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Handling and Processing of Blood Specimens from Patients with COVID-19 for Safe Studies on Cell Phenotype and Cytokine Storm

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The pandemic caused by severe acute respiratory syndrome coronavirus 2 heavily involves all those working in a laboratory. Samples from known infected patients or donors who are considered healthy can arrive, and a colleague might be asymptomatic but able to transmit the virus. Working in a clinical laboratory is posing several safety challenges. Few years ago, International Society for Advancement of Cytometry published guidelines to safely analyze and sort human samples that were revised in these days. We describe the procedures that we have been following since the first patient appeared in Italy, which have only slightly modified our standard one, being all human samples associated with risks.

• **Key terms**
SARS-CoV-2; Covid-19; coronavirus; biosafety; cytokines; cytometry

THE dramatic epidemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes Corona Virus Disease-2019 (Covid-19), started in China in late 2019 and has rapidly spread worldwide (1). In Italy, the first patients with severe pneumonia were observed in Lombardy, and the first confirmed case dated February 21, 2020. The exponentially growing number of infected people can now be traced in a website that is continuously updated (2).

As of the end of March 2020, the city of Modena is dealing with >1,500 cases and about one-fifth are hospitalized. We have been deeply involved in monitoring the immune system of patients at different stages of the disease, including those asymptomatic, taking novel therapies, requiring intensive care. The analysis that was requested was related to lymphocyte phenotype along with a few functional assays to identify skewing toward T helper type 1 (TH1) or T helper type 2 (TH2) differentiation. The purpose of this report is to provide the first experience of the Modena Covid-19 Working Group (MoCo19) on handling, processing and analyzing by flow cytometry blood specimens obtained from patients with Covid-19. Here we describe our procedures in studying peripheral blood mononuclear cells (PBMCs) isolated from infected samples with the intent to provide indications for performing relatively simple immunological studies and reassure the flow cytometry community, currently on the frontline to the fight against the virus (3), since there are no particular risks if all precautions are taken.

Names of the Modena Covid-19 Working Group (MoCo19) are listed at the end of the manuscript.

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Laboratory Biosafety

Risk Assessment

We first conducted a local risk assessment to address safety or security risks. At this level, risks were defined and characterized, and mitigation measures were implemented accordingly. All laboratory processes, including locations, procedures, and equipment used, were discussed and defined by the risk assessment team of our University. Since panic is often the first reaction of those who are not in the lab but work in the same area, it is strongly recommended to contact and reassure them and the administration personnel explaining how the safety procedure are respected when starting studies on this topic.

SARS-CoV-2 belongs to the Coronaviridae family and is taxonomically related to the subgenus Sarbecovirus (4). This is an enveloped virus containing a single-stranded positive-sense RNA as viral genome. Virions are spherical, with the spiked glycoprotein embedded in the envelope. Additional viral proteins include envelope, matrix, and nucleocapsid. The presence of SARS-CoV-2 RNA across different specimens, that is, bronchoalveolar lavage fluid, fibrobronchoscope brush biopsy, sputum, nasal swabs, pharyngeal swabs, feces, blood, and urine, has been quantified by real-time transcriptase-polymerase chain reaction (RT-PCR) (5). According to these first data, a small percentage (1%) of blood specimens had positive PCR test results for viral RNA. Moreover, the median PCR cycle threshold value reported was 34.6 (range: 34.1–35.4, 95% confidence interval [CI]: 0.0–36.4) suggesting that a low concentration of viral RNA is present in the blood.

In principle, finding viral RNA in a fluid does not mean that RNA has the original length, nor that it works. No solid information is currently available regarding the detection of infective SARS-CoV-2 particles in the blood, nor on the real meaning of viral RNA present in plasma. In fact, the natural route of transmission is person-to-person, and there are no reports of laboratory infections. In fact, the infection occurs primarily via direct contact or through droplets spread by coughing or sneezing from infected individuals (6). However, a recent study reports that the viral load in nasal and throat swabs from an asymptomatic patient was similar to that of symptomatic patients, indicating that infected persons with no symptoms can transmit the virus, likely with the same infectivity (7). Regarding the stability of SARS-CoV-2 in aerosols and on various surfaces, it has been reported that the virus can remain viable and infectious in aerosols for hours, and on surfaces up to days (depending on the inoculum shed) (8). Even if finding viral RNA dos not mean finding an infectious virus, this suggests that surfaces must be accurately cleaned with hypochlorite and ethanol.

