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ORIGINAL ARTICLE

Intermediate monocytes expansion and homing markers expression in COVID-19 patients associate with kidney dysfunction

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Abstract

Patients with severe SARS-CoV-2 infection have an overwhelming inflammatory response characterized by remarkable organs monocyte infiltration. We performed an immunophenotypic analysis on circulating monocytes in 19 COVID-19 patients in comparison with 11 naïve HIV-1 patients and 10 healthy subjects. Reduced frequency of classical monocytes and increased frequency of intermediate monocytes characterized COVID-19 patients with respect to both HIV naïve patients and healthy subjects. Intensity of C–C motif chemokine receptor 2 (CCR2) monocyte expression highly correlated with parameters of kidney dysfunction. Our data indicate that highly activated monocytes of COVID-19 patients may be pathogenically associated with the development of renal disease.

Keywords Covid-19 · SARS-CoV-2 · Monocytes · Innate immunity · C–C motif chemokine receptor 2

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection can lead to different clinical pictures (from asymptomatic/pauci-symptomatic infection to moderate/severe forms of disease), suggesting that the clinical manifestations might strictly depend on the outcome of the SARS-CoV-2 immune system interaction in the patient. The factors that trigger severe illness in SARS-CoV-2 infected individuals are not completely understood. Immune system dysregulation, leading to an excessive inflammatory response to SARS- CoV-2, is thought to be a major cause of disease severity and death in patients with Coronavirus Disease (COVID-19) [1]. This condition is associated with high levels of circulating cytokines as well as by substantial mononuclear cell infiltration in the lungs, heart [2], spleen, lymph nodes and kidney [3, 4]. Among mononuclear cells, a key pathogenic role for COVID-19 inflammation has been attributed to monocytes [5, 6]. In particular, expansion of CD14 + CD16 + + intermediate monocytes [7] has

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been observed in SARS-CoV-2 infected patients [8]. This particular subset of circulating monocytes has been previously found associated with inflammation and viral diseases as HIV infection [7, 9]. In order to better characterize the phenotype of monocytes in patients with SARS-CoV-2 infection, we analyzed circulating monocytes of COVID-19 patients in comparison with corresponding cells from healthy subjects and HIV-1 naïve patients. Moreover, we searched for associations between phenotypic monocyte abnormalities in COVID-19 patients and serum clinical markers of disease, finding a strict correlation between C–C motif chemokine receptor 2 (CCR2) expression and parameters of kidney functionality.

Materials and methods

Patients and healthy donors

This was a descriptive observational cross-sectional clinical study. Peripheral blood was collected from 19 consecutives patients from March 2020 affected by moderate/severe COVID-19 who were enrolled at the Division of Infectious Diseases and the Internal Medicine and Clinical Immunology Unit of the Policlinic San Martino University Hospital in Genoa (Supplementary Table 1). Diagnosis of COVID-19 was confirmed in all patients by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) positive from a nasal and/or throat swab. We collected also peripheral blood from 11 HIV-1 naïve patients, SARS-CoV-2 negative (Supplementary Table 2), as well as from 10 healthy donors (HD). The study was carried out in compliance with the Helsinki Declaration and approved by the Ethical Committee of the San Martino Hospital in Genoa (N. CER Liguria 114/2020—ID 10,420 and P.R.251REG2014).

