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T Cell Senescence by Extensive Phenotyping: An Emerging Feature of COVID-19 Severity

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Abbreviations: ARDS, acute respiratory distress syndrome; HCV, hepatitis C virus; HBV, hepatitis B virus; HIV-1, human immunodeficiency virus; NLR, neutrophil-to-lymphocyte ratio; AUC, area under the curve; LYM, total lymphocytes; ROC, receiver operating characteristic; N, naïve; CM, central memory; EM, effector memory; TEMRA, terminal effector memory.

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ABSTRACT

Objective: To identify the potential prognostic value of lymphocyte subsets in COVID-19 patients, where lymphopenia is a common finding.

Methods: In 353 COVID-19 inpatients and 40 controls T cell subsets with markers of senescence and exhaustion were studied by flow cytometry.

Results: In severe illness, total lymphocytes B, NK, and all T subsets were dampened. Senescent CD4+, but mainly CD8+ T cells, increased in patients with respect to controls. The most significant index predicting fatal outcome was neutrophils/CD3+ T ratio.

Conclusion: In conclusion, an altered T cell pattern underlies COVID-19 severity and is involved in predicting the outcome.

Since late December 2019, the COVID-19 pandemic caused by SARS-CoV-2 registered more than 244.3 million of cases and more than 4.9 million deaths as of October 27, 2021, according to the WHO Coronavirus (COVID-19) Dashboard. A wide range of clinical manifestations is associated with SARS-CoV-2 infection, which causes few and mild symptoms in the vast majority of cases but potentially results in severe pneumonia and acute respiratory distress syndrome (ARDS), mainly in frail and/or aged patients.[1]

Advanced age and comorbidities concur in enhancing disease severity: elderly patients are more likely to have severe disease with a higher death rate compared to younger individuals.[2] This age-related outcome might depend in part to age-related differences in the immune response: while children express more naïve T cells, elders are characterized by increased levels of memory cells.[3]

A reduction of lymphocytes was demonstrated in the most severe COVID-19 cases,[1] and several studies reported a significant, although not univocal, specific reduction of T cell subsets,[4] which are pivotal in the immune response against viral infections.[5]

The aim of this study was to identify the potential prognostic value of lymphocyte subsets in COVID-19 patients by extensive phenotyping of differentiation, senescence, and exhaustion markers on the surface of CD4+ and CD8+ T cells.

Materials and Methods

In this retrospective study, approved by the local ethic committee (number 27444), a total of 353 COVID-19 patients consecutively admitted at the University Hospital of Padova were studied. Included patients from March to October 2020 had a maximum of 10 days' disease history and were comprised of 221 males (mean age, 57 ± 16.47 years; range, 21–95 years) and 132 females (mean age, 60 ± 19.06 years; range, 19–98 years) with no difference in age (Student *t*-test, $P = .0882$). Peripheral blood immune cell data were analyzed the same day or the day after hospital admission. For comparison, 40 unselected healthy blood donors (HDs) were included as a control group. In accordance with Italian law, HD are screened at each donation with NAAT for hepatitis C virus (HCV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV-1); moreover, they must be free from any symptoms, including COVID-19–related symptoms, for at least 14 days.

The studied inpatients were referred to a semi-intensive and intensive care unit (ICU, n = 74) or to the Tropical and Infectious diseases care unit (NOT-ICU, n = 279). A total of 24 patients (7%) died within 3 months from hospital admission due to COVID-19–related causes.

Full blood cell count with differential were obtained from K2-EDTA tubes (Sysmex XE-series, Sysmex). Lymphopenia was defined as lymphocytes <1.10 × 10^9/L. Neutrophil count was used to obtain the neutrophil-to-lymphocyte ratio (NLR) and neutrophil-to-T cell indexes.

Lymphocyte subsets were analyzed within 2 days from sample collection. Samples were loaded on AQUIOS CL Flow Cytometer (Beckman Coulter), stained with AQUIOS TETRA-1 panel (CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5) and AQUIOS TETRA-2 panel (CD45-FITC/(CD56+ CD16)-RD1/CD19-ECD/CD3-PC5), and analyzed using the Tetra Combo analytical mode for the detection of CD3+/CD4+/CD8+ T cells, CD19+ B cells, and CD16+ 56+ NK cells.