This provides the first rule: at work, including during breaks, lab meetings, and data discussions, if individuals are in close proximity or just if more than one person is present in the same room, everybody must always wear a simple surgical mask (not a Filtering Face Piece Type-2 [FFP2] mask, which does not filter exhaled air). Unlike disposable gloves, surgical masks can be used several times along multiple days.

Laboratory Working Areas

Any handling, processing, and testing of blood specimens from Covid-19 patients need to be performed in appropriately equipped laboratories by competent personnel, previously trained on the technical and safety procedures. National guidelines on the laboratory biosafety should be followed in all circumstances, and general information is also available in the World Health Organization (WHO) Laboratory Biosafety Manual (9). In Modena, blood specimens from patients with Covid-19 are handled in Biosafety Level (BLS)-2 laboratory supplied with Class II biological safety cabinets (BSC). All cabinets are daily equipped with an internal waste (containing 0.5% bleach) where any possible contaminated biological material is discarded.

All Laboratory workers must wear personal protective equipment. In details, when working in the laboratory area, personnel need to mandatory wear disposable gloves, laboratory coat, and surgical mask, required to prevent the spread of unwanted droplets. This precaution is also important to prevent the infection spreading in case a researcher is asymptomatically infected. Laboratory clothing is maintained in the lab and should never be used outside. Laboratory doors are kept closed during all experiments in progress.

A distance of at least 1m is maintained between people inside the lab and, if possible, the presence in each room should be limited to one person only. If not possible, it is important not to have two operators using the same instrument (e.g., like a cabinet 180 cm large), nor two researchers sitting too close in front of the same flow cytometer or of the same computer.

Manipulation of Blood and Analysis and PBMCs from Covid-19 Patients

Packaging and Transport

Blood specimens from confirmed cases, collected by adequately protected and trained physicians at the patients' bed are transported to and between laboratories as UN3373, "Biological Substance, category B," and are placed in two secondary containers to minimize the potential for breakage. Opening of containers is performed inside a certified Class II BSC in a manner that reduces the risk of exposure to an unintended sample release.

Handling and Processing

During specimen manipulation in a Class II BSC, personnel wear two pairs of disposable gloves, laboratory coats, surgical mask, and eye protection. The use of two pairs of gloves is mandatory

to work in BSC, so that at the end of the procedure the external layer of gloves is removed and discarded into the waste located inside the BSC. FFP-2 masks are also available and are used for personnel protection during specific procedures, including cell sorting or stimulation/activation of living cells. It is better to perform these procedures alone, and thus this type of mask can be used—only when operating and not close to other people.

According to the WHO Laboratory Biosafety Manual, for procedures with a high likelihood to generate aerosols or droplets (e.g., vortexing, mixing, sonication or centrifugation), a certified Class II Type A1 or A2 BSC should be used. During the procedure for the isolation of PBMCs from peripheral blood, centrifugation steps are at high risk to generate fine-particulate aerosols and droplets. However, centrifuge buckets are sealed for centrifugation, and specimens are centrifuged in securely capped polypropylene tubes that are loaded and unloaded in a Class II BSC. As additional precaution, every step of the procedure is performed in a Class II BSC to minimize the risk of exposure to an unintentional sample release. Only disposable plasticware and pipettes are used, which are decontaminated into the internal waste.

On completion of work, the internal waste is closed and discarded into a biosafety waste. Surfaces are decontaminated typically with 0.5% bleach and then with 70% ethanol.

Handling of PBMCs for Phenotype Analysis

To date, no data are available regarding the ability of SARS-CoV-2 to infect PBMCs. If SARS-CoV-2 behaved like all respiratory viruses, the blood from Covid-19 patients should not contain infective particles. However, waiting for definitive reports, and according to standard precautions, we prefer to take into consideration the fact that in principle plasma and mononuclear cells obtained from blood may contain transmissible infectious agents, and must be handled in a Class II BSC. Indeed, this is what we have been doing for many years when analyzing human blood from patients with different physiological conditions (for example, age, from 0 to 110 years, or pregnancy) that is always treated as if it were infected with a pathological agent like human immunodeficiency virus (HIV) or hepatitis B virus. So, many years ago—I would say more than 30—we started to strictly follow first safety procedures, then the indications given by the International Society for Advancement of Cytometry (ISAC) (10,11), with the recent updates (see: https://isac-net.org/page/Biosafety).