Monoclonal antibodies and immunofluorescence analyses

Cell expression of membrane antigens was tested by immunofluorescence analysis performed with 100 µl of peripheral blood. Samples were incubated with specific fluorochrome-conjugated monoclonal antibodies (mAbs) at room temperature for 20 min in the dark. The following panel was used: phycoerythrin (PE) conjugated anti-CD38, Peridinin Chlorophyll Protein Complex-Cyanin 5.5 PerCP-Cy5.5conjugated anti-HLA-DR, allophycocyanin (APC) conjugated anti-CD11b, brilliant violet (BV) 421 conjugated anti-CCR2, BV605 conjugated anti-CD16, BV711 conjugated anti-CD14, BV785 conjugated anti-CD3 (Becton Dickinson, (BD) Biosciences, San Josè CA). For lysing red blood cells and fixing leukocytes following direct immunofluorescence staining of human peripheral blood, samples were resuspended in 4 ml of FacsLysing buffer (containing formaldehyde, BD) and then centrifuged and resuspended in 300 µl of FacsLysing. Following the staining and lysing procedures, the cells were analyzed by a BD LRSFortessa X-20 flow cytometer (BD Biosciences) using FACS DIVA software 8.0 (BD Biosciences). Levels of expression of HLA-DR, CD38, CCR2 markers were shown as mean fluorescence intensity (MFI) on the monocyte subsets. Since HLA-DR, CD38 and CCR2 molecules resulted absent on neutrophils, we used the MFI of HLA-DR, CD38 and CCR2 molecules on this population as an internal negative control. Cytometer performances were checked weekly with CS&T beads (BD Biosciences) to determine cytometer settings and performance measurements for reproducible application.

Gating strategy for monocyte identification

The gating strategy to identify monocytes, as described in Supplementary Fig. 1, was the following: a) debris and dead cell exclusion in forward-scatter (FSC-Height) vs side-scatter channel (SSC-Height) plot (Panel A); b) doublet exclusion in FSC-Area vs FSC-Height plot (Panel B); c) gating for monocytes in HLA-DR vs SSC plot to select them as HLA-DR + cells with higher SSC than HLA-DR + and HLA-DR- lymphocytes; in this plot monocytes were distinguished from neutrophils based on the higher SSC and HLA-DR negativity of these latter cells (Panel C); d) confirmation of monocyte population as CD3-CD11b + in CD3 vs CD11b plot (Panel D). The differentiation between monocytes and neutrophils was corroborated by the analyses of HLA-DR (that are molecules not present on the surface of neutrophils), CD16 (brighter expression on neutrophils) and CD11b (brighter expression on monocytes).

We used HLA-DR instead of CD14 as identifier marker for monocytes in order to not underestimate the subpopulation of nonclassical monocytes (that exhibit CD14 low/neg expression). Then, the HLA-DR + CD3-CD11b + monocyte population was analyzed by CD14 and CD16 markers to identify the three subpopulations of monocytes, as follows: classical (CD14 + + CD16-), intermediate (CD14 + + CD16 +) and nonclassical (CD14 ± CD16 + +) (Supplementary Fig. 1, Panel E). The levels of CD16 positivity within the monocyte population were discriminated through comparison with those of CD3 + T and putative B lymphocytes, NK CD16 + cells and neutrophils; each of them references for negative, intermediate and bright expressions, respectively (Supplementary Fig. 2).

The percentage of monocytes, evaluated as HLA-DR + CD11b + CD3- (Supplementary Fig. 1, Panel D), was referred to the total leukocytes (lymphocytes-monocytes-neutrophils) identified based on their FSC-H and SSC-H features in Panel A. The frequencies of different monocyte subsets (Supplementary Fig. 1, Panel E) were

determined as percentages of the total monocyte population defined in Supplementary Fig. 1, Panel D.

Multidimensional data reduction analysis

To visualize the different clustering of monocyte subpopulations in the three groups of subjects (healthy donors, Covid-19 and HIV + naïve patients), flow cytometric data derived from a representative subject for each group were exported with compensated parameters to FCS express software v6.03.0011 (DeNovo software) in order to perform a multidimensional data reduction analysis. Monocytes were defined based on their FSC vs SSC physical parameters and HLA-DR + CD11b + expression (as shown in Supplementary Fig. 1, Panel D): 144,000 monocytes per subject were merged into a new FCS file. A t-dependent Stochastic Neighbor Embedding (t-SNE) map was generated, using FCS express software 119 v6.03.0011 (DeNovo Software), in the merged file among 1000 iterations with Barnes-Hut 287 approximation and 40 perplexity value for following markers: FSC-A, SSC-A, CCR2, HLA- DR, CD38. This generated 2-D plots that clustered the cells on the basis of marker expression profiles.

