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XLP1 inhibitory effect by 2B4 does not affect DNAM-1 and NKG2D activating pathways in NK cells

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X-linked lymphoproliferative disease 1 (XLP1) is a rare congenital immunodeficiency caused by *SH2D1A* (Xq25) mutations resulting in lack or dysfunction of SLAM-associated protein adaptor molecule. In XLP1 patients, upon ligand (CD48) engagement, 2B4 delivers inhibitory signals that impair the cytolytic activity of NK (and T) cells. This causes the selective inability to control EBV infections and the occurrence of B-cell lymphomas. Here, we show that in the absence of SLAM-associated protein, co-engagement of 2B4 with different activating receptors, either by antibodies or specific ligands on target cells, inhibits different ITAM-dependent signaling pathways including activating killer Ig-like receptors. In XLP1 NK cells, 2B4 affected both the cytolytic and IFN-γ production capabilities, functions that were restored upon disruption of the 2B4/CD48 interactions. Notably, we provide evidence that 2B4 dysfunction does not affect the activity of DNAM-1 and NKG2D triggering receptors. Thus, while CD48$^+$ B-EBV and lymphoma B cells devoid of NKG2D and DNAM-1 ligands were resistant to lysis, the preferential usage of these receptors allowed XLP1 NK cells to kill lymphomas that expressed sufficient amounts of the specific ligands. The study sheds new light on the XLP1 immunological defect and on the cross-talk of inhibitory 2B4 with triggering NK (and T) receptors.

Keywords: 2B4 · Ligands · NK cells · Triggering receptors · XLP1

See accompanying article by Watzl and Claus

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Introduction

Human NK cells are equipped with an array of surface receptors that either trigger or dampen NK-cell activity [1, 2]. Activating receptors include NKp46, NKp30, NKp44 (collectively termed natural cytotoxicity receptors (NCR)), NKG2D, and DNAM-1 whose function is under the control of inhibitory receptors such as inhibitory killer Ig-like receptors (iKIRs, CD158) and CD94/NKG2A heterodimers that recognize HLA-A, -B, -C or -E molecules, respectively. KIRs present also isoforms that transduce activating signals (activating KIRs (aKIRs)) [3, 4], whose expression is mostly confined to individuals characterized by the group B KIR haplotype [5]. While NCR are mostly NK restricted, both NK and $CD8^+$ T cells express NKG2D and DNAM-1 receptors that recognize MICA/B and ULBPs, or poliovirus receptor (PVR, CD155) and Nectin-2 (CD112), respectively, cellular ligands that are upregulated or de novo expressed by transformed cells [6–8]. In general, virus-infected or neoplastic cells become susceptible to NK-cell-mediated lysis because they downregulate HLA-class I ("missing self-recognition") and upregulate ligands for activating NK receptors ("induced self-recognition") [9].

X-linked lymphoproliferative disease 1 (XLP1, Duncan disease, OMIM#308240) is a rare congenital immunodeficiency caused by mutations in *SH2D1A* (Xq25), the gene encoding the signaling lymphocyte activation molecule (SLAM) associated protein (SAP) [10, 11]. SAP is a cytoplasmic adaptor protein that associates with members of the SLAM family, which includes SLAM (CD150), LY9 (CD229), CRACC (CD319), CD84, NTB-A (CD352), and 2B4 (CD244) [12]. 2B4 is a surface molecule expressed in NK and T lymphocytes specifically recognizing CD48, which is present solely on hematopoietic cells. 2B4 engagement causes tyrosine phosphorylation of immunoreceptor tyrosine-based switch motifs present in its cytoplasmic tail and recruitment of SAP, which transduces activating signals via Fyn-dependent processes [13, 14]. In the presence of SAP, 2B4 potentiates the NCR-mediated signaling thus acting as a co-receptor molecule [15]. In the absence of SAP, 2B4 associates with protein tyrosine phosphatases and delivers inhibitory signals [16, 17]. In NK cells, this occurs in physiological conditions such as during NK-cell development [18] and pregnancy [19]. On the other hand, in XLP1, the lack (or dysfunction) of SAP causes the selective inability to control infection by EBV, a γ-herpes virus that infects and sequesters itself in B cells [16, 20–22]. This causes severe manifestations including fulminant mononucleosis and B-cell lymphomas [23, 24]. Among the immunological defects identified, the defective NK and $CD8^+$ T-cell-mediated cytolytic responses against EBV-infected cells have been shown to account for B-cell accumulation and persistence of reactive inflammatory responses [25]. The lack of NK-cell-mediated cytotoxicity mainly relies on the inhibitory function of 2B4, which, upon engagement by CD48 (upregulated on B-EBV cells), impairs the activity of receptors involved in the recognition of infected cells such as NCR [16, 26]. Importantly, in vitro experiments demonstrated that the defect is reverted by mAb-mediated blocking of 2B4/CD48 interactions [16].

