European Journal of Immunology

Immunodeficiencies and autoimmunity

Clinical

Research Article

Inhibitory 2B4 contributes to NK cell education and immunological derangements in XLP1 patients

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X-linked lymphoproliferative disease 1 (XLP1) is an inherited immunodeficiency, caused by mutations in *SH2D1A* encoding Signaling Lymphocyte Activation Molecule (SLAM)-associated protein (SAP). In XLP1, 2B4, upon engagement with CD48, has inhibitory instead of activating function. This causes a selective inability of cytotoxic effectors to kill EBV-infected cells, with dramatic clinical sequelae. Here, we investigated the NK cell education in XLP1, upon characterization of killer Ig-like receptor (KIR)/KIR-L genotype and phenotypic repertoire of self-HLA class I specific inhibitory NK receptors (self-iNKRs). We also analyzed NK-cell cytotoxicity against CD48$^+$ or CD48$^-$ KIR-ligand matched or autologous hematopoietic cells in XLP1 patients and healthy controls. XLP1 NK cells may show a defective phenotypic repertoire with substantial proportion of cells lacking self-iNKR. These NK cells are cytotoxic and the inhibitory 2B4/CD48 pathway plays a major role to prevent killing of CD48$^+$ EBV-transformed B cells and M1 macrophages. Importantly, self-iNKR defective NK cells kill CD48$^-$ targets, such as mature DCs. Self-iNKR$^-$ NK cells in XLP1 patients are functional even in resting conditions, suggesting a role of the inhibitory 2B4/CD48 pathway in the education process during NK-cell maturation. Killing of autologous mature DC by self-iNKR defective XLP1 NK cells may impair adaptive responses, further exacerbating the patients' immune defect.

Keywords: XLP1 · SAP · SLAM · 2B4 · CD48 · NK cells · KIR · HLA class I · NK receptors · NK-cell education

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Introduction

X-linked lymphoproliferative disease 1 (XLP1; OMIM #308240) is a rare congenital immunodeficiency resulting in selective vulnerability to Epstein–Barr virus infection, with clinical complications as fulminant infectious mononucleosis, malignant lymphoma, dysgammaglobulinemia or hemophagocytic lymphohistiocytosis (HLH) [1–4]. XLP1 is caused by hemizygous mutations in the gene *SH2D1A* (Xq25), encoding the adaptor molecule Signaling Lymphocyte Activation Molecule (SLAM)-associated protein (SAP) [5, 6]. SAP, a small cytoplasmic adaptor protein, consists in a single SH2 domain that binds to tyrosine-based switch motifs located in the cytoplasmic domains of SLAM-family receptors (SFRs) [7]. While most SFRs as SLAMF6 (NTB-A) [8] and SLAMF7 (CRACC) are homotypic receptors [9], SLAMF4 (CD244 or 2B4, as thereafter named) interacts with SLAMF2 (CD48), a GPI-anchored Ig-like protein [10]. SLAMs are solely expressed on hematopoietic cells. Upon receptor engagement, tyrosine-based switch motifs are phosphorylated and SAP recruits protein tyrosine kinase Fyn, thereby enabling activation of downstream signaling proteins. SAP also prevents coupling of SFRs to inhibitory tyrosine phosphatases [11]. In SAP deficiency, tyrosine phosphatases are recruited, leading to inhibitory signals of SLAM receptors, resulting in immune dysfunction [6, 12]. We recently proposed a diagnostic algorithm, based on the combined intracytoplasmic staining of SAP and a 2B4 functional assay in NK cells, to rapidly distinguish subjects with XLP1 from other types of patients presenting with HLH features [13]. In XLP1, the 2B4-mediated inhibition affects the receptors that transduce via ITAM-containing molecules, such as natural cytotoxicity receptor (NCR; i.e. NKp46, NKp30, and NKp44), which are the major triggering NK receptors [14]. In contrast, the function of DNAX accessory molecule-1 (DNAM-1) and NKG2D is not impaired [15, 16]. This applies also to XLP1 T cells, where TCR function is impaired, while DNAM-1 and NKG2D pathways are unaffected. This explains the peculiar defect in patients with XLP1, who are unable to eliminate EBV-transformed B cells (B-EBVs), which are characterized by high expression of CD48 and lack of ligands of both DNAM-1 (i.e. PVR and Nectin-2) and NKG2D (i.e. MICA/B and ULBPs) [17].

NK cells are regulated by an array of receptors that tune their effector functions, including cytotoxicity and cytokine production [18, 19]. HLA class I specific inhibitory receptors prevent the NK-cell mediated attack against normal autologous cells [20]. Three types of inhibitory receptors exist: (i) killer Ig-like receptors (KIRs), specific for epitopes shared by groups of HLA-A, -B, or -C allotypes (termed KIR-L); (ii) CD94/NKG2A, a heterodimer recognizing nonclassical HLA-E; (iii) leukocyte Ig-like receptor subfamily B member 1 (LILRB1) with a broad HLA class I specificity [21]. Inhibitory KIR (iKIR) include KIR2DL1 specific for HLA-C alleles characterized by Lys^{80} (termed C2 epitope), KIR2DL2/L3 recognizing HLA-C alleles with Asn^{80} (termed C1 epitope), and KIR3DL1 specific for some HLA-B and HLA-A molecules sharing the Bw4 epitope [14, 22]. In addition, KIR2DL2/L3 also bind C2 with low affinity, while KIR3DL2 recognizes HLA-A3 and HLA-A11 [23, 24]. Activating KIR isoforms exist (aKIR), characterized by short cytoplasmic tail (KIR2DS and KIR3DS), whose ligands and function have not been completely established [25, 26]. The genes encoding KIR and HLA class I are present in different chromosomes and independently segregate. KIR expression is clonally distributed and, together with CD94/NKG2A and LILRB1, creates stochastic but tolerant repertoires of NK cells. During maturation, the NK cell receptor repertoire is selected in a way that each NK cell expresses at least one inhibitory receptor for self-HLA class I molecules, otherwise being hyporesponsive. This phenomenon is termed licensing or education [27–31].

