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Selective downregulation of HLA-C and HLA-E in childhood acute lymphoblastic leukaemia

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Keywords

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We have recently shown that susceptibility to childhood B-cell acute lymphoblastic leukaemia (B-ALL) is influenced by the presence of *HLA-C* encoded ligands C1 and C2 for killer cell immunoglobulin-like receptors (KIR), (Babor *et al*, 2014). B-ALL patients exhibited an increased frequency of the C2 ligand and, moreover, C2 was associated with increased risk of late relapse. The study suggested that the expression of *HLA* class I-encoded KIR ligands on tumour cells influences disease susceptibility and Natural Killer (NK) cell-mediated control of B-ALL. In order to extend our knowledge about expression of the major inhibitory NK cell receptor ligands in childhood B-ALL, Bw4, *HLA-C*-encoded C1 and C2 epitopes, and HLA-E were assessed at the surface and mRNA level in leukaemic cells. To this end, we employed antibodies for specific detection of Bw4 (MUS4H4) and Bw6 (OUW4F11) as previously described (Verheyden *et al*, 2009), for HLA-C/E (DT9), (Braud *et al*, 1998, Thomas *et al*, 2009) and HLA-E (3D12, BioLegend, San Diego, CA, USA) in peripheral blood samples in 31 children (age 0.3–19.3 years, mean 7.2) with newly diagnosed B-ALL (common ALL: n=18; preB-ALL: n= 11; proB-ALL: n=2). In order to

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Author contributions

F.B. and M.U. contributed equally to this work. S.B.R. and A.R.M. designed the project, performed the experiments and wrote the manuscript; J.E. and J.C.F. performed experiments and reviewed the manuscript; A.M., F.H.C., and M.C. contributed essential reagents and reviewed the manuscript, A.B. contributed clinical samples and reviewed the manuscript; F.B. and M.U. designed the project and wrote the paper.

Conflict of interest

The authors state that they have no conflict of interest.

accommodate for possible differences in cell size between leukaemic blasts and unaffected B cells, mean fluorescence intensity was normalized to mean forward scatter levels of the respective cell population. All HLA class I-specific antibodies were unconjugated and subsequently stained with secondary fluorescein isothiocyanate-coupled anti-IgG antibodies to avoid a systematic bias of directly conjugated antibodies due to differences in coupling efficiencies and flow cytometric compensation.

Overall levels of HLA class I surface expression, which were measured with a panHLA class I antibody (W6/32, BioLegend), were comparable and within the same range in CD19⁺ B cells of patients and healthy controls (Fig 1A). Similarly, we could not detect B-ALL-specific changes in overall expression levels of HLA-B allotypes carrying the Bw4 epitope, which constitutes the ligand for the inhibitory KIR3DL1 receptor, nor the Bw6 epitope, which is not recognized by inhibitory KIR. In all cases, the flow cytometry results were consistent with the HLA class I typing status, confirming the specificity of the Bw4 and Bw6 reagents (data not shown). Whereas panHLA class I and Bw4/Bw6 expression remained unchanged, strong and consistent downregulation of HLA-C and HLA-E surface expression was observed in patients (Fig 1A). At initial diagnosis, leukaemic patients but not healthy controls exhibited B cell populations with very low HLA-C and -E expression levels (Fig 1B) that could be consistently allocated to the CD19⁺CD45⁻ leukaemic subset (Fig 1C). The differences in HLA-C and -E expression levels between non-leukaemic and leukaemic B cells were highly significant (Fig 1D). Upon complete remission, B cell populations with low HLA-C and HLA-E levels were no longer detectable, consistent with the disappearance of the leukaemic blast population (Fig 1B, C). In contrast, Bw4- and Bw6-bearing allotypes led to homogenous staining in leukaemic and control samples (Fig 1B).

To determine whether downregulation of HLA-C and -E expression on the cell surface was due to repression of transcription, B cells were separated into leukaemic and non-leukaemic subsets by flow cytometric cell sorting and subsequently subjected to reverse transcription polymerase chain reaction analysis. Indeed, the mRNA levels for C1 and C2-bearing *HLA-C* alleles as well as for *HLA-E* were clearly lower in CD34⁺ leukaemic blasts than in the residual non-leukaemic B cells (Fig 2A). Nonetheless, except one case where no *HLA-E* message could be detected (data not shown), the reduction of mRNA levels was moderate (2- to 4-fold compared to normal B cells) pointing to additional post-transcriptional mechanisms that might explain the many cases exhibiting an almost complete lack of surface expression. Notably, the distribution of the well-characterized *HLA-E* dimorphism at position 107 (Geraghty *et al*, 1992) was not different from the control cohort (data not shown).