Statistical analyses

The existence of statistically significant differences between means of data was analyzed by Mann–Whitney t test for nonparametric values. The existence of statistically significant correlations between variable parameters was analyzed by Spearman test for nonparametric values. Calculation was performed by GraphPad Prism v.5 software (GraphPad Software, San Diego, California, USA).

Results

Monocyte phenotypic characterization

COVID-19 patients showed monocyte circulating frequencies comparable to those of healthy subjects and HIV + naive patients (Fig. 1A). However, the relative distributions of the three different monocyte subsets, namely classical (CD14 + + CD16-), intermediate (CD14 + + CD16 +) and nonclassical (CD14 ± CD16 + +) types [10], were peculiar of COVID-19 patients. In fact, the frequency of classical monocytes was decreased and that of intermediate monocytes was increased in COVID-19 patients with respect to both healthy donors and HIV + naïve patients (Figs. 1B and 1C). It should be noted that the frequency of intermediate monocytes was higher in COVID-19 patients than in HIV + naive patients (Fig. 1C). No differences were observed concerning nonclassical monocytes (Fig. 1D). Interestingly, when monocyte morpho-cytometric features were comparatively analyzed in the three subgroups of subjects, again a peculiarly altered morphology, characterized by an increase in side scatter (SSC) dimension (index of cytoplasmic complexity), hallmarked the monocyte population of COVID-19

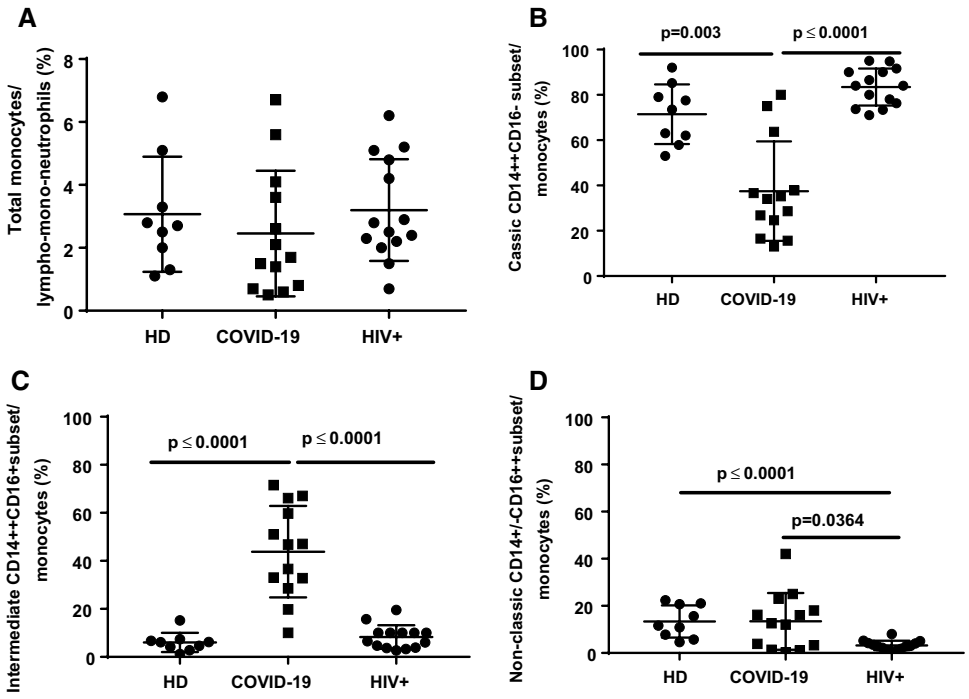

Fig. 1 Frequency of circulating monocytes. The frequency of total monocytes out of circulating leucocytes **A** and those of classical **B**, intermediate **C** and nonclassical **D** monocyte subsets were comparatively analyzed in COVID-19 patients, in HIV + naïve patients and in healthy donors

patients compared to HD and HIV patients (Fig. 2). The differences among the three subgroups further emerged when data, relative to monocyte expression of CD38 and HLA-DR activation markers and CCR2 homing receptor of a representative subject for each group, were merged and evaluated applying t-dependent Stochastic Neighbor Embedding (t-SNE) analysis. The t-SNE maps showed that monocytes derived from COVID-19 patient #8, HIV + naïve patient #1 and healthy donor #2 clusterized differently, accordingly with their morphologic features and expression profiles of CD38, HLA-DR and CCR2 molecules (Supplementary Fig. 3, Panels A-C). This analysis showed a different clustering of monocyte subsets among different subjects, confirming the enrichment

