Natural killer cell licensing after double cord blood transplantation is driven by the self-HLA class I molecules from the dominant cord blood

Natural killer (NK) cells gain functionality through an educational process ("licensing") that depends on killer immunoglobulin-like receptor (KIR) recognition of self-HLA class I molecules. In allogenic double umbilical cord blood stem cell transplantation (2CBT), the respective roles of the two cord blood (CB) units and the recipient's HLA class I molecules in NK cell licensing of the dominant CB (DCB) unit have yet to be characterized. In this study, following 2CBT, NK cells from the DCB unit were found to be licensed by their own HLA class I molecules (HLA-C1/C2 allotypes). HLA molecules from the recipient and the non-dominant CB (ndCB) did not show a significant role.

In allogenic hematopoietic stem cell transplantation (allo-HSCT), NK cells are able to recognize and eliminate leukemic cells.[1] NK cells constitute the first lymphocyte subset to be reconstituted after allo-HSCT, with the rapid expansion of the $CD56^{bright}$ NK cell subpopulation and the subsequent expansion of the dominant $CD56^{dim}$ subset with a high cytotoxic function. The activity of NK cells is regulated by the balance between activating and inhibitory signals. The latter are mediated by the binding of major histocompatibility complex class I (MHC-I) molecules to the target cell on NK cell inhibitory receptors, including, in humans, the lectin-like heterodimer CD94/NKG2A (which interacts with HLA-E) and some of the polymorphic KIRs. The KIRs specifically bind to HLA-C allotypes with asparagine 80 (group C1 alleles), HLA-C allotypes with lysine 80 (group C2 alleles), and Bw4-expressing HLA-A and HLA-B allotypes.[2] Observations in patients having undergone HLA-haploidentical HSCT highlighted a correlation between donor KIR and host KIR ligand expressions in tumor relapse occurrence.[1,3]

Recognition of self-MHC-I molecules by inhibitory KIRs is involved in the calibration of the NK cell effector capacities during differentiation and maturation. This enables cells lacking MHC-I to subsequently be recognized as a "missing-self" target.[4] We have shown that after KIR ligand-mismatched conventional allo-HSCT in humans, the NK cell licensing reiterates the responsiveness pattern determined by the donor's HLA ligands.[5] Two retrospective studies of the effect of KIR ligand incompatibility in single[6,7] and double[7] unrelated CB transplantation for hematological malignancies showed conflicting results in terms of leukemia-free survival and incidence of relapse. Usually, one CB unit dominates the other in the 2CBT. It is presumed that alloreactive graft-versus-graft rejection occurs, depending on the status of $CD4^+$ T cells[8] or $CD8^+$ T cells.[9] The dose of $CD34^+$ cells[10] and the cell banking procedures[11] may also influence CB unit dominance which appears to be independent of the KIR-HLA mismatch.[12] The co-existence of 3, and then 2, allogeneic partners after 2CBT raises the issue of the NK cell education. The objective of this study was to assess *ex vivo* NK cell licensing after 2CBT in humans, without studying the *in vivo* graft-versus-leukemia effect. This study focused on KIR2DL1/S1 and 2DL2/L3/S2 receptor function that recognize C2/C1 HLA-C allotypes.

This study included 20 patients having received a non-manipulated 2CBT to treat a hematological disorder (patients and transplant characteristics are summarized in *Online Supplementary Table S1*; methods in *Online Supplementary Methods*). We first analyzed the distribution of NK cell subpopulations after transplantation in *ex vivo* samples. $CD3^-CD56^{dim}$ $NKG2A^+$ expressing NK cells were much more abundant than $CD3^-CD56^{dim}$ $NKG2A^-$ NK cells (mean: 103 +/- 65 /µl *vs.* 36 +/- 33/µl). Distribution of NK cell subsets was estimated in thawed samples as below: in $CD3^-CD56^{dim}$ $NKG2A^+$ population, monoKIR2DL1/S1$^+$ and monoKIR2DL2/L3/S2$^+$ cells represent 9.5±5.8% and 13.6±9.1%, respectively. In $CD3^-CD56^{dim}$ $NKG2A^-$ population, monoKIR2DL1/S1$^+$ and monoKIR2DL2/L3/S2$^+$ cells represent 14.1±12.4% and 18.3±17.2%, respectively (*Online Supplementary Table S2*).