In this study, we provide evidence that in XLP1 NK cells the 2B4 dysfunction does not impair the activity of NKG2D and DNAM-1 triggering receptors. This results in the ability of XLP1 NK cells to kill $CD48^+$ lymphomas that express one or more ligands of these activating receptors.

Results

In XLP1 NK cells the 2B4 cross-linking does not impair the activity of NKG2D and DNAM-1 receptors

XLP1 patients were characterized by different *SH2D1A* mutations, defective expression of SAP (evaluated by intracytoplasmic flow cytometry), and inhibitory 2B4 function (in reverse antibody-dependent cellular cytotoxicity (R-ADCC) assay against the $Fc\gamma Rc^+$ P815) (Table 1) [16, 22, 27]. To analyze the capability of 2B4 to inhibit various activating pathways, polyclonal-activated NK-cell populations from XLP1 patients were used as effectors in R-ADCC assays in the presence of the anti-2B4 mAb used in combination with mAbs specific for one or another activating NK receptor (Fig. 1). In healthy NK cells, 2B4 cross-linking sustained activation mediated by all activating receptors (Supporting Information Fig. 1). On the contrary, in XLP1 NK cells, 2B4 engagement inhibited the triggering signal mediated not only by NCR (i.e. NKp46, NKp30, and NKp44) and CD16 as previously described [16] but also by aKIR (Fig. 1A and B). Interestingly, however, 2B4 cross-linking did not affect the NKG2D- and DNAM-1-dependent cytotoxicity (Fig. 1A). To confirm this observation, we evaluated the effect of 2B4 engagement on decreasing concentrations of anti-NKG2D or anti-DNAM-1 triggering antibodies (from 500 to 6 ng/mL) in R-ADCC assay using the $Fc\gamma Rc^+$ L1210 cell line as target, which is more resistant to NK-cell lysis than P815. As shown

Table 1. Characterization of XLP1 patients

Patients	*SH2D1A* mutations	SAP expression NK cells	2B4 function NK cells R-ADCC
UPN360	c.138_201del (entire exon 2 deletion)	Negative	Inhibitory
UPN590	Del exons 2–4	Negative	Inhibitory
UPN627	c.138(−2)A >G (skipping of exon 2)	Negative	Inhibitory
UPN674	c. 84C >G p. S28R	Weak[a)]	Inhibitory
UPN722	c.137+3_+6delGACT (loss of transcript)	Negative	Inhibitory

[a)] SAP expression significantly lower than healthy donors.

