To the editor:

**CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cell phenotype can be induced by cryopreservation**

Natural killer (NK) cells can contribute to the control of different viruses.\textsuperscript{1,2} Recently, Azzi et al described the role of early differentiated NK cells in Epstein-Barr virus–driven infectious mononucleosis (IM).\textsuperscript{3} Besides the conventional CD56\textsuperscript{bright}CD16\textsuperscript{+} and CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cell populations, a phenotypically distinct CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cell population was transiently observed during acute IM.

We have also observed this CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cell phenotype in cryopreserved peripheral blood mononuclear cells (PBMCs) from hematopoietic stem cell transplantation (HSCT) recipients,

**Figure 1.** CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cell phenotype can be induced by cryopreservation. (A-B) Representative flow cytometry plots of fresh and cryopreserved NK cells from HSCT recipients at 4 weeks after transplantation (A) and healthy donors (B). (C-D) Longitudinal NK cell reconstitution in 9 HSCT recipients, based on the paired evaluation of fresh (C) and cryopreserved (D) PBMCs. Shown is the mean ± SEM of fresh healthy donors. (F-G) Contribution of early differentiated NKG2A\textsuperscript{+}KIR\textsuperscript{−} NK cells (F) and cryopreservation-induced CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cells (G) to the total NK cell population, expressed as a function of time after HSCT. (H) Differentiation of NK cells based on the expression of NKG2A and KIRs on NK cells in fresh (F) and cryopreserved (C) PBMCs. Shown is the percentage of NKG2A\textsuperscript{+}KIR\textsuperscript{−} (light gray), NKG2A\textsuperscript{−}KIR\textsuperscript{+} (white), NKG2A\textsuperscript{−}KIR\textsuperscript{−} (dark gray), and NKG2A\textsuperscript{+}KIR\textsuperscript{−} (black) NK cells divided over the CD56\textsuperscript{bright}CD16\textsuperscript{+} and CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cell phenotypes. Bars represent mean ± SEM of 4 healthy donors or 2 HSCT recipients at 4 weeks after transplantation. Flow cytometry-data were acquired on a Becton Dickinson LSRII flow cytometer and analyzed using Beckman Coulter (BC) Kaluza software. Gating strategy (A-E,G): forward scatter vs DAPI (Sigma-Aldrich)→ CD45 (FITC, clone 2D1, BD) vs CD14 (PE, MOP9, BD) vs CD3 (PE, 7E12, BD) vs and CD235a (PE, KC16, BC)→ CD19 (APC, J4.119, BC)→ CD3 (Brilliant Violet 421, UCHT1, BD) vs CD7 (Alexa 700, M-701, BD)→ CD56 (PE-Texas Red, N901, BC) vs CD16 (Brilliant Violet 711, 3G8, BD). Gating strategy (F,H): forward scatter vs DAPI (Sigma-Aldrich)→ CD45 (FITC, clone 2D1, BD) vs CD14 (APC, MOP9, BD) and CD19 (APC, J4.119, BC)→ CD3 (Brilliant Violet 421, UCHT1, BD) vs CD7 (Alexa 700, M-701, BD)→ CD56 (PE-Texas Red, N901, BC) vs CD16 (Brilliant Violet 711, 3G8, BD)→ NKG2A (PE-Cy7, Z199, BC) vs KIR (PE, combination of CD158a +a/h +i +j +b) [DX9, BD + E66, BC + FES172, BC + GL183, BC].
especially early after transplantation. However, only the conventional CD56brightCD16+ and CD56dimCD16+ NK cells were detected in freshly analyzed PBMCs, indicating that the CD56dimCD16- phenotype is induced by cryopreservation (Figure 1A).

