Alterations of Blood Immune Cells in Elderly Patients with COVID-19 and with or without Pre-Existing Type 2 Diabetes

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Abstract

Elderly individuals, especially those with pre-existing conditions like type 2 diabetes mellitus (T2DM), have a high risk for developing severe cases of COVID-19. The aim of this work was to characterize the alterations of blood immune cells (BIC) in patients with symptomatic COVID-19 and confirmed SARS-CoV-2 infection, ≥60 years and who needed hospitalization in the Centro de Salud Hospital of Tucuman, Argentina, during the second peak of the pandemic in Argentina. Ten patients were enrolled from December 2020 to May 2021. Blood samples were taken at the time of admission (day 0) and five days after (day 5) for routine laboratory tests and the characterization of BIC by flow cytometry. Most of the patients were men (70%) aged between 60 and 78 years. The 70% of patients had T2DM while 50% had arterial hypertension. At day 0, all the patients had increased neutrophils and inflammatory markers (C reactive protein and D-dimers) and reduced numbers of lymphocytes, HLA-DR* monocytes, CD16*CD56* NK cells, CD3*HLA-DR*CD25* cells, CD4* and CD8* T cells in blood. Patients received a standard treatment
for COVID-19 care (O₂, corticosteroids and antibiotics). The hospital treatment normalized the levels of BIC (day 5) in 30% of patients who were those with no comorbidities. In patients with T2DM, BIC recovery was variable. In T2DM patients who required administration of plasma (30%), prolonged O₂ therapy (40%) or referral to the intensive care unit (10%) significant reductions of CD16⁺CD56⁺, CD3⁺HLA DR⁺CD25⁺, CD4⁺ and CD8⁺ cells were observed between days 0 and 5. In line with previous studies, our results show that absolute counts of major lymphocyte subsets in blood are significantly and substantially decreased during the course of severe COVID-19 disease in elderly patients. These BIC alterations may persist despite clinical care in elderly patients with T2DM. Further studies are needed to investigate the utility of early lymphocyte subset measurements as prognostic biomarkers of disease severity, mortality, and response to treatment in COVID-19 elderly patients with T2DM.

Keywords
COVID-19, SARS-CoV-2, Diabetes, Elderly, Blood Leukocytes, Immune Response

1. Introduction
Diabetes is a global health problem due to its high morbidity and mortality associated to the increased risks of cardiovascular diseases and infections. The relationship between type 2 diabetes mellitus (T2DM) and infection has long been recognized [1]. Non-controlled or poorly controlled T2DM enhances the risk for skin, bone, eye, and mucosal infections, with significantly increased hospitalization and mortality rates. In fact, it was reported that infectious diseases substantially diminish life expectancy of patients with T2DM [2]. The hazard ratio for a person with T2DM dying from any infection was 2.39, and this is more likely to happen when the glycemic control is poor [3].

Respiratory infections, particularly pneumonia, are more often common and serious in elderly patients with T2DM [4]. Studies performed during influenza epidemics suggested that individuals with T2DM were highly susceptible to severe infections compared to healthy individuals [5]. In addition, T2DM has been recognized as a risk factor for severe disease progression and mortality in respiratory infections caused by the severe acute respiratory syndrome coronavirus (SARS-CoV) [6], Middle East respiratory syndrome-related coronavirus (MERS-CoV) [7], and the novel SARS-CoV-2 [8]. In this regard, multi-center studies of the coronavirus infectious disease 2019 (COVID-19) found that patients with T2DM required more medical interventions and had a significantly higher mortality rates [9]. The severe cases of COVID-19 can rapidly progress to acute respiratory distress syndrome (ARDS), septic shock, and multiple organ dysfunction syndrome [10] [11] [12]. It was suggested that the deregulated immune response caused by T2DM would be responsible for the increased disease severity
of SARS-CoV-2 since higher ratios of lymphopenia and increased levels of neutrophils, serum C reactive protein (CRP), and inflammatory cytokines were observed in the patients with COVID-19 and pre-existing T2DM [9].

Changes in blood immune cells (BIC) could be extrapolated to understand how the specific cell subsets are responsible for severe outcomes in patients with viral respiratory infections and expanding the knowledge on how these subsets contribute to the overall immune response, in particular to the cases associated with severe complications. In fact, several previous studies have already described the potential role of blood leucocytes counts to evaluate the progression towards a more severe disease and to predict the risk of death among COVID-19 patients [13] [14] [15] [16] [17]. The majority of these studies were conducted in the northern hemisphere during the first phases of the COVID-19 pandemic. Studies involving patients from different geographical areas and later during the pandemic are needed to confirm and extend those results.