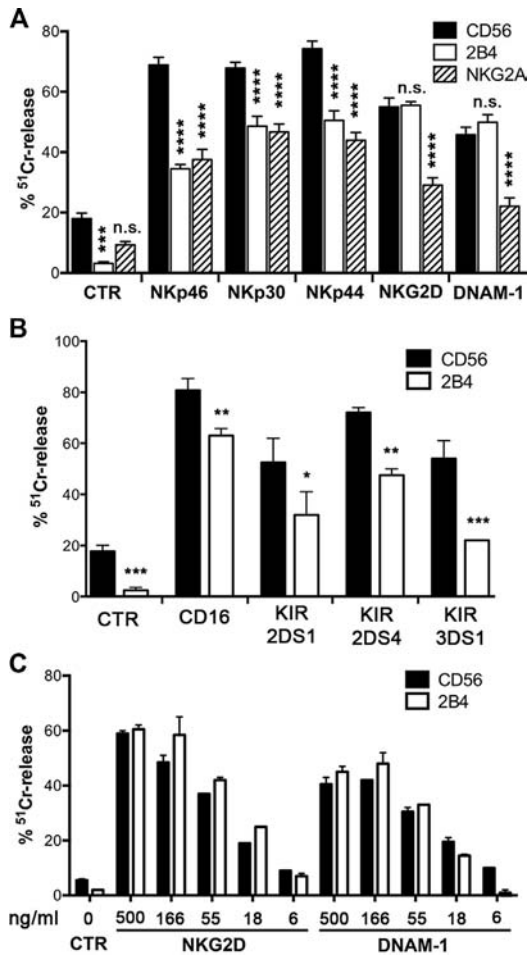

Figure 1. In XLP1 NK cells, 2B4 does not inhibit the NKG2D- and DNAM-1-mediated triggering pathways. (A) Polyclonal-activated NK cells from UPN360, UPN590, and UPN627 were analyzed in R-ADCC against P815 in the absence (CTR) or presence of mAbs to one or another triggering receptor (indicated in X axis) used in combination with mAbs to CD56 (negative control), 2B4, or NKG2A. Data are shown as mean + SEM and are pooled from three independent experiments. $^{***}p < 0.001$, $^{****}p < 0.0001$; one-way ANOVA for repeated measures followed by Bonferroni's multiple comparison test. (B) Activated NK cells from UPN627, characterized by B/x KIR genotype, were tested against P815 in the absence (CTR) or presence of mAb to CD16 or aKIR in combination with mAbs to CD56 (negative control) or 2B4. Data are shown as mean + SEM and are pooled from two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Student's *t*-test. (C) Activated NK cells from UPN360 and UPN674 were tested in R-ADCC against L1210 cell line in the presence of decreasing concentrations of anti-NKG2D or anti-DNAM-1 mAbs (serial 1:3 dilutions starting from 500 ng/mL), used in combination with anti-CD56 (negative control) or anti-2B4 mAbs (500 ng/mL). Data are shown as mean + SEM and are pooled from two independent experiments. No statistical difference at any dilution tested (Student's *t*-test). In all experiments, the E:T ratio was 4:1.

in Fig. 1C, the NK-cell triggering mediated by NKG2D or DNAM-1 receptors was unaffected by the co-engagement of 2B4 at any mAb concentration used. The anti-NKG2A mAb used as control, inhibited all the triggering receptors analyzed including NKG2D and DNAM-1 (Fig. 1A). These data suggested that in XLP1 NK cells, the NKG2D and DNAM-1 activating pathways were functionally independent from the inhibitory 2B4 co-receptor.

Defective functional activity against NKG2D and DNAM-1 ligand negative B-EBV and B-cell lymphomas

In healthy donors, NK cells efficiently kill ($CD48^+$) EBV-infected B cells and B-cell lymphomas (Supporting Information Fig. 2) and target cell recognition is mostly dependent on the co-engagement of NCR (NKp46 or NKp30) and 2B4 by the specific ligands expressed on hematopoietic targets [28]. Polyclonal NK-cell populations from XLP1 patients displayed poor cytolytic activity against 721.221 (thereafter termed 221) B-EBV cells and the defect could be reverted by mAb-mediated masking of 2B4 (Fig. 2A) [16]. Moreover, the co-culture of XLP1 NK cells with B-EBV cells did not result in increments of IFN-γ production, while it strongly increased upon mAb-mediated disruption of 2B4/CD48 interactions reaching levels significantly higher than healthy controls (Fig. 2B). Similar to 221, the Namalwa lymphoma cell line was highly resistant to lysis by XLP1 NK cells (Fig. 2C). It is of note that both $CD48^+$ cell lines showed defective expression of NKG2D and DNAM-1 ligands. In particular, 221 cells did not express any ligands and only very low surface densities of Nectin-2 were detected in Namalwa (Supporting Information Fig. 3).

NKG2D and/or DNAM-1 ligand expression in $CD48^+$ lymphomas overcomes 2B4 dysfunction

Polyclonal-activated XLP1 NK cells were analyzed for cytolytic activity against $CD48^+$ target cells expressing high surface density of ligands for NKG2D and/or DNAM-1 activating receptors (Supporting Information Fig. 3). The cytolytic activity of XLP1 NK cells against the $ULBP1^+$ DAUDI lymphoma was comparable to that of healthy controls at high E:T ratios, whereas a significant decrease in cytolytic activity was documented at low E:T ratios (Fig. 3A). In these cases, optimal cytotoxicity was restored by mAb-mediated disruption of inhibitory 2B4/CD48 interactions. In healthy NK cells, killing of DAUDI depended on the combined action of NCR and NKG2D and only the simultaneous blocking of both triggering receptors resulted in a significant reduction of lysis (Fig. 3B). On the other hand, in XLP1 NK cells, NKG2D played a major role. Indeed, mAb-mediated masking of NKG2D alone significantly reduced the NK-cell-mediated killing of DAUDI (Fig. 3B).