Here, we provide evidence that in some XLP1 patients, a consistent proportion of NK cells lacks inhibitory receptors specific for self-HLA class I molecules. At variance from healthy individuals, these cells are fully functional against $CD48^-$ targets, thus suggesting a role of inhibitory 2B4 in their maturation and education.

Results

Analysis of KIR and KIR-L genotypes in XLP1 patients and healthy donors

We selected six XLP1 patients, carrying different *SH2D1A* mutations, characterized by defective SAP expression and inhibitory 2B4 function (Table 1) [13, 15]. Five patients developed clinical manifestations following EBV infection, while the sixth patient (unique patient number [UPN]674) was diagnosed in preclinical phase due to familial recurrence of XLP1. These patients were analyzed for KIR gene profile by SSP-PCR, to define their *KIR* genotype. Also, the presence of KIR-L (i.e. C1, C2, and Bw4) was evaluated, either by the analysis of high-resolution HLA class I typing or by SSP-PCR (Fig. 1). Through combined KIR/KIR-L typing, we evaluated, for each patient, the presence of genes coding for educated or uneducated iKIR. UPN360 was C2/C2 but lacked *KIR2DL1* (coding for the C2-specific iKIR), suggesting the absence of KIR-mediated NK-cell tolerance in this patient. The other patients carried one or more *iKIR* specific for self-HLA class I allotypes (termed self-iKIR). In particular, the two C1/C2, Bw4 patients (UPN627 and UPN590) were positive for iKIR recognizing these KIR-L (*KIR2DL1*, *KIR2DL2/L3,* and *KIR3DL1*), but UPN590 was characterized by a *KIR3DL1* allele (having T at nucleotide 320) coding for a nonsurface-expressed receptor [32, 33]. All self-iKIR were present in the C1/C2 patient UPN674 (*KIR2DL1* and *KIR2DL2/L3*) and in the C2/C2, Bw4 patients UPN722 and UPN1064 (*KIR2DL1* and *KIR3DL1*). We then selected ten healthy controls (H1–H10) matching for KIR-L typing with one or another patient. These samples were also analyzed for *KIR* gene profile (Fig. 1).

In some XLP1 patients, sizeable percentages of NK cells lack self-inhibitory NK receptors

Due to the very limited availability of blood sample from XLP1 patients, we mainly focused our study on in vitro expanded NK cells. Polyclonal-activated NK cell populations from XLP1

Table 1. Characteristics of XLP1 patients

Patient	Age at diagnosis (years)	Clinical picture	Evidence of previous EBV infection	Stem cell transplant (age)	Current status (age)	*SH2D1A* mutations	SAP expression[a)]	2B4 function NK cells R-ADCC[a)]
UPN360	5.8	HLH (5.8 years) with severe encephalopathy and secondary autism; entero-colitis (13 years) requiring resection.	Yes	Not performed due to severe neurologic sequelae	Alive (15 year)	c.138_201del (entire exon 2 deletion)	Negative	Inhibitory
UPN590	2.8	HLH (2.8 years)	Yes	Yes (3 years)	Alive, cured (8 years)	Del exons 2—4	Negative	Inhibitory
UPN627	13.1	Infectious mononucleosis (9 years); Hypogamma-globulinemia (11 years).	Yes	Yes (15 years)	Alive, cured (18 years)	c.138(-2)A>G (skipping of exon 2)	Negative	Inhibitory
UPN674	2.0	Asymptomatic, diagnosis in preclinical phase[b)].	No	Yes (2 years)	Alive, cured (6 years)	c.84C>G p.S28R	Weak	Inhibitory
UPN722	12	Burkitt lymphoma (4 years); encephalopathy (11 years); HLH (12 years).	Yes	Yes (12 years)	Dead of SCT-related complica-tions (13 years)	c.137+3_+6delGACT (loss of transcript)	Negative	Inhibitory
UPN1064	17.8	Intestinal B-lineage non-Hodgkin lymphoma (10 years); HLH (17.8 years) with severe encephalopathy.[c)]	Yes	Not performed due to severe neurologic sequelae	Dead of EBV reactivation and HLH (19 years)	c.192G>A p.W64X	Negative	Inhibitory

UPN in the HLH registry; SCT, stem cell transplantation.
[a)]SAP expression was evaluated by intracytoplasmic immunofluorescence and flow cytometry; 2B4 function was assessed by R-ADCC assays as previously described [13].
[b)]Brother of two males with the same *SH2D1A* mutation who died of EBV infection and Burkitt lymphoma.
[c)]Two months before HLH onset, his 20-year-old brother died of encephalitis, non-EBV-related.

		Inhibitory KIR					Activating KIR						KIR-ligand				
		2DL1	2DL2	2DL3	3DL1	3DL2	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	HLA-C C1 (N80)	HLA-C C2 (K80)	HLA-B Bw4 (T80)	HLA-B Bw4 (I80)	HLA-A Bw4
XLP1	UPN360				C					S4/d				*04:01,*06:02			
	UPN590				T					d			*03:03	*05:01			*32:01
	UPN627				C					S4			*12:03	*06:02		*38:01,*57:01	
	UPN674				C					d			*08:03	*04:01			
	UPN722				C/T					S4/d				*06,*06	*13:02	*57:01	
	UPN1064				C/T					S4/d				*04:01,*06:02	*13:02		
Healthy	H1				C/T					d				*04:01/*04:01			
	H2				C					S4/d			*07:02	*05:01	*44:02		*24:02
	H3				C					d			*12:02	*04:01		*52:01	
	H4				C					d			*08:02	*02			
	H5				C/T					d				*06,*15:02	*13	*51:08	
	H6				C					S4/d				*06:02,*16:02		*51:01	
	H7				C					d				*02:10,*05:01			
	H8				C					S4			*08	*04:01			
	H9				C/T					d			*07:01	*04:01			*23
	H10				T					d			*07	*04			*25
	H15				C					d				*02:02,*04:01			

Figure 1. KIR gene and KIR-L analyses. Presence (gray boxes) or absence (white boxes) of KIR genes and KIR-L in patients (top) and healthy controls (bottom) are indicated. KIR2DS4 alleles coding for surface receptors are reported as "S4," while alleles coding for putative soluble molecules as "d." KIR3DL1 "C" or "T" indicate alleles coding for surface and intracellular retained receptors, respectively [33]. Dark gray boxes indicate educated KIR.

