Anti-Human CD117	Biolegend	Cat# 313206 Clone: 104D2 (APC) (1:200 dilution)
Anti-Human CD11c	Biolegend	Cat# 301604 Clone: 3.9 (FITC) (1:200 dilution)
Anti-Human CD123	Biolegend	Cat# 306014 Clone: 6H6 (FITC) (1:200 dilution)
Anti-Human CD127	Biolegend	Cat# 351320 Clone: A019D5 (PE/Cyanine7) (1:200 dilution)
Anti-Human CD14	Biolegend	Cat# 325604 Clone: HCD14 (FITC) (1:200 dilution)
Anti-Human CD16	Biolegend	Cat# 980104 Clone: 3G8 (APC) (1:400 diltuion)
Anti-Human CD19	Biolegend	Cat# 302206 Clone: HIB19 (FITC) (1:200 dilution)
Anti-Human CD1a	Biolegend	Cat# 300104 Clone: HI149 (FITC) (1:200 dilution)
Anti-Human CD20	Biolegend	Cat# 302304 Clone: 2H7a (FITC) (1:200 dilution)
Anti-Human CD22	Biolegend	Cat# 363508 Clone: S-HCL-1 (FITC) (1:200 dilution)
Anti-Human CD3	Biolegend	Cat# 317306 Clone: OKT3 (FITC) (1:200 dilution)
Anti-Human CD34	Biolegend	Cat# 343504 Clone: 581 (FITC) (1:200 dilution)
Anti-Human CD4	Biolegend	Cat# 317428 Clone: OKT4 (PerCP/Cyanine5.5) (1:200 dilution)
Anti-Human CD4	Biolegend	Cat# 317408 Clone: OKT4 (FITC) (1:200 dilution)
Anti-Human CD45	BD	Cat# 506178 Clone: 2D1 (APC/H7) (1:200 diltuion)
Anti-Human CD56	Biolegend	Cat# 318306 Clone: HCD56 (PE) (1:200 dilution)
Anti-Human CD8	Biolegend	Cat# 300924 Clone: HIT8a (PerCP/Cyanine 5.5) (1:200 dilution)
Anti-Human CRTH2	Biolegend	Cat# 350116 Clone: BM16 (PerCP/Cyanine5.5) (1:200 dilution)
Anti-Human FcεR1α	Biolegend	Cat# 334608 Clone: AER-37 (FITC) (1:200 dilution)
Anti-Human TBX21	ebioscience	Cat# 25-5825-82 Clone: ebio4B10 (PE/Cyanine7) (1:200 dilution)
Anti-Human TCRα/β	Biolegend	Cat# 306706 Clone: IP26 (FITC) (1:200 dilution)
Anti-Human TCRγ/δ	Biolegend	Cat# 331208 Clone: B1 (FITC) (1:200 dilution)

Table S2: Race and Ethnicity of Cohorts

Characteristic	Uninfected N=86	Hospitalized N=40	Outpatient N=51
Race or ethnic group – number (%)			
White	25 (29.1)	17 (42.5)	46 (90.2)
Black or African American	0 (0)	6 (15)	2 (3.9)
Asian	1 (1.2)	1 (2.5)	1 (2)
Unknown or not reported	60 (69.8)	16 (40)	2 (3.9)
Hispanic or latinx	1 (1.2)	11 (27.5)	3 (5.9)

* Percentages may not equal 100 because of rounding

Table S3: Change in Cell Abundance Due to Age, Sex, and COVID-19 Severity

Fold difference (log2) [±95%CI]

	Lymphocytes†
Age	-0.008*** [-0.012, -0.004]
Male	-0.267*** [-0.400, -0.135]
Hospitalized	-0.411*** [-0.572, -0.250]
Outpatient	0.129 [-0.030, 0.289]
R^2	0.416

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

† per 10^6 PBMCs ‘

Table S4: Change in Cell Abundance Due to Age, Sex, and COVID-19 Severity

Fold difference (log2) [±95%CI]