The aim of this work was to characterize the alterations of BIC in patients with symptomatic COVID-19 and confirmed SARS-CoV-2 infection, older than 60 years, with or without pre-existing T2DM and who needed hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina.

2. Materials and Methods

2.1. Study Design

This study was conducted in accordance with ethical principles of the Declaration of Helsinki and Good Clinical Practice guidelines and received approval from the authorized institutional review board SIPROSA (Sistema Provincial de Salud de la Provincia de Tucuman), Exp. N. 3629-410-V-2020 protocol number approval CI N37-2020. Participants or legally authorized representatives provided informed consent.

A single-center study involving patients with SARS-CoV-2 infection was performed at the Centro de Salud Hospital of Tucuman (San Miguel de Tucumán, Tucuman, Argentina) during the second peak of the pandemic (from December 2020 to May 2021). The ten patients enrolled were elderly adults (≥60 years), hospitalized for COVID-19, with a positive reverse transcription polymerase chain reaction (RT-PCR) for SARS-CoV-2 on a nasopharyngeal swab. The original SARS-CoV-2 virus infected the patients while no variants were detected. All patients were tested for bacterial and viral respiratory pathogens including Influenza Viruses A and B and Streptococcus pneumoniae. Participants were excluded if requiring invasive mechanical ventilation at study entry.

The following information on each patient was extracted from the hospital medical records: age, sex, medical history, symptoms, severity assessment on admission, laboratory findings, chest computed tomography (CT), radiograph findings, treatment, and efficacy.

All participants received a background standard of care for COVID-19 in keep-
ing with the local clinical practice for COVID-19 management, which include corticosteroids and non-invasive O2 therapy for 10 days, and antibiotics for 7 days. Healthy age-matched subjects (non-infected) were used as controls.

2.2. Flow Cytometry

Blood samples were obtained in heparinized tubes and stained with different mixtures of the following mouse anti-human antibodies from Inmuino Tools: Fluorescein isothiocyanate (FITC) CD3 antibody (clone HIT3b); phycoerythrin (PE) CD4 antibody (clone EDU-2); peridinin-chlorophyll-protein (PerCP) CD8 antibody (clone UCHT-4); Al-lophycocyanin (APC) CD11b antibody (clone HI11b); PE CD11c antibody (clone BU15); FITC CD16 antibody (clone HI16a); APC CD19 antibody (clone LT19); APC CD25 antibody (clone MEM-181); PerCP CD45 antibody (clone HI30); PE CD56 antibody (clone B-A19); FITC HLA-DR antibody (clone LT-DR); and annexin V APC-conjugated. Blood samples from COVID-19 patients (50 µL) were incubated with different antibody mixtures for 30 min at 4˚C protected from light. Samples stained with a single color were used to set the compensation, and an unstained sample was stored under the same conditions. Red blood cells lysis and white blood cells fixation were done by adding 500 µL of Lyse/Fix Buffer (BD Biosciences) into each tube. Cells were acquired on a FacsCalibur flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). Healthy volunteers of aged between 60 and 71 years provided blood samples and served as the non-infected control group.

2.3. Statistical Analysis

Differences between groups or days of sampling were assessed using the Mann-Whitney U test, Kruskal–Wallis test (continuous variable) or Chi2 test (categorical variables), as appropriate. A two-sided p value of <0.05 was considered statistically significant.

3. Results

3.1. Symptoms and Routine Laboratory Tests in Elderly Patients with COVID-19

In all selected patients, CT scans that were performed at the time of admission revealed abnormal results. The most common patterns on chest CT were ground-glass opacity and bilateral patchy shadowing. Thus, the main clinical condition on admission for all the enrolled patients was SARS-CoV-2-related pneumonia. Most of the patients were men (70%) aged between 60 and 78 years. The majority of the enrolled population had at least one comorbidity. The 70% of patients had T2DM while 50% had arterial hypertension. The rate of ICU admission was 10% and no deaths were registered.

