Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Technical Note

Evaluation of circulating leucocyte populations both in subjects with previous SARS-COV-2 infection and in healthy subjects after vaccination

Vincenzo Grimaldi a,*, Giuditta Benincasa b, Giusi Moccia a, Annunziata Sansone a, Giuseppe Signoriello c, Claudio Napoli a, b

a U.O.C. Division of Clinical Immunology, Immunohematology, Transfusion Medicine and Transplant Immunology. Clinical Department of Internal Medicine and Specialistic Units, University of Campania "L. Vanvitelli", 80138 Naples, Italy
b Department of Advanced Medical and Surgical Sciences (DAMSS), University of Campania "Luigi Vanvitelli", 80138 Naples, Italy
c Statistical Unit, Department of Mental Health and Preventive Medicine, University of Campania "Luigi Vanvitelli", 80138 Naples, Italy

ARTICLE INFO

Keywords:
COVID-19 infection
COVID-19 mRNA BNT162b2 (Pfizer-BioNTech) vaccine
T cell immune response
Flow cytometry characterization

ABSTRACT

Innate immune mechanisms are central players in response to the binding of pathogens to pattern-recognition receptors providing a crucial initial block on viral replication. Moreover, innate immune response mobilizes cells of the cellular-mediated immune system, which develop into effector cells that promote viral clearance. Here, we observed circulating leukocyte T cell response in healthy subjects, COVID-19 infected, and in healthy vaccinated subjects. We found a significant CD8+ T cells (p < 0.05) decrease and an augmented CD4+/CD8+ ratio (p < 0.05) in COVID-19 infected group compared with vaccinated subjects. In addition, healthy vaccinated subjects have a significant increased expression of CD8+ T cells, and a reduction of CD4+/CD8+ ratio with respect to subjects previously COVID-19 infected. Central Memory and Terminal Effector Memory cells (TEMRA) increased after vaccine but not among groups.

1. Introduction

T cells play a relevant role in the cellular-mediated immune response to viral infection (Napoli et al., 2021; Lund et al., 2008; Swain et al., 2012; Liston et al., 2021; Yatim et al., 2021). In particular, viruses can activate CD4+ T cells which are strong effectors of the cellular-mediated immune responses for their ability to help B cell-related antibody production and to interact with CD8+ T cells driving the cytotoxic response able to kill the infected cells (Swain et al., 2012). Indeed, CD8+ T cells directly recognize viral peptides which are presented at the surfaces of infected cells causing apoptosis and preventing the virus from spreading further (Napoli et al., 2021; Swain et al., 2012). When the infection is resolved, the majority of CD4+ T cells die but a smaller population of memory CD4+ T cells persists long-term (Williams et al., 2008). These memory CD4+ T cells respond more rapidly and effectively during viral re-infection and give their potential contribution to immunity when they are induced by either infection or vaccination. In the viral pneumonia context, T cell characterization of patients infected with SARS-CoV (2002/03) demonstrated that CD4+ T cell responses strongly correlated with improved clinical outcomes (Channappanavar et al., 2014; Li et al., 2008). Recent evidence has shown that SARS-CoV-2-specific T cells are present relatively early and increase over time (Chen and John Wherry, 2020; Marfella et al., 2022). Besides, the presence of pre-existing SARS-CoV-2-reactive T cells in a subset of SARS-CoV-2 naïve healthy subjects is of high interest in the prevention of COVID-19; however, larger-scale prospective cohort studies are needed to assess whether their presence may correlate with the protection or severity of COVID-19. First, we evaluated T cell response in subjects who have been infected by SARS-CoV-2 both asymptomatic and/or with middle/severe symptoms and in healthy subjects. Then, we also documented T cell response in a group that received COVID-19 mRNA BNT162b2 (Pfizer-BioNTech) vaccine.

2. Methods

2.1. Subjects

In this study, we selected three groups of subjects who were enrolled in our ongoing DEMETRA clinical trial (NCT04746521). Basic demographic informations are summarized in Supplementary Table S1. In
the first group, we included 10 subjects who were never infected with SARS-CoV-2 (Healthy); the second group included 10 subjects who manifested symptoms after infection (Infected); the third group included 10 healthy subjects who received the vaccine (Vaccinated). Our observations are made about 1 month from second dose of vaccine for healthy subjects and after 2 months from negative nasopharyngeal swabs for COVID-19 disease in infected subjects. Since the kits used in the present study are commercially available and routinely employed in the normal clinical practice, the protocol was just approved by the institutional review board and registered in the NHC web control sites as NCT04746521. In any case, we received the written informed consent from each participant and the study has conducted by the Helsinki declaration.