	CD4⁺ T†	**ILC†**	**CD8⁺ T†**	**CD16⁺ NK†**
Age	-0.012***	-0.043***	-0.009*	0.021***
	[-0.018, -0.005]	[-0.053, -0.033]	[-0.016, -0.002]	[0.010, 0.032]
Male	-0.409***	-0.334*	-0.177	0.184
	[-0.618, -0.201]	[-0.659, -0.010]	[-0.406, 0.051]	[-0.169, 0.538]
Hospitalized	0.168	-0.835***	0.227	-1.205***
	[-0.084, 0.421]	[-1.228, -0.441]	[-0.050, 0.503]	[-1.633, -0.778]
Outpatient	0.332*	-0.088	-0.023	-0.522*
	[0.082, 0.581]	[-0.478, 0.302]	[-0.298, 0.253]	[-0.948, -0.095]
R^2	0.275	0.478	0.070	0.232

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

† per 10^6 lymphocytes

Figure S1

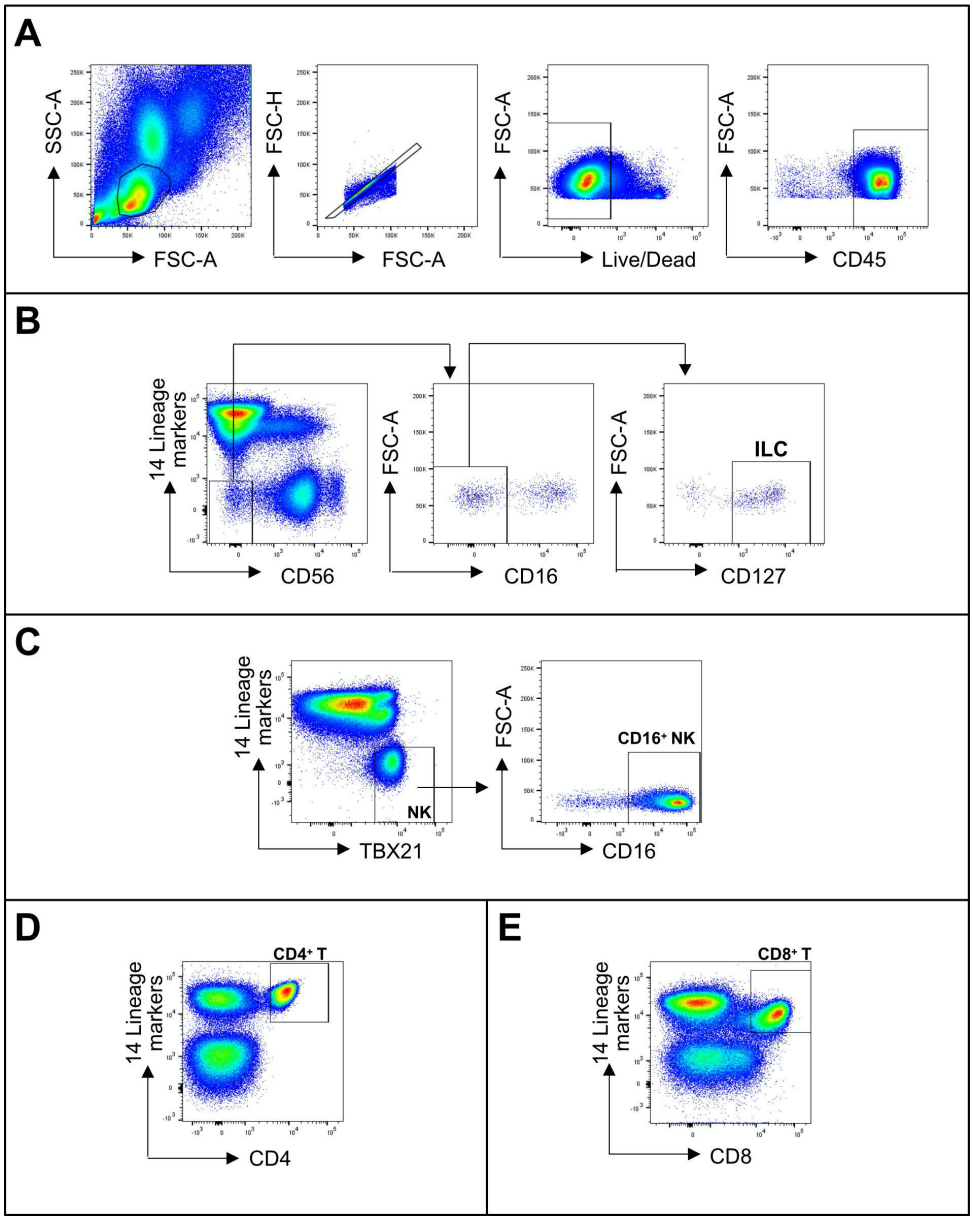

Figure S1: Representative gating strategy. All cell subsets were first gated on lymphoid cells, singlets, live/dead, and CD45$^+$ (A). ILCs were identified as Lin$^-$CD56$^-$CD16$^-$CD127$^+$ using 14 lineage markers (B). CD16$^+$ NK cells were identified as Lin$^-$TBX21$^+$CD16$^+$ (C). CD4$^+$ T cells were identified as Lin$^+$CD4$^+$ (D), and CD8$^+$ T cells were identified as Lin$^+$CD8$^+$ (E).

Figure S2

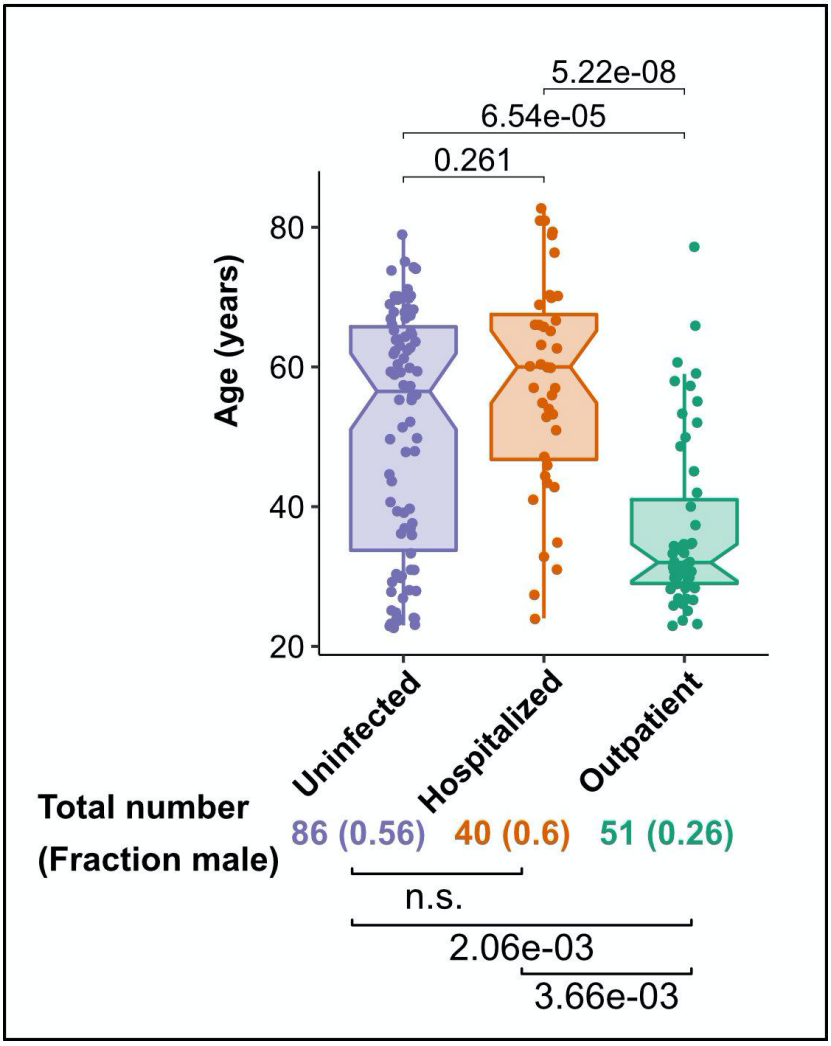

Figure S2: Age and fraction male of groups included in the study with P-values from pairwise two-sided Wilcoxon rank-sum and Fisher's exact test, respectively, with Bonferroni correction for multiple comparisons. Box plots represent the distribution of the data with the center line drawn through the median with the upper and lower bounds of the box at the 75th and 25th percentiles respectively. The upper and lower whiskers extend to the largest or smallest values within 1.5 x the interquartile range (IQR). The notch spans roughly the 95% confidence interval around the median as given by 1.58 x IQR/n^0.5.

