Deep Immune Status Monitoring with Multi-color Flow Cytometry

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With the development of immune therapy, the role of immune monitoring during treatments has been emphasized. Flow cytometry is the best method for immune status monitoring because of its characteristic of multi-parameter analysis. To broadly monitor immune status, we established and validated a multi-color comprehensive panel of 29 antibodies to cover 90 immune cell subsets and the research work was published in the issue of ILSH recently [1]. The article reported that the clinical applications of 10-color flow cytometry to monitor the immune status of patients before or after clinical treatments in 2 ml whole blood samples.

The paper described the proper sample collection and sample preparation. 2 ml EDTA anticoagulant peripheral blood (PB) was collected and flow testing was performed within 24 h. The white blood cell (WBC) count and lymphocyte percentage were obtained from Sysmex-KX-21N, which data were used to prepare single cell suspension with cell count in the range of 1 to $2 \times 10^6/\mu L$ for antibody staining. The following fluorescent labelled antibodies were used: CD3-APC (UCHT1), CD3-APC-AF750 (UCHT1), CD4-FITC (13B8.2), CD5-PB (BL1a), CD8-ECD (B9.11), CD8-APC-AF700 (B9.11), CD11c-PE (BU15), CD13-PC7 (366(My7)), CD14-FITC (RMO52), CD16-PB (3G8), CD19-ECD (J3-119), CD19-APC-AF700 (J3-119), CD25-PE (B1.49.9), CD27-PC7 (1A4CD27), CD45-KrO (J33), CD45RA-ECD (2H4LDH11LDB9), CD45RO-PC7 (UCHL1), CD56-PC5.5 (N901/NKH-1), CD69-PE (TP1.55.3), CD123-ECD (SSDCKY107D2), CD127-PC7 (R34.34), PD1 (CD279)-PC5.5(PD1.3), HLA-DR-APC-AF750 (L243), HLA-DR-PB (Immu-357), Kappa-FITC (Polyclonal), Lambda-PE (Polyclonal), IgD-APC (IA6-2), TCRαβ-APC (IP26A), TCR γδ-PC5.5 (IMMU510). All of the antibodies were purchased from Beckman coulter (USA). The article also introduced the antibody titration,

data analysis, gating strategy, and flow cytometry settings, including PMT voltage and compensation. Stained cells were analyzed with Navios 10-color flow cytometer and Kaluza software (Beckman Coulter, USA).

The major immune cell populations detected by 29 antibody panel included T cell subsets (CD3+ total T, CD4+Th, CD8+Tc, Treg, CD8hi T, CD8dim T, αβTCR cells, γδTCR cells, naïve T and memory T cells), T cell activation markers (percentage and MFI of CD25, CD69 and HLA-DR) and one immune checkpoint PD1(CD279), B cell subsets (CD5+B1, switched memory, non-switched, naïve B, CD27-IgD- B cells, and the light chain Kappa or Lambda), neutrophils, basophils, four monocytic cell subsets (CD13+CD14hi CD16- Mo1, CD13+CD14lowCD16+ Mo2, CD13+CD14hiCD16+ Mo3, and CD13+CD14lowCD16- Mo4), dendritic cells (pDCs and mDCs), four NK cell subsets (CD16+CD56- NK1, CD16+CD56+ NK2, CD16-CD56low NK3, CD16low/-CD56hi NK4), and NKT (CD3+CD16+or CD3+CD56+). In addition to the percentage of all cell subsets, we calculated the cell absolute number (cells/μl) using dual-platform. Based on WBC and lymphocytes percentage from Sysmex-KX- 21N, and cells subset percentages from flow cytometer, we can calculate the absolute number of 90 cell subsets. These panels of antibodies had been applied to monitor immune status (percentage and absolute number) in total 303 clinical cases with various diseases before or after treatments, such as leukemia, lymphoma, cancers, immune deficiencies, and autoimmune diseases.

