Received: 12 March 2021 | Revised: 22 September 2021 | Accepted: 30 September 2021
DOI: 10.1002/cyto.a.24510

OMIP

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

OMIP-080: 29-Color flow cytometry panel for comprehensive evaluation of NK and T cells reconstitution after hematopoietic stem cells transplantation

Sarka Vanikova | **Abhishek Koladiya** | **Jan Musil**

Department of Immunomonitoring and Flow Cytometry, Institute of Hematology and Blood Transfusion, Prague, Czech Republic

Correspondence
Jan Musil, Department of Immunomonitoring and Flow Cytometry, Institute of Hematology and Blood Transfusion, U Nemocnice 2094/1, 128 00 Prague, Czech Republic.
Email: jan.musil@uhkt.cz

Funding information
European Regional Development Fund and the state budget of the Czech Republic, project AIIHHP, Grant/Award Number: CZ.02.1.01/0.0/0.0/16_025/0007428 OP RDE MEYS; Ministerstvo Zdravotnictví Ceské Republiky; H CZ - DRO (Institute of Hematology and Blood Tranfusion - IHBT), Grant/Award Number: IN 00023736

Abstract

This 29-color panel was developed and optimized for the monitoring of NK cell and T cell reconstitution in peripheral blood of patients after HSCT. We considered major post-HSCT complications during the design, such as relapses, viral infections, and GvHD and identification of lymphocyte populations relevant to their resolution. The panel includes markers for all major NK cell and T cell subsets and analysis of their development and qualitative properties. In the NK cell compartment, we focus mainly on CD57 + NKG2C+ cells and the expression of activating (NKG2D, DNAM-1) and inhibitory receptors (NKG2A, TIGIT). Another priority is the characterization of T cell reconstitution; therefore, we included detection of CD4+ RTEs based on CD45RA, CD62L, CD95, and CD31 as a marker of thymus function. Besides that, we also analyze the emergence and properties of major T cell populations with a particular interest in CD8, Th1, ThCTL, and Treg subsets. Overall, the panel allows for comprehensive analysis of the reconstituting immune system and identification of potential markers of immune cell dysfunction.

1 | BACKGROUND

Hematopoietic stem cell transplantation (HSCT) remains the only curative treatment available to patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) [1, 2]. Furthermore, it is indicated as a treatment for other malignant and nonmalignant diseases [3–5]. Although HSCT represents the most effective immunotherapy to date, it is associated with severe post-transplant complications that are the result of improper immune system function. These include relapse of the original disease, graft-versus-host-disease (GvHD) and infectious complications, such as reactivation of latent human cytomegalovirus (HCMV) infection [6]. Therefore, monitoring markers of immune system dysfunction is highly sought as it opens up the possibility for early therapeutic intervention. For example, the transfer of virus-specific T cells is actively investigated in clinical trials for the treatment of viral complications [7].

Flow cytometry represents the method of choice when evaluating the status of the post-transplant immune system as it can identify quantitative and qualitative differences between the regenerating and healthy immune system (Table 1).

Therefore, we have developed a 29-color panel to monitor the reconstitution of NK (natural killer) cell and T cell subpopulations as they are the major drivers of anti-leukemia and anti-pathogen responses as well as gatekeepers of tissue tolerance.

NK cells represent the first lymphocyte population to recover after HSCT and play an essential role in controlling viral infections and mediating the graft-versus-leukemia (GvL) effect [8–10].

Sarka Vanikova and Abhishek Koladiya contributed equally to this work.

TABLE 1 Summary table for application of OMIP-080

Purpose	T cells and NK cells
Species	Human
Cell types	PBMC
Cross-reference	OMIP-004,007,013,015,017,024,030,036,037,039,042,050,058,060