The role of DNAM-1 in XLP1 NK cells was appreciated when the PVR^+, $MICA^+$, $ULPB\text{-}1^{dull}$, and $ULBP\text{-}3^{dull}$ L540 lymphoma were used as target. XLP1 NK cells were virtually unaffected by the inhibitory 2B4/CD48 interactions, and indeed they lysed L540 similarly to healthy NK cells (Fig. 3C). In XLP1 NK cells, mAb-mediated blocking of NKG2D and DNAM-1 resulted in a reduction of lysis more marked as compared to healthy NK cells ($p < 0.001$) and was sufficient to virtually abrogate the lysis suggesting a dominant role of these receptors in target cell recognition (Fig. 3D). In healthy NK cells, the abrogation of lysis also required mAb-mediated masking of NCR.

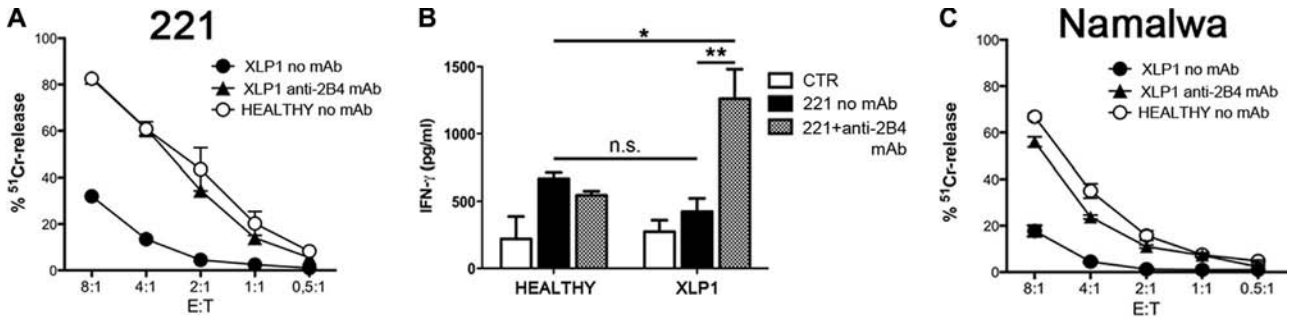

Figure 2. Decreased effector function of XLP1 NK cells against 221 B-EBV and Namalwa lymphoma cell lines. (A) Polyclonal-activated NK cells from UPN722 and a healthy donor were analyzed for cytolytic activity against 221 cells at different E:T ratios in the absence or presence of the anti-2B4 mAb. Data are shown as mean ± SEM and are pooled from two independent experiments. (B) Activated NK cells from UPN590, UPN627, and three healthy donors were cultured either alone (CTR) or with 221 cells (with or without anti-2B4 mAb). Supernatants were analyzed by ELISA for the presence of IFN-γ. Data are shown as mean ± SEM and are pooled from two different experiments. $^{*}p < 0.05$; one-way ANOVA followed by Dunnett's multiple comparison test. (C) Activated NK cells from UPN360, UPN722, and two healthy donors were analyzed for cytolytic activity against Namalwa cells (preincubated with anti-HLA class I mAb) at different E:T ratios in the absence or presence of the anti-2B4 mAb. Data are shown as mean ± SEM and are pooled from three independent experiments; two-way ANOVA and Bonferroni test.

Prevalent usage of NKG2D and DNAM-1 pathways when inhibitory 2B4/CD48 interaction occurs

XLP1 NK cells efficiently killed nonhematological malignancies such as the melanoma FO-1 cell line, which lacks HLA-class I and CD48 while expressing ligands of NCR, NKG2D, and DNAM-1 receptors (Fig. 4 and Supporting Information Fig. 3) [6, 29].

