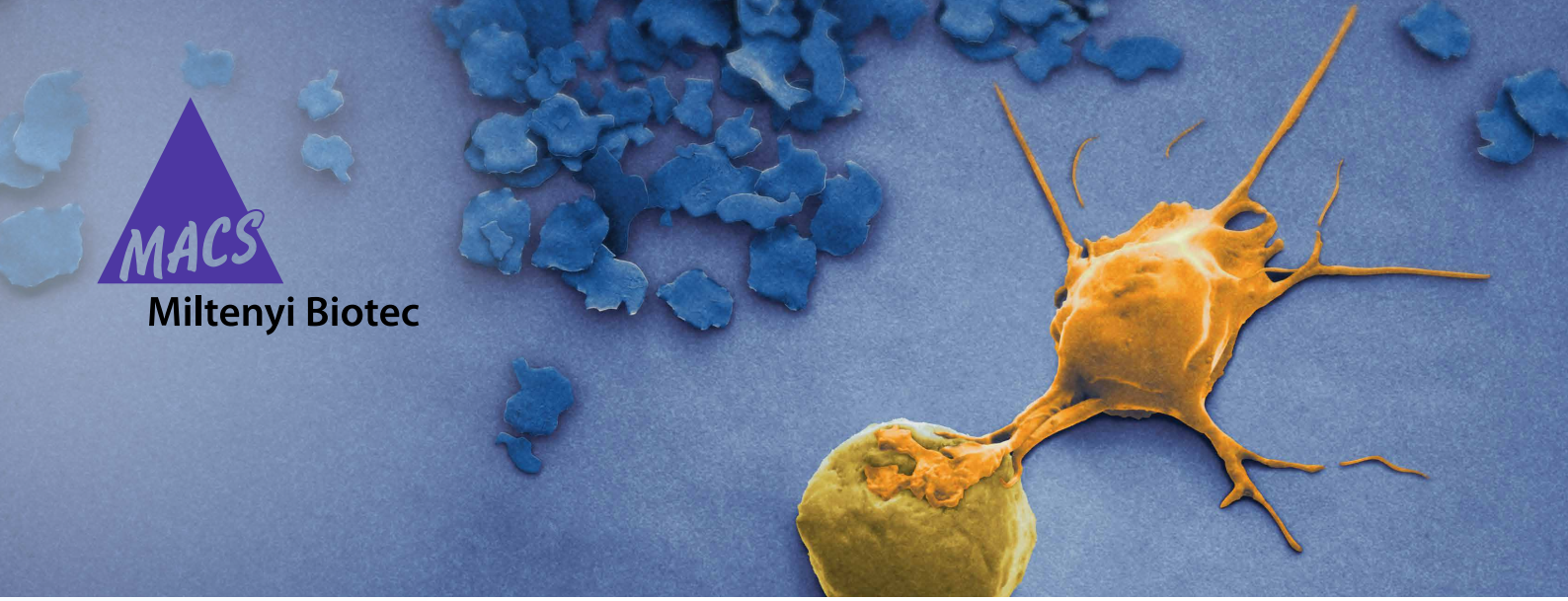

Extensive characterization of NK cells using mass cytometry

Amir Horowitz, Ph.D.
The Horowitz Laboratory
Icahn School of Medicine at Mount Sinai

Introduction

Natural killer (NK) cells were primarily noted for their ability to kill tumor cells[1,2]. Since their initial exposure, other important NK cell functions have become evident, such as elimination of virus-infected cells. Researchers have investigated these remarkable immune cells and their biology, leading to the knowledge that is available today about NK cell classification. Emerging data in clinical settings of immunotherapy for cancers demonstrate critical roles for NK cells in contributing to early tumor killing, as well as potentiating effective anti-tumor T cell functions. Miltenyi Biotec is actively working with academic NK cell experts to develop superior reagents to resolve phenotypic heterogeneity and identify subsets of NK cells with enhanced immunotherapeutic potential.

Human NK cells have been divided into two major subsets. They are identified according to the surface density of the CD56 antigen[3]. $CD56^{bright}$ NK cells are found at highest frequency in tissues, where they dominantly express NKG2A and NKp46. They are poorly cytolytic but secrete critical cytokines and chemokines. $CD56^{dim}$ NK cells mainly reside in peripheral blood and express NKG2A or killer immunoglobulin-like receptors (KIR), or both. Upon activation they display potent cytolytic activity and produce cytokines and chemokines. Further, deeper analyses into NK cells have revealed that mature NK cells are highly heterogenous in phenotypes and functions, where genetic and environmental determinants collectively shape the repertoire of human NK cells[4,5].