T-, B- and NK cells play crucial roles in immune regulation. Therefore, monitoring lymphocyte subsets could lead to the diagnosis of wide variety of immunologic disorders and immune conditions. For examples, the near-complete absence of T cells in PB contribute to the diagnosis of severe

combined immunodeficiency (SCID); lymphocytosis with increased percentage of double negative T-cells (DNT) and DNT αβ-T-cells is the diagnostic criterion of autoimmune lymphoproliferative syndrome (ALPS); quantitative assessment of B cell populations and subpopulations is useful for the diagnosis of X-linked agammaglobulinemia (XLA) and Common variable immunodeficiency (CVID). Decreased T cell numbers and low CD45RA$^+$ naïve T cells were often detected in DiGeorge syndrome characterized by thymic dysplasia, and while B cells in DiGeorge syndrome show impaired maturation, with low switched memory B cells and a wide spectrum of antibody deficiencies or dysgammaglobulinemia (dysfunctional maturation B cells).

As for the monitoring of targeted therapy, Treg is key regulator in immune response and also one of the candidate target therapies for autoimmune diseases and cancers; PD1 is an immune checkpoint target for immunotherapy, PD1 inhibitors, drugs that block PD1, can activate the immune system to attack tumors and has already been used to treat certain type lymphoma (Hodgkin lymphoma) and various cancers (Melanoma, lung cancer, stomach cancer). The CAR-T therapy products were approved by Food and Drug Administration (FDA) in 2017 and numerous clinical trials are starting to evaluate CAR-T therapy as a first line or second line of treatment for cancers. To measure the level of persistent CAR-T cells, the ratio of CD4$^+$CAR-T and CD8$^+$CAR-T may assistant predict the effectiveness of CAR-T therapy.

The 10-color panel also includes the antibodies for innate immune cells, such as neutrophils, monocytes, DCs, and NK cells. Neutrophils are the most abundant phagocytic cells and play critical roles in immune response. Each of the four monocyte subsets has a relatively unique function and plays different roles in the immune response and inflammatory process. For examples, the CD14^{hi}CD16$^-$ classical monocytes (Mo1) play largely phagocytic and antitumor roles; decreased Mo1 monocytes while increased nonclassical (CD14^{low}CD16$^+$ Mo2) and intermediate (CD14^{hi}CD16$^+$ Mo3) cells were detected in acute myocardial infarction (AMI) patients. DCs are antigen-presenting cells that express MHC molecules. DCs can also secrete cytokines, such as interferon-I and interferon-α. NK cells are innate lymphocytes and unlike T cells and B cells that require antigen presentation. There are obvious differences in phenotype and function among the four subsets of NK. We listed CD56^{low}CD16$^+$ NK1 as an individual population because of the differential surface markers expression. The main subset CD56^{dim}CD16$^+$ NK2 cell was demonstrated cytotoxicity enhancement. A significant predominance of CD56^{dim}CD16$^+$ NK3 cell was found in blood samples of papillary thyroid cancer (PTC), NK3 tissue infiltration positively correlated with advanced stages while CD56^{hi}CD16$^{low/-}$NK4 was negatively

associated with tumor stages of PTC. NK cells especially CD56^{hi}CD16$^{low/-}$NK4 associated with the incidence of acute graft versus host disease (aGVHD) and correlated with the severity of aGVHD.

These panels were used to monitor immune status of different clinical cases, and we found that T cell percentage of bone marrow lymphocytes at the time of AML diagnosis associated with overall survival [2]. Besides, we showed that AML patients with GVHD post-transplant have a lower Th cell percentage and higher Tc cell percentage at diagnosis. This is to say, decreased Th/Tc ratio in BM of initial diagnosed AML patients may predict a high risk of GVHD post-transplant [3].

Furthermore, the designed panel can also be used for immune monitoring of COVID-19 infection, known as a global pandemic crisis. Decreased CD3$^+$T, CD4$^+$ Th, CD8$^+$Tc cells and increased NK cells and CD8$^+$ activation markers such as CD38 and HLA-DR were detected in COVID-19 patients and immune responses changed with infection severity in COVID-19 patients [4].

Taken together, the article reported an immune monitor panel of flow cytometry, provided the details of samples stain and instrument setup, and furthermore, validated with clinical cases. This may contribute to the successful development of immune therapy and will benefit further development of personalized treatment.

Conflict of Interest Disclosure

The authors declare no competing financial interests.

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