Blood samples were taken at the time of admission (day 0) and five days after (day 5) for routine laboratory tests and the characterization of BIC by flow cy-
tometry. At day 0, sight increases in serum lactate dehydrogenase (LDH) levels were observed in most patients (Figure 1A) while serum creatinine was significantly higher than non-infected controls in only one patient (Figure 1B). The levels of blood ferritin were augmented in most of the SARS-CoV-2-infected subjects (Figure 1C). In addition, all the patients had increased levels of the inflammatory markers C reactive protein (data not shown) and D-dimers (Figure 1D). Blood hemoglobin values were under the normal values in 60% of the patients (Figure 1E) while blood creatine phosphokinase (CPK) was significantly increased in 80% of the subjects (Figure 1F).

Patients received a standard treatment for COVID-19 care that included O2 therapy, corticosteroids and antibiotics. These treatments normalized the levels of routine laboratory tests on day 5 in 30% of patients who were those with no comorbidities. In subjects with T2DM, recovery was variable. Patients with T2DM required administration of plasma (30%), prolonged O2 therapy (40%) or referral to the intensive care unit (10%). Thus, the patients with T2DM needed

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**Figure 1.** Laboratory test of elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. Lactate dehydrogenase activity (LDH) (A), creatinine (B), ferritin (C), D-dimers (D), hemoglobin (E) and creatine phosphokinase (CPK) (F) were determined in blood samples at the time of admission (d0). The light blue boxes in each graph indicate the average value for each laboratory parameter found in healthy age-matched individuals.
significantly more intensive integrated treatments to manage their symptoms of COVID-19 than the subjects with no diabetes. All the patients with T2DM presented HBA1c values within normal ranges and were receiving standard medication for the treatment of their disease (metformin) at the time of hospital admission. No relationship was found between the severity of COVID-19 and HBA1c values or medication in our study.

3.2. Blood Immune Cells Variations in Elderly Patients with COVID-19

The numbers of BIC populations were also evaluated at days 0 and 5 as shown in Figure 2. At the time of admission to the hospital, the patients with COVID-19 presented blood leukocytes values within the normal range (Figure 2A). Nevertheless, when discriminating the populations of granulocytes and lymphocytes, it was observed a significant alteration of the normal proportions of blood leukocytes (Figure 2B) characterized by increased numbers of granulocytes (Figure 2C, Figure 2D) and reduced numbers of lymphocytes (Figure 2E, Figure 2F) when compared with non-infected controls.

The treatment given in the hospital to the patients reduced the number of granulocytes in most of the subjects at day 5 with the most remarkable effect observed in patient in which the granulocytes fell from 37.7 to 8.5 G/L (Figure 2D). In addition, in most of the patients with COVID-19 a significant reduction in the numbers of blood lymphocytes was observed at day 5 (Figure 2F). Of note, patients with no T2DM significantly increased their numbers of blood lymphocytes when values from days 0 and 5 where compared.

The expression of annexin was analyzed in CD45+ cells in order to assess the impact of SARS-CoV-2 infection of BIC apoptosis (Figure 3A, Figure 3B). At day 0, most of the patients with COVID-19 had percentages of blood CD45+Annexin+ cells that were significantly higher than non-infected controls. No differences were found in the MFI of blood CD45+Annexin+ cells when days 0 and 5 were compared. However, the analysis of the absolute number of CD45+Annexin+ cells demonstrated a significant decrease from days 0 to 5 in most of the patients. Of note, patients with no T2DM had no variations in the absolute number of CD45+Annexin+ cells from days 0 to 5 (Figure 3B).

The down-regulation of blood HLA-DR expression has been reported in the context of acute clinical inflammation, especially sepsis [18]. Thus, we also aimed to evaluate the variations of blood CD45+HLA-DR+ cells in elderly patients with COVID-19. As shown in Figure 3C, the percentages of blood CD45+HLA-DR+ cells were significantly reduced in SARS-CoV-2-infected patients when compared to non-infected controls at day 0. No significant variations were detected in the percentage of blood HLA-DR+ cells when values of days 0 and 5 were compared (Figure 3C). However, when absolute numbers were analyzed, it was detected that most of the patients with COVID-19 reduced the numbers of blood CD45+HLA-DR+ cells from days 0 to 5 (Figure 3D). Only
patients with no T2DM were able to increase the numbers of blood HLA-DR+ cells after the hospital treatment.