TABLE 2 Reagents used for OMIP-080

	Antigen	Fluorochrome	Clone	Purpose
1	CD62L	BUV395	DREG-56	T cell differentiation
2	Viability	Live-Dead Blue	—	Viability
3	CD69	BUV496	FN50	NK and T cell activation marker
4	CCR6 (CD196)	BUV563	11A9	Chemokine receptor; Th subset identification
5	CD27	BUV615	M-T271	T cell differentiation
6	PD1 (CD279)	BUV661	EH12.1	T cell inhibitory receptor
7	CD25	BUV737	2A3	T cell activation; Treg identification
8	CD8	BUV805	RPA-T8	CD8 T cell and NKT-like cell lineage marker
9	NKG2A (CD159a)	BV421	131411	NK and NKT-like cell inhibitory receptor
10	CD45RA	PB	HI100	T cell differentiation
11	Tim3	BV480	7D3	T cell inhibitory receptor
12	CD4	BV570	RPA-T4	CD4 T cell lineage marker
13	CD57	BV605	QA17A04	T cell and NK cell differentiation
14	CD95	BV650	DX2	T cell activation and differentiation
15	γδTCR	BV711	11F2	γδ T cells
16	DNAM-1 (CD226)	BV750	DX11	T cell and NK cell activating receptor
17	CD31	BV786	WM59	Adhesion molecule; identification of RTE
18	CCR10	BB515	1B5	Chemokine receptor; Th subset identification
19	CCR4 (CD194)	BB700	1G1	Chemokine receptor; Th subset identification
20	TIGIT	BB750	741182	T cell and NK cell inhibitory receptor
21	NKG2D (CD314)	BB790	1D11	NK cell activating receptor
22	NKG2C (CD159c)	PE	FAB138P	NK cell activating receptor
23	CD56	PE-CF594	NCAM16.2	NK cell and NKT-like cell lineage marker
24	CXCR3 (CD183)	PE-Cy5	1C6/CXCR3	Chemokine receptor; Th subset identification
25	FoxP3	PE-Cy5.5	PCH101	Master transcription factor for Tregs
26	CD39	PE-Cy7	A1	Treg activation marker
27	CD3	AF647	UCHT1	T cell and NKT-like cell lineage marker
28	CD16	AF700	3G8	NK cell differentiation
29	Perforin	APC Fire750	B-D48	Cytolytic function

Abbreviations: AF, Alexa Fluor; APC, Allophycocyanin; BB, Brilliant Blue; BUV, Brilliant Ultraviolet; BV, Brilliant Violet; Cy, cyanine; PB, Pacific blue; PE, R-phycoerythrin.

However, their function, that is, cytotoxicity and cytokine production, is heavily impaired, and it can take up to 6 months until full functionality is restored [11, 12]. To be able to comprehensively monitor the reconstitution of the NK cell compartment, we have included several types of maturation, functional and qualitative markers (Table 2).

Maturation of the NK cell compartment is monitored based on the identification of the four major subsets. These include CD56hiCD16− cells representing producers of cytokines such as interferon-gamma (IFNγ) and tumor necrosis factor alpha (TNFα) [13], CD56loCD16+ highly differentiated cytotoxic cells, CD56hiCD16+ cells representing a transitional phenotype sharing functional characteristics with CD56hiCD16− and CD56loCD16+ such as cytotoxicity and cytokine production, and CD56loCD16− which is a heterogeneous population composed of activated NK cells that downregulate CD16 and potential precursors of the CD56loCD16+ subset [14, 15].

HSCT does not only affect the distribution of NK cell subsets but has also been shown to affect their functional properties, such as the production of perforin and IFNγ [16]. Therefore, we have included perforin and Tim3 as a proposed regulator of IFNγ production in NK cells [12]. Furthermore, regarding relapse of the original disease expression of activating receptors NKG2D and DNAM-1 and inhibitory receptors, NKG2A and TIGIT are monitored due to their role in leukemia immune escape [17–19].

T cells represent the orchestrators of adaptive immunity and are indispensable for long-lasting protective immune responses. In comparison to NK cells, their reconstitution is more complex as it is highly influenced by the type of graft, that is, umbilical cord blood, bone marrow, mobilized blood stem cells [20]. Two pathways of reconstitution exist, namely homeostatic expansion and de novo differentiation via the thymus [11].

In this panel, we define two main subsets of T cells based on the type of T cell receptor (TCR), namely γδTCR+ cells and γδTCR−, which we consider to be αβ T cells. We further subtype αβ T cells based on CD56 expression into CD56+ T cells and classical T cells (Figure 1).

γδ T cells represent a bridge between innate and adaptive responses, they recognize infected or malignant cells either through the expression of activating receptors which they share with NK cells like NKG2D, NKG2C and DNAM-1 or by recognition through their TCR, that recognizes phosphoantigens including intermediates from the isoprenoid biosynthesis pathway [21, 22]. In our panel, we subtype γδ T cells based on the expression of CD57 and NKG2A into CD57 + NKG2A− terminally differentiated highly cytotoxic cells, CD57−NKG2A− cytotoxic cells and cytokine-producing CD57-NKG2A + cells. Furthermore, we are able to evaluate the expression of costimulatory molecules such as NKG2D, which mediates TCR unrestricted effector functions, and CD27 which has been proposed to co-stimulate and thereby boost recognition of target cells via TCR [23].