Duraclone IMT cell panel (CD45RA-FITC/CCR7-PE/CD28-ECD/PD1-PC5.5/CD27-PC7/CD4-APC/CD8-A700/CD3-APC-A750/CD57-PB/CD45-KO) was used for the detailed analysis of T cell phenotype on a 10-color NAVIOS EX Flow Cytometer (Beckman Coulter).

Kaluza Analysis Software (Beckman Coulter) was used to set a gate for identification of T lymphocytes (CD45+ CD3+ cells) and then to identify CD4+ (helper) or CD8+ (suppressor) lymphocytes. Within the CD4+ or CD8+ population we analyzed markers of differentiation (CD45RA, CCR7, CD28, CD27), senescence, and exhaustion (CD57 and PD1).

Procedures for calibration, internal quality controls, and external quality assessment schemes were performed in accordance with ISO 15189 accreditation requirements for clinical laboratories, being the laboratory accredited since 2006.

Statistical analyses were performed using Stata v13.1 (StataCorp) and GraphPad Prism version 9.2.0 (GraphPad Software). Student *t*-test and 1-way ANOVA or multivariate ANOVA were used to assess differences between 2 or more groups, using Bonferroni's adjustment for multiple testing. Nonparametric test for trend was used to evaluate increasing or decreasing trends. Nonparametric receiver operating characteristic (ROC) analyses were used for assessing predictive performances of studied variables and estimating the area under the curve (AUC), with the Benjamini–Hochberg procedure for adjusting *P* value. Multivariate analyses were performed including age and sex as covariates.

Results

Total Lymphocytes and Lymphocyte Subsets Decline as Age and Disease Severity Increases

Lymphopenia occurred in 64% (225/353) of all patients and in 75% (117/157) of those >60 years. Total lymphocytes (LYM), CD4+ and CD8+ T cells, B cells, and NK cells were significantly lower in patients (F = 45.38 $P < .0001$ for LYM; F = 42.44, $P < .0001$ for CD4+; F = 28.27 $P < .0001$ for CD8+; F = 29.11, $P < .0001$ for CD19+ B cells; F = 21.83, $P < .0001$ for NK cells) than in controls. A decreasing trend in CD3+ T cells was observed, although the variation was not significant (F = 0.87, P = ns).

The following age-related categories were defined for further statistical analysis: <40 years (52 patients and 13 HDs), 40–60 years (144 patients and 23 HD), and >60 years (157 patients and 4 HDs).

As shown in **FIGURE 1A**, among COVID-19 patients a significant age-related decrease was found in LYM, CD3+ T cells, and their subsets and B cells; whereas no difference was seen in healthy controls (F = 14.23, $P < .0001$ vs F = 0.99, P = ns for LYM; F = 8.22, $P = .0003$ vs F = 1.21, P = ns for CD3+ T cells; F = 18.98, $P < .0001$ vs F = 0.65, P = ns for CD4+; F = 17.86, $P < .0001$ vs F = 0.37, P = ns for CD8+; F = 4.83, $P = .0086$ vs F = 0.96, P = ns for B cells). No age-related change was found for NK cells (F = 0.64, P = ns vs F = 1.14, P = ns). For any age class, the decrease in LYM and subsets that was observed was more pronounced in ICU than NOT-ICU, except for CD3+ and B cells (F = 11.92, $P = .0006$ for LYM; F = 0.93, P = ns for CD3+; F = 13.68, $P = .0003$ for CD4+; F = 8.41, $P = .004$ for CD8+; F = 1.62, P = ns for B cells; F = 4.73, $P < .05$ for NK).

In healthy controls NLR, neutrophil-to-CD3+ cells ratio (N3R), neutrophil-to-CD4+ cells ratio (N4R), and neutrophil-to-CD8+ cells ratio (N8R) did not differ significantly between age classes (**FIGURE 1B**). Conversely, ICU care for >60 year-old patients showed a sustained increase of NLR, N3R, N4R, and N8R; F = 13.43, $P = .0003$ for NLR; F = 8.28, $P = .0043$ for N3R; F = 10.76, $P = .0011$ for N4R; F = 4.90, $P = .0275$ for N8R).

ROC curve analyses examining whether the lymphocyte count and subsets were associated with the referring hospitalization unit (NOT-ICU vs ICU) based on patients age classes are shown in **TABLE 1**.

Pairwise comparisons of ROC showed that, although NLR presented the higher AUC value, the difference was not significant with respect to other AUC values.