3.3. Blood Monocytes Variations in Elderly Patients with COVID-19

We next concentrated the analysis of BIC in monocytes (Figure 4). A remarkable decrease in the percentages of blood monocytes was observed when patients

![Figure 2](image-url). Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. Total leukocytes count (A, B), granulocytes (C, D) and lymphocytes (E, F) were determined in blood samples at the time of admission (day 0) and five days after (day 5). The light blue box in the graphs indicates the average value for the parameter found in healthy age-matched patients (non-infected). Red, blue and grey lines indicate significant decreases, increases and no variations for the same patient between days 0 and 5, respectively. Asterisks indicate significant differences when compared to the non-infected control group (*P < 0.05).
with COVID-19 at day 0 were compared with non-infected controls (Figure 4A). At day 5, most of the subjects increased the percentages of blood monocytes when compared to the values at day 0 (Figure 4A). The same trend was observed when the absolute values of monocytes were analyzed (Figure 4B). Of note, some patients showed a remarkable reduction in the absolute numbers of blood monocytes from days 0 to 5, all these patients belonged to the group of T2DM. The variation of HLA-DR in monocytes has been proposed as marker of infections and prognosis in various conditions including sepsis [19], trauma [20], and burns [21]. Thus, we also evaluated the variations of blood HLA-DR\textsuperscript{hi} monocytes (Figure 4C, Figure 4D). As expected, the percentages of blood HLA-DR\textsuperscript{hi} monocytes in patients with COVID-19 at day 0 was significantly lower than non-infected controls (Figure 4C). When the percentages of these blood immune cells at days 0 and 5 were compared it was ob-served that most of the patients increased this parameter (Figure 4D). However, when the absolute number of HLA-DR\textsuperscript{hi} monocytes was analyzed 60% of the patients had significant reductions from day 0 to 5 while 40% of the subjects, most from the non-T2DM group, increased the values of these cells.
Figure 4. Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. Monocytes (A, B), and HLA-DR$^{\text{hi}}$ monocytes (C, D) were determined in blood samples at the time of admission (day 0) and five days after (day 5). Healthy age-matched patients (non-infected) were used for comparisons. Red, blue and grey lines indicate significant decreases, increases and no variations for the same patient between days 0 and 5, respectively. Asterisks indicate significant differences when compared to the non-infected control group (*P < 0.05).

To correlate the alterations of the studied blood cell populations with differences in age, sex, comorbidities and severity of the COVID-19, we made an analysis of the relative variations of all the parameters evaluated and represented them in a heat-map (Figure 5). Two groups of patients were clearly differentiated with this analysis considering the variations of whole blood leukocytes, granulocytes, lymphocytes, CD45$^{\text{+}}$Annexin$^{\text{+}}$ cells, CD45$^{\text{+}}$HLA-DR$^{+}$ cells and HLA-DR$^{\text{hi}}$ monocytes. Most of the patients with T2DM, arterial hypertension and/or other comorbidities clustered together and showed alterations in BIC that were not significantly improved by the standard hospital treatment. Furthermore, the individuals who required administration of plasma, prolonged O$_2$ therapy (more than 10 days) or referral to the intensive care unit were in this group (Figure 5). No clear association of BIC alterations with sex or age was detected.

3.4. NK Cells Variations in Elderly Patients with COVID-19

The variations of blood NK cells in patients with COVID-19 were evaluated using the CD3, CD16 and CD56 markers as shown in the Figure 6. A great variation in the percentages of CD3$^{\text{+}}$CD16$^{\text{+}}$CD56$^{\text{+}}$ NK cells was observed in the SARS-CoV-2-infected subjects at day 0. While 60% percent of the elderly pa-
Patients with COVID-19 presented values within the normal range, 40% had increased or decreased values with respect to the non-infected controls (Figure 6A). A great variability was also observed in the percentages of CD3−CD16+CD56+ NK cells when the values at days 0 and 5 were compared. However, when the absolute number of CD3−CD16+CD56+ NK cells was analyzed it was shown that 60% of the patients had significant reductions from days 0 to 5 while 40% of the subjects, most from the non-T2DM group, increased the values of NK cells (Figure 6B). We also evaluated the variations in the cytokine-producing CD56hi NK cells and the cytotoxic CD56lo NK cells [22]. The same trend variation of total NK cells was observed when the cytokine producing CD16+CD56hi NK cell population was analyzed (Figure 6D). Of note, when the cytotoxic CD16+CD56lo NK cell population was studied it was observed that their percentages and their absolute numbers increased or remained unaltered in most of the patients (Figure 6C). Only three patients, all belonging to the T2DM group, had a significant reduction of cytotoxic CD16+CD56lo NK cells from days 0 to 5.