NKT-like cells represent another subset of T cells that share similarities with NK cells. In our panel, we define them as CD3 + γδTCR-CD56+ cells. It has to be noted that this population is highly heterogeneous, and besides, NKT-like cells contain CD1d-restricted type I (iNKT) and type II NKT cells; however, these represent a minority [24]. Currently, it has been proposed that NKT-like cells arise from classical T-cells that start to express NK cell markers after prolonged antigen stimulation [25]. Their physiological role is currently under investigation. As there is no clear agreement about the nomenclature of these cells, we further refer to them as CD56+ T cells. In our panel, we identify three subsets of CD56+ T cells: CD4+, CD8+, and CD4− CD8− (DN) cells. We further subdivide the CD8 subset into NKG2C− and NKG2C+ cells as these might represent cells with specific roles for HCMV. These cells are dominated by the CD45RA+ CD62L− effector phenotype, are highly cytotoxic and lack expression of the NKG2A receptor. CD4+ CD56+ T cells are also highly differentiated with an effector memory phenotype. These cells contain a substantial population of cytotoxic cells, as evidenced by the expression of perforin and CD57.

Classical T cells are defined in our panel as CD3 + CD56 − γδTCR−, however it has to be noted that the population of CD3 + CD56 − γδTCR− is actually heterogeneous and includes true classical αβT cells and mucosal-associated invariant T (MAIT) cells characterized by the expression of an invariant αβTCR [26]. αβT cells are the centerpiece of adaptive immune responses. However, they are heavily affected by HSCT. The most prominent change, when compared to healthy, is the inverse ratio of CD4:CD8 T cells which is caused by faster regeneration of CD8+ T cells that reach normal levels in 100 days, whereas CD4+ T cell regeneration can take up to a year [11, 27]. As mentioned above, reconstitution in the months after HSCT is driven by the homeostatic expansion of T cells contained in the graft. However, the only way of reconstituting a broad TCR repertoire that can efficiently protect against invading pathogens is through the thymus [28]. To monitor the thymic output, we use the measurement of recent thymic emigrants (RTE) in the naïve CD4 compartment based on the expression of CD31 [29]. CD8+ T cells reconstitute faster, but there is currently no validated marker for CD8+ RTEs; therefore, we monitor only the emergence of naïve CD8+ T cells defined as CD45RA + CD62L + CD95−.

As early post-transplant responses heavily rely on CD8+ T cells, we have included several types of markers for assessing the functional status of the CD8+ T cell compartment. First of all, we monitor the distribution of different memory subsets such as CD45RA + CD62L + CD95+ memory stem cells (Tscm), CD45RA − CD62L+ central memory cells (Tcm), CD45RA − CD62L− effector memory cells (Tem), and finally CD45RA + CD62L− effector cells (Teff). Besides that, we have also included activation markers such as the early activation marker CD69, CD25, and perforin as a marker for effector function.

Furthermore, we included markers of T cell exhaustion and senescence, that is, PD1, TIGIT, Tim3, and CD57, as these are important when considering AML relapse [30–32].

CD4+ T cells are the main regulators of innate and adaptive immune responses. We have designed our panel to separate the main T helper (Th) subsets, that is, CD25 + FoxP3 regulatory T cells (Tregs), perforin+ CD27− Th cytotoxic lymphocytes (ThCTL), CCR10-CCR4-CXCR3+ Th1, CCR10 + CCR4 + CCR6+ Th22, CCR10 − CCR4 + CCR6+ Th17, CCR10 − CCR4 + CCR6− Th2, CCR10 + CCR4 + CCR6− Th granulocyte-macrophage colony-stimulating factor (ThGM-CSF), and finally CCR10 − CCR4 − CXCR3 − CCR6+ Th9 cells.

Monitoring of Tregs in this panel is a priority as they have been shown to adversely affect anti-leukemic responses and can influence the occurrence of GvHD [33–35]. We monitor Th-like Treg phenotypes, as it has been proposed that Tregs mirror the effector cells they suppress [36]. Additionally, we included markers of highly suppressive Tregs, i.e., CD39, PD1, Tim3 and TIGIT [37, 38].

Another priority of our panel is the identification of exhaustion markers on Th1 and ThCTL cells, as these represent important mediators of antiviral and anti-leukemic responses [39, 40].

Other non-Treg subsets like Th17 and Th22 can play an important role in immunity against bacterial pathogens causing post-HSCT complications such as *Pseudomonas aeruginosa*, *Streptococcus pneumonie or Pneumocystis jirovecii* [41–43]. Finally, we utilized the EmbedSOM algorithm to identify important cell subsets from live single cells (Figure 1H). [20].