We examined $CD56^{dim}$ CD94/$NKG2A^{+/-}$ $KIR^{+/-}$ NK cell subsets by degranulation assays at several time points

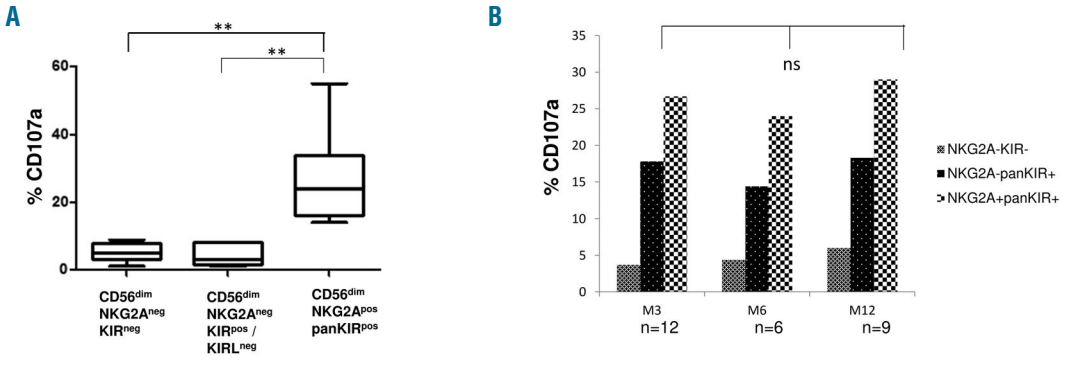

Figure 1. Responsiveness of NK cell subsets after 2CBT. (A) The degranulation capacity of NK cell subsets was evaluated in all samples after incubation with K562 targets. The $NKG2A^{neg}$ KIR^{neg} and the $NKG2A^{neg}$ monoKIR NK cell subsets, in which the 2 cord blood units and the recipient lack a cognate KIR ligand ($KIRL^{neg}$), are hyporesponsive in comparison to the $NKG2A^{pos}$ $panKIR^{pos}$ subsets. $^{**}P<0.001$ (Kruskal-Wallis). (B) Degranulation at 3 months (M3), 6 months (M6) and 12 months (M12) of the $NKG2A\text{-}KIR^-$ $NKG2A\text{-}panKIR^+$ and $NKG2A^+panKIR^+$ NK cell subsets was respectively evaluated in 12, 6 and 9 samples. ns.: not significant (Kruskal-Wallis).

after 2CBT (*Online Supplementary Table S1*). CD56^{dim} NK cells were divided into four subsets by flow-cytometry, according to their CD94/NKG2A and KIR expressions: NKG2A$^-$KIR$^-$ cells; NKG2A$^+$ panKIR$^+$ cells (defined as KIR2DL1/S1$^+$ KIR2DL2/L3/S2$^+$ KIR3D$^+$); KIR2DL1/S1 monoKIR NK cells (defined as KIR2DL1/S1$^+$ KIR2DL2/L3/S2$^-$) and KIR2DL2/L3/S2 monoKIR NK cells (defined as KIR2DL2/L3/S2$^+$ KIR2DL1$^-$) (*Online Supplementary Figure S1*). The NK cell populations were analyzed for degranulation activity (CD107a is mobilized to cell surface following a co-culture with the K562 cell line) at 3, 6 and 12 months post-2CBT. As a baseline measurement, we evaluated the degranulation of CD56^{dim} NKG2A$^-$monoKIR cells lacking their cognate HLA-C ligand in the two CB units and the recipient. Accordingly, CD56^{dim} NKG2A$^-$KIR2DL1/S1$^+$ and CD56^{dim} NKG2A$^-$KIR2DL2/L3/S2$^+$ NK cells were unlicensed in the absence of HLA-C2 and HLA-C1 ligands, respectively (degranulation mean ± SD after K562 challenge: 3.1±3.3%). As expected, CD56^{dim} NKG2A$^-$ KIR$^-$ NK cells also responded poorly during the first year post-2CBT (Figure 1A). In contrast, the CD56^{dim} NKG2A$^+$ panKIR$^+$ NK subset was the most responsive (degranulation mean: 28.1±8.8%) independently of the KIR/KIR ligand status of the recipient and CB units. We examined NK cell subsets at several time points after 2CBT (*Online Supplementary Table S1*). The licensing pattern was stable at two of the time points tested (Figure 1B).