This is the first comprehensive analysis of *HLA-C* and *HLA-E*-encoded NK cell ligands in childhood ALL. It is important to mention that the HLA-C-specific reagent (DT9) cross-reacts with HLA-E, which might partially account for the decrease in DT9 signals. Moreover, the HLA-E-specific antibody (3D12) weakly recognizes certain HLA-B and HLA-C alleles (Corrah *et al*, 2011). However, these cross-reactions would not explain our observation that both reagents gave vastly diminished signals on leukaemic blasts whereas the same leukaemic cells showed normal levels of HLA-A and -B expression. Our observations are in line with a previous study showing downregulation of HLA-C in a small

ALL cohort, employing a panel of antibodies for single HLA-C allotypes (Verheyden *et al*, 2009). Furthermore, our data show that HLA-E is expressed at the mRNA but not cell surface level on leukaemic blasts, which is in contrast to a previous study, which reported complete absence of *HLA-E* mRNA level (Majumder *et al*, 2006).

The downregulation of HLA-C and HLA-E as reported here should theoretically lead to ‘missing-self’ recognition of leukaemic cells by NK cells expressing the respective cognate inhibitory receptor. However, CD107 mobilization of short-term stimulated NK cells from healthy donors remained low, even on NK cell subsets expressing a single inhibitory KIR for C2 (KIR2DL1), C1 (KIR2DL1), Bw4 (KIR3DL1) or HLA-E (NKG2A) (Fig 2B). The observation that B-ALL blasts are not efficiently recognized by NK cells despite their consistent downregulation of NK cell receptor ligands might be explained by the lack of appropriate ligands for stimulatory receptors, such as NCR, NKG2D, and DNAM1 (Pende *et al*, 2005). However, the driving force behind the downregulation of HLA-C and HLA-E is yet unknown. Possible explanations involve the escape from T cells that recognize peptides presented in the context of HLA-C or HLA-E. A thought-provoking but speculative explanation would be that the presence of HLA-C and HLA-E-negative leukaemic cells in haematopoietic stem cell niches, such as bone marrow, might drive NK cells into a hyporesponsive state. In this regard, it was shown that TAP-deficient patients that express almost no HLA class I (Sleiman *et al*, 2014) have NK cells that are not licensed and exhibit decreased cytotoxicity and cytokine production. Further experiments analysing the functional state of patient NK cells will hopefully help to clarify the underlying mechanism behind the specific downregulation of NK cell receptor ligands in childhood B-ALL.