It is worth noting that in our studied population the patient deaths (n = 24) occurred in the >60 year age class, therefore the prognostic role of laboratory findings on survival, estimated with ROC curve analysis, was made in patients belonging to this age class only. The most discriminant index was N3R with an AUC of 0.877.

Disease Severity Is Associated with T Cell Senescence and Exhaustion

Markers of T cell differentiation, senescence, and exhaustion were further analyzed in 68 COVID-19 patients (61 NOT-ICU and 7 ICU) and 20 HD. The following CD4+ and CD8+ subsets were identified: naïve (N) (CCR7+ CD45RA+), central memory (CM) (CD45RA– CCR7+), effector memory (EM) (CD45RA– CCR7–), and terminal effector memory (TEMRA) (CD45RA+ CCR7–).

None of CD4+ T cell subsets varied between HD, NON-ICU, and ICU. On the contrary, CD8+ CM ($P < .001$) and EM ($P = .012$) subsets significantly decreased in ICU patients (Supplemental Figure 1A). No significant age-related differences were found for any of the studied subsets (data not shown).

Both CD4+ and CD8+ senescent cells, that is CD57+ PD1– and CD57+ PD1+, showed a trend toward increasing values in NON-ICU and ICU patients with respect to HD ($P < .05$) (Supplemental Figure 1B). Among these, the variations of CD8+ senescent and exhausted CD57+ PD1+ cells were independent from age ($P = .089$), while increasing age was correlated with CD4+ CD57+ cells ($P = .015$), CD4+ CD57+ PD1+ cells ($P = .025$) and CD8+ CD57+ cells ($P = .04$).

When analyzing both markers in each subset (N, CM, EM, and TEMRA) among HD, NOT-ICU and ICU groups, naïve CD4+ CD57–PD1+ and CD4+ CD57+ PD1+ cells tended to progressively increase among NON-ICU and ICU patients ($P = .011$ and $P = .005$, respectively). A similar and age-independent pattern was found for EM CD4+ CD57+ PD1+, CM, and EM CD8+ CD57+ PD1+ cells ($P = .006$, $P = .044$, and $P = .014$, respectively) (Supplemental Figures 2 and 3). On the contrary, the increase of naïve CD8+ CD57+ PD1+ cells observed in more severe cases ($P = .048$), was in part correlated with age ($P = .010$). No significant trend was identified for CM, TEMRA CD4+, and TEMRA CD8+.

Discussion

During viral infection, T cells are crucial to viral clearance; CD8+ cytotoxic T cells directly attack and kill virus-infected cells, whereas CD4+ helper T cells assist cytotoxic T cells and B cells and enhance their ability to eliminate pathogens. In contrast to HIV-1, cytomegalovirus, and Epstein-Barr virus infections, which lead to proliferative lymphocyte response, one prominent feature of SARS-CoV-2 is lymphopenia due to pulmonary recruitment from the blood and direct virus killing.[6]

Previous studies in COVID-19 patients reported lymphopenia to affect all subsets (T, B, and NK cells), with the lowest lymphocyte counts found in severe disease that needed ICU care being reversed in almost all convalescents.[7]

In our study, lymphopenia was confirmed to be a common finding in COVID-19 patients, being more severe among those >60 years old; however, we should mention as a limitation of our study that only 4 HDs aged >60 were enrolled. This pattern was also found when considering the absolute number of CD4+ and CD8+ T cells and B cells, being the lowest levels detected in older patients of the ICU group. Furthermore, in COVID-19 patients, lymphopenia associated with normal/increased neutrophils explained the observed increase in NLR, a marker of systemic inflammation and infection; again, the highest NLR was found in the >60 year olds in ICU care. Interestingly, other hematologic features were noted in the COVID-19 cohort, such as an average eosinophilic count lower in COVID-19 patients than in HD ($0.02 \pm 0.04 \times 10^9$/L vs $0.17 \pm 0.09 \times 10^9$). This finding is in agreement with recent data reported by Outh et al.[8]

Qin et al[9] proposed some parameters derived from lymphocyte subsets to predict disease severity and survival. To confirm these findings, we performed ROC curve analysis to evaluate the role of total lymphocyte (CD3+, CD4+, and CD8+) counts and NLR/N3R/N4R/N8R for association with hospitalization unit and survival prediction—the last one estimated only for patients >60 years, since no death occurred among those younger. The most significant index of disease severity was NLR, while N3R was the best in predicting survival.