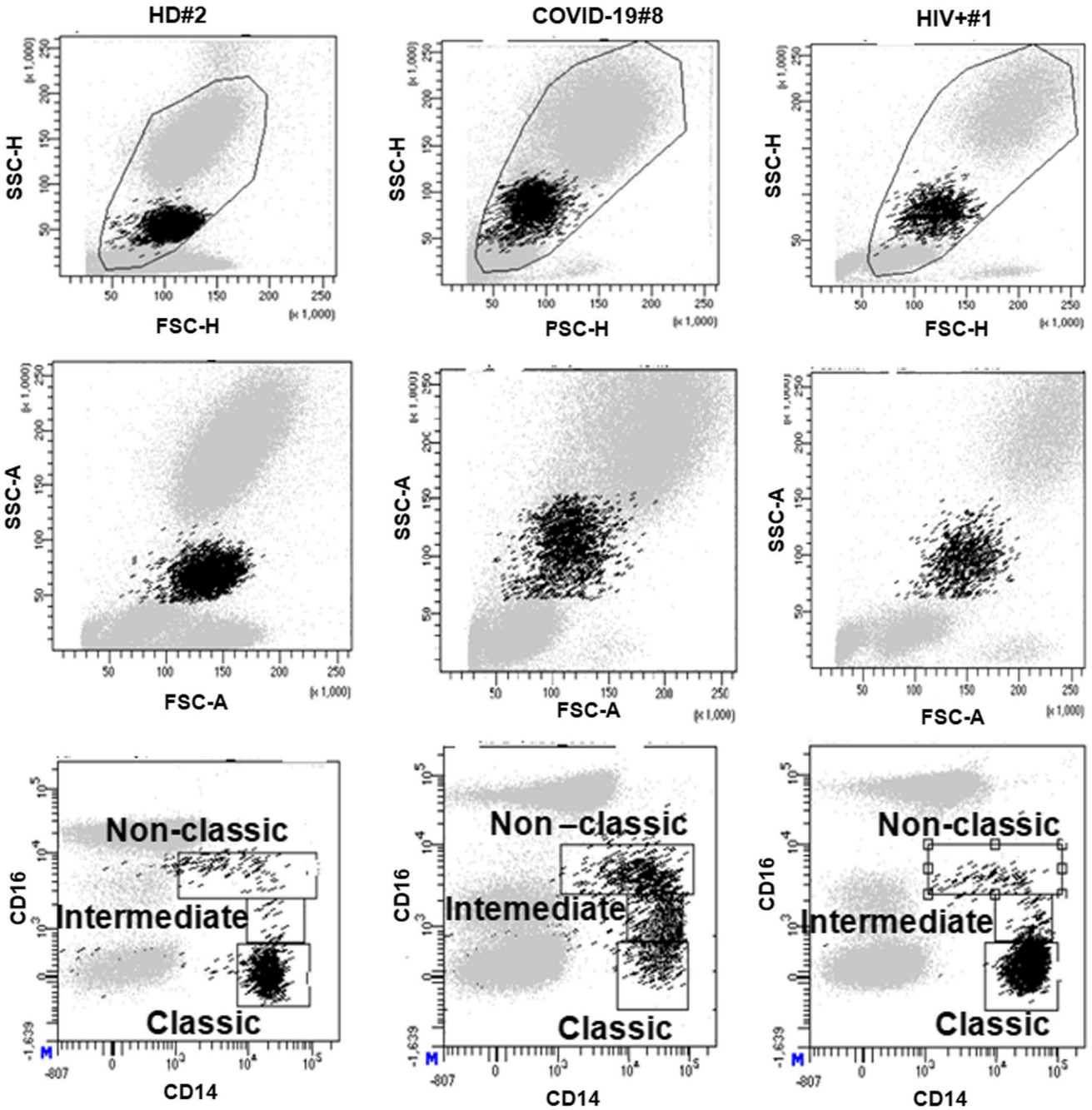

Fig. 2 Cytometric features of circulating monocytes. The figure shows the morpho-cytometric characteristics (upper and middle rows) and the relative distribution among the three monocytes subsets (classical, intermediate and nonclassical monocytes) (lower panels) of circulating monocytes on representative samples derived from COVID-19 patient # 8 (middle column), healthy donor #2 (HD, left column) and HIV + naïve patient #1 (right column)

of intermediate monocytes in the sample derived from the COVID-19 patient with respect to the other groups (Supplementary Fig. 3, Panel B). Moreover, the t-SNE maps of mean fluorescence intensity of CD38 and HLA-DR activation markers showed their increased expression on monocytes subsets derived from both patients with respect to the healthy donor. Higher CCR2 monocyte expression was only observed in the clusters of the COVID-19 patient with respect to those of the other groups (Supplementary Fig. 3, Panel C). These differences in CD38, HLA-DR and CCR2 MFI between COVID-19 patients and both HIV + naïve patients and HD, suggested by the multidimensional analyses performed on cells from representative subjects of each group, were confirmed by conventional cytometry analyses (Supplementary Fig. 4 and Fig. 3).

Correlations among monocyte and clinical parameters

In order to verify whether monocyte expression profiles of CD38, HLA-DR and CCR2 on different monocyte subsets may have a clinical impact in COVID-19 patients, we correlated MFI of CD38, HLA-DR and CCR2 molecules with the serum levels of the following clinical indexes: creatinine, glomerular filtration rate (GFR), azotemia, troponin I, D-dimer, ferritin, fibrinogen, pro-calcitonin, C-reactive protein, lactate dehydrogenase, creatinine phosphokinase (Supplementary Table 3). We found that CCR2 expression on classic, intermediate and nonclassic monocyte subsets highly correlated with kidney function parameters (Fig. 4, Box A, B and C, respectively). In fact, we observed a direct correlation between CCR2 MFI on classical, intermediate and nonclassic monocytes with azotemia (Fig. 4 upper panels) and creatinemia (Fig. 4, middle panels), and an inverse correlation with GFR (Fig. 4, lower panels). Moreover, CD38 expression on intermediate

