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{+}/2 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.
especially early after transplantation. However, only the conventional CD56<sup>bright</sup>CD16<sup>+</sup> and CD56<sup>dim</sup>CD16<sup>+</sup> NK cells were detected in freshly analyzed PBMCs, indicating that the CD56<sup>dim</sup>CD16<sup>+</sup> 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<sup>−</sup>CD3<sup>−</sup>CD56<sup>−/−</sup>CD16<sup>−/−</sup> cells within the live (DAPI<sup>−</sup>)CD4<sup>+</sup>CD14<sup>−</sup>CD33<sup>−</sup>CD235a<sup>−</sup> lymphocyte gate. 5,6 In fresh PBMCs, CD56<sup>dim</sup>CD16<sup>+</sup> cells constituted <3% of NK cells, mainly caused by overspill from CD56<sup>bright</sup>CD16<sup>+</sup> or CD56<sup>dim</sup>CD16<sup>−</sup> NK cells. 5,6 In contrast, CD56<sup>dim</sup>CD16<sup>+</sup> 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 CD56<sup>dim</sup>CD16<sup>+</sup> NK cell population was identified with various monoclonal antibodies (CD56-clones N901/HCD56; CD16-clones 3G8/B73.1). Interestingly, the cryopreservation-induced CD56<sup>dim</sup>CD16<sup>+</sup> 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 CD56<sup>dim</sup>CD16<sup>+</sup> NK cells originated from either CD56<sup>bright</sup>CD16<sup>−/−</sup> or CD56<sup>dim</sup>CD16<sup>−</sup> NK cells as a consequence of reduced CD56 or CD16 expression, respectively. At 3 weeks after HSCT, predominantly the largest CD56<sup>bright</sup>CD16<sup>−/−</sup> NK cell population was decreased after cryopreservation (mean 67% to 46% of NK cells, P = .002), coinciding with the appearance of the CD56<sup>dim</sup>CD16<sup>−</sup> phenotype. One week later, both a reduction of CD56<sup>bright</sup>CD16<sup>−/−</sup> (45% to 36%, P = .01) and CD56<sup>dim</sup>CD16<sup>−</sup> (54% to 41%, P = .01) NK cells was observed. At 1 year after HSCT and in healthy donors, CD56<sup>dim</sup>CD16<sup>+</sup> 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 CD56<sup>bright</sup>CD16<sup>−/−</sup> and CD56<sup>dim</sup>CD16<sup>−</sup> NK cells could contribute to the CD56<sup>bright</sup>CD16<sup>−/−</sup> phenotype.

A skewing toward the early differentiated NKGA2<sup>+</sup> KIR<sup>−</sup> 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<sup>+</sup> KIR<sup>−</sup> NK cells and the occurrence of CD56<sup>dim</sup>CD16<sup>−</sup> NK cells showed different kinetics (Figure 1F-G). In multivariate analysis, the percentage of CD56<sup>dim</sup>CD16<sup>−</sup> cells was only correlated with time after HSCT (P < .001 vs P = .35, linear regression on log-transformed data). Importantly, no significant differences were observed between the NKGA2<sup>+</sup>KIR phenotype of CD56<sup>dim</sup>CD16<sup>−</sup> and CD56<sup>bright</sup>CD16<sup>−/−</sup> 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 CD56<sup>dim</sup>CD16<sup>−</sup> NK cell phenotype, which was transiently observed during the acute phase of IM. 3 Both during acute IM and early after HSCT, CD56<sup>dim</sup>CD16<sup>−</sup> 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 CD56<sup>dim</sup>CD16<sup>−</sup> NK cells can originate from both conventional NK cell populations, we recommend assessing the cryopreservation-induced CD56<sup>dim</sup>CD16<sup>−</sup> 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.

Gertjan Lugthart
Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands
Monique M. van Ostainen ten Dam
Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands
Maarten J. D. van Tol
Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands
Arjan C. Lankester
Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands
Marco W. Schilham
Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands

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Correspondence: Gertjan Lugthart, Department of Pediatrics, Leiden University Medical Center, PO Box 9600, 2300 RC, Leiden, The Netherlands; e-mail: g.lugthart@lumc.nl.

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Gertjan Lugthart, Monique M. van Ostaijen-ten Dam, Maarten J. D. van Tol, Arjan C. Lankester and Marco W. Schilham