patients were analyzed by immunofluorescence, using a combination of mAb-recognizing self-iKIR (possibly excluding the activating counterpart), NKG2A, and LILRB1, thereafter referred to as self-inhibitory NK receptors (self-iNKRs; Fig. 2). The only methodological limitation was related to the unavailability of mAbs able to discriminate between KIR2DL2 and KIR2DS2, encoded by genes in strong linkage disequilibrium. Thus, in *KIR2DL2/S2* and C1 patients (UPN590, UPN627, UPN674), as well as in H3 and H4 controls, the percentage of educated NK cells could be overestimated by including KIR2DS2$^+$ cells either lacking any inhibitory receptor or co-expressing only uneducated iKIR (i.e. KIR3DL1 in UPN674 and H4).

Phenotypic analysis of C2/C2 UPN360, who lacked KIR2DL1, revealed that only 52% NK cells were stained using a mixture of anti-NKG2A and anti-LILRB1 mAb (Fig. 2). Since KIR2DL2 has been demonstrated to recognize C2 at low affinity, we also performed the staining adding the anti-KIR2DL2/L3/S2 mAb. The percentage of positive cells increased, but still up to 25% of NK cells lacked self-iNKR (Supporting Information Fig. 1A). Notably, similar data were obtained analyzing resting NK cells (Supporting Information Fig. 1B). We could not find any healthy individual, even among the family members of UPN360, being *KIR2DL1*$^-$ and having C2 as only KIR-L. In the KIR-L matched control (H1) the vast majority (94%) of NK cells were stained with a mAb combination recognizing KIR2DL1, NKG2A, and LILRB1 (Fig. 2), with a negligible contribution of KIR2DL2/L3/S2 in both activated and resting cells (Supporting Information Fig. 1A and C).

NK cells from the other patients, as well as from the healthy controls, were stained with a combination of mAb-recognizing self-iNKR. While in healthy controls the percentage of positive cells was 94.5 ± 3 (mean ± SD, $n = 10$), a substantial fraction of cells lacking self-iNKR was evident in patients, particularly in UPN627 and UPN722 (26 and 20%, respectively; Fig. 2). The addition of the anti-KIR2DL2/L3/S2 mAb in C2/C2 individuals did not significantly modify the results (Supporting Information Fig. 1D).

In XLP1 NK cells with defective iNKR repertoire, 2B4 dominates the B-EBV cell recognition

Polyclonal NK cell populations from patients and healthy controls were tested for cytolytic activity using several B-EBV cell lines expressing different KIR-L (Fig. 3). To simulate the autologous setting, appropriate KIR-L matched effector/target combinations were chosen. Experiments were performed either in the absence or in the presence of mAbs blocking 2B4/CD48 and/or self-iNKR/HLA class I interactions, relevant for NK-cell mediated recognition of B-EBV cells.

Polyclonal NK cell populations from both patients and healthy donors displayed poor spontaneous cytolytic activity against KIR-L matched B-EBV cell lines. In NK cells from healthy donors, mAb-mediated masking of HLA class I made target cells highly susceptible to lysis, whereas masking of the activating 2B4 had no

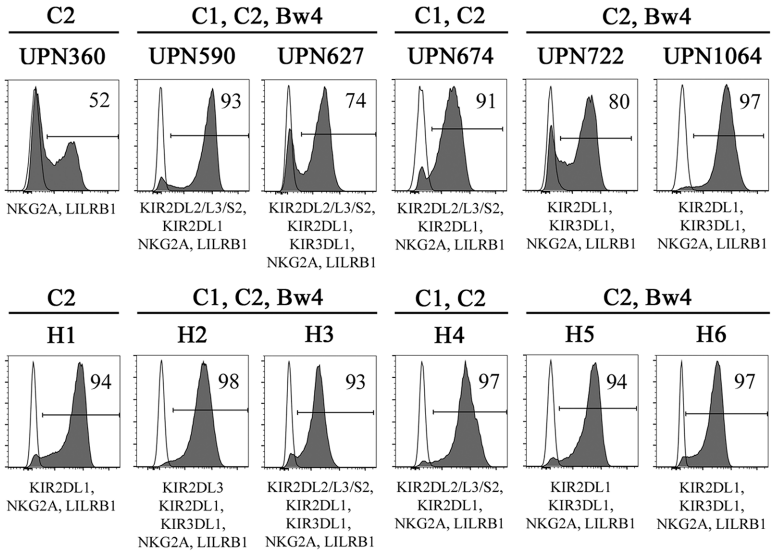

Figure 2. Defective self-iNKR repertoire in XLP1 NK cells. Phenotypic receptor repertoire of polyclonal-activated NK cells from XLP1 patients (UPN, top) and representative healthy controls (H, bottom) was analyzed by immunofluorescence, staining with mAbs specific for the indicated KIR(s), NKG2A and LILRB1, as described in Materials and methods. For each individual the KIR-L(s) are indicated. Empty profiles represent negative control. The percentage of positive cells is indicated within each graph. Data shown are representative of at least two independent experiments for each sample.

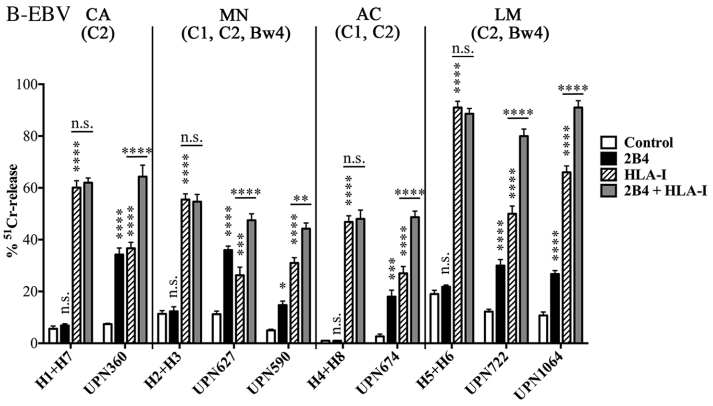

Figure 3. Relevance of inhibitory 2B4/CD48 interaction in B-EBV recognition. Polyclonal-activated NK cells from XLP1 patients (UPN) and healthy controls (H) were tested against ^{51}Cr-labeled KIR-L matched B-EBV cell lines (CA, MN, AC, and LM), in the absence (Control) or in the presence of mAbs to the indicated molecules. The KIR-L of target cells are indicated within brackets. E:T was 10:1. Data pooled from three independent experiments; mean ± SEM (each healthy group $n = 8$; each XLP1 $n = 6$) and significance are shown (two-way ANOVA and Tukey's multiple comparison test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and $^{****}p < 0.0001$.