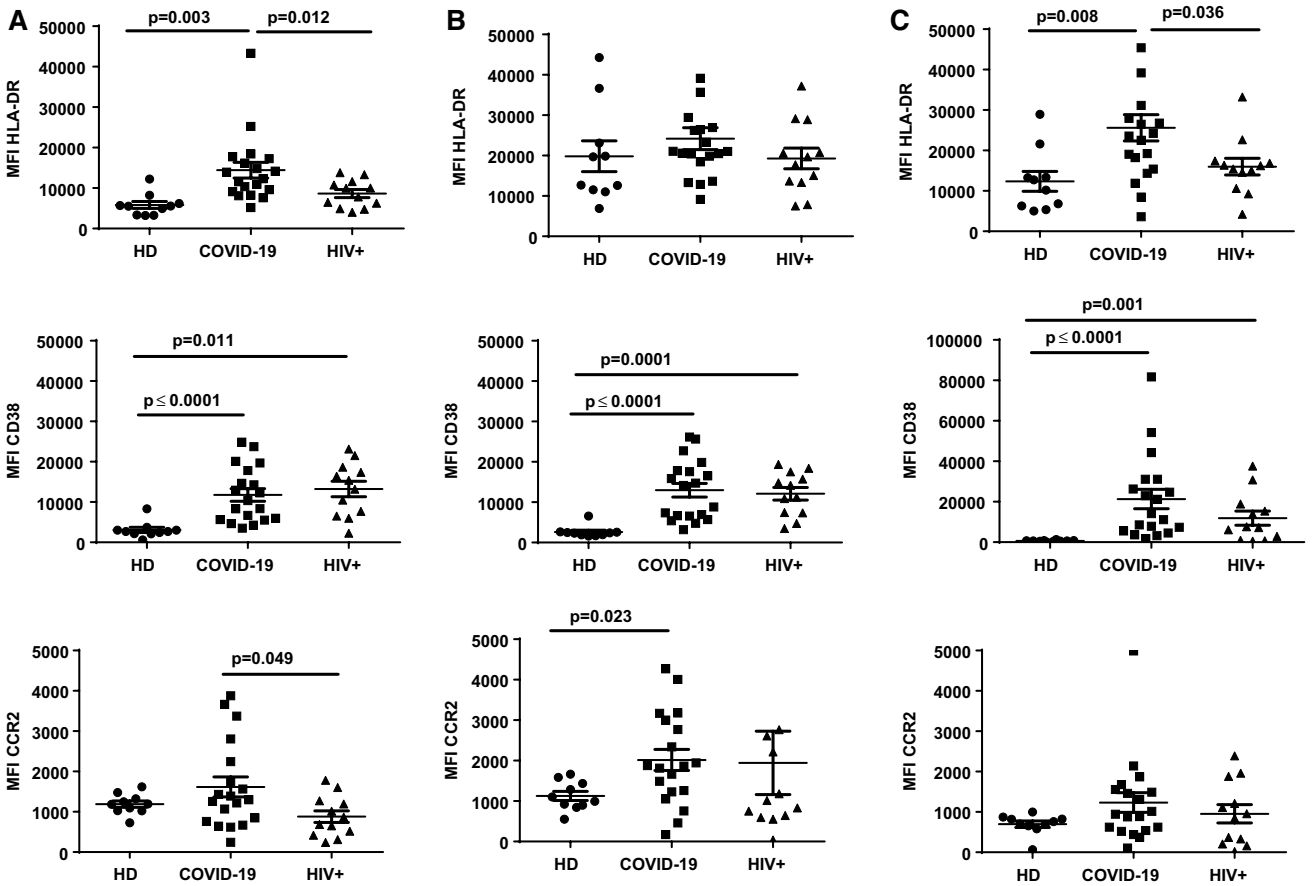

Fig. 3 Comparison of HLA-DR, CD38 and CCR2 MFI on monocyte subsets derived from healthy donors (HD), COVID-19 and HIV + naïve patients. Box A, Box B and Box C show the comparison of HLA-DR, CD38 and CCR2 MFI (upper row, middle row, lower row, respectively) on classical monocytes (Box A), intermediate monocytes (Box B) and nonclassical monocyte (Box C) derived from healthy donors (HD), COVID-19 patients and HIV + naïve patients, respectively