FIGURE 1. A, Age-related decrease of total lymphocytes and lymphocyte subsets in COVID-19 patients compared to healthy donors (F = 14.23, *P* < .0001 vs F = 0.99, *P* = ns for total lymphocytes; F = 8.22, *P* = .0003 vs F = 1.21, *P* = ns for total T cells; F = 18.98, *P* < .0001 vs F = 0.65, *P* = ns for CD4+; F = 17.86, *P* < .0001 vs F = 0.37, *P* = ns for CD8+; F = 4.83, *P* = .0086 vs F = 0.96 *P* = ns for B cells; not significant for NK cells F = 0.64, *P* = ns vs F = 1.14, *P* = ns); age-related decrease both in NOT-ICU and ICU for total lymphocytes, CD4+ and CD8+ subsets only (F = 11.92, *P* = .0006 total lymphocytes; F = 0.93, *P* = ns total T cells; F = 13.68, *P* = .0003 CD4+; F = 8.41, *P* = .004 CD8+; F = 1.62, *P* = ns B cells; F = 4.73, *P* < .05 for NK).

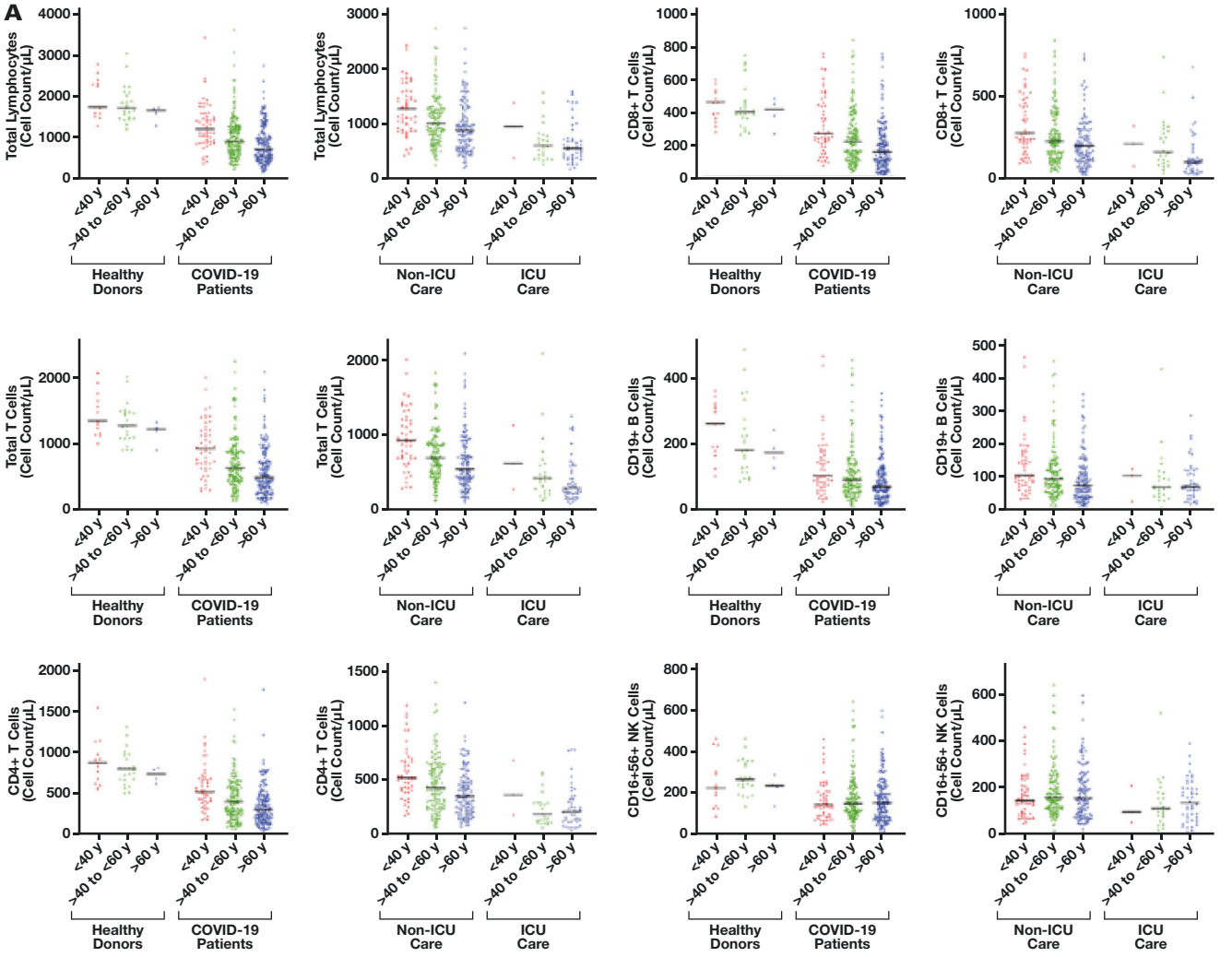