Blood samples were obtained from 9 pediatric HSCT recipients and 4 healthy donors with written informed consent and approval by the Institutional Review Board (protocol P02.099). PBMCs were isolated using Ficoll-Isopaque separation within 6 hours after blood withdrawal and analyzed by flow cytometry. The remainder of cells was cryopreserved in liquid nitrogen, thawed, and reanalyzed. NK cells were defined as CD19-CD3-CD7-CD56-/CD16- cells within the live (DAPI-) CD45-CD14-CD33-CD235a- lymphocyte gate.4

In fresh PBMCs, CD56dimCD16− cells constituted <3% of NK cells, mainly caused by overspill from CD56brightCD16+/or CD56dimCD16− NK cells.5,6 In contrast, CD56dimCD16+ cells represented a larger and distinct population in cryopreserved PBMCs (Figure 1A-B). These cells accounted for 17% to 36% of cryopreserved NK cells at 3 weeks after HSCT (fresh vs cryopreserved: P < .001, paired Student t test), rapidly decreasing in the weeks thereafter (Figure 1C-D). This CD56dimCD16− NK cell population was identified with various monoclonal antibodies (CD56-clones N901/HCDS6; CD16-clones 3G8/B73.1). Interestingly, the cryopreservation-induced CD56dimCD16− population was also enlarged when healthy donor blood was stored for 24 hours at room temperature before PBMCs were isolated (Figure 1E), suggesting a relationship with the vitality of PBMCs prior to cryopreservation.7,8

We hypothesized that the cryopreservation-induced CD56dimCD16− NK cells originated from either CD56brightCD16+/ or CD56dimCD16+ NK cells as a consequence of reduced CD56 or CD16 expression, respectively. At 3 weeks after HSCT, predominantly the largest CD56brightCD16+/− NK cell population was decreased after cryopreservation (mean 67% to 46% of NK cells, P = .002), coinciding with the appearance of the CD56dimCD16− phenotype. One week later, both a reduction of CD56brightCD16+/− (45% to 36%, P = .01) and CD56dimCD16+ (54% to 41%, P = .01) NK cells was observed. At 1 year after HSCT and in healthy donors, CD56dimCD16+ NK cells formed the majority of NK cells and mainly this phenotype was reduced after cryopreservation (Figure 1C-E). Thus, depending on time after HSCT, both CD56brightCD16+/− and CD56dimCD16+ NK cells could contribute to the CD56brightCD16+/− phenotype.

A skewing toward the early differentiated NKGA2−KIR− NK cell phenotype was observed early after HSCT as well as during acute IM,3,9 raising the possibility that these cells might be particularly sensitive to cryopreservation. However, the presence of NKGA2−KIR− NK cells and the occurrence of CD56dimCD16− NK cells showed different kinetics (Figure 1F-G). In multivariate analysis, the percentage of CD56dimCD16− was only correlated with time after HSCT and not to the percentage of NKGA2−KIR− cells (P < .0001 vs P = .35, linear regression on log-transformed data). Importantly, no significant differences were observed between the NKGA2−KIR phenotype of CD56dimCD16− and CD56brightCD16−/− NK cells when analyzed before and after cryopreservation (Figure 1H).

Altogether, NK cells are temporarily more sensitive to cryopreservation-induced phenotype changes in the first weeks after HSCT. This might relate to the CD56dimCD16− NK cell phenotype, which was transiently observed during the acute phase of IM.3 Both during acute IM and early after HSCT, CD56dimCD16− NK cells were observed under inflammatory conditions. This raises the possibility that cryopreservation-induced phenotypic changes are related to activation-associated cellular mechanisms.

Clinical situations like the absence of T cells after HSCT or during viral infections provide unique opportunities to study human NK cell biology. It should be taken into account that under such circumstances, NK cells may be transiently susceptible to cryopreservation-induced phenotype changes. Because the CD56dimCD16− NK cells can originate from both conventional NK cell populations, we recommend assessing the cryopreservation-induced CD56dimCD16− NK cells separately to avoid phenotype-skewing of the conventional populations. Our data emphasize the importance of validating flow cytometric results with fresh PBMC samples in order to become aware of potential cryopreservation-induced phenotypic changes.

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