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Review Article

2B4 dysfunction in XLP1 NK cells: More than inability to control EBV infection

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ABSTRACT

X-linked lymphoproliferative disease 1 (XLP1) is a monogenic disorder caused by mutations in *SH2D1A*, resulting in the absence/dysfunction of the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP). Consequently, SLAM receptors as 2B4 (CD244) and NTB-A (SLAMF6), upon ligand engagement, exert inhibitory instead of activating function. This causes an immune dysfunction that is worsened by the selective inability of NK and T cells to kill EBV-infected B cells with dramatic clinical sequelae (e.g. fulminant mononucleosis, hyperinflammation, lymphoma). Here we outline recent findings on the interplay between inhibitory 2B4 and the various activating receptors in NK cells. 2B4 engagement selectively blocks ITAM-dependent activating receptors as NCR and CD16, while it does not affect NKG2D and DNAM-1. Furthermore, inhibitory 2B4 participates to NK cell education, as highlighted by the existence in XLP1 patients of a large subset of fully functional NK cells that lack self-HLA specific inhibitory receptors and exert autoreactivity against mature dendritic cells.

1. Introduction

X-linked lymphoproliferative disease 1 (XLP1, Duncan disease, OMIM # 308240) is a primary immunodeficiency (PID) due to hemizygous mutations of the *SH2D1A* gene (Xq25) in male infants [1,2]. *SH2D1A* encodes the Signaling Lymphocyte Activation Molecule (SLAM)-associated protein (SAP), a small cytoplasmic adaptor expressed by T and NK lymphocytes. SAP consists of a single SH2 domain that associates with the immunoreceptor tyrosine-based switch motif (ITSM, TxYxxI/V) present in the cytoplasmic tail of the SLAM family receptors (SFRs). The family comprises nine members [3], including 2B4 (SLAMF4, CD244), expressed by $CD8^+$ T and NK cells, and NTB-A (SLAMF6, CD352) that is also present in B lymphocytes and macrophages [4–7]. While NTB-A, as most SFRs, displays homophilic interaction, 2B4 recognizes CD48 (SLAMF2), a GPI-anchored Ig-like protein exclusively present on hematopoietic cells. After ligand recognition, the ITSMs present in the cytoplasmic tail of 2B4 and NTB-A become phosphorylated, and associate with SAP, which in turn activates downstream signaling pathways that result in cell activation. 2B4 and NTB-A support the function of main activating receptors, thus acting as co-receptors that improve the recognition and killing of hematopoietic targets. In particular, Epstein Barr virus (EBV) infected B (B-EBV) cells are highly susceptible to the activity of cytolytic lymphocytes since they express NTB-A and very high levels of CD48, which has been called "super-induced" antigen [8].

In XLP1 patients, in the absence of SAP, the engaged 2B4 and NTB-A associate with protein tyrosine phosphatases and deliver potent inhibitory signals, which result in impaired cytolytic responses of NK and $CD8^+$ T cells against B cell targets [4–6,9–11]. Thus, as unique trait among PID, XLP1 patients have the selective inability of controlling

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; AICL, activation-induced C-type lectin; APCs, antigen presenting cells; CTL, cytotoxic T lymphocytes; DNAM-1, DNAX accessory molecule-1; FC, flow cytometry; FIM, fulminant infectious mononucleosis; HLH, hemophagocytic lymphohistiocytosis; HSCT, hematopoietic stem cell transplant; iNKR, inhibitory NK receptors; ITAM, immunoreceptor tyrosine-based activating motif; ITSM, immunoreceptor tyrosine-based switch motif; ITT, immunoreceptor tyrosine tail; KIR, killer Ig-like receptor; LAMP-1, lysosomal-associated membrane protein-1; LILRB1, Leukocyte Immunoglobulin Like Receptor B1; mDC, mature dendritic cells; ML, malignant lymphoma; NCR, natural cytotoxicity receptor; NF-κB, nuclear factor-κB; NK, natural killer; XLP, X-linked lymphoproliferative disease; PBMC, peripheral blood mononuclear cells; PID, primary immunodeficiency; RICD, restimulation-induced cell death; SAP, SLAM-associated protein; EBV, Epstein-Barr virus; SFRs, SLAM family receptors; SLAM, signaling lymphocyte activation molecule

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EBV infection [1,9], which causes dramatic clinical sequelae [12,13].