We subsequently focused on the NKG2A$^-$KIR$^+$ population to determine the influence of the KIR ligand source on NK cell education. To that end, we evaluated the degranulation activity of the monoKIR-expressing NK cells according to the presence, or absence, of the corresponding KIR ligand in the DCB, the ndCB and/or the recipient.

Firstly, to evaluate the recipient's involvement in NK cell licensing, we studied monoKIR NK cell subsets in the context of HLA-C group-matched or -mismatched transplantation between the recipient and the DCB. In the HLA-C group-matched transplantation, the presence of KIR ligand in the recipient and in the DCB (R+DCB+) was associated with a significantly higher percentage of degranulation for NKG2A$^-$ monoKIR$^+$ NK cells (19±7.5%; P<0.001) compared to NKG2A$^-$KIR$^-$ cells (Figure 2A). We then studied cases with one HLA-C group mismatch between the recipient and the DCB. The presence of KIR ligand in the DCB alone (R-DCB+; n = 6 cases) conferred responsiveness on the corresponding NKG2A$^-$ monoKIR$^+$ NK cells (15.8±5.6%) similar to that of the matched situation (Figure 2B). In contrast, in the presence of KIR ligand only in the recipient (R+DCB-; n = 4 cases), the NKG2A$^-$monoKIR$^+$ subset showed a lower degranulation capacity than NKG2A$^-$monoKIR$^+$ cells in an HLA-C group-matched situation (8.8±3.2% and 18.3±8.4%,

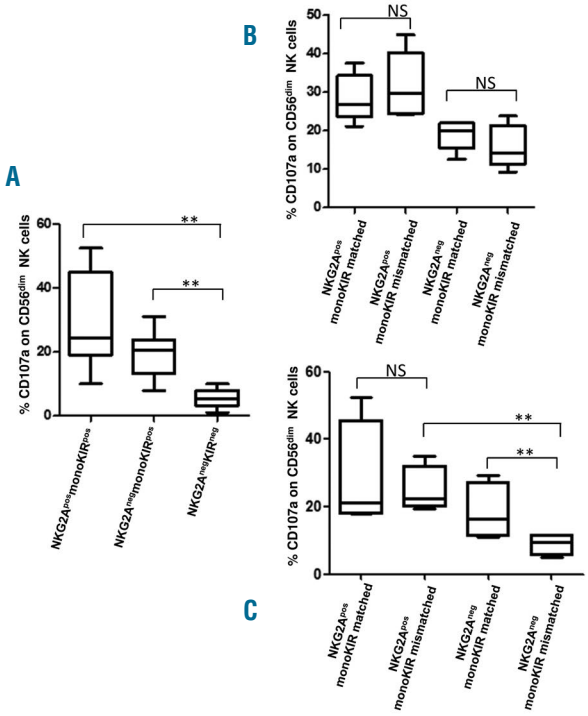

Figure 2B
R = Recipient
DCB = Dominant Cord Blood

Cases	HLA-C allotypes (R/DCB)	HLA-C mismatch (R-/DCB+)	KIR mismatched	KIR matched
15	C2C2 / C1C2	C1	KIR2DL2	KIR2DL1/S1
16	C2C2 / C1C2	C1	KIR2DL2/L3/S2	KIR2DL1
17	C2C2 / C1C2	C1	KIR2DL3/S2	KIR2DL1/S1
18	C2C2 / C1C2	C1	KIR2DL2/L3/S2	KIR2DL1
19	C1C1 / C1C2	C2	KIR2DL1/S1	KIR2DL2/L3/S2
20	C1C1 / C1C2	C2	KIR2DL1	KIR2DL3