effect. On the contrary, in XLP1 NK cells masking of the inhibitory 2B4 resulted in significant increment of lysis when used either alone or in combination with the anti-HLA class I mAb. In particular, in UPN360 and UPN627 masking of 2B4 alone resulted in an increase in NK-mediated killing comparable with or even higher than that obtained upon disruption of self-iNKR/HLA class I interactions ($p < 0.0001$). This result was consistent with the presence in these patients of a large fraction of NK cells lacking self-iNKR (Fig. 2; Supporting Information Fig. 1), suggesting that this subset expressed "only" 2B4 as inhibitory receptor. In NK cells from patients characterized by lower percentage of NK cells lacking self-iNKR (UPN590, UPN674, UPN722, and UPN1064) the inhibitory role of 2B4 was still evident, although it was inferior to that played by inhibitory HLA-specific receptors. Nevertheless, in all XLP1 samples, we detected a collaboration of both pathways in B-EBV recognition.

2B4 regulates the interaction of XLP1 NK cells with M1 macrophages

We next investigated the susceptibility to lysis of M1-polarized macrophages by XLP1 NK cells (Fig. 4). Monocytes were obtained from healthy individuals with KIR-L matching those of XLP1 patients, in vitro differentiated into macrophages and polarized toward M1 with TLR stimulation. M1 macrophages expressed CD48 and HLA class I molecules, although at levels lower than those expressed by B-EBV (Supporting Information Fig. 2A and B). The experiments were performed either in the absence or in the presence of 2B4 and/or HLA class I specific mAbs.

M1 macrophages were poorly susceptible to the spontaneous lysis mediated both by healthy and XLP1 NK cells. In healthy controls, M1 macrophages were protected from lysis by self-iNKR/HLA class I interactions. Indeed, killing of M1 macrophages was unleashed by mAb-mediated masking of HLA class I molecules, whereas masking of 2B4 had no effect. On the contrary, in XLP1 patients such as UPN360 and UPN627, who presented high percentage of NK cells lacking self-iNKR, 2B4/CD48 and inhibitory receptors/HLA class I interactions equally contributed to target cell protection (Fig. 4). In particular, in UPN360 the NK-mediated killing of M1 was significantly increased even by the disruption of a single inhibitory pathway, whereas in UPN627 NK cells, which displayed lower cytolytic activity, a significant increment of NK-mediated cytotoxicity was detectable only upon the blocking of both pathways. In UPN1064, who presented low percentage of NK cells lacking self-iNKR, HLA class I molecules played a dominant role in target cell protection, whereas the contribution of the inhibitory 2B4/CD48 interactions was negligible.

XLP1 NK cells with defective self-iNKR repertoire kill mature DC

We addressed the role of 2B4/CD48 and self-iNKR/HLA class I pathways in the interaction between XLP1 NK cells and DC. Monocytes obtained from KIR-L matched donors were in vitro differentiated into immature DCs (iDCs) and mature DCs (mDCs). For UPN360, it was also possible to derive autologous mDCs.

iDCs were HLA class I^{+}, and their expression was upregulated upon maturation [34]. The expression of CD48 was low in iDCs and virtually absent in mDCs (Supporting Information Fig. 2C). According to previous data obtained in an autologous setting [35], iDCs were susceptible to lysis mediated by NK cells from healthy donors, whereas HLA class I^{high} mDCs were highly resistant to lysis and were killed only after mAb-mediated masking of HLA class I molecules (Fig. 5A and B).

We analyzed the role of self-iNKR/HLA class I interactions in DC lysis mediated by XLP1 NK cells, neglecting 2B4, which plays a minor role due to the poor/absent expression of CD48 on target cells. As in healthy donors, NK cells from UPN590, characterized by low percentage of NK cells lacking self-iNKR, killed iDCs but spared mDCs that were protected by high levels of HLA class I molecules (Fig. 5A). In contrast, NK cells from patients with high percentage of NK cells lacking self-iNKR (i.e. UPN627 and UPN360) efficiently killed not only iDCs but also mDCs (Fig. 5A and B). Remarkably, in UPN360, we could confirm data in an autologous setting. Consistent with results obtained in the KIR-L matched allogeneic NK/DCS setting, UPN360 NK cells were highly cytotoxic against autologous mDCs (Fig. 5C).

We could also derive NK cell clones from UPN360, which were grouped according to the presence or absence of the HLA-E-specific CD94/NKG2A heterodimer, the main self-iNKR present in this patient. Interestingly, NKG2A^{-} clones displayed the highest capability of killing autologous mDCs (Fig. 5D), which was not

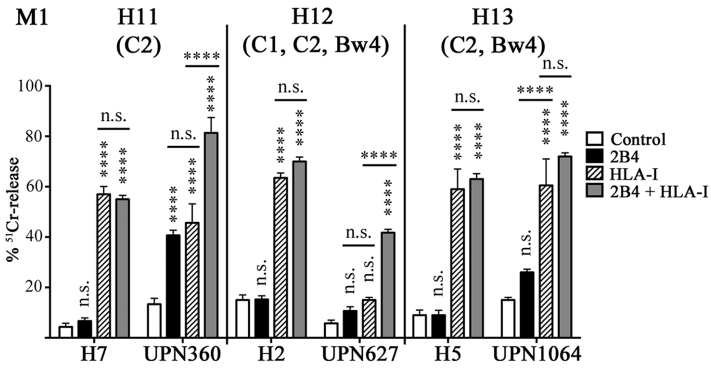

Figure 4. Differential inhibitory pathways involved in NK/macrophage interaction. Cytolytic activity of polyclonal-activated NK cells from XLP1 patients (UPN) and healthy controls (H) was analyzed against M1-polarized macrophages derived from KIR-L matched individuals (H11, H12, and H13), in the absence (control) or in the presence of mAbs to the indicated molecules. The KIR-L of target cells are indicated within brackets. E:T was 10:1. Data pooled from two independent experiments; mean ± SEM (each healthy $n = 4$; each XLP1 $n = 4$) and significance are shown (two-way ANOVA and Tukey's multiple comparison test). $^{****}p < 0.0001$.