We generated a stable FO-1/CD48 cell transfectant that expressed levels of CD48 comparable to lymphoma cell lines (Supporting Information Fig. 3). FO-1 and FO-1/CD48 cells were analyzed for susceptibility to lysis mediated by XLP1 NK cells (Fig. 4). The XLP1 NK-cell-mediated killing of FO-1/CD48 cells was comparable to that of FO-1 cells. However, the evaluation of the relative contribution of the various triggering receptors

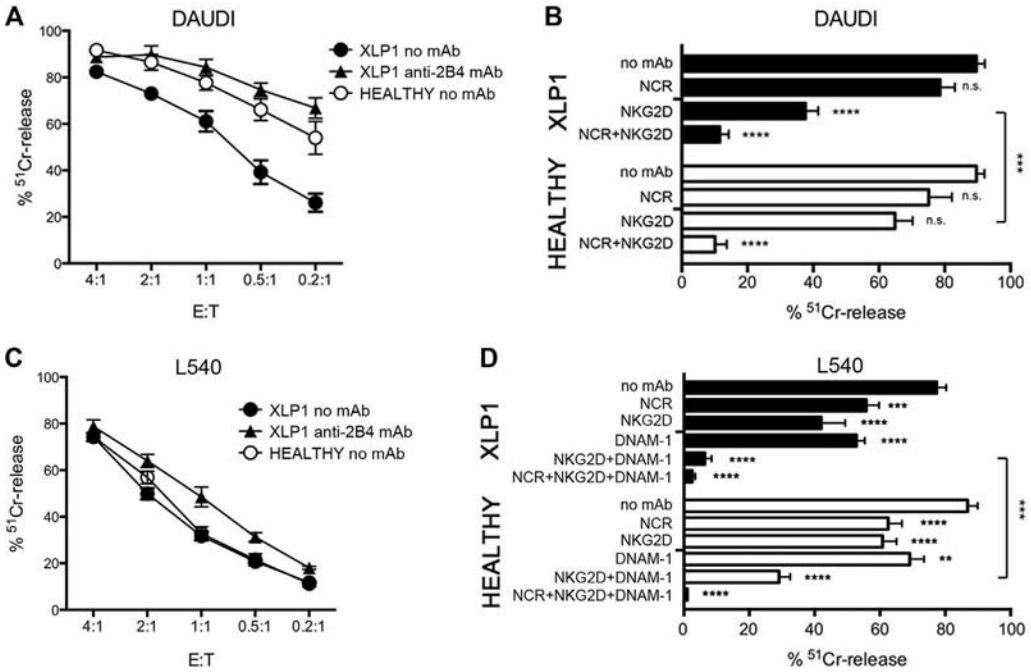

Figure 3. XLP1 NK cells kill CD48^{+} lymphomas expressing DNAM-1 and/or NKG2D ligands. (A) Polyclonal-activated NK cells from UPN360, UPN674, UPN590, UPN722, and four healthy donors were analyzed for cytolytic activity against DAUDI cell line at different E:T ratios in the absence or presence of anti-2B4 mAb. (B) Activated NK cells from UPN360, UPN489, UPN590, UPN722 (E:T ratio 4:1), and four healthy donors (E:T ratio 1:1) were analyzed for cytolytic activity against DAUDI cell line in the absence or presence of mAbs to the indicated activating receptors. (C) Activated NK cells from UPN360, UPN722, and two healthy donors were analyzed for cytolytic activity against L540 cells (preincubated with anti-HLA class I mAb) at different E:T ratios in the absence or presence of the anti-2B4 mAb, and (D) in the absence or presence of mAbs to the indicated activating receptors (E:T ratio 4:1). (A–D) Data are shown as mean ± SEM and are pooled from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; two-way ANOVA and Bonferroni test.

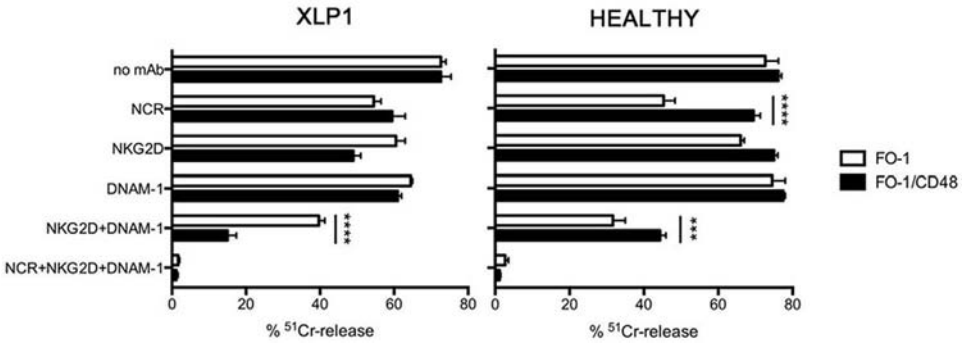

Figure 4. Prevalent role of NKG2D and DNAM-1 activating pathways in the presence of inhibitory 2B4/CD48 interactions. Polyclonal-activated NK cells from UPN360, UPN674, UPN722, and three healthy donors were analyzed for cytolytic activity against FO-1 and FO-1/CD48 (E:T ratio 2:1) in the presence of mAbs to the indicated molecules. Data are shown as mean ± SEM and are pooled from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$; two-way ANOVA and Bonferroni test.