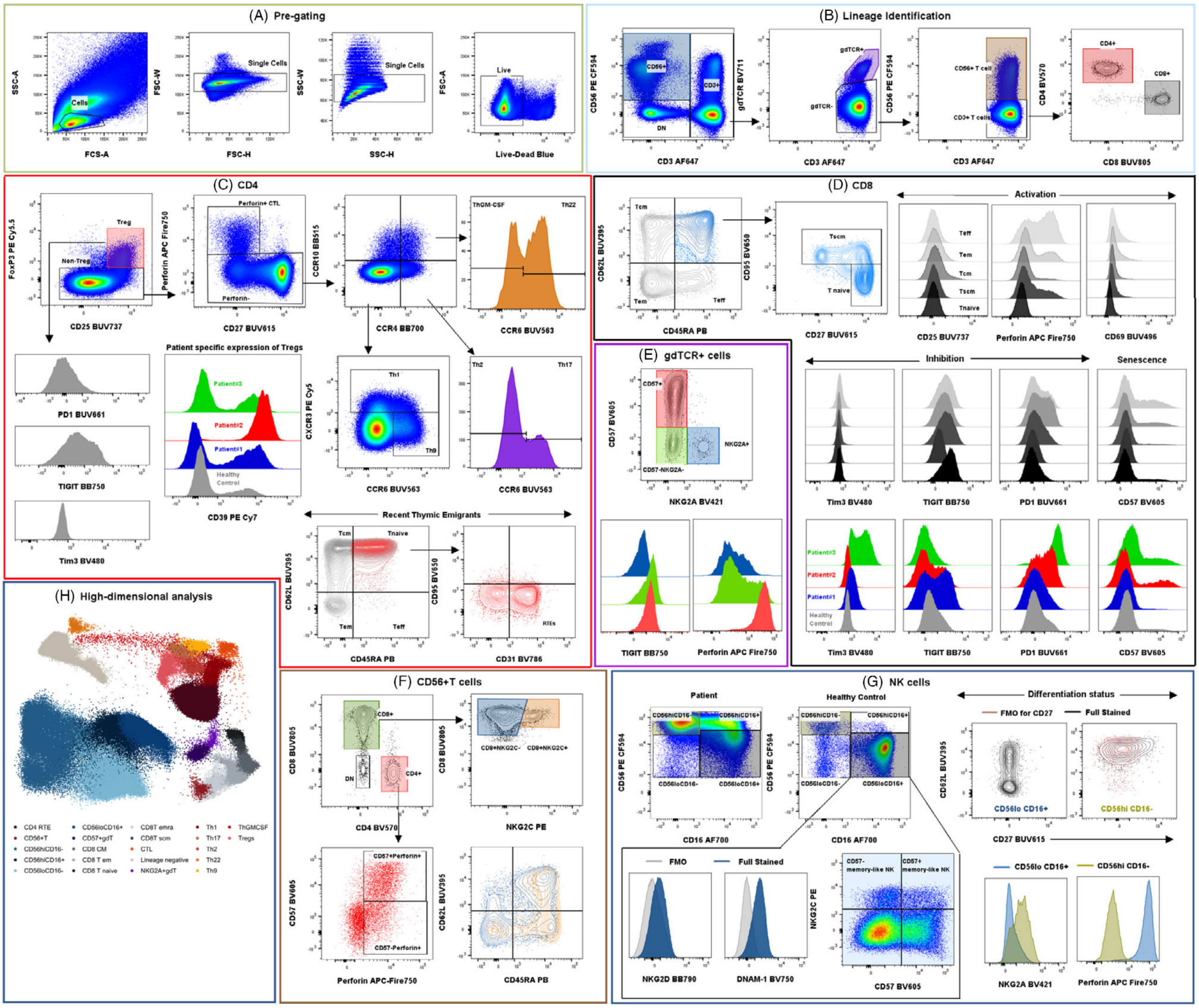

FIGURE 1 Manual gating strategy and algorithmic analysis. (**A**) Before the identification of individual subsets, debris, doublets and dead cells were removed. (**B**) Cell lineages were identified based on the expression of the following phenotypes: CD56+ (NK cells), CD56−CD3+ γδTCR+ (γδT cells), CD3+γδTCR-CD56+ T cells, CD3+γδTCR−CD56−CD4+ (T helper cells) and CD3+γδTCR−CD56−CD8+ (T cytotoxic cells). (**C**) CD4+ T cells were delineated into CD25+FoxP3+ Tregs and non-Treg cells. Activation of Tregs was evaluated based on the expression of CD39. Furthermore, expression of PD1, TIGIT, and Tim3 identifying highly suppressive Tregs was evaluated. Cells in the non-Treg gate were divided into individual T helper subsets based on the combination of chemokine receptors. These cells were first gated for perforin+CD27−CTL cells and perforin− cells. The perforin− cells were gated for CCR10+ CCR4+ CCR6+ Th22 cells, CCR10+ CCR4+CCR6− ThGM-CSF, CCR10−CCR4+CCR6− Th2 cells, CCR10−CCR4+CCR6+ Th17 cells, CCR10-CCR4-CCR6+CXCR3− Th9 cells and CCR10−CCR4−CCR6+/−CXCR3+ Th1 cells. Thymic function was evaluated on CD4+ gated cell via identification of CD45RA+CD62L+CD95−CD31+ cells. (**D**) Gated CD8+ cells were subtyped into individual memory subsets based on CD45RA and CD62L and expression of inhibitory receptors, activation markers and perforin was evaluated. Inhibiting receptors are only weakly expressed on T cells from healthy donors, and therefore we show a comparison between healthy and patients after HSCT. (**E**) Gating of γδT cells based on CD57−NKG2A+ cytokine-producing cells, CD57+NKG2A− terminally differentiated cytotoxic cells and CD57−NKG2A− with intermediate phenotype. Expression of perforin and TIGIT on these cells is depicted using a histogram. (**F**) NK cells were subtyped based on the expression of CD56 and CD16, and a comparison of a healthy donor and post-HSCT patient is shown. CD56hiCD16- cells represent less differentiated cytokine-producing cells characterized by low expression perforin and high expression of CD27, whereas CD56loCD16+ cells express high levels of perforin and lack NKG2A and CD27. These cells include a subset of CD57+NKG2C+ memory-like NK cells. (**G**) CD56+ T cells can be divided into three main lineages based on CD4 and CD8. The CD4+ cells are enriched with cytotoxic perforin expressing cells mostly terminally differentiated, as evident by expression of CD57. The CD8+ cell can be split into two subsets based on the expression of NKG2C. These subsets show distinct differentiation status, as evident by the expression patterns of CD45RA and CD62L. (**H**) Identification of individual cell types was performed on gated live single cells using the Embedding Guided by Self-Organizing Map (EmbedSOM) algorithm [20]. Expression pattern of markers on individual cell subsets is show in Figure S8. FSC, forward-scatter; SSC, side-scatter [Color figure can be viewed at wileyonlinelibrary.com]