influenced by HLA class I expression on target cells. Moreover, in NKG2A$^-$ but not in NKG2A$^+$ clones 2B4 inhibited B-EBV cell lysis (Fig. 5E). Data at the clonal cell level further support the notion that in XLP1, NK cell subpopulation lacking self-iNKR are functionally competent and mainly use inhibitory 2B4 pathway to regulate killing of autologous HLA class I$^+$ cells. Thus, mDCs represent quite an exception among the hematopoietic cells, being susceptible to lysis due to the lack of CD48.

In XLP1 resting NK cells lacking self-iNKR display optimal degranulation capacity

Our data strongly suggested that XLP1 patients were characterized by high percentage of functionally competent NK cells lacking self-iNKR. However, experiments were performed using cytokine-activated polyclonal or clonal NK cells. To exclude that in vitro activation and expansion could provide functional competence to these cells, we analyzed the function of self-iNKR$^-$ NK cells in freshly derived PBMC of UPN360, who did not receive stem cell transplant (Table 1).

NK cells, identified by gating on CD3$^-$ CD56$^+$ cells, were analyzed for degranulation properties either in the absence or in the presence of the prototypic NK susceptible K562 tumor cell line. As previously described, self-iNKR$^-$ NK cells from healthy donors displayed poorly functional capabilities demonstrating the lack of in vivo education [28]. On the contrary, self-iNKR$^-$ NK cells of XLP1 showed optimal degranulation properties upon stimulation with target cells (Fig. 6). Notably, the overall NK cell activity of UPN360 NK cells was significantly higher as compared to healthy controls, including the KIR-L matched H15 donor ($p < 0.0001$; Fig. 6A). This is in line with data by Wu et al. describing that NK-cell degranulation in R-ADCC assay (P815 and anti-NKp46 mAb) was higher in XLP1 than in healthy donors [36].

Discussion

In this study, we provide evidence that the inhibitory receptor repertoire may be disrupted in XLP1, with subpopulations of NK cells lacking self-iNKR. In these NK cells, 2B4 is the main inhibitory receptor and, through CD48 engagement, is responsible

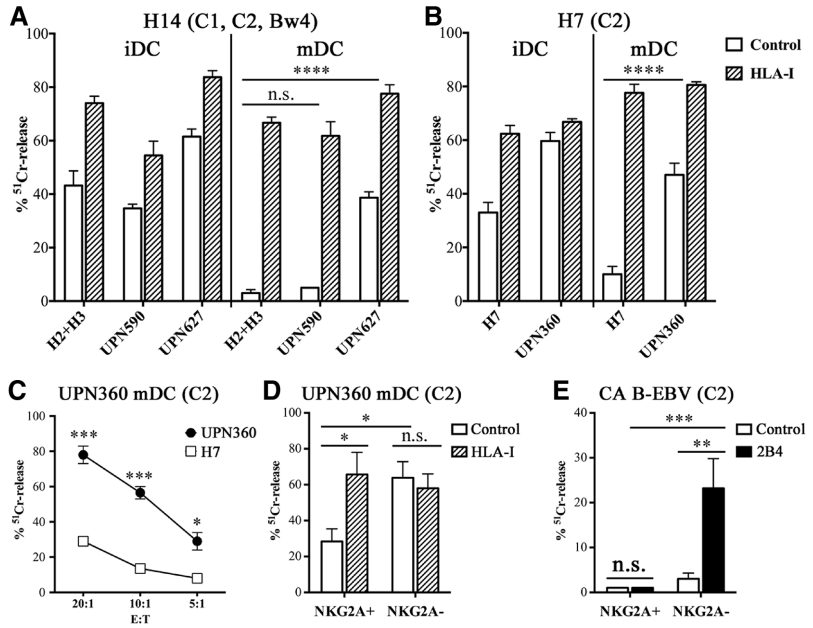

Figure 5. Susceptibility to lysis of mDCs by XLP1 NK cells. (A and B) Polyclonal-activated NK cells from XLP1 patients (UPN) and healthy controls (H) were tested against KIR-L matched or autologous iDCs and mDCs (H14 and H7), in the absence (Control) or in the presence of anti-HLA class I mAb, as indicated, at E:T ratio 10:1. Data pooled from two independent experiments; mean ± SEM (each healthy $n = 4$; each XLP1 $n = 4$) and significance are shown (two-way ANOVA and Tukey's multiple comparison test). (C) Polyclonal-activated NK cells from UPN360 were tested against autologous mDCs at different E:T ratio as indicated, in comparison to NK from H7. Data pooled from two independent experiments; mean ± SEM (each $n = 4$) and significance are shown (two-way ANOVA and Sidak's multiple comparison test). (D and E) NK cell clones, grouped as NKG2A$^+$ ($n = 5$) or NKG2A$^-$ ($n = 5$), were derived from UPN360 and tested against autologous mDCs (D) and KIR-L matched B-EBV (E), in the absence (Control) or in the presence of mAbs to the indicated molecules, at E:T ratio of 10:1. Mean ± SEM and significance are shown (two-way ANOVA and Sidak's multiple comparison test). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and $^{****}p < 0.0001$. The KIR-L of target cells are indicated within brackets.