In approximately 40% of individuals seropositive for cytomegalovirus (CMV) infection, NK cells expressing the activating isoform of NKG2A : NKG2C disproportionately expand and can represent as much as 70% of the total repertoire[6]. This subset of $NKG2C^+$ NK cells upregulates CD57, loses expression of Eat-2 and FcεR1γ adaptor proteins and uniquely expresses self-HLA-C-reactive KIR receptors. Recent studies suggest that these $NKG2C^+$ NK cells can display adaptive or 'memory-like' features[7,8], and differentially recognize CMV-derived HLA-E-specific peptides from different CMV strains[9]. The specific expansion, together with other adaptive characteristics, is compatible with the memory-like function of these cells, which can allow for prompt control of CMV reactivation[10] and potential targets to exploit for cancer immunotherapies.

This application note describes the applicability of mass cytometry, or CyTOF®, as a novel platform for high-dimensional, phenotypic analysis of single cells using REAfinity™ Recombinant Antibodies. The combination of 16 markers sheds light into the complex and diverse NK cell subset repertoire present in three healthy donors.

Methods

Donors

Cryopreserved peripheral blood mononuclear cells (PBMCs) from three healthy donors were thawed and recovered overnight at 37 °C with 5% CO_2 in RPMI medium containing heat-inactivated fetal bovine serum (10%) without exogenous cytokines. DNA was genotyped for HLA class I and KIR genes in order to accurately validate anti-KIR antibodies and predicted frequencies of KIR-positive NK cells.

Antibodies and gating strategy

Commercial antibodies targeting CD3, CD14, CD16, CD19, CD33 and HLA-DR were obtained pre-conjugated to metal-loaded polymers and used to gate down to NK cells (Fluidigm, Inc). All NK cell-specific antibodies were provided in purified form from Miltenyi Biotec unless otherwise specified. Importantly, all antibodies were provided in phosphate-buffered saline (PBS) solution without addition of carrier proteins that could interfere with the conjugation process. The pure antibodies were conjugated to metal-loaded polymers using Maxpar® kits (Fluidigm, Inc).

Gating of NK cell subsets

Lymphocytes were selected by gating on singlets (figure 1A) and by excluding dead cells (figure 1B). NK cells were identified by the sequential gating of the following markers: expression of CD56 and the absence of CD14 and CD19 (figure 1C). Then total HLA-DR^{dim} CD56^{+} cells were gated (figure 1D) and subsequently CD3^{+} cells were excluded (figure 1E). NK cell subsets were identified using the following combinations: CD56^{bright} CD16^{-} NK cells (figure 1F.1), CD56^{dim} CD16^{+} NK cells (figure 1F.2), CD56^{-} CD16^{+} NK cells (figure 1F.3) and CD56^{dim} CD16^{-} NK cells (figure 1F.4). Commercial antibodies used were CD14, CD19, HLA-DR, CD3, CD16 (Fluidigm, Inc.).

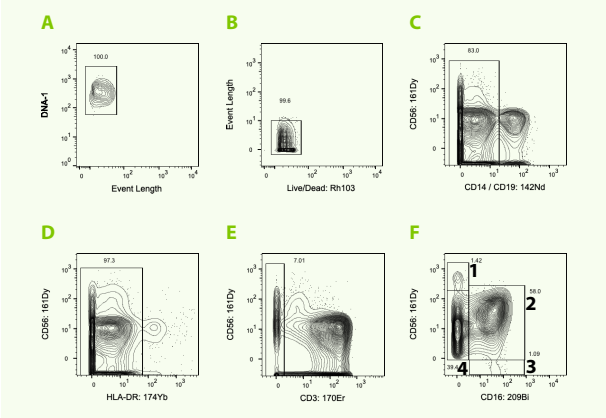

Figure 1: Gating strategy of NK cell subsets.

Gating of adaptive or 'memory-like' NK cells

Adaptive or 'memory-like' NK cells were identified by the sequential gating of the following markers: CD3^{-} CD56^{total} cells (figure 1E) expression of NKG2C and absence of NKG2A, followed by expression of CD57 and absence of FCεR1γ (figure 2).