Figure 5. Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. Heat-map analysis of total blood leukocytes counts, blood granulocytes, blood lymphocytes, CD45+Annexin+ cells, CD45+HLA-DR+ cells and HLA-DRhi monocytes variations between the time of admission (day 0) and five days after (day 5). The differences between the patients considering sex, age, the presence of type 2 diabetes (T2DM), arterial hypertension (AHT) and other comorbidities (CMB), and the need of plasma, O2 or intensive care unit (ICU) are shown in green and orange.
Figure 6. Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. CD3−CD16+CD56+ NK cells (A, B), CD3-CD16+CD56lo NK cells (C) and CD3−CD16+CD56hi NK cells (D) were determined in blood samples at the time of admission (day 0) and five days after (day 5). Healthy age-matched patients (non-infected) were used for comparisons. Red, blue and grey lines indicate significant decreases, increases and no variations for the same patient between days 0 and 5, respectively.

We also performed a heat-map analysis with the values of total CD3−CD16+CD56+ NK cells, cytokine producing CD56hi cells and cytotoxic CD56lo cells (Figure 7). No clearly defined group was observed among the elderly patients with COVID-19. It was also not observed a clear association between blood NK cells variations and the differences in age, sex, comorbidities or severity of the COVID19.

3.5. Lymphocytes Variations in Elderly Patients with COVID-19

Finally, we focused in the variation of the different populations of blood lymphocytes. The 40% of the patients with COVID-19 had percentages of blood CD3−CD19+ B cells that were below the normal range found in the non-infected group (Figure 8A, Figure 8B). In addition, all the SARS-CoV-2-infected patients had values of blood CD3+CD19+ T cells that were lower than non-infected individuals (Figure 8C, Figure 8D). Most of the patients did not present significant variations in the percentages or in the absolute numbers of blood CD19+ B cells, with the exception of one patient who showed a reduction from 2.2 to 0.3 G/L when days 0 and 5 were compared (Figure 8B). When analyzing the variations of CD3+ T cells, it was observed that half of the patients increased the absolute values of these cells from days 0 to 5, while the other half showed significant reductions (Figure 8D).
Figure 7. Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. Heat-map analysis of CD3⁻CD16⁺CD56⁺, CD3⁻CD16⁺CD56⁻ and CD3⁻CD16⁺CD56⁻ NK cells variations between the time of admission (day 0) and five days after (day 5). The differences between the patients considering sex, age, the presence of type 2 diabetes (T2DM), arterial hypertension (AHT) and other comorbidities (CMB), and the need of plasma, O₂ or intensive care unit (ICU) are shown in green and orange.

In addition, we evaluated the activation of T cell by assessing the expression of the markers HLA-DR and CD25 (Figure 9). Most of the patients showed percentages of CD3⁺HLA-DR⁺ T cells that were higher than the uninfected controls (Figure 9A), while the percentages of CD3⁺CD25⁺ cells were slightly increased in COVID-19 patients, but without statistically significant differences with the controls (Figure 9C). When the variations of CD3⁺HLA-DR⁺ and CD3⁺CD25⁺ T cells between days 0 and 5 were analyzed, it was observed that most of the patients decreased the absolute values of both immune cell populations (Figure 9B, Figure 9D).

The study of CD4⁺ and CD8⁺ T cells in blood samples of the elderly patients with COVID-19 revealed that both lymphocyte populations were decreased when compared with non-infected controls (Figure 10). When the percentages of CD3⁺CD4⁺ (Figure 10B) and CD3⁺CD8⁺ (Figure 10D) T cells were compared between days 0 and 5, it was observed that most of the patients increased these immune cell populations. However, the study of the absolute values revealed that half of the patients actually decreased the values of CD4⁺ and CD8⁺ lymphocytes.

A population of double positive T cells was also found in the blood of elderly patients with COVID-19 (Figure 11). The percentages of CD4⁺CD8⁺ T cells were lower in patients in comparison with the values observed in non-infected subjects. Most of the patients did not present variations in the percentages of
these cells between days 0 and 5. Only four patients showed decreases in the percentages of CD4+CD8+ T cells, all belonging to the group of T2DM. However, when the absolute numbers of CD4+CD8+ T cells was analyzed, it was shown the most of the patients significantly reduced these immune cells from day 0 to 5 (Figure 11B).