FIGURE 1. (cont) B, Neutrophil-to-lymphocyte ratio (NLR), neutrophil-to-CD3+ cells ratio (N3R), neutrophil-to-CD4+ cells ratio (N4R), and neutrophil-to-CD8+ cells ratio (N8R) between age classes in healthy donors (*P* = ns) vs COVID-19 patients (highest ratios in >60-year-old patients. F = 14.95, *P* < .0001 for NLR, F = 11.97, *P* < .0001 for N3R, F = 11.97, *P* < .0001 for N4R, F = 11.20, *P* < .0001 for N8R) and NOT-ICU vs ICU care (highest ratios in >60 years ICU F = 13.43, *P* = .0003 for NLR; F = 8.28, *P* = .0043 for N3R; F = 10.76, *P* = .0011 for N4R; F = 4.90, *P* = .0275 for N8R).

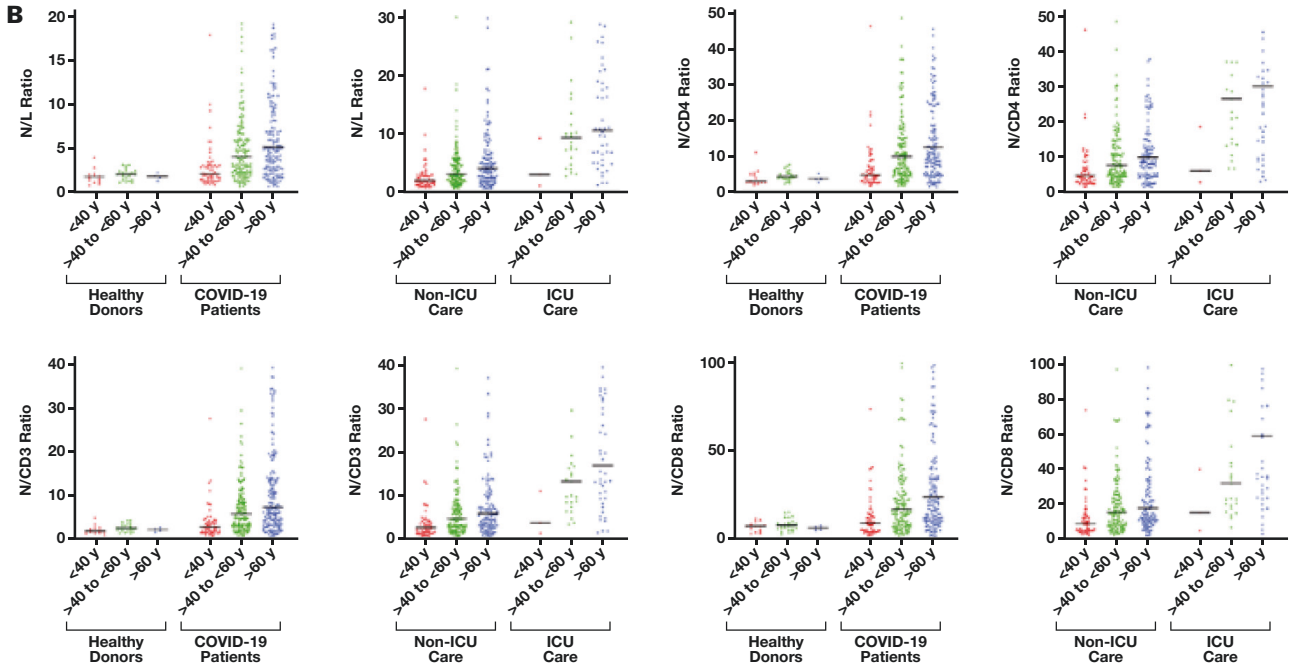

TABLE 1. ROC Curve Analyses for Lymphocyte Count and Subsets Associated with Referring Hospitalization Unit (NOT-ICU vs ICU) and in Survival Prediction Based on Patients Age Classes