As we well know, for the analysis of cell phenotype by flow cytometry sample preparation typically includes isolation of PMBCs, staining with monoclonal antibodies (mAbs), incubation for a short period, washing, and fixation. Then, fixed samples are acquired by using an instrument that, in our case, is located in a locked BLS-2 room. Personnel involved in sample preparation handle PBMCs specimens in a Class II BSC and wear laboratory coat, gloves, surgical mask, and eye protection.

As additional precaution, even if not required, those involved in sample acquisition can even wear FFP2 instead of surgical mask. After the acquisition, the flow cytometer is washed for 15 min with 0.5% bleach, 15 min with cleaning solution and finally 15 min with deionized water. At the end of the acquisition, the entire working area is cleaned-up by using disinfectant solution (1/10 volume dilution of 0.71 M sodium hypochlorite, then 70% ethanol). Disposable materials (collection tubes, gloves, pipettes, tips) are discarded into appropriate biohazard containers with hypochlorite and all work surfaces are wiped off.

A potential exposure to infectious materials, or any sort of accident has to be immediately reported to the head of the laboratory for the appropriate evaluation. Needless to say, activities like eating, drinking, smoking, handling of contact lens, applying cosmetics, playing with the phone or chatting

Table 1. Summary of the personal protective equipment and collective protective devices for handling and processing blood specimens and PBMCs from Covid-19 patients

PROCEDURE	PERSONAL PROTECTIVE EQUIPMENT	COLLECTIVE PROTECTIVE DEVICES
Handling of blood	• Surgical mask • Two pairs of gloves (the external to be used only when working in the BSC) • Eye protection • Lab coat	• Class II BSC in a BLS-2 lab
Staining of PBMCs	• Surgical mask • Two pairs of gloves (the external to be used only when working in the BSC) • Eye protection • Lab coat	• Class II BSC in a BLS-2 lab
Acquisition at the flow cytometer (fixed cells)	• Surgical mask • Gloves • Eye protection • Lab coat	• BLS-2 lab
Acquisition of unfixed cells: requires cell sorting procedures	See https://isac-net.org/page/Biosafety	• BLS-3 lab