Figure 2C
R = Recipient
DCB = Dominant Cord Blood

Cases	HLA-C allotypes (R/DCB)	HLA-C mismatch (R+/DCB-)	KIR mismatched	KIR Matched
11	C1C2 / C1C1	C2	KIR2DL1/S1	KIR2DL2/S2
12	C1C2 / C1C1	C2	KIR2DL1	KIR2DL2/L3/S2
13	C1C2 / C1C1	C2	KIR2DL1	KIR2DL2/S2
14	C1C2 / C2C2	C1	KIR2DL3/S2	KIR2DL1/S1

Figure 2. Role of KIR ligand of the dominant CB unit and the recipient in NK cell licensing after 2CBT. Recipient KIR ligand involvement in NK cell licensing was evaluated in a total of 33 assays performed on 27 samples. (A) The responsiveness of CD56^{dim} NKG2A^{pos} monoKIR^{pos} and CD56^{dim} NKG2A^{neg} monoKIR^{pos} NK cells was evaluated when appropriate KIR ligands (HLA-C allotypes) were present in the recipient and the two cord bloods when compared to CD56^{dim} NKG2A^{neg} KIR^{neg} NK cells; (n = 31 tests; **P<0.001, Kruskal-Wallis). (B) The responsiveness of CD56^{dim} NKG2A$^{pos/neg}$ monoKIR^{pos} NK cells was evaluated when appropriate KIR ligands (HLA-C allotypes) were absent in the recipient but present in the DCB (R- DCB$^+$, n=6 tests; cases 15, 16, 17, 18, 19, 20). MonoKIR mismatched cells represent NK KIR^{pos} cells which recognize HLA-C present only in the DCB. MonoKIR matched cells represent NK KIR^{pos} cells which recognize HLA-C present in the recipient and the DCB. NS: Not Significant (P>0.05). (C) The responsiveness of CD56^{dim} NKG2A^{pos} or neg monoKIR^{pos} NK cells was evaluated when appropriate KIR ligands (HLA-C allotypes) were present in the recipient but absent in the DCB (R+ DCB-, n=4 tests; cases 11, 12, 13, 14). MonoKIR mismatched cells represent NK KIR^{pos} cells which recognize HLA-C present only in the recipient. MonoKIR matched cells represent NK KIR^{pos} cells which recognize HLA-C present in the recipient and the DCB. CD56^{dim} NKG2A^{neg} monoKIR mismatched compared to CD56^{dim} NKG2A^{neg} monoKIR matched NK cells were hyporesponsive (**P<0.001 ; Kruskal-Wallis). NS: Not Significant (P>0.05).

respectively; $P<0.001$) (Figure 2C). This led us to the conclusion that recipient KIR ligand played a minor role in the NK education process after 2CBT.

In 2CBT, the HLA class I environment transiently includes the ndCB HLA class I molecules. Indeed, in our cohort, whilst all patients displayed full DCB chimerism at three months after 2CBT, 8 patients underwent mixed ndCB/DCB chimerism for at least one month post-graft (See *Online Supplementary Table S1*), thus potentially impacting licensing of the DCB. We therefore evaluated the education pattern for monoKIR$^+$ NK cells according to HLA-C group-matched or -mismatched CB units (i.e. the presence of HLA-C1 or HLA-C2 in the DCB or ndCB unit alone) at our earliest available time point, 3 months after 2CBT. The presence of KIR ligands only in the ndCB was unable to confer responsiveness to NKG2A$^-$ monoKIR$^+$ NK cell subsets whilst the presence of KIR ligand in the DCB was associated with a significantly higher percentage of NK cell degranulation. The presence, for example, of C2 ligands in the ndCB did not license monoKIR2DL1/S1 cells (Figure 3).

In the present study, NK cell education was investigated 3, 6 and/or 12 months after KIR ligand matched or mismatched 2CBT. NKG2A$^-$KIR$^-$ NK cells had a very low CD107a expression after stimulation by target cells consistent with the known hyporesponsive state of NK cells lacking inhibitory receptors.[4] In contrast, the NKG2A$^+$ panKIR$^+$ CD56^{dim} NK cell subset was the most responsive, with a stable licensing pattern at two of the time points tested, highlighting the pivotal role of CD94/NKG2A and KIRs in maintaining a functional NK cell repertoire after transplantation. Regarding the more mature NKG2A$^-$KIR$^+$ subset, the presence of KIR ligand only in the recipient conferred a poor responsiveness on the corresponding monoKIR$^+$ NK cells in contrast to its presence in DCB units.