involved in killing allowed to detect differences between the NK cells from XLP1 patients and those from healthy controls. In XLP1 NK cells, the contribution of NKG2D and DNAM-1 was emphasized due to the presence of inhibitory 2B4/CD48 interactions, which dampen the NCR function. Indeed, mAb-mediated masking of NKG2D and DNAM-1 inhibited the lysis of FO-1/CD48 more efficiently than that of FO-1 ($p < 0.0001$). An opposite effect was observed in healthy NK cells in which the role of NKG2D and DNAM-1 was minimized due to the presence of the activating 2B4/CD48 interactions that support the function of NCR. Indeed, blocking of NKG2D and DNAM-1 inhibited lysis of FO-1/CD48 less efficiently than FO-1 ($p < 0.001$).

Thus, in XLP1 NK cells, the inhibitory 2B4/CD48 interactions do not significantly affect the NKG2D and DNAM-1 activation pathways; this confirms the functional independence of these receptors from the 2B4 co-receptor.

Discussion

In this study, we show that in the absence of SAP, combining 2B4 with different activating receptors, either cross-linked with antibodies or engaged with targets expressing the specific ligands, inhibits NK-cell-mediated cytotoxicity. Importantly, this affects different ITAM-dependent signaling pathways including activating KIR, while does not impair the function of NKG2D and DNAM-1 receptors. In XLP1 patients, this resulted in the preserved capability of NK cells to kill $CD48^{+}$ lymphomas that express sufficient amounts of NKG2D and/or DNAM-1 ligands. In healthy donors during NK to B-EBV cell interactions, NCR and 2B4 transduce activating signals leading to both cytotoxicity and IFN-γ production [28, 30]. In XLP1 NK cells, the 2B4 dysfunction caused not only defective killing of EBV-infected B cells (that express high levels of CD48) but also defective IFN-γ production. However, the disruption of the inhibitory 2B4/CD48 interactions resulted in the release of amounts of IFN-γ significantly higher than in healthy controls. This suggests that B-EBV cells being resistant to lysis could induce a preliminary "priming" of XLP1 NK cells, which would be able to produce an excess of cytokines when further stimulated by either soluble factors or cell-to-cell contacts. In this context, DNAM-1 is involved in the cross-talk between NK cells and DC or macrophages and its engagement results in the release of high amounts of IFN-γ [31, 32]. This phenomenon might contribute to the uncontrolled systemic inflammatory response observed in vivo in XLP1 [33] as in familial hemophagocytic lymphohistiocytosis [34]. The enhanced IFN-γ production could be also the result of an increased number of licensed NK cells in polyclonal XLP1 NK-cell populations [35]. Although further studies are needed to address this hypothesis, the persistence of an inhibitory 2B4 receptor during NK-cell development might render functionally competent NK cells lacking self-HLA class I inhibitory receptors.

NK cells express various activating receptors that are similar to TCR associate with homo- or heterodimers of ITAM-bearing signaling molecules (CD3ζ, FcεRγ, and DAP12) [36, 37] and are functionally coupled with 2B4/SAP/LAT complexes that provide a co-activating signal [30, 38]. Interestingly, the first ITAM of CD3ζ has been shown to directly bind SAP [39]. Both the activating [40] and inhibitory function of 2B4 target ITAM-bearing receptor complexes that include NCR, TCR, and CD16 [14, 16, 41], as well as aKIRs/DAP12, which seem to be involved in the control of herpes virus infections [42]. In the presence of SAP, 2B4 has been shown to co-operate also with activating receptors that either directly transduce signals (i.e. DNAM-1) or use chains devoid of classical (xYxxI/Lx_{6-8}YxxI/L) ITAM (i.e. NKG2D/DAP10) [2, 43]. On the other hand, our present data demonstrate that while DNAM-1 and NKG2D activation signals are blocked by ITIM-bearing receptors such as CD94/NKG2A, they are exempt from the inhibition mediated by 2B4 in the absence of SAP. How these receptors transduce activation signals and at which point they are inhibited might explain their independency from inhibitory 2B4. In this context, DNAM-1 and NKG2D have been shown to synergize with 2B4 through the selective phosphorylation in the downstream SLP-76 adaptor molecule of a tyrosine residue (Y128) complementary to that targeted by 2B4/SAP/Fyn complexes (Y113) [2, 43, 44]. Moreover, while ITIM preferentially bind the tyrosine phosphatases SHP-1 and SHP-2, which act at a very early step thus preventing rather than terminating NK-cell