2.2. Chemiluminescent microparticle immunoassay for SARS-CoV-2 IgG and IgM detection

For healthy and infected subjects, qualitative detection of IgG and IgM was performed by chemiluminescent microparticle immunoassay (CMIA) using SARS-CoV-2 IgG (6R86) and IgM (6R87) Reagent Kit (Abbott Laboratories, Diagnostics Division, Abbott Park, IL 60064 USA). In addition, for healthy vaccinated subjects, quantitative measurement of IgG antibodies against the spike receptor-binding domain (RBD) of SARS-CoV-2 was performed by using SARS-CoV-2 IgG II Quant assay (Abbott Laboratories, Diagnostics Division, Abbott Park, IL 60064 USA).

2.3. Fluorescence reagents for flow cytometer staining

The expression of CD3, CD4, CD8, CD19, and CD16-CD56 on cells in peripheral blood samples was detected by using BD Multitest 6-Color TBNK Reagent (BD Biosciences San Jose, CA, USA) a 6-color direct immunofluorescence reagent to identify and determine the percentages and absolute counts of T, B, and natural killer (NK) cells, as well as the CD4 and CD8 subpopulations of T cells in peripheral blood. Cell counts of lymphocytes were calculated using BD Trucount tubes.

In addition, whole blood samples were stained using preconfigured lyophilized reagent tubes (BD Lyotube TM 8-color CD4 and CD8 bundle, BD Biosciences San Jose, CA, USA including CD4 specific and CD8 specific “Lyotubes”): CD4 Lyotube: CD95 FITC/CCR7 PE / CD3 PerCPCy 5.5 / CD25 PE-Cy7/CD127 Alexa Fluor 647/CD45 APC-H7 / CD45RA V450/CD4 V500-C; CD8 Lyotube: CD38 FITC/CCR7 PE / CD3 PerCPCy 5.5/CD69 PE-Cy7/CD127 Alexa Fluor 647/CD45 APC-H7 / CD45RA V450/CD8 V500-C.

2.4. Blood sampling and staining procedure

Blood was drawn via sterile venipuncture into EDTA vacutainers (Becton Dickinson, BD). Then, 50 μL of blood were incubated for 15 min with 20 μL of BD Multitest 6-Color TBNK Reagent in Trucount tubes. Samples were lysed with 2 mL of lysis buffer 1× (BD PharmLyse) at room temperature in the dark for 10 min and processed immediately.
V. Grimaldi et al.

Journal of Immunological Methods 502 (2022) 113230

(caption on next page)
The second Lyotubes staining procedure carried out with rehydration by using 100 µL of blood and incubated for 5 min before mixing. After an additional 30 min of incubation at room temperature, samples were resuspended with 800 µL of buffer (FACS Flow, BD), and lysed with 2 mL of lysing buffer 1× in the dark. After washing, samples were resuspended with 800 µL of buffer (FACS Flow, BD), and processed within 1 h.

2.5. Instrument and data analysis details

Sera sample CMIA was performed by using the ARCHITECT i2000SR system (Abbott Laboratories, Diagnostics Division, Abbott Park, IL 60064 USA) (data not shown).

Flow cytometry characterization was performed by using the BD FACSLyric Flow Cytometer and the analysis was made in conjunction with BD FACSuite Clinical software (v. 1.4) for BD Multitest 6-Color TBNK Reagent with BD Trucount Tubes and BD FACSuite software v. 1.4 for BD Lytocyte CD4 and CD8 kit (BD Biosciences San Jose, CA, USA).

BD CS&T beads are used for control and setting of flow cytometer (Colacurci et al., 2020). These beads allow the FACSuite software to automatically characterize, track, and report measurements of supported digital flow cytometers. CS&T beads are dyed with fluorochromes which are excited by the cytometer's lasers. In addition, setting by using BD FC beads for all fluorochromes with in conjunction with FACSuite Clinical software and CS&T beads, is performed to establish standardized fluorescence compensation for the FACSLyric flow cytometer.

The different flow cytometry gating strategy for each test were described in the Figs. 1 and 2.

2.6. Statistical analysis

One-way ANOVA analysis of variance was used for the comparison among the groups (Table 1 and Table 3), and the Bonferroni t-test was used for multiple comparisons among groups (Table 2). Statistical significance was set at 5%. The normal distribution of data was evaluated with the Kolmogorov-Smirnov test. P values less than 0.05 were considered statistically significant.