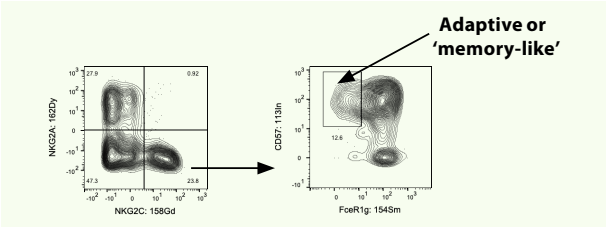

Figure 2: Gating of adaptive or 'memory-like' NK cells.

viSNE analysis

viSNE analysis was performed on 20,000 NK cells from each donor. NK cells were clustered according to expression of the REAfinity™ Recombinant Antibodies CD56 (REA196), CD122 (REA167), Tim-3 (REA635), TIGIT (REA1004), DNAM-1 (REA1040), NKG2A (REA110), NKG2C (REA205), KIR2DL1 (REA284), KIR2DL1/S1 (REA1010), KIR2DL2/L3 (REA1006), CD57 (REA769), KIR2DL3 (REA147), KIR3DL1 (REA1005) as well as NKp46 (9E2) and commercially available FcεR1γ.

Results

Adaptive or 'memory-like' NK cells

During the screening of donors, a sample from a CMV seropositive donor displayed a minor expansion of NKG2C^{+} NK cells with 24% proportional makeup comprised of adaptive or 'memory-like' NK cells (figure 3A).

A donor with HLA-C1 (C1/C1) haplotype has NK cells that express KIR2DL3 and a homozygous donor with HLAC-C2 (C2/C2) haplotype has NK cells that express KIR2DL1 (figure 4).

Representative plots of the two donors are depicted in figure 3C and 3D. In line with figure 4, KIR2DL3 was expressed in 100% of the adaptive or 'memory-like' NK cells from a donor homozygous for HLA-C1 (C1/C1) (figure 3C). Similarly, a representative donor homozygous with HLA-C2 (C2/C2) has 100% of adaptive or 'memory-like' NK cells (data not shown), 80% of them expressing KIR2DL1 (figure 3D).

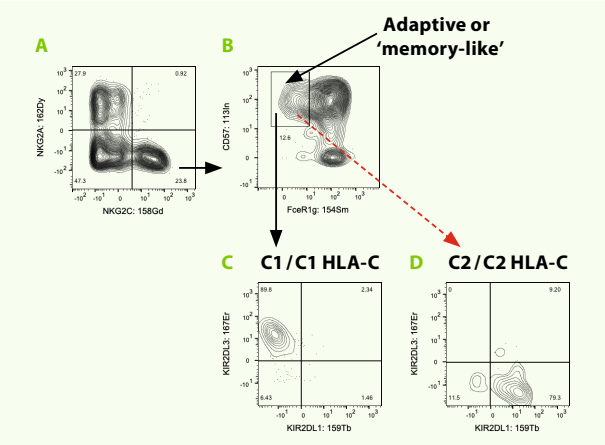

Figure 3: A, B. Representative plots of two donors. C. Adaptive or 'memory-like' NK cells from a donor homozygous with HLA C1 (C1/C1). D. Adaptive or 'memory-like' NK cells from a donor homozygous with HLA C2 (C2/C2).

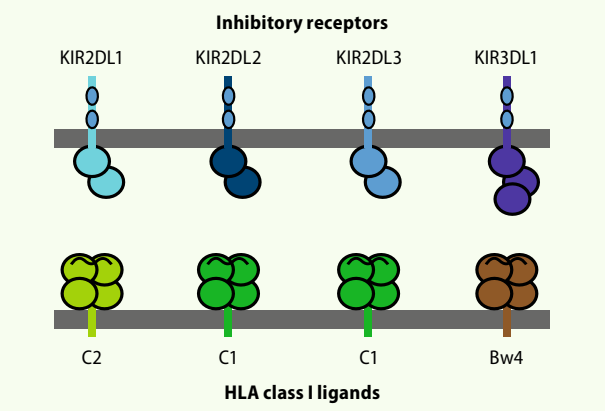

Figure 4: Inhibitory KIRs and HLA class I ligands[5].

NK cell diversity among donors

Activating and inhibitory receptors

NK cells were analyzed from two donors. Wide diversity was found between donors, for instance, donor 1 has a complete different distribution of CD56 and activation markers compared to donor 2 (figure 5). Similarly, donor 1 has a specific subset of cells expressing NKG2C, KIR2DL3 and KIR2DL2/L3 (figure 6).

Education / exhaustion receptors

A KIR3DL1 population could be identified in donor 1 that was absent in donor 2 (figure 7). Different populations expressing exhaustion markers can be identified and compared amongst donors (figure 7). The biological implications of these populations are beyond the scope of this work, but together with epidemiological data they grant interesting findings.