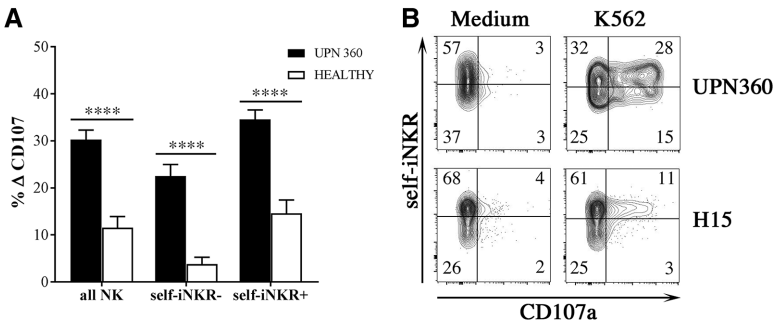

Figure 6. Degranulation capability of resting XLP1 NK cells. (A and B) Gating on CD3$^-$ CD56$^+$ NK cells (total, self-iNKR negative or positive), PBMCs of UPN360 and three healthy controls (including H15) were tested by flow cytometry for degranulation capability (CD107a assay) either in the absence or in the presence of K562 target cells. ΔCD107 represents the difference between the percentage of CD107a$^+$ cells after K562 stimulation and the percentage of CD107a$^+$ cells after incubation with medium alone. (A) Data pooled from three independent experiments for healthy (n = 9) and two independent experiments for UPN360 (n = 8); mean ± SEM and significance are shown in (A) (two-way ANOVA and Sidak's multiple comparison test). ****$p < 0.0001$. (B) A representative experiment is shown.

of blocking lysis of HLA class I$^+$, KIR-L matched hematopoietic cells. This has been shown in functional assays using B-EBV or M1 macrophages as targets. Remarkably, XLP1 NK cells with high percentage of self-iNKR negative population efficiently killed in vitro derived mDCs (HLA class I^{high} and CD48$^-$). This was confirmed at the clonal level and autologous setting. A diagram summarizing these data is shown in Fig. 7.

In physiology, each individual shows a highly stochastic HLA class I specific receptor repertoire, which can be shaped by his immunological history, but always maintaining self-tolerance. By investigating the KIR and HLA class I alleles gene profile of six XLP1 patients, we could discriminate, in each patient, educated or uneducated iKIR(s). Educated iKIR(s) together with CD94/NKG2A and LILRB1 were considered to completely define the self-iNKR repertoire at the phenotypic level. We also selected a group of healthy donors KIR-L matched with the patients, to compare the self-iNKR repertoire and effector activity in normal versus XLP1, and to get adequate amount of target cells to be used in functional assays mimicking the autologous setting. Since in XLP1 patients, functional studies are usually limited by the availability of small blood sample volume due to young age, and sequential studies are often unfeasible due to the therapeutic options, including early stem cell transplantation, we focused our analyses on activated NK cells, in vitro expanded in the presence of IL-2. The combined genetic and phenotypic analysis showed that in three of six XLP1 patients (i.e. UPN360, UPN627, and UPN722) a substantial fraction of NK cells lacked self-iNKR, largely exceeding that observed in healthy controls. The existence of self-iNKR defective NK cells even upon activation is a remarkable finding, since it has been reported that cytokine stimulation induces de novo expression of CD94/NKG2A and/or KIRs (through epigenetic modification of KIR promoters) [37]. Thus, hyporesponsive NK cells gain functional competence only if they acquire at least one self-iKIR (in case of CD94/NKG2A$^-$), to avoid autoreactivity. Consistent with this study, in healthy controls virtually all activated NK cells were self-iNKR positive. At variance in XLP1, NK cells were activated and functional even in the absence of self-iNKR, implying an additional mechanism to ensure self-tolerance. Our present data suggest that the 2B4/CD48 inhibitory pathway can fulfill this role. Claus et al. [10] demonstrated that in NK cells 2B4 not only binds in trans to CD48 on neighboring cells but also interacts in cis with CD48 on the same cell. In XLP1, this cis interaction is inhibitory, representing a possible mechanism to preserve the viability and sustain the activation of fully functional NK cells lacking any inhibitory self-HLA class I specific receptor. Thus, the 2B4 inhibitory receptor can participate to NK cell education, allowing the mobilization from the bone marrow and the maintenance in periphery of these particular mature NK cells. In physiological conditions, SAP deficiency and inhibitory 2B4 function are confined to immature stages of NK cell development and to decidual NK cells [38–40]. Indeed, at an early stage of differentiation, NK cells are characterized by the NCR^{dull}, CD94/NKG2A$^-$, KIR$^-$ phenotype, and the inhibitory 2B4 represents a fail-safe mechanism preventing killing of surrounding autologous cells. In addition to 2B4/CD48, NTB-A (SLAMF6) homotypic interactions appear relevant contributing to NK and

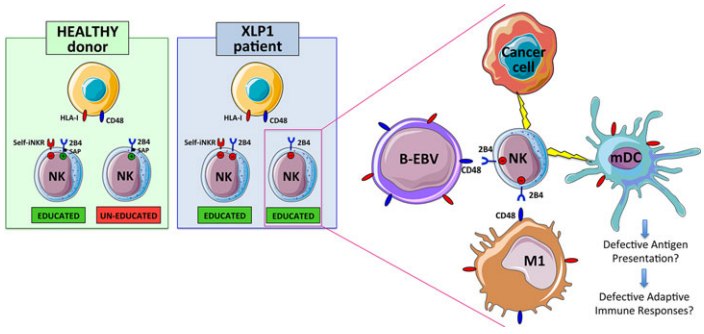

Figure 7. NK cell education in XLP1 as compared to healthy controls. In healthy donors, NK cells expressing inhibitory receptors for self-HLA class I molecules (self-iNKR$^+$) are educated, being responsive to activating receptor stimulation, including the CD48-specific 2B4 receptor which is coupled with SAP. Self-iNKR$^-$ NK cells, representing a minor subset, are uneducated and thus hyporesponsive. In XLP1 patients, SAP deficiency results in inhibitory signals of 2B4 (as other SFRs) after receptor engagement. Self-iNKR$^-$ NK cells, which can be well-represented in the whole NK cell pool, are educated and thus fully functional against HLA class I$^-$ and CD48$^-$ cancer cells as K562. When encountering different hematopoietic HLA class I$^+$ autologous cells, self-iNKR$^-$ NK cells cannot kill EBV-infected B cells and M1 macrophages due to the 2B4/CD48 inhibitory interactions, while CD48$^-$ mDCs are susceptible to lysis. The elimination of mDCs can lead to immunological derangements caused by defective antigen presentation and adaptive immune responses.