Figure S3

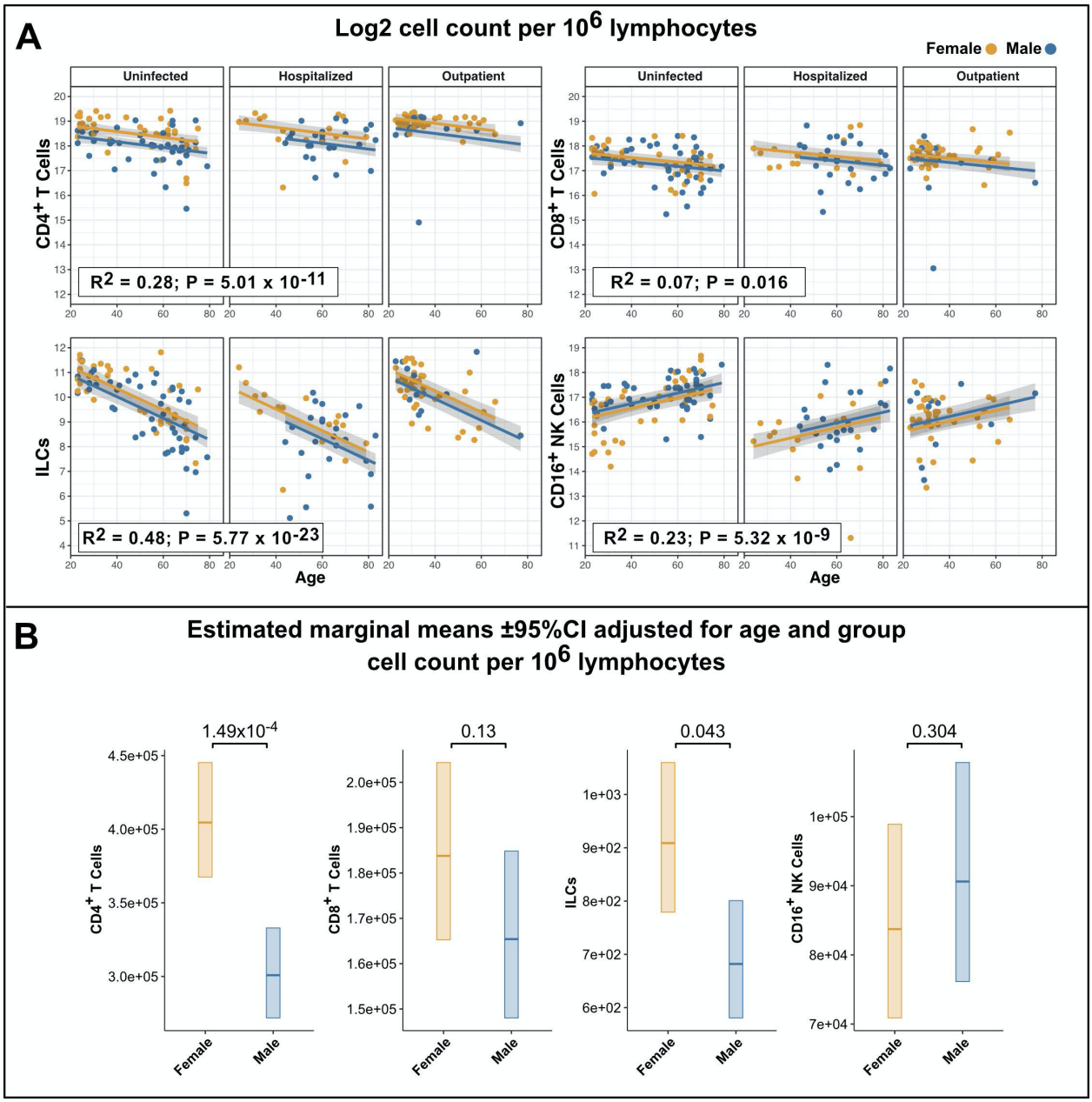

Figure S3: Visualization of change in log2 abundance of lymphoid cell populations with age as determined with the regression analysis in Table S4. Regression lines, R^2, and P-values are all from the associated multiple regression analyses and shading represents the 95%CI (A). Sex differences in lymphoid cell abundance shown as estimated marginal means ±95CI generated from the multiple linear regressions in Table S4 and averaged across age and group.