We previously reported that in conventional allogeneic HSCT, including 11 single CBT, NK cell education in the recipient is supported by the donor HLA-genotype for at least 3 years post-transplantation.[5] To our knowledge, only the study by Foley *et al*. has addressed the issue of NK cell education after 2CBT showing that NK cells expressing self-KIR were (i) fully educated after 2CBT in a CD107a degranulation assay and (ii) expressed IFNγ.[13] However, Foley *et al.* did not report the source of the ligand (recipient *vs.* DCB or ndCB) involved in NK cell licensing. In view of the reported plasticity of the education process, the potential impact of the ndCB on this process also needs to be assessed. Indeed, in murine models, unlicensed NK cells became functional after transfer into a MHC-I-sufficient environment, whereas previously educated NK cells became hyporesponsive in an MHC-I-deficient environment.[14] However, reports on patients having undergone allo-HSCT showed the existence of a stable donor-type licensing pattern[5] as observed here, suggesting that the cell population required for NKG2A$^-$NK cell education is transferred with the graft which will successfully reconstitute the recipient.[5,15]

In conclusion, although based on a small cohort of patients, these data provide proof of principle rules governing NK licensing after 2CBT with a potential impact on CB choice.

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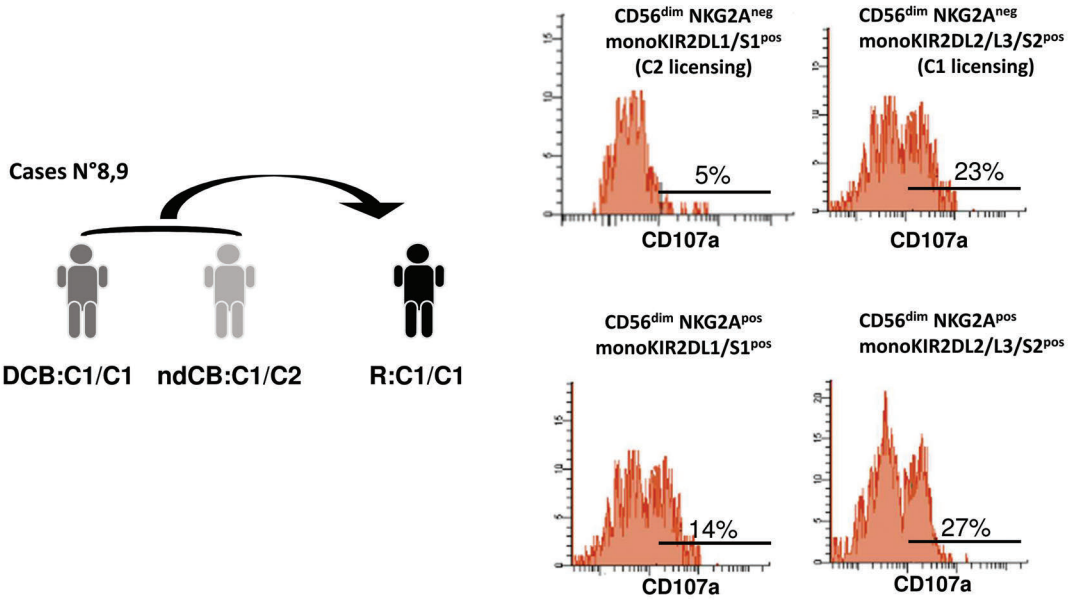

Figure 3. KIR ligand involvement of the ndCB in NK cell licensing after 2CBT. The responsiveness of CD56^{dim} NKG2A$^{pos/neg}$ monoKIR2DL1/S1^{pos} or monoKIR2DL2/L3/S2^{pos} NK cells in a degranulation assay was evaluated as a function of the presence of KIR ligands HLA-C2 only in the ndCB. CD56^{dim} NKG2A^{neg} monoKIR2DL1/S1^{pos} mismatched were hyporesponsive. We present one representative example of two cases (cases 8, 9 see *Online Supplementary Table S1*).

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