T dysfunction in XLP1 [8, 41]. Recently, Wu et al. [36] documented a contribution of NTB-A (SLAMF6) to NK cell education, demonstrating that human and murine NK cells lacking SAP showed enhanced response against nonhematopoietic cells due to stronger SLAMF6-mediated education. Several murine models show that SFR and self-iNKR/MHC-I are two pathways that can influence each other controlling NK-cell responsiveness. NK cells lacking SAP are unable to mediate missing self-recognition, mainly due to SFRs inhibitory function [42]. In addition, in the absence of MHC-I dependent inhibition as in MHC-I mosaic mice, NK-cell tolerance can be explained by an acquired dysfunction of SFRs [43]. In the absence of efficient inhibitory control, a chronic engagement of activating SFR leads to NK-cell anergy, following a "disarming model" for NK cell education [44, 45]. Thus, NK cell education, which occurs constantly during lifetime, relies on a fine tuning of inhibitory and activating thresholds, through MHC-I dependent and independent mechanisms [46]. In XLP1, triggering receptors are fully competent and SFRs, by recruiting tyrosine phosphatases, can sustain self-tolerance even without HLA-I dependent inhibition.

We tested the interplay of XLP1 NK cells with different hematopoietic cells. Since the inefficient defense against EBV infection is the major clinical problem in XLP1, we used KIR-L matched B-EBV as target cells to analyze the function of XLP1 NK cells [9, 12, 13]. B-EBV cells were not lysed by XLP1 NK lymphocytes due to both 2B4/CD48 and self-iNKR/HLA inhibitory interactions. In patients with high proportions of NK cells lacking self-iNKR, 2B4 played a major role and the disruption of its interaction with CD48 restored the capability of NK cells to kill target cells. CD48 is considered a pan-leukocyte antigen. A possible consequence of the existence in XLP1 of functional NK cells with defective self-iNKR repertoire might be autoreactivity against $CD48^{dim}$ or $CD48^{-}$ cells. We then investigated the susceptibility to lysis by XLP1 NK cells of hematopoietic cells that are relevant in the immune responses, such as macrophages and DC. Also in this set of experiments, we derived target cells from healthy individuals, selected as KIR-L matched with XLP1 patients. Although macrophages express CD48 and HLA class I molecules at lower density than B-EBV, the intervention of both 2B4 and iNKR was detected especially in patients with defective self-iNKR repertoire. Noteworthy, the relative contribution of the two pathways might change during viral infection. In this context, it has been shown that murine cytomegalovirus dampened the surface expression of several SLAM receptors during the course of macrophage infection and that m154 (a murine CMV gene product) leads to proteolytic degradation of CD48 [47]. Thus, CD48 is a target of immune evasion by viruses.

At variance from other mononuclear hematopoietic cells, certain types of DCs lack the expression of CD48. Myeloid DCs from inflamed lymph nodes and plasmocytoid DCs do not express CD48, whereas myeloid DCs in BM, blood, and thymus are $CD48^{+}$ [48, 49]. In vitro studies documented that CD48 is rapidly downregulated in monocytes differentiating toward DCs [49]. Consistent with these data, we showed that CD48 was weakly expressed by iDCs and absent on mDCs (Supporting Information Fig. 2) that also lack NTB-A [50]. NK/DC crosstalk has been described leading to NK-cell activation and DC maturation. In healthy donors, activated NK cells can lyse autologous DC that fail to undergo proper maturation, a phenomenon called "DC editing" [34]. On the contrary, mDCs are resistant to NK cell lysis, due to the potent protection mediated by high levels of expression of classical and nonclassical (HLA-E) HLA class I molecules [35]. Remarkably, we provide evidence that, in some XLP1 patients, NK cells efficiently killed both KIR-L matched and autologous (UPN360) mDCs. This correlated with the insufficient repertoire of self-iNKR in NK cells and with the lack of CD48 on mDCs. It should be also considered that mDCs express levels of PVR and Nectin-2, the ligands of DNAM-1 receptor, higher than M1 macrophages and iDCs (Supporting Information Fig. 2). Moreover, in XLP1 this activating pathway is not affected by the inhibitory function of 2B4 [15]. All these features would render these mDCs potent stimulators of both NK-cell cytotoxicity and IFN-γ production (Supporting Information Fig. 3). The observation that XLP1 NK cells can efficiently eliminate autologous mDCs can lead to a defect in antigen presentation and adaptive responses, further exacerbating the patients' immune defect.

Clinical manifestations of XLP1 may vary widely. In our cohort, beyond UPN674 who was diagnosed in the presymptomatic phase due to familial recurrence, the other patients analyzed had evidence of EBV infections and quite typical clinical manifestations. Three patients (UPN360, UPN722, and UPN1064) developed severe encephalopathy. UPN360 also developed a GvHD-like picture with gastritis and colitis resulting in intestinal occlusion requiring resection. Gastrointestinal symptoms are quite rare in XLP1, but already described by Booth et al. [3] in three patients with large SAP gene deletions. Accordingly, UPN360 was characterized by an entire exon 2 deletion (Table 1). UPN360 also showed a very peculiar genetic setting, characterized by C2 KIR-L only and by lack of *KIR2DL1*. The lack of *KIR2DL1* has been observed only in 3–5% of the population and the concomitant presence of C2/C2 without Bw4 appears very rare [51, 52]. UPN360 lacked KIR-mediated tolerance and showed the most striking defective self-iNKR repertoire. Whether these clinical manifestations may be related to the high proportion of fully functional NK cells with defective iNKR-mediated education remain to be assessed.

Materials and methods

Patients and healthy subjects

Six XLP1 patients, referred to the HLH registry [53], were included in this study; *SH2D1A* mutations and clinical features are summarized in Table 1. Patients and healthy adult donors gave informed consent, according with the Declaration of Helsinki. This study was approved by the Institutional review board at the A.O.U. Meyer (Florence, Italy), Istituto G. Gaslini (Genoa, Italy), and Ospedale Bambino Gesù (Rome, Italy).