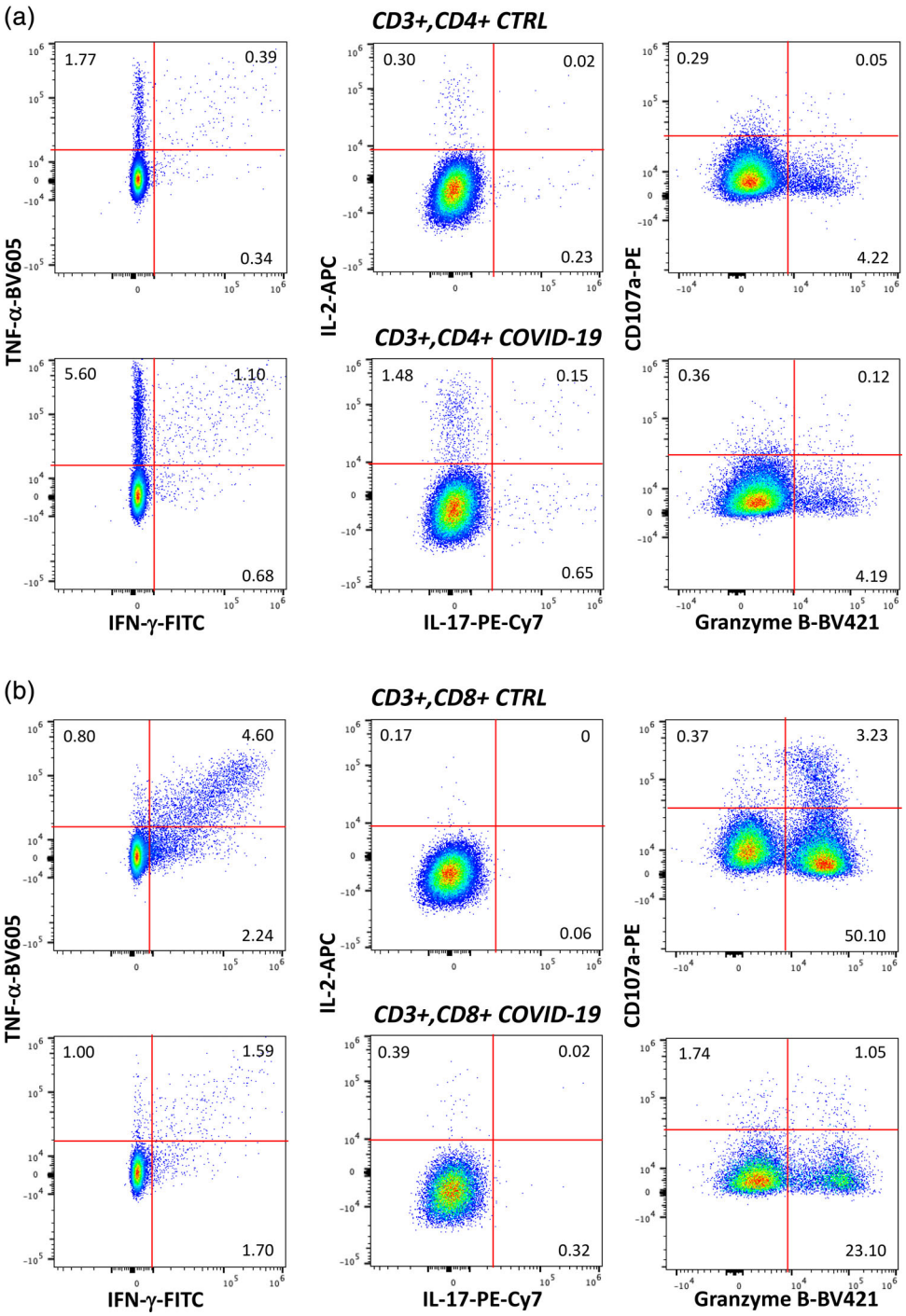

Figure 1. Representative example of cytokine production by CD4+ and CD8+ T cells from a Covid-19 patient with severe pneumonia after in vitro stimulation after in vitro stimulation with anti-CD3/CD28 (1ug/mL) for 16 h in the presence of anti-CD107a-PE (Biolegend, San Diego, CA).(14,15) PBMC were stained with viability marker (AQUA Live Dead, ThermoFisher) and anti-CD4-AF700 and CD8-APC-Cy7 (Biolegend). Cells were fixed and permeabilized with Cytofix/Cytoperm (Becton Dickinson, San Josè, CA) according to manufacturer protocols. Finally, cells were stained with anti-IFN-γ-FITC, anti-TNF-α-BV605, anti-IL-17A-PE-Cy7, anti-IL-2-APC, and anti-Granzyme B-BV421 (all from Biolegend). Data were acquired by using attune NxT acoustic flow cytometer. (A) Intracellular staining of different cytokines in previously gated living CD3+,CD4 + in a healthy donor (upper plots) and in a patient (lower panels); (B) intracellular staining of different cytokines in previously gated living CD3 +,CD8+ in a healthy donor (upper plots) and in a patient (lower panels); (C) analysis of the polyfunctionality of CD8+ T cells by using "Simplified Presentation of Incredibly Complex Experiments (SPICE),", kindly provided by Dr. Mario Roederer (NIH, Bethesda, MD).(16) Arcs represent the total production of each cytokine, pie slices the polyfunctional capacity of cells. For the functional analysis of CD8+ T cells, that in theory can provide 64 populations of cells producing different combination of cytokines, a threshold of 0.5% was set on the basis of the distribution of negative values generated after background subtraction. Note that, as expected, in patient and control no CD8+ T cell was able to produce IL-2. [Color figure can be viewed at wileyonlinelibrary.com]