Figure 8. Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. CD3-CD19+ B cells (A, B), and CD3+CD19- T cells (C, D) were determined in blood samples at the time of admission (day 0) and five days after (day 5). Healthy age-matched patients (non-infected) were used for comparisons. Red, blue and grey lines indicate significant decreases, increases and no variations for the same patient between days 0 and 5, respectively. Asterisks indicate significant differences when compared to the non-infected control group (*P < 0.05).
Figure 9. Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. CD3+HLA-DR+ T cells (A, B), and CD3+CD25+ T cells (C, D) were determined in blood samples at the time of admission (day 0) and five days after (day 5). Healthy age-matched patients (non-infected) were used for comparisons. Red, blue and grey lines indicate significant decreases, increases and no variations for the same patient between days 0 and 5, respectively. Asterisks indicate significant differences when compared to the non-infected control group (*P < 0.05).

Figure 10. Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. CD3+CD4+CD8- T cells (A, B), and CD3+CD4-CD8+ T cells (C, D) were determined in blood samples at the time of admission (day 0) and five days after (day 5). Healthy age-matched patients (non-infected) were used for comparisons. Red, blue and grey lines indicate significant decreases, increases and no variations for the same patient between days 0 and 5, respectively. Asterisks indicate significant differences when compared to the non-infected control group (*P < 0.05).
Figure 11. Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. Double positive CD3+CD4+CD8+ T cells (A, B) were determined in blood samples at the time of admission (day 0) and five days after (day 5). Healthy age-matched patients (non-infected) were used for comparisons. Red, blue and grey lines indicate significant decreases, increases and no variations for the same patient between days 0 and 5, respectively. Asterisks indicate significant differences when compared to the non-infected control group (*P < 0.05).

Finally, we constructed a heat-map to summarize the variations of the different blood lymphocytes populations and to associate them with age, sex, presence of comorbidities or the severity of the COVID-19 in elderly patients (Figure 12). No clear association of blood lymphocytes alterations with sex or age was detected. In contrast, the simultaneous comparison of the variations of the different blood lymphocytes populations allowed a good separation between patients with and without T2DM. Furthermore, the patients who presented the most severe COVID-19 were separated from the rest of the patients with T2DM (Figure 12).

4. Discussion

Research from the last two years has clearly demonstrated that age is a critical determinant in the outcome of SARS-CoV-2 infection. Older people is more prone to suffer severe symptoms of COVID-19 as well as to develop deregulate respiratory and systemic inflammatory responses that worsen their prognosis. More severe symptoms in elderly patients with COVID-19 are often accompanied by significant increases of inflammatory and biochemical markers including C-reactive protein, D-dimer, LDH, erythrocyte sedimentation rate, ferritin.
Figure 12. Blood leukocytes variations in elderly patients infected with SARS-CoV-2 (COVID-19+) with or without type 2 diabetes, who required hospitalization in the Centro de Salud Hospital of Tucuman during the second peak of the pandemic in Argentina. Heat-map analysis of blood CD3^+CD19^-, CD3^+CD4^+CD8^-, CD3^+CD4^-CD8^+, CD3^+HLA-DR^+, CD3^+CD25^+ and double positive CD3^+CD4^+CD8^+ T cells variations between the time of admission (day 0) and five days after (day 5). The differences between the patients considering sex, age, the presence of type 2 diabetes (T2DM), arterial hypertension (AHT) and other comorbidities (CMB), and the need of plasma, O2 or intensive care unit (ICU) are shown in green and orange.

and fibrinogen [23]. In addition to inflammatory serum markers, it was shown that variations in blood leukocytes counts are significantly affected in patients with severe COVID-19, especially in non-survivors [17]. The increased susceptibility to SARS-CoV-2 infection in the elderly could be significantly enhanced by the presence of a pre-existing T2DM. In fact, studies have reported that patients with COVID-19 and pre-existing T2DM had much higher mortality rates than patients without diabetes [8] [24] [25]. Therefore, the scientific community has focused on the study of the cellular and molecular processes mediated by leukocytes that drive progression from mild to severe/fatal COVID-19 to identify predictive biomarkers and therapeutic targets.