KIR gene profile and KIR-L analyses

DNA of the tested samples was extracted using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). KIR gene profiles were analyzed by sequence-specific primer-PCR (SSP-PCR) using KIR genotyping kit (GenoVision, Saltsjoebaden, Sweden) [54]. KIR-L were attributed analyzing high-resolution HLA class I typing, using the website http://www.ebi.ac.uk/ipd/kir/ligand.html. Analysis of KIR-L was also performed by SSP-PCR using KIR HLA ligand kit (GenoVision).

Cells

Polyclonal-activated NK cell populations, NK cell clones, iDC, mDCS, and M1-polarized macrophages were obtained from PBMC as previously described [55–57]. B-EBV cell lines were derived from HLA class I typed donors, either in our laboratory (MN, AC, and LM) [24] or kindly provided by ECBR cell-line bank (http://bioinformatics.hsanmartino.it/ecbr/ecbrsite.html; Calogero, hereafter termed CA). The $CD48^-$ HLA class I^- K562 cell line was also used. All cell lines were routinely screened for mycoplasma infection.

Cytofluorimetric analysis

Surface expression of HLA-specific inhibitory receptors on polyclonal-activated NK cells was assessed by indirect immunofluorescence using the appropriate mAb, followed by FITC-conjugated isotype specific goat anti-mouse second reagent (Southern Biotechnology, Birmingham, AL, USA), together with anti-CD3 VioBlue (Miltenyi-Biotec, Bergish Gladbach, Germany) to exclude any T-cell contaminants. The mAbs used for phenotypic characterization of self-iNKR were: GL-183 (IgG1, anti-KIR2DL2/S2/L3), EB6b (IgG1, anti-KIR2DL1/S1), Z27 (IgG1, anti-KIR3DL1/S1), F278 (IgG1, anti-LILRB1), Z270 (IgG1, anti-NKG2A) were produced in our laboratory; 143211 (IgG1, anti KIR2DL1) was obtained by R&D (Minneapolis, MN, USA), and FITC-conjugated DX9 (IgG1, anti KIR3DL1) by Miltenyi Biotec (Bergisch Gladbach, Germany). The analysis of resting NK cells was performed in PBMCs gating on $CD3^-CD56^+$ cells, by using the anti-CD3 BW264/56-VioBlue (Miltenyi Biotec) and anti-CD56 N901-PC7 (Beckman-Coulter, Brea, CA, USA) mAbs. Data were acquired on FACSCalibur (Becton-Dickinson, San Jose, CA, USA) or MACSQuant (Miltenyi-Biotec, Bergish Gladbach, Germany) cytometers and analyzed using FlowJo Version 10.7 (TreeStar).

Functional assays

Cytolytic activity of polyclonal NK cell populations or NK cell clones was evaluated in standard 4 h ^{51}Cr-release assays. Masking experiments were performed using saturating amounts of MA344 mAb (anti-2B4, IgM) and/or A6-136 mAb (anti-HLA class I, IgM). The effector-target (E/T) ratio was 10:1, unless otherwise indicated.

Resting NK cell degranulation assay was performed using PBMC (overnight incubated in RPMI supplemented with 10% FCS), upon stimulation with K562 (or medium as control), as previously described [58]. Surface expression of CD107a (H4A3-PE, BD Bioscience) was evaluated by cytofluorimetric analysis on $CD3^-CD56^+$ NK cells, also differentiating between self-$iNKR^+$ or self-$iNKR^-$ subsets using appropriate combinations of the following mAbs: Z199-APC or REA110-FITC (anti-NKG2A, Beckman-Coulter or Miltenyi Biotec, respectively), HP-F1-APC (anti-LILRB1, Thermo Fisher, Waltham, MA, USA), EB6-APC (anti-KIR2DL1/S1, Beckman-Coulter), 143211-FITC (anti-KIR2DL1, R&D), CH-L-FITC (anti-KIR2DL2/L3/S2, BD Bioscience), and DX9-FITC (anti-KIR3DL1, Miltenyi Biotec). Data were acquired on Gallios (Beckman-Coulter) flow cytometer and analyzed using FlowJo Version 10.7 (TreeStar).

Statistical analysis

Statistical analyses were performed using GraphPad software Version 6.0, utilizing two-way ANOVA and Tukey or Sidak's multiple comparison test, as recommended. Not significant (n.s.); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and $^{****}p < 0.0001$.

Acknowledgments: This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC) IG-10643 (A.M.), IG-10225 (L.M.), IG-16764 (D.P.), and special project 5 × 1000–9962 (F.L., A.M., and L.M.); 5xmille 2013 Ministero della Salute and RF-2010-2316606 (D.P.); Ministero dell'Istruzione, dell'Università e della Ricerca (A.M., L.M., and C.B.); Associazione Italiana Ricerca Istiocitosi (AIRI) O.N.L.U.S., Ministero della Salute (RF-TOS-2008-1219488), Seventh Framework Programme (FP7) of the European Commission ("FIGHT-HLH" Project Number 306124; M.A.).

Conflict of interest: A.M. is a cofounder and shareholder of Innate-Pharma (Marseille, France). The remaining authors declare no financial or commercial conflict of interest.

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Abbreviations: **B-EBV**: EBV-transformed B cell · **DNAM-1**: DNAX accessory molecule-1 · **HLH**: hemophagocytic lymphohistiocytosis · **iDCs**: immature DCs · **iNKRs**: inhibitory NK receptors · **KIR**: killer Ig-like receptor · **LILRB1**: leukocyte Ig-like receptor subfamily member 1 · **mDCs**: mature DCs · **NCR**: natural cytotoxicity receptor · **SAP**: SLAM-associated protein · **SFR**: SLAM-family receptor · **SLAM**: Signaling Lymphocyte Activation Molecule · **UPN**: unique patient number · **XLP1**: X-linked lymphoproliferative disease 1

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Received: 15/12/2016
Revised: 20/2/2017
Accepted: 28/3/2017
Accepted article online: 6/4/2017