3. Results

Since data obtained from cell counts and percentages of lymphocyte expression did not show differences, statistical analysis is performed and presented according to the expression percentages of differentiation clusters observed. Statistical variance analysis revealed a significant variation in expression of CD8+ T cells (p = 0.035) and CD4+ /CD8+ ratio (p = 0.030) (Table 1). In particular, subjects who received the COVID-19 mRNA BNT162b2 vaccine have shown an increased expression of CD8+ T cells (p = 0.048), and a reduction of CD4+ /CD8+ ratio (p = 0.048) compared to subjects previously infected (Table 2 and Fig. 3a). In infected subjects we found a significant reduction of CD8+ T cells (p = 0.048) and a significant increase of CD4+ /CD8+ ratio (p = 0.048) respect to vaccinated subjects (Table 2 and Fig. 3a). No significant difference was observed for Naïve, Central Memory, Effector Memory, and Terminal Effector Memory both CD4+ and CD8+ and Treg CD4+ cells comparing groups (Table 3 and Fig. 3b and c).

4. Discussion

The role of T cells and their potential to provide long-term protection from reinfection with SARS-CoV-2 remains debated, yet. Here, we report T cell characterization by using commercial kits which have been widely used in infectious disease studies (Chen and John Wherry, 2020) and recent observations on SARS-CoV-2 infection (Bordini et al., 2012). Our results are in agreement with recent evidence (Huang et al., 2020; Chen et al., 2020; Wang et al., 2020; De Biasi et al., 2020). We observe that: 1) T cells subset remains within the normal range in all groups, 2) an increased expression of CD8+ T cells and a decreased CD4+ /CD8+ ratio in vaccinated respect to infected subjects and 3) a reduction of CD8+ T cells and an increase of CD4+ /CD8+ ratio in infected subjects compared to vaccinated group. Although our results revealed different CD4+ and CD8+ T maturation profiles, no significant difference was found comparing groups. Of note, our clinical approach could be improved in the future with additional data on other immune cell types and/or comprehensive data for circulating inflammatory mediators for a larger cohort of subjects with different age (Napoli et al., 2020).

Table 1

One-way ANOVA variance analysis for TBNK results, p-value ≤ 0.05 is statistically significant

| Factor | Healthy | Infected | Vaccinated | p-value |
|--------|---------|----------|------------|---------|
| N      | 10      | 10       | 10         |         |
| CD4+, mean (SD) | 47.8 (8.5) | 50.7 (7.3) | 43.8 (5.4) | 0.12    |
| CD8+, mean (SD) | 28.9 (7.2) | 20.8 (5.3) | 30.5 (11.5) | 0.035   |
| CD4+/CD8+, mean (SD) | 1.8 (0.6) | 2.7 (1.2) | 1.7 (0.8) | 0.030   |
| NK, mean (SD) | 13.5 (4.5) | 13.5 (6.0) | 14.5 (7.0) | 0.90    |

Table 2

Bonferroni t-test for multiple comparisons between groups, p-value ≤ 0.05 is statistically significant

| Factor | (I) soort | (J) soort | Mean Difference (I-J) | Std. Error | P | 95% Confidence Interval |
|--------|----------|----------|-----------------------|------------|---|-----------------------|
|        | Healthy  | Infected |                       |            |   |                       |
| CD8+   |          |          |                       |            |   |                       |
| Healthy |          |         | 8.1400                | 3.7655     | 0.119 | –1.471 17.751          |
| Infected |          |         | –1.5500               | 3.7655     | 1.000 | –11.161 8.061          |
| Vaccinated |          |         | –8.1400               | 3.7655     | 0.119 | –17.751 1.471          |
| Vaccinated |          |         | –9.6900               | 3.7655     | 0.048 | –19.301 –0.079         |
| Vaccinated |          |         | –9.6900               | 3.7655     | 0.048 | –19.301 –0.079         |
| Vaccinated |          |         | –1.5500               | 3.7655     | 0.048 | –19.301 –0.079         |
| Vaccinated |          |         | –8.1400               | 3.7655     | 0.048 | –19.301 –0.079         |
| CD4+/CD8+ |          |          |                       |            |   |                       |
| Healthy |          |         | –0.90800              | 0.39017    | 0.083 | –1.9039 0.0879        |
| Infected |          |         | –0.90800              | 0.39017    | 1.000 | –0.9019 1.0899        |
| Vaccinated |          |         | –0.90800              | 0.39017    | 0.083 | –0.8799 1.0939        |
| Vaccinated |          |         | –0.90800              | 0.39017    | 0.083 | –0.8799 1.0939        |
Fig. 3. Dot plots summarize data analysis of: a, CD4⁺, CD8⁺ and NK cells comparing the three different groups: healthy (no Covid), infected (Covid) and healthy vaccinated (Vaccinated) subjects. b, T CD4⁺ maturation profile (naive, central memory T cells (Tcm), effector memory T cells (Tem) and terminal differentiated effector memory T cells (Temra)) including Treg cells and c, T CD8⁺ maturation profiles.