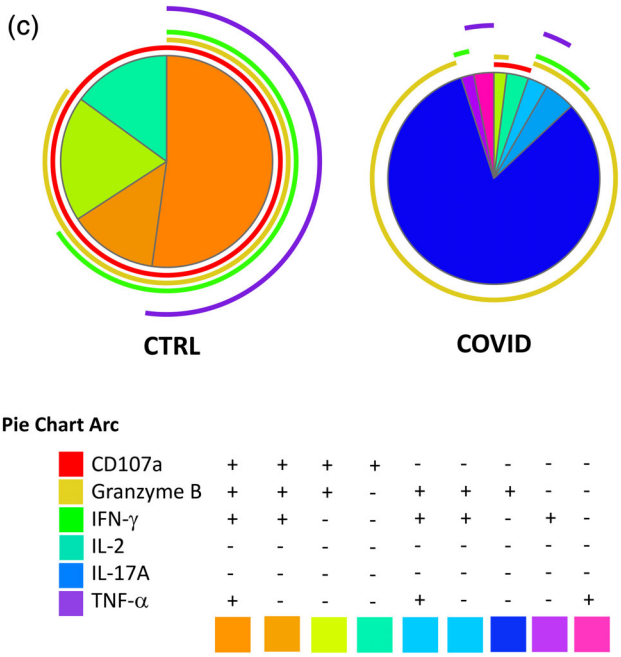

Figure 1. (Continued)

on social networks are absolutely prohibited. In Table 1, the main personal protective equipment and collective protection devices are summarized.

Detecting Cells Responsible for the Cytokine Storm

Short term stimulation is now assuming a pivotal importance in the fight against Covid-19. Indeed, several reports have described abnormally increased levels of cytokines in plasma from patients infected by SARS-CoV-2 (12), that has been defined "cytokine storm," similarly to what has been described in bacterial sepsis (13). This condition is driven by, and causes inflammation, and molecules like interleukin (IL)-1, tumor necrosis factor (TNF)-α, and especially IL-6 are strongly produced by a variety of cells. Likely, since most infected people remain asymptomatic, this is not happening in all infected individuals. Interestingly, it is now known that children and pregnant women usually experience a mild form of Covid-2 if not a fully asymptomatic one. These categories of persons are characterized by an immune response skewed toward a TH2 profile (i.e., activities of the so-called T-helper Type 2 cells), with a preferential production of cytokines like IL-4 and IL-10. Typically, production of the aforementioned inflammatory cytokines is a feature of TH1 cells. Thus, it could be of interest to investigate whether profiling immune cells for their ability to produce TH1 or TH2 cytokines could be useful in the management of Covid-19 patients. This in vitro assay is typically based upon isolation of PBMCs, stimulation with different *stimuli* (i.e., anti-CD3/CD28, superantigens like *Staphylococcus aureus* enterotoxins, phorbol myristate acetate plus ionomycin, peptide pools) and quantification of intracellular cytokines.

In the last weeks this assay has been extensively used from our group to study CD4+ and CD8+ T cells. The experimental procedures that we follow require that PBMCs have to be maintained for a few hours (or, in some cases, for 2 days) in an incubator, at 37°C in a humidified atmosphere with 5% CO_2. For the analysis of polyfunctionality by the detection of intracellular production of cytokines, PBMCs are thus incubated with different *stimuli* inside capped tubes. In these conditions, aerosol particles or droplets can be generated inside the tube. However, tubes are loaded in a Class II BSC, transferred to the incubator, kept for some hours, unloaded and treated with Brefeldin A under the BSC as described before, and reincubated. At the end of the last incubation period tubes are unloaded in a BSC. Then, cells are fixed, permeabilized, stained with mAbs, and acquired. It is to note that the dedicated incubator is also located in the same BLS-2 laboratory. In the case reported by Figure 1 (from a study that has been approved by the "Area Vasta Emilia Nord" Ethical Committee on March 10, 2020), we used the Attune NxT acoustic flow cytometer (ThermoFisher Scientific, Eugene, OR). For this type of analysis, we first applied the classical methods for intracellular staining and rare event detection (14,17). Then, we found that a relevant difference in cytokine production was present between CD8+ T cells from a patient with Covid-19 pneumonia and an age- and sex-matched donor. Indeed, most CD8+ T cells from this patient were able to produce Granzyme B but not interferon-γ or TNF-α, and were CD107a negative. This type of assay is now under deep investigation to understand the clinical importance of a polyfunctional response that in viral infections like that by HIV plays a major defensive role and can predict, at least in part, the course of the infection (18,19).