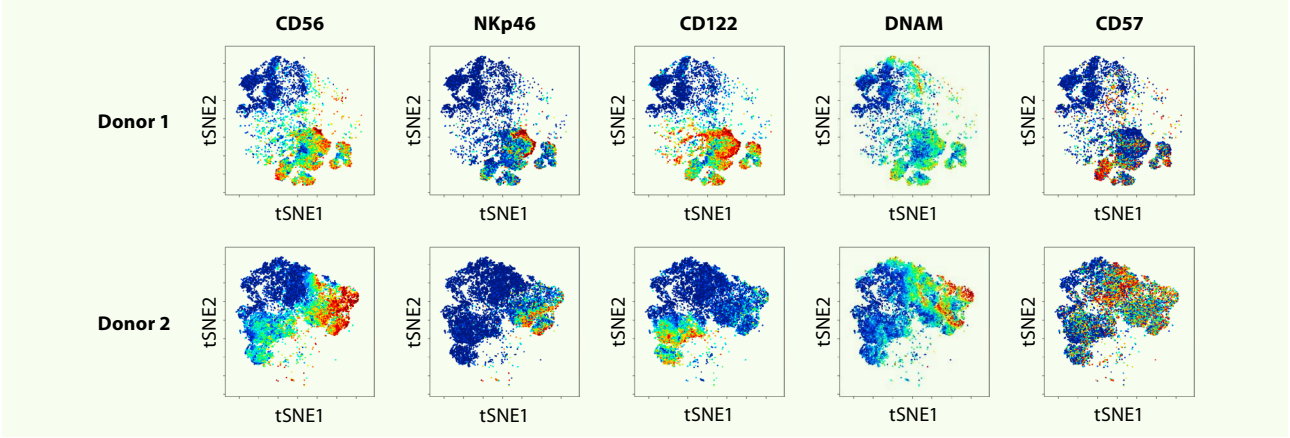

Figure 5: Expression pattern of CD56, NKp46, CD122, DNAM and CD57 in human PBMCs. After gating on all living cells, expression of the respective markers was visualized by viSNE analysis. The clustering was performed on 16 different antibodies. The REAfinity™ Recombinant Antibodies used for staining are described in the methods section.

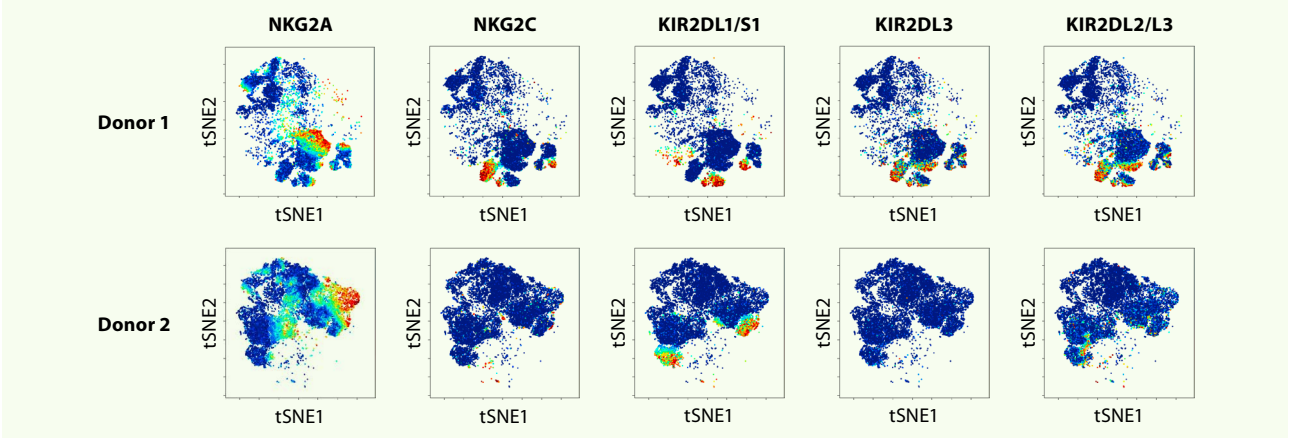

Figure 6: Expression pattern of NKG2A, NKG2C, KIR2DL1/S1, KIR2DL3 and KIR2DL2/L3 in human PBMCs. After gating on all living cells, expression of the respective markers was visualized by viSNE analysis. The clustering was performed on 16 different antibodies. The REAfinity Recombinant Antibodies used for staining are described in the methods section.