Additional details regarding cell phenotypes and markers can be found in the online material.

2 | SIMILARITIES TO PUBLISHED OMIPs

Currently, there is no OMIP tailored explicitly to the need of monitoring a regenerating immune system post-HSCT. OMIP-080 represents a unique combination allowing for comprehensive monitoring of NK cell, NKT-like cell and both γδT and αβT cell reconstitution using qualitative and functional markers. It identifies NK cell subsets similarly to OMIP 007, 039, 070 and evaluates the expression of activating and inhibitory receptors. However, OMIP-080 also includes DNAM-1, which plays roles in the regulation of relapses. Furthermore, it evaluates functional properties of NK cells such as perforin production.

T cell reconstitution is also a priority of the panel and therefore it maps the emergence of RTE defined similarly to OMIP 013 and monitors the differentiation status Th subsets similarly to OMIP 017 and 030. In contrast to OMIP 030, it defines memory subsets using CD62L and identifies cytotoxic CD4 T cells as a unique subset. Furthermore, our panel allows for deeper analysis of Treg function through evaluation of CD39 expression similarly to OMIP 004 and 015, and identification of highly suppressive populations based on expression of PD1, TIGIT, Tim3. Similarly, to OMIP 037 and 050 our panel detects markers of exhaustion, senescence and activation, but also allows to map these markers to individual Th subsets.

ACKNOWLEDGMENTS

The authors would like to thank Miroslav Kratochvíl for providing constructive feedback for the unsupervised analysis of data. Special thanks go to Patrik Dvořák for instrument maintenance and calibration.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Sarka Vanikova: Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing – review and editing (equal). **Abhishek Koladiya:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing – review and editing (equal). **Jan Musil:** Conceptualization (equal); formal analysis (equal); funding acquisition (lead); methodology (equal); project administration (equal); resources (equal); supervision (lead); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/cyto.a.24510.

ORCID

Jan Musil https://orcid.org/0000-0002-9378-5956

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Vanikova S, Koladiya A, Musil J. OMIP-080: 29-Color flow cytometry panel for comprehensive evaluation of NK and T cells reconstitution after hematopoietic stem cells transplantation. Cytometry. 2022;101:21–6. https://doi.org/10.1002/cyto.a.24510