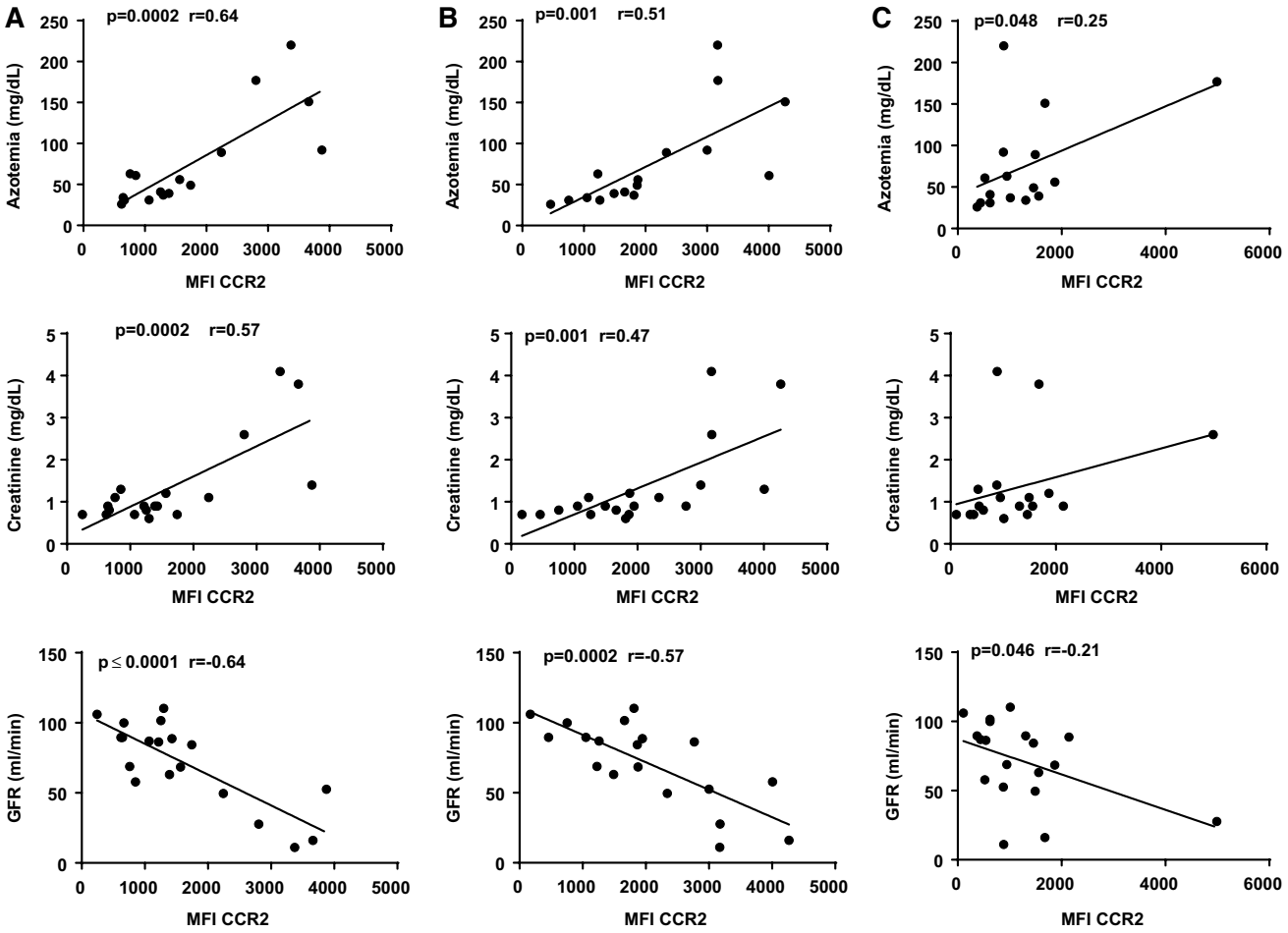

Fig. 4 Correlations between CCR2 MFI on circulating monocyte subsets and parameters of renal function in COVID-19 patients. Box A, Box B and Box C show the correlations of serum azotemia (upper row), creatinine (middle row) and GFR (lower row) with CCR2 MFI on classical (Box A), intermediate (Box B) and nonclassical monocytes (Box C), respectively, in COVID-19 patients

monocyte subset revealed a direct correlation with ferritin and fibrinogen serum concentrations (Supplementary Fig. 5).

Concerning the days before a negative swab (median days = 10), we found a direct correlation ($r = 0.017$) with % of classical monocytes and with the CCR2 MFI expression on nonclassical monocytes ($r = 0.01$). For duration of clinical manifestations of symptoms (median days = 22), we found a positive correlation ($r = 0.023$) with % of classical monocytes and with the CCR2 MFI expression on classical ($r = 0.02$) and on nonclassical monocytes ($r = 0.04$).