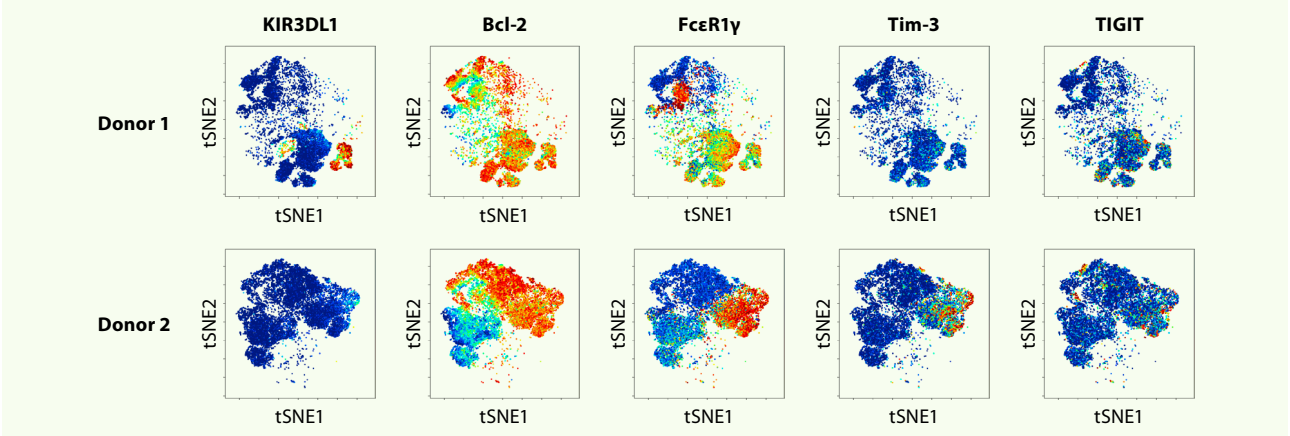

Figure 7: Expression pattern of KIR3DL1, Bcl-2, FcεR1g, Tim-3 and TIGIT in human PBMCs. After gating on all living cells, expression of the respective markers was visualized by viSNE analysis. The clustering was performed on 16 different antibodies. The REAfinity Recombinant Antibodies used for staining are described in the methods section.

Conclusions

- We have successfully explored the applicability of mass cytometry as a novel platform for high-dimensional phenotypic analysis of NK cells.
- REAfinity™ Recombinant Antibodies enable the reliable detection of multiple NK cell subsets and populations.

Product	Clone
Recombinant REAfinity Antibodies	
CD56 pure, human	REA196
CD122 (IL-2Rβ) pure, human	REA167
Tim-3 pure, human	REA635
TIGIT pure, human	REA1004
CD226 (DNAM-1) pure, human	REA1040
CD159a (NKG2A) pure, human	REA110
CD159c (NKG2C) pure, human	REA205
CD158a (KIR2DL1) pure, human	REA284
CD158a/h (KIR2DL1/DS1) pure, human	REA1010
CD158b (KIR2DL2/DL3) pure, human	REA1006
CD158e (KIR3DL1) pure, human	REA1005
CD57 pure, human	REA769
CD158b2 (KIR2DL3) pure, human	REA147
Hybridoma Antibodies	
CD335 (NKp46) pure, human	9E2

Note: Some of the antibodies have been ordered via the custom antibody design service (CADS).

References

1. Kiessling, R. *et al.* (1975) Eur. J. Immunol. 5(2): 117–21.
2. Kiessling, R., Klein, E., and Wigzell, H. (1975) Eur. J. Immunol. 5(2): 112–7.
3. Caligiuri, M.A. (2008) Blood 112(3): 461–9.
4. Horowitz, A. *et al.* (2013) Sci Transl Med. 5(208): 208ra145.
5. Horowitz, A. *et al.* (2016) Sci Immunol. 1: eaag1672.
6. Beziat, V. *et al.* (2013) Blood 121(3): 2678–88.
7. Lee, J. *et al.* (2015) Immunity 42(3): 431–42.
8. Schlums, H. *et al.* (2015) Immunity 42(3): 443–56.
9. Hammer, Q. *et al.* (2018) Nat. Immunol. 19(5): 453–56.
10. Della Chiesa, M. *et al.* (2015) Front Immunol. 6: 573.

Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG | Phone +49 2204 8306-0 | Fax +49 2204 85197 | macs@miltenyibiotec.de | **www.miltenyibiotec.com**

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

V.02