**Funding**

This study was funded by: PRIN2017F8ZB89 from the Italian Ministry of University and Research (MIUR) (Claudio Napoli) and Ricerca Corrente (RC) 2019 from the Italian Ministry of Health (Claudio Napoli); grants GR-2016-02364785 from the Italian Ministry of Health (Vincenzo Grimaldi and Claudio Napoli).

**Declaration of Competing Interest**

None.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2022.113230.
Table 3
One-way ANOVA variance analysis for T lymphocyte results.

| Factor   | Healthy | Infected | Vaccinated | p-value |
|----------|---------|----------|------------|---------|
| N        | 10      | 10       | 10         |         |
| Naive, mean (SD) | 31.5 (12.2) | 39.2 (11.6) | 31.8 (11.7) | 0.28    |
| Tcm, mean (SD)   | 37.0 (9.1)  | 39.7 (5.7)  | 34.3 (10.7) | 0.39    |
| Tem, mean (SD)   | 24.5 (9.8)  | 16.9 (7.7)  | 24.0 (10.1) | 0.14    |
| Temra, mean (SD) | 6.3 (7.4)   | 2.1 (2.0)   | 7.8 (11.3)  | 0.26    |
| Treg, mean (SD)  | 3.3 (1.2)   | 3.4 (1.0)   | 2.4 (0.9)   | 0.079   |
| Naive, mean (SD) | 21.9 (12.1) | 24.9 (10.6) | 19.0 (13.4) | 0.56    |
| Tcm, mean (SD)   | 10.8 (5.4)  | 11.1 (4.5)  | 11.1 (5.5)  | 0.99    |
| Tem, mean (SD)   | 24.9 (11.2) | 25.1 (9.4)  | 28.9 (12.5) | 0.67    |
| Temra, mean (SD) | 39.0 (15.2) | 35.6 (14.2) | 39.0 (18.6) | 0.86    |

References

Bordoni, V., et al., 2012. A novel 8-color flow cytometry panel to study activation, maturation and senescence of CD4 and CD8 T lymphocytes in HIV-infected individuals at different stages of disease. Int. J. Immunopathol. Pharmacol. 25 (2), 415-424. https://doi.org/10.1177/039463201202500211.

Channappanavar, R., Zhao, J., Perlman, S., 2014. T cell-mediated immune response to respiratory coronaviruses. Immunol. Res. 59 (1–3), 118–128. https://doi.org/10.1007/s12026-014-8534-z.

Chen, Z., John Wherry, E., 2020. T cell responses in patients with COVID-19. Nat. Rev. Immunol. 20 (9), 529-536. https://doi.org/10.1038/s41577-020-0402-6.

Chen, G., et al., 2020. Clinical and immunologic features in severe and moderate coronavirus disease 2019. J. Clin. Invest. 130 (3), 2620-2629. https://doi.org/10.1172/JCI97244.

Colacurci, N., et al., 2020. Flow cytometry characterization of pluripotent transmembrane glycoproteins on resident cervix uteri cells in patients screened for cervical cancer. Cancer Investig. 38 (4), 228–239. https://doi.org/10.1080/07357907.2020.1742349.

De Biasi, S., et al., 2020. Marked T cell activation, senescence, exhaustion and skewing towards TH17 in patients with COVID-19 pneumonia. Nat. Commun. 11 (1), 3434. https://doi.org/10.1038/s41467-020-17292-4.

Huang, W., et al., 2020. Lymphocyte subset counts in COVID-19 patients: a meta-analysis. Cytometry. A 97 (8), 772-776. https://doi.org/10.1002/cyto.a.24172.

Lund, J.M., et al., 2008. Coordination of early protective immunity to viral infection by regulatory T cells. Science 320 (5880), 1220–1224. https://doi.org/10.1126/science.1155209.

Marfella, R., et al., 2022. Does poor glycaemic control affect the immunogenicity of the COVID-19 vaccination in patients with type 2 diabetes: the CAVEAT study. Diabetes Obes. Metab. 24 (1), 160–165. https://doi.org/10.1111/dom.14547.

Napoli, C., et al., 2020. Immune reactivity during COVID-19: implications for treatment. Immunol. Lett. 231, 28–34. https://doi.org/10.1016/j.imlet.2021.01.001.

Swain, S.L., McKinstry, K.K., Strutt, T.M., 2012. Expanding roles for CD4+ T cells in immunity to viruses. Nat. Rev. Immunol. 12 (2), 136–148. https://doi.org/10.1038/nri3152.

Williams, M.A., Ravkov, E.V., Bevan, M.J., 2008. Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory. Immunity 28 (4), 533-545. https://doi.org/10.1016/j.immuni.2008.02.014.

Yatim, N., et al., 2021. Immune checkpoint inhibitors increase T cell immunity during SARS-CoV-2 infection. Sci. Adv. 7 (34), eabg4081. https://doi.org/10.1126/sciadv. abg4081.