NK cells are important players of innate immunity and are involved in early immunity against hematological and non-hematological malignancies, as well as viral infections, in particular those mediated by herpes viruses [14]. NK cells exert a potent cytolytic activity and release immunostimulatory cytokines such as IFN-γ that potentiate both innate and adaptive immune responses. Human NK cells are regulated by an array of activating and inhibitory receptors that tune their effector functions [15]. Besides CD16, the low affinity Fcγ receptor, the principal activating NK receptors include NKp46, NKp30, and NKp44, collectively termed Natural Cytotoxicity Receptors (NCR), DNAM-1 and NKG2D [6,16], which recognize ligands upregulated or de novo expressed at the cell surface of target cells [17–19]. The main inhibitory receptors recognize HLA class I molecules and include inhibitory Killer-cell immunoglobulin-like receptors (iKIRs), CD94/NKG2A and Leukocyte Immunoglobulin Like Receptor B1 (LILRB1), preventing attack of normal autologous cells [20,21].

In this review, we briefly describe the clinical manifestation and the diagnostic methodology in XLP1 patients; we also outline recent knowledge on the role of inhibitory 2B4 in the cross-talk with the various NK cell receptors and in the education process.

1.1. Clinical manifestations in XLP1

EBV is a ubiquitous infectious agent with tropism to B cells, where it remains latent for the remainder of the host's life. Epidemiological studies conducted during the ‘80s had showed that virtually all subjects were expected to develop seroconversion, i.e. had met EBV, by the age of 18 years [22]. Immune response against EBV is a multifactorial chain of events, which provides the organism an effective protection against the adverse outcome due to acute infection but also against possible reactivation.

In a small minority of male subjects, EBV infection may be followed by severe complications. On the basis of the review of 100 cases, in 25 kindreds Purtilo et al. [23] defined the Duncan's syndrome, later redefined as XLP1. Four main clinical phenotypes were defined: fulminant infectious mononucleosis (FIM), malignant lymphoma (ML), dysgammaglobulinemia and hemophagocytic lymphohistiocytosis (HLH) [2,12,13,24,25]. It is now recognized that XLP1 patients have a complex immune dysregulation that includes both EBV-dependent and independent clinical manifestations [26]. Booth et al. retrospectively reviewed 91 patients with genetically confirmed XLP1 [13]. The most common presenting feature (39.6% of patients) was HLH, and the most common shared feature was dysgammaglobulinemia, observed during the clinical course in 50% of patients. Twenty-two patients had malignant lymphoproliferative disease, including 18 with B-cell non-Hodgkin lymphoma. EBV-positivity was documented in 64.6% of patients tested, who had a higher frequency of HLH, a complication associated with 65.6% mortality rate. Of the 91 patients, 43 had been treated with hematopoietic stem cell transplant (HSCT) and their survival was 81.4%, but only 50% in transplanted patients with HLH.

In XLP1 patients, a broad spectrum of *SH2D1A* mutations has been described, including deletions, nonsense or missense single nucleotide substitutions, and splice-site abnormalities. Although there is no clear correlation between the different mutations and the type or severity of clinical phenotype [27], all XLP1 subjects are unable to eliminate B-EBV cells. This exposes the organism, usually a male child, to development of a life-threatening clinical picture characterized by cytokine overproduction and multi-tissue damage. Genotype-phenotype correlation studies documented that XLP1 subjects may be identified among patients with HLH but also among males with non-Hodgkin lymphoma, in most cases associated with EBV infection. Thus, wider knowledge of these correlations is warranted among pediatric immunologists and oncologists.