activation, a crucial effector of 2B4-mediated inhibition is represented by SHIP-1 (and possibly SHIP-2), which negatively regulates downstream signals such as PLC-γ-mediated Ca^{2+} fluxes [14, 43].

According to our observation, in $SAP^{-/-}$ mice, members of the SLAM receptor family have been shown to inhibit NKG2D that, different to humans, associates with both DAP10 and DAP12 [45, 46].

It is of note that the functional independency of NKG2D and DNAM-1 from 2B4 could be relevant also in NK-cell physiology and in XLP1 T-cell biology. Indeed, in peculiar microenvironments such as during NK-cell development [18] and pregnancy [19], SAP^{-} immature or decidual NK lymphocytes might interact with bystander $CD48^{+}$ hematological cells expressing different repertoires of NKG2D and DNAM-1 ligands. Moreover, in XLP1 patients, NKG2D and DNAM-1 could be also involved in T-cell-mediated responses against EBV-infected cells and lymphoma cells [21]. In this context, also the dysfunction of the NTB-A co-receptor has been shown to contribute to the inability of NK and T cells to clear EBV infection. In particular, in XLP1 NK cells the maximal recovery of the NK-mediated lysis of B-EBV targets was obtained with the simultaneous disruption of both 2B4/CD48 and NTB-A/NTB-A interactions [26]. NTB-A is expressed by NK, T, and B cells, displays homophilic recognition [47], and upon engagement associates with either SAP or tyrosine phosphatases [26, 39, 48]. Different from 2B4, however, NTB-A does not associate with LAT [26], possibly explaining the minor role played by this molecule in both co-stimulation and co-inhibition.

In conclusion, we provide evidence that in SAP^{-} NK cells, 2B4 inhibits ITAM-dependent signaling pathways while does not affect the activity of NKG2D and DNAM-1 receptors. This reflects on the specific defect of XLP1 to lyse various target cells depending on ligand expression, and may have implication in the capacity of patients with XLP1 to prevent lymphomagenesis.

Materials and methods

Patients

Five patients referred to the hemophagocytic lymphohistiocytosis (HLH) registry [49], for confirmation of XLP1 clinical diagnosis, were included in this study. UPN674 was diagnosed in the absence of clinical symptoms due to affected brothers. The UPN627 KIR gene profile was characterized as described [50]. Participants gave written informed consent and the study was approved by the Institutional Review Board at the AOU Meyer (Florence, Italy) and Istituto G. Gaslini (Genoa, Italy).

Mutation analysis

Genomic DNA was isolated from peripheral blood samples using Qiacube Workstation (Qiagen, Jesi, Italy). The four coding exons and exone–intron boundaries of the *SH2D1A* gene were amplified and directly sequenced, using ABI Prism 3130XL Sequence Detection System (Applied Biosystems). In patients suspected for deletion, multiplex ligation-dependent probe amplification (P205 SALSA MLPA probe set) was performed on genomic DNA according to the manufacturer's instructions (http://www.mrc-holland.com). To detect transcript alterations, total RNA was extracted from PBMCs by the QIAamp® RNA Blood mini kit (Qiagen). SAP mRNA was reverse transcribed, amplified, and sequenced in both directions.