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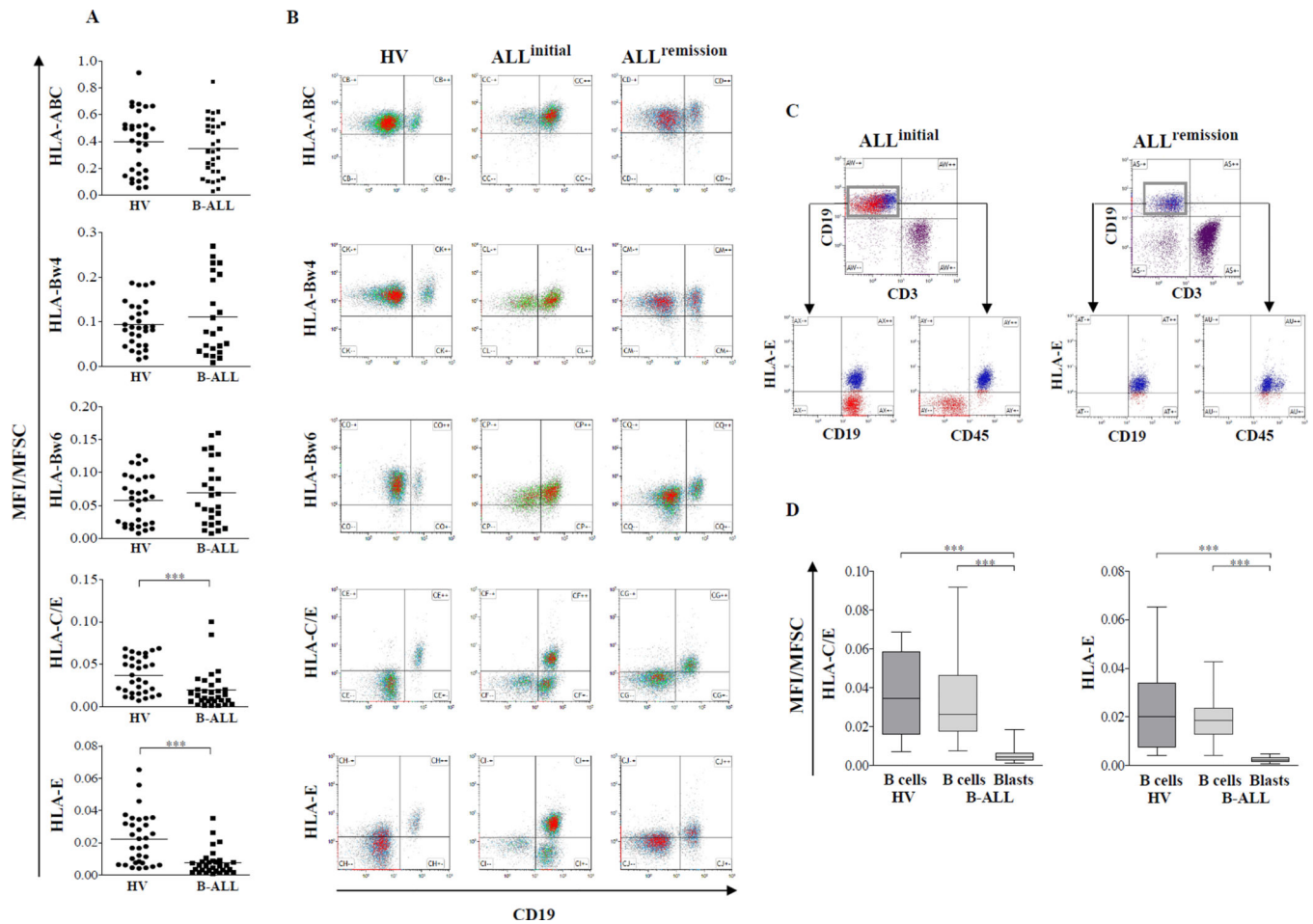


Figure 1. Selective downregulation of HLA-C and HLA-E in paediatric B-ALL patients
 Peripheral blood mononuclear cells (PBMC) from 31 newly diagnosed B-ALL patients and 32 healthy volunteers (HV) were stained with HLA class I-specific human monoclonal antibodies (mAbs) for HLA-ABC, HLA-Bw4, HLA-Bw6, HLA-C/E and HLA-E. Measurements of all HLA class I-specific antibodies were made with the same instrument settings. The mean fluorescence intensity (MFI) was normalized to mean forward scatter (MFI/MFSC) to adjust for possible differences in cell size. **(A)** Plots representing the surface expression of the respective HLA class I molecules on B cells (CD19⁺ subset) of healthy volunteers and leukaemic patients. Each dot represents one individual and horizontal bars represent mean values. **(B)** Flow cytometric data are shown for a representative leukaemic patient (middle panel) at initial diagnosis and complete remission (right panel) following chemotherapy in comparison to a healthy volunteer (left panel). **(C)** Representative flow cytometric analysis illustrating the presence of two CD19⁺ subsets with either high (CD45⁺ non-leukaemic B cells) or low (CD45⁻ leukaemic blasts) HLA-E expression at initial diagnosis (upper panel) and the subsequent disappearance of the leukaemic HLA-E-negative B cell subset following chemotherapy and complete remission (lower panel). **(D)** Box plots representing surface expression of HLA-C and HLA-E for non-leukaemic and leukaemic B cells in comparison to B cells from healthy volunteers. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test (***= p<0.001).