In this work, we characterized the changes in BIC of elderly patients with symptomatic COVID-19 and with or without pre-existing T2DM. In our study, no deaths were recorded despite the fact that the recruited individuals had pneumonia, remarkable alterations of inflammatory parameters and required hospitalization. This may be because the work was done during the second peak
of the pandemic in Argentina and the Centro de Salud Hospital of Tucuman was able to efficiently apply protocols that preserved the lives of patients.

When we analyzed the BIC populations in our study, we observed that the SARS-CoV-2 infection in elderly patients induced a significant increase in granulocyte populations. These results are in line with several reports indicating alterations of the granulocytes counts in patients with COVID-19, especially in those who required hospitalization [8] [26] [27] [28] [29] [30]. The significant increase in blood neutrophils counts in the first days after admission to hospitals together with the presence of immature granulocytes including myelocytes and metamyelocytes in blood has been reported in patients with severe COVID-19 of all ages [26]. Single-cell RNA sequencing studies described the appearance of immature neutrophils in the blood of patients with severe COVID-19 that were absent patients with mild disease as well as morphological abnormalities [31]. In addition to granulocytes morphological abnormalities [26], studies described an up-regulation of the neutrophil-degranulation gene program [27] and the appearance of dysfunctional neutrophils expressing PD-L1 and exhibiting an impaired oxidative burst response [31] in patients with severe COVID-19 in comparison with individuals with mild or moderate disease. In addition to blood neutrophils alterations, studies in patients with COVID-19 also described changes in monocytes. The decrease in blood monocytes observed in patients with COVID-19 has been associated to the recruitment of CD16⁺CD14⁺ monocytes from the blood to infiltrate the inflamed tissues like the infected lung. A high expression of HLA-DRA, HLA-DRB1, and the co-stimulatory molecule CD83 was described in CD14⁺ monocytes of patients with mild COVID-19 [31]. HLA-DR³⁺CD11chi and HLA-DR³⁺CD83hi CD14⁺ monocytes with a strong antiviral IFN-signature have been linked to the expansion of antigen-specific T cells that are protective during viral infections. In contrast, monocytes from patients with severe COVID-19 have a low expression of HLA-DR [31]. In agreement with these previous results, we observed that the absolute number of HLA-DR³⁺ monocytes was reduced during the analyzed period while some patients, most from the non-T2DM group, increased the values of these cells. Thus, alterations of HLA-DR³⁺ monocytes numbers and emergency myelopoiesis with release of immature neutrophils, which are characteristics of severe COVID-19 contributing to ARDS development [31], were more notable in elderly patients with pre-existing T2DM.

Studies evaluating the numbers and activities of blood NK cells in patients with T2DM have reported some discrepancies. Some reports suggested that the activity of NK cells is altered in patients with T2DM, and it was shown that the low cytotoxic CD16⁺CD56hi NK cells are increased while the high cytotoxic CD16⁺CD56low cells are diminished [32]. Moreover, it was observed that NK cell activity is lower in patients with T2DM and it is significantly affected by the quality of glucose control [32]. These alterations are aggravated when patients are infected by SARS-CoV-2 [33] [34] [35] [36]. Reports described reduction of blood NK cell numbers in patients with moderate and severe COVID-19 [34] [35], which were markedly diminished in non-survivors [17]. Single-cell RNA
sequencing studies revealed that the CD56hi population was depleted in all COVID-19 patients but the CD56low population was depleted only in patients with severe disease [34]. In addition, it was shown that blood NK cells in severe disease had enhanced expression of the inhibitory receptor NKG2A, and lower capacity to produce IFN-γ, TNF-α, and granzyme B [33]. It was also observed that the population of blood NK cells as well as their cytotoxicity was rapidly normalized in patients with mild COVID-19, but these parameters recovered slowly in patients with severe disease [36]. Our results are in line with these previous studies describing the reduction of blood NK cells absolute counts in patients infected with SARS-CoV-2. However, although the most notable alterations in the reduction of CD16+CD56low NK cells were observed in patients with T2DM, studies of the variations of the different NK cell subpopulations were not able to clearly discriminate the patients with or without pre-existing T2DM in our study (Figure 7). These findings may be due to several factors including the number of individuals studied, the time at which the samples were taken, and the baseline status of the patients with respect to their glycemic control and its impact on NK cells.