Finally, we underline that sorting of cells from Covid-19 patients requires a completely different approach. In fact, the simple procedures that we have described above are easily applicable to studies where cells are finally fixed, like those on cell phenotype or detection of intracellular molecules, or other assays. For unfixed, living cells (as, e.g., in the case of analysis of the functionality of different organelles or of calcium fluxes, among others) we recommend to use the same measures required for cell sorting. At this regard, the ISAC Biosafety Committee has just released (March 26, 2020) novel procedures recently approved by the NIH-Institutional Biosafety Committee for CoV-2 cell sorting. The procedures are extremely clear and well written, and we invite those interested in visiting ISAC website at: https://isac-net.org/page/Biosafety.

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References

1. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 2020;579:270–273.
2. https://gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6
3. Cossarizza A, De Biasi S, Guaraldi G, Girardis M, Mussini C. SARS-CoV-2, the virus that causes COVID-19: cytometry and the new challenge for global health. Cytometry A 2020;97:340–343. https://doi.org/10.1002/cyto.a.24002.
4. Khan S, Siddique R, Shereen MA, Ali A, Liu J, Bai Q, Bashir N, Xue M. The emergence of a novel coronavirus (SARS-CoV-2), their biology and therapeutic options. J Clin Microbiol 2020 (in press). https://doi.org/10.1128/JCM.00187-20.
5. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, Tan W. Detection of SARS-CoV-2 in different types of clinical specimens. JAMA 2020 (in press). https://doi.org/10.1001/jama.2020.3786.
6. Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. J Autoimmun 2020 (in press);102433. https://doi.org/10.1016/j.jaut.2020.102433.
7. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, Yu J, Xia J, Guo Q, Yen H-L, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. N Engl J Med 2020;382:1177–1179.
8. van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, Tamin A, Harcourt JL, Thornburg NJ, Gerber SI, et al. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. N Engl J Med 2020 (in press). https://doi.org/10.1056/NEJMc2004973.
9. World Health Organization WHO Laboratory Biosfety Manual, 3rd ed. Geneva, Switzerland: WHO Library; 2004. https://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf?ua=1
10. Schmid I, Lambert C, Ambrozak D, Marti GE, Moss DM, Perfetto SP, International Society of Analytical Cytology. International Society for Analytical Cytology biosafety standard for sorting of unfixed cells. Cytometry A 2007;71:414–437.
11. Holmes KL, Fontes B, Hogarth P, Konz R, Monard S, Pletcher CH Jr, Wadley RB, Schmid I, Perfetto SP. International Society for the Advancement of Cytometry cell sorter biosafety standards. Cytometry A 2014;85:434–453.
12. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan. China Lancet 2020;395(10223):497–506.
13. Rubio I, Osuchowski MF, Shankar-Hari M, Skirecki T, Winkler MS, Lachmann G, La Rosée P, Monneret G, Venet F, Bauer M, et al. Current gaps in sepsis immunology: New opportunities for translational research. Lancet Infect Dis 2019;e422–e436. https://doi.org/10.1016/S1473-3099(19)30567-5.
14. Cossarizza A, Chang HD, Radbruch A, Andrä I, Annunziato F, Bacher P, Barnaba V, Battistini L, Bauer WM, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies. Eur J Immunol 2017;47:1584–1797.
15. Mousset CM, Hobo W, Woestenenk R, Preijers F, Dolstra H, van der Waart AB. Comprehensive phenotyping of T cells using flow cytometry. Cytometry A 2019;95:647–654.
16. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A 2001;79:167–174.
17. Cossarizza A, Cousins D. Overcoming challenges in cellular analysis: Multiparameter analysis of rare cells (Webinar). Science 2015;347(6220):443. https://doi.org/10.1126/science.347.6220.443-c.
18. Duvall MG, Precopio ML, Ambrozak DA, Jaye A, McMichael AJ, Whittle HC, Roederer M, Rowland-Jones SL, Koup RA. Polyfunctional T Cell responses are a hallmark of HIV-2 infection. J Exp Med 2008; 38:350–363.
19. Cossarizza A, Bertoncelli L, Nemes E, Pinti M, Nasi M, De Biasi S, Gibellini L, Montagna JP, Vecchia M, Manzini L, et al. T cell activation but not polyfunctionality after primary HIV infection predicts control of viral load and length of the time without therapy. PLOS One 2012;7(12):e50728.