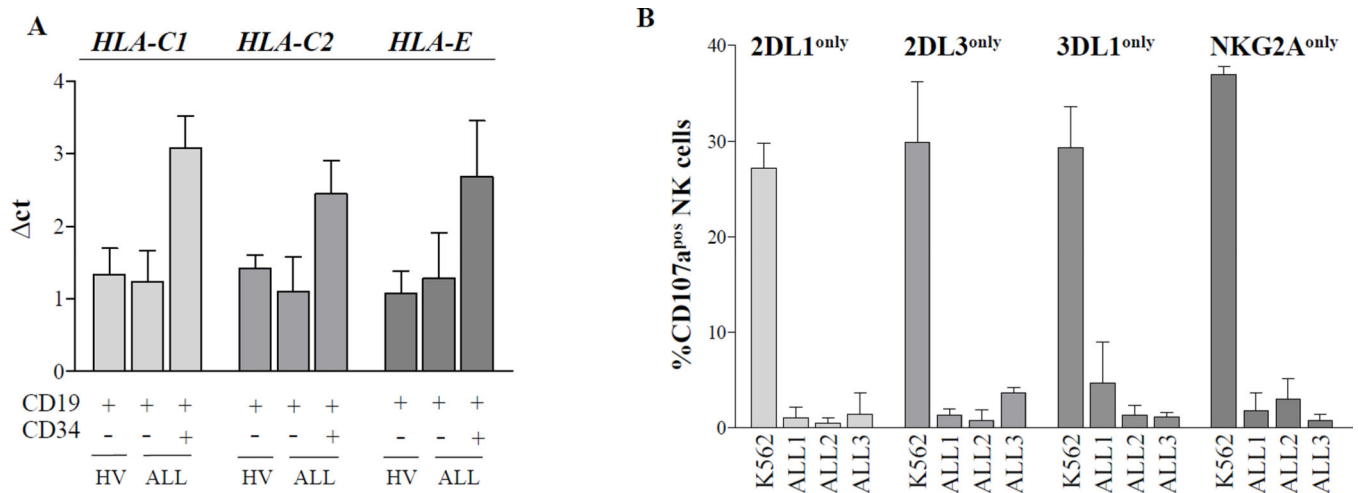


Figure 2. Reduced *HLA-C* and *HLA-E* mRNA expression and lack of NK cell activation by leukaemic cells

(A) Semiquantitative reverse transcription (RT) PCR analysis of *HLA-C1*, *HLA-C2* and *HLA-E* mRNA expression. Leukaemic blasts (CD34⁺CD19⁺) and B cells (CD34⁻CD19⁺) from acute lymphoblastic leukaemia (ALL) patients as well as B cells from healthy volunteers were separated by flow cytometric cell sorting. mRNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by cDNA synthesis (Moloney murine leukaemia virus, Promega, Madison, WI, USA). Transcriptional levels were analysed by SYBR green-based real-time PCR (StepOnePlus PCR-System, Applied Biosystems, Foster City, CA, USA) and normalized to expression levels of *ACTB*. Bars represent the mean expression level of three healthy (far left bar in each grouping) and three leukaemic individuals (middle and right bars in each grouping), each heterozygous for *HLA-C1* and *HLA-C2*, respectively; error bars represent standard error of the mean (SEM). Primers for *HLA-C*-encoded ligands were from (Babor *et al*, 2014). Primers for *HLA-E* were as follows: forward: 5'-GCACATGGCACGTGTATCTC-3', reverse: 5'-CCTTCTGGAGAGGAGCAGAG-3'. (B) CD107a mobilization assay of healthy NK cells stimulated overnight with interleukin 2 (1000 u/ml) against paediatric B-ALL blasts (frequency >80% of peripheral blood mononuclear cells) indicated as ALL1-3 or against K562 (erythroleukaemic HLA-class I deficient cell line) as a positive control (effector/target ratio of 10:1). CD107a monoclonal antibody (mAb) (H4A3, BioLegend) was added prior to incubation. Subsequent flow cytometric staining included mAbs for CD56 (HCD56, Biolegend), CD3 (UCHT1, BioLegend), CD159a (NKG2A, Z199, Beckman Coulter, Brea, CA, USA), CD158a/h (KIR2DL1/S1, 11PB6, Miltenyi Biotec, Bergisch Gladbach, Germany), CD158b1/b2/j (KIR2DL2/L3/S2, GL183, Beckman Coulter) and CD158e1 (KIR3DL1, DX9, BioLegend). CD107a expression was determined on CD56^{dim}CD3⁻ NK cells expressing the indicated inhibitory receptor. Bars represent the mean expression of three healthy individuals; error bars represent standard error of the mean (SEM).