Discussion

Collectively, the results of our study show that monocytes of COVID-19 patients are highly activated and that their distribution among the three circulating subsets of monocytes is quite peculiar, since it is different from that of both healthy donors and HIV + naive patients. This finding, together with that related to the peculiar morpho-cytometric parameters of intermediate monocytes in COVID-19 patients, suggests that SARS-CoV-2 infection induces a robust stimulation of these cells. Such stimulation selectively expands the intermediate monocytes that constitute

a cell subset provided with pro-inflammatory features and that has been associated with infective and inflammatory diseases [7]. Interestingly, these cells in COVID-19 patients highly expressed CCR2, a chemokine receptor that likely drives them toward the tissue site of inflammation [11, 12]. Accordingly, elevated levels of CCL2, the chemokine specific for the CCR2, have been observed in the bronco-alveolar fluid of COVID-19 patients with pneumonia [13], thus likely explaining its rich monocyte content [14]. Hence, we searched for an association between CCR2 MFI and the serum levels of several clinical indexes in our series of COVID-19 patients. We found that CCR2 MFI on intermediate monocytes correlated with all the clinical parameters of renal function (creatinine, GFR, azotemia). This unprecedented finding suggests that intermediate monocytes may be also pathogenically related to renal alterations and acute kidney insufficiency, clinical manifestations of SARS-CoV-2 infection observed in about 14% and 5% of patients [15], respectively. This is not surprising since SARS-CoV-2 may target renal tissues due to the presence of the ACE2 receptor on the epithelial cells of the proximal tubules [16]. Flow cytometry analyses of blood samples of COVID-19 demonstrate that monocyte percentage did not change, while a different distribution of peripheral blood monocyte subsets is observed. These results add new information about the alteration in monocyte subset dynamic that could support the role of infected circulating monocytes to induce acute inflammatory responses and cause cytokine storm which enhances the pathogenicity of the virus and disease worsening in patients. Moreover, the studies of CCR2 expression level on classic and intermediate monocytes only in COVID-19 patients showing an increase in this molecule confirm the role of CCR2 in viral replication and immune activation as inducer factor to the employment of inflammatory monocytes from the blood circulation to tissues.

Our data show that the intensity of CD38 expression by intermediate monocytes of COVID-19 patients correlated with biomarkers of inflammation, as ferritin and fibrinogen, whose production is dependent by IL6 [17, 18], a cytokine highly released by monocytes [19] and found at high concentration in COVID-19 patient serum [20].

Collectively, our data show that SARS-CoV-2 infection determines peculiar alterations of monocytes targeting morpho-phenotypic and maturation features and that elevated CCR2 MFI mainly on intermediate monocytes associates with parameters of renal function.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10238-022-00927-9.

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Author contributions AP, DF, GF, MF, TA, were in charge of the study design. AP, DF, TA, FB carried out the phenotyping experiments and analyzed the data. AP, DF, FF, TA, FB performed laboratory work and experiments. CD, ADM, MB, ADB, AV, PP, RDP, DRG managed the patients, their withdrawals and the clinical aspect; MG managed the data collection site. All authors contributed to the article and approved the submitted version.

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Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Outside the submitted work: C.D. has received speaker honoraria from Angelini, Novartis, Gilead, ViiV, Shinogi, D. R. G. reports personal consultation fees from Stepstone Pharma GmbH and grants from MSD Italia and Correvio Italia. A.D.B. reports hospital grant from Gilead and consultancy for ViiV, Janssen, MSD, Gilead, Abbvie. M. B. serves on scientific advisory boards for Angelini, AstraZeneca, Bayer, Cubist, Pfizer, Menarini, MSD, Nabriva, Paratek, Roche, Shionogi, Tetraphase, The Medicine Company, and Astellas Pharma Inc, and has received funding for travel or speaker honoraria from Algorithm, Angelini, Astellas Pharma Inc, AstraZeneca, Cubist, Pfizer, MSD, Gilead Sciences, Menarini, Novartis, Ranbaxy, and Teva.

Availability of data material The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics approval The study was carried out in compliance with the Helsinki Declaration and approved by the Ethical Committee of the San Martino Hospital in Genoa (N. CER Liguria 114/2020—ID 10420 and P.R.251REG2014).

Consent to participate All participants provided written informed consent to participate.

Consent for publication All participants have consented to publication of their data.

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