In addition to the increase of blood granulocytes counts, several studies reported that lymphopenia is a consistent finding in SARS-CoV-2-infected patients [28]. The decrease of blood lymphocytes counts has been associated with disease severity and therefore, this parameter has been proposed as predictive marker of enhanced risk of hospitalization and mortality [29] [30]. In fact, several previous studies highlighted the potential role of blood lymphocyte counts to evaluate the progression and to predict the risk of death among COVID-19 patients [13] [14] [15] [16] [17]. Of note, studies comparing patients with COVID-19 and with or without pre-existing T2DM reported that subjects with T2DM more frequently had lymphopenia compared with non-diabetic persons [24]. In fact, blood total T lymphocytes, CD4+ T cells and CD8+ T cells counts were markedly lower in diabetic cases than in non-diabetic cases. Our results confirmed the findings of these previous works, high-lighting the potential of the study of lymphocytes, particularly T lymphocytes, to predict the severity of COVID-19 in elderly patients with and without comorbidities.

Detailed studies of lymphocytes subpopulations in patients with COVID-19 have shown that the absolute lymphocyte counts decrease is associated to the reduction of both CD4+ helper T cells and CD8+ cytotoxic T cells [16] [23], particularly in subjects with severe disease [24]. In addition, it was shown that the CD3+CD4+CD25+ T cell subset was significantly lower in the severe disease group when compared to patients with mild or moderate COVID-19 [23]. In agreement, we observed significant reductions of CD4+, CD8+ and CD4+CD25+ T cells in blood samples of elderly patients, with the most remarkable effect found in patients with pre-existing T2DM.

Our flow cytometry studies with the CD4 and CD8 markers also revealed alterations in the population of double positive T cells in the blood of elderly patients with COVID-19. CD4+CD8+ T cells were lower in patients with COVID-19
in comparison with the values observed in non-infected subjects and most of the patients significantly reduced these immune cells from day 0 to 5. In line with our results, a work comparing double positive T cells in the blood of patients infected with SARS-CoV-2 of different ages described that CD4⁺CD8⁺ cells were significantly diminished in the elderly group when compared to young patients [23]. Moreover, the magnitude of the reduction in this immune cells population seemed to be associated with the severity of the disease. Similarly, it was shown that in hospitalized patients with COVID-19 the absolute counts of double positive CD4⁺CD8⁺ lymphocytes progressively diminished in patients with more severe disease [17]. It is not yet clear what role these immune cells would play during SARS-CoV-2 infection. It was reported that CD4⁺CD8⁺ T cells are present in blood in small numbers and some studies have suggested that they are differentiated effect or memory cells with anti-viral effects, which are increased in the blood of individuals with chronic viral infections [37]. This fact would explain why lower values of these cells were found in the blood of patients with more severe COVID-19 but their exact function is under ongoing debate.

Our results showed that the study of the absolute values of the different populations of T lymphocytes would be more useful than their percentage values to predict the course of COVID-19. Similarly, it was reported that the differences in the absolute counts of T lymphocyte subsets would have a greater clinical relevance compared to their percentages in the context of SARS-CoV-2 infection [17]. Moreover, our work and those previously mentioned indicate that the study of blood T cells absolute counts in the early stages of COVID-19 might represent a useful tool for identifying subjects at enhanced risk of unfavorable outcomes.

5. Conclusion

In line with previous studies, we observed profound alterations in granulocytes, monocytes, NK cells and the T cell compartment. Our results show that absolute counts of major lymphocyte subsets in blood are significantly and substantially decreased during the course of severe COVID-19 disease in elderly patients. These BIC alterations may persist despite clinical care in elderly patients with T2DM. Our study had several limitations, chief among them its observational design and its small size. All patients had mild COVID-19 and, therefore, our data cannot be extrapolated to more severe disease. Further studies are needed to investigate the utility of early lymphocyte subset and the M1 and M2 subpopulations of macrophages measurements as prognostic biomarkers of disease severity, mortality, and response to treatment in a higher number of COVID-19 elderly patients with T2DM with mild and severe disease in our region.

Authors’ Contributions

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Institutional Review Board Statement

This study protocol received full approval from the local Ethics Committee of the Hospital Centro de Salud “Zenon Santillan”, from the Provincial Health System (SIPROSA) of Tucumán, Argentina, and was conducted in compliance with the Declaration of Helsinki (as amended in 2000) Approval Code: N37-2020. Expte. 3629-410-V-2020; Approval Date: 25/08/2020.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement

Data is contained within the article.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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