Variable	AUC (95% Confidence Interval)			
Hospitalization Unit	**Age < 40 y**	**40 y < Age < 60 y**	**Age > 60 y**	**Overall**
LYM	0.7143 (0.31485–1.0000)	0.7339 (0.61017–0.85760)	0.7007 (060979–0.79153)	0.7358 (0.67041–0.80126)
CD3+ T cells	0.6871 (0.25313–1.0000)	0.7189 (0.59572–0.84206)	0.7425 (0.65739–0.82762)	0.7666 (0.70496–0.82822)
CD4+ T cells	0.6803 (0.23878–1.0000)	0.7556 (0.64209–0.86916)	0.7098 (0.62083–0.79876)	0.7637 (0.70385–0.82352)
CD8+ T cells	0.7279 (0.36817–1.0000)	0.6034 (0.46939–0.73751)	0.7379 (0.65531–0.82056)	0.7363 (0.67081–0.80183)
NLR	0.7958 (0.73857–0.85296)	0.8467 (0.75735–0.93606)	0.7466 (0.66600–0.82727)	0.8050 (0.74953–0.86038)
N3R	0.6939 (0.39524–0.99251)	0.8325 (0.74269–0.92222)	0.7597 (0.67991–0.83952)	0.7950 (0.73676–0.85317)
N4R	0.7007 (0.42987–0.97149)	0.8349 (0.73976–0.93003)	0.7443 (0.66337–0.82528)	0.8047 (0.74708–0.86239)
N8R	0.7483 (0.47900–1.0000)	0.7365 (0.62245–0.85057)	0.7349 (0.65215–0.81764)	0.7581 (0.69561–0.82058)
Survival				
LYM			0.734 (0.628–0.840)	
CD3+ T cells			0.866 (0.784–0.947)	
CD4+ T cells			0.831 (0.747–0.915)	
CD8+ T cells			0.821 (0.735–0.907)	
NLR			0.849 (0.754–0.943)	
N3R			0.877 (0.793–0.961)	
N4R			0.858 (0.765–0.950)	
N8R			0.826 (0.726–0.926)	

AUC, area under the curve; LYM, total lymphocytes; NLR, neutrophil-to-lymphocyte ratio.

In our patients, a decreasing, not age-related, trend of CM and EM CD8+ from HD to NON-ICU and ICU care patients was found. Although we could not determine whether lower percentages of CM and EM CD8+ might be a preexisting condition or a direct effect of COVID-19 infection, these results indicate that patients with lower percentages at admission might need ICU care and confirm reports

that T cell compartment displays several alterations.[10] Indeed, known conditions of decreased numbers of CD8+ memory T cells include solid cancer[11] and autoimmune diseases[12] that affected our extended immunophenotyped patients in 7% and 15%, respectively. Given the not-so-infrequent occurrence of these disorders in the general population, a planned phenotyping at selected time points during clinical follow-up might enhance infection-related risk stratification in chronic diseases.

We observed that the same populations, CM and EM CD8+ T cells, along with naïve and EM CD4+, express markers of senescence and exhaustion (CD57 and PD1) in higher percentages than in HD, as already noted[13]; this exhausted phenotype, peculiar to T cells that are chronically stimulated by antigens from viral infection and malignancies, might worsen the already impaired response capability of those cell populations. The prognostic impact of T cell exhaustion in chronic viral illnesses is debated: as PD-1 expression on T cells seems to correlate with disease progression in HIV-1 infection, its role as a target of specific therapy is considered in different animal models of HCV and HBV.[14]

Conclusion

T cell subsets along with lymphocyte and neutrophil counts at the time of admission of COVID-19 patients might allow an early identification of individuals at risk of developing critical illness. Extensive phenotyping of T cell subsets ensures a deeper insight of SARS-CoV-2 pathological mechanisms, highlighting that an exhausted phenotype associated with a severe disease could lead to a state of low-cell responsiveness impairing immune response.

Supplementary Data

Supplemental figures and tables can be found in the online version of this article at www.labmedicine.com.

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