Cytofluorimetric analysis

To evaluate intracellular SAP expression, polyclonal-activated NK cells, obtained as previously described [50], were fixed, permeabilized (cytofix/cytoperm, BD Bioscience, San Jose, CA, USA), and stained with anti-SAP (1C9, IgG_{2a}, Abnova, Taipei, Taiwan) or isotype-matched control mAbs, followed by PE-conjugated goat anti-mouse IgG_{2a} (Southern Biotechnology Associates, Birmingham, AL, USA). The various cell lines were stained with the following mAb: L95 (IgG_1, anti-PVR), L14 (IgG_{2a}, anti-Nectin-2), BAM195 (IgG_1, anti-MICA) [50–52]; M295 (IgG_1, anti-ULBP1), M310 (IgG_1, anti-ULBP2), M551 (IgG_1, anti-ULBP3) kindly provided by Amgen (Seattle, WA, USA); W632 (IgG_{2a}, anti-HLA-class I), followed by PE-conjugated AffiniPure $F(ab')_2$ goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA). To detect CD48, CO202 (IgM) was used followed by $F(ab')_2$ goat anti-mouse IgM-PE (Beckman Coulter, Milan, Italy) [47]. Mean relative fluorescence intensity indicates the ratio between MFI of cells stained with the relevant mAb and that of cells stained with the isotype-matched negative control. Data were acquired on a FACSCalibur cytometer (BD) and analyzed using FlowJo Version 8.8.6 (TreeStar).

Cytolytic assays

To evaluate 2B4 function, activated NK cells were used as effectors in R-ADCC assays against ^{51}Cr-labeled either P815 or L1210 (both $Fc\gamma Rc^{+}$) murine targets, in the presence of functional grade purified anti-2B4 mAb (PP35, IgG1; e-Bioscience, San Diego, CA, USA) at a concentration of 500 ng/mL, either alone or in combination with suboptimal doses of mAb (all of IgG_1 isotype) to one or another triggering NK receptor: c127 (anti-CD16), BAB281 (anti-NKp46), Z231 (anti-NKp44), A76 (anti-NKp30), F22 (anti-DNAM-1), ON72 (anti-NKG2D), 11PB6 (anti-KIR2DL1/S1), PAX180 (anti-KIR2DS4), and Z27 (anti-KIR3DL1/S1) [50–52]. We also used c218 (IgG_1, anti-CD56) and Z270 (IgG_1, anti-NKG2A). Activated NK cells were analyzed for cytolytic activity against the HLA-class I^{-} cell lines, including the B-EBV 221, the lymphoma DAUDI, the melanoma FO-1, and FO-1/CD48-transfected cells. Other target cells were the HLA-class I^{+} Namalwa and L540 lymphomas used after preincubation with saturating amounts of A6–136 mAb (anti-HLA-class I, IgM) to abolish any

possible disturbance due to HLA-class I expression and specific receptor interactions (differing from donor to donor). Masking experiments were performed with the addition of saturating amounts of the following mAb: MA344 (IgM, anti-2B4), KL247 (IgM, anti-NKp46), KS38 (IgM, anti-NKp44), F252 (IgM, anti-NKp30), F5 (IgM, anti-DNAM-1), and BAT221 (IgG_1, anti-NKG2D) [50–52]. The E:T ratios are indicated in the text.

Analysis of IFN-γ production

Polyclonal NK-cell populations from patients and healthy donors (1.5×10^5/well) were cultured overnight either alone or with 221 cell line (5×10^4/well) in U-bottom plastic plates in the presence or absence of MA344 mAb (10 μg/mL), each conditions in a total volume of 200 μL/well. The supernatants were collected and analyzed for the presence of IFN-γ by ELISA (Invitrogen, Camarillo, CA, USA).

Production of FO-1/CD48 cell transfectant

FO1 cell line was transfected with CD48 coding pCDNA 3.1 plasmids, using nonliposomial FuGene HD reagent (Roche, Monza, Italy). The selection of the transfected cells was performed using G418 sulfate (Calbiochem) at 1 mg/mL, the bulk FO1 $CD48^{+/-}$ cell line was cloned and the clones were screened using the anti-CD48 CO202 mAb [47].

Statistical analysis

Statistical analyses were performed using Graphpad software Version 6.0. The utilized tests are indicated in the figure legends. Not significant (n.s.); $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$; and $^{*}p < 0.05$.

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Abbreviations: **aKIR**: activating KIR · **KIR**: killer Ig-like receptor · **NCR**: natural cytotoxicity receptor · **R-ADCC**: reverse antibody-dependent cellular cytotoxicity · **SAP**: SLAM-associated protein · **SLAM**: signaling lymphocyte activation molecule · **XLP1**: X-linked lymphoproliferative disease

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