Treatment of XLP1 is aimed at preventing the risk of fatality, which is particularly high in children presenting with HLH. For them, indication to HSCT is warranted. Yet, rare patients may survive initial IM and then reach a stable condition of agammaglobulinemia in which replacement therapy allows normal life. For them, indication to HSCT should be individually discussed, in the light of the risk of malignancy and of available donor options. Autologous T-cell gene therapy might offer an alternative therapeutic option for patients with XLP1. Recently, in SAP-deficient mice, 20% to 40% engraftment of gene-modified T cells led to improved cytotoxicity and T follicular helper cell function in vitro. In lymphoma model in NSG mice, adoptive transfer of gene-corrected patient-derived cytotoxic T lymphocytes (CTL) reduced tumor burden to the same level as healthy donor CTL [28]. In the near future, also gene editing techniques as CRISPR/Cas9 may offer a solution for PID patients, particularly for XLP1 that is a monogenic disease virtually restricted to the hematopoietic compartment.

1.2. Early XLP1 diagnosis based on NK cell assays

XLP1 patients can present with clinical manifestations similar to other PID associated with HLH. Differential diagnosis is based on the genetic testing, which represents the gold standard but is costly and time-consuming, due to the high numbers of involved genes and mutations described.

Recently, a diagnostic algorithm has been proposed to rapidly identify XLP1 among various inherited forms [29,30]. This is based on the combined phenotypic and functional cytofluorimetric analysis of NK cells present in peripheral blood (PB) of patients (Fig. 1). In particular, SAP expression can be measured by intra-cytoplasmic staining of patients' PB mononuclear cells (PBMCs) using 1C9 anti-SAP mAb and flow cytometry (FC), gating on $CD3^-$ $CD56^+$ NK lymphocytes. XLP1 patients may harbor different *SH2D1A* mutations, including deletions, nonsense and missense mutations, which can lead to lack of SAP expression or to expression levels significantly lower than those detected in healthy individuals [31]. Interestingly, in some patients carrying point mutations, the evidence of somatic revertant memory $CD8^+$ SAP^+ T cells, functional against B-EBV cells, has been described; in the same cases, no or very few SAP^+ NK cells were detected [32]. FC analysis of SAP can also be used for the detection of carrier status of females in XLP1 families [33]. PB NK cells of female carriers show a bimodal distribution of the SAP molecule, resulting from SAP^+ and SAP^- NK cell subsets, due to random X inactivation. Interestingly, although patients' mothers are expected to be obligate carriers, a patient has been described whose mother lacks the mutation detected in her son, possibly due to either a de novo mutation or a germinal mosaicism [30].

Notably, at least one single aminoacid change in *SH2D1A* (i.e. R55L) has been described to impair SAP function despite preserved expression at FC, by using 1C9 mAb. Thus, to avoid this possible pitfall of insufficient sensitivity in the differential diagnosis of XLP1, in the case of normal results of phenotypic analysis in a male with robust diagnostic suspect, this should be complemented by data showing the inhibitory function of 2B4 [30]. PBMCs from XLP1 and healthy controls are cultured overnight with rIL-2 and used as effectors in reverse antibody-dependent cellular cytotoxicity assays (R-ADCC) against the $FcγRc^+$ P815 murine target cell line, either in the absence or in the presence of anti-2B4 and anti-NKp46 mAbs, used alone or in combination. The degranulation capability is then analyzed by FC evaluating the surface expression in NK cells of the lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) (CD107a assay) (Fig. 1) [34]. In healthy individuals both 2B4 and NKp46 induce NK cell degranulation and synergize when simultaneously engaged. On the contrary, in XLP1 patients the engagement of 2B4 inhibits the spontaneous degranulation of NK cells against target cells, and significantly decreases that induced by NKp46-engagement.

Combination of SAP expression with 2B4 functional assay is a rapid, low-cost diagnostic strategy that requires very small blood samples, identifies all XLP1 cases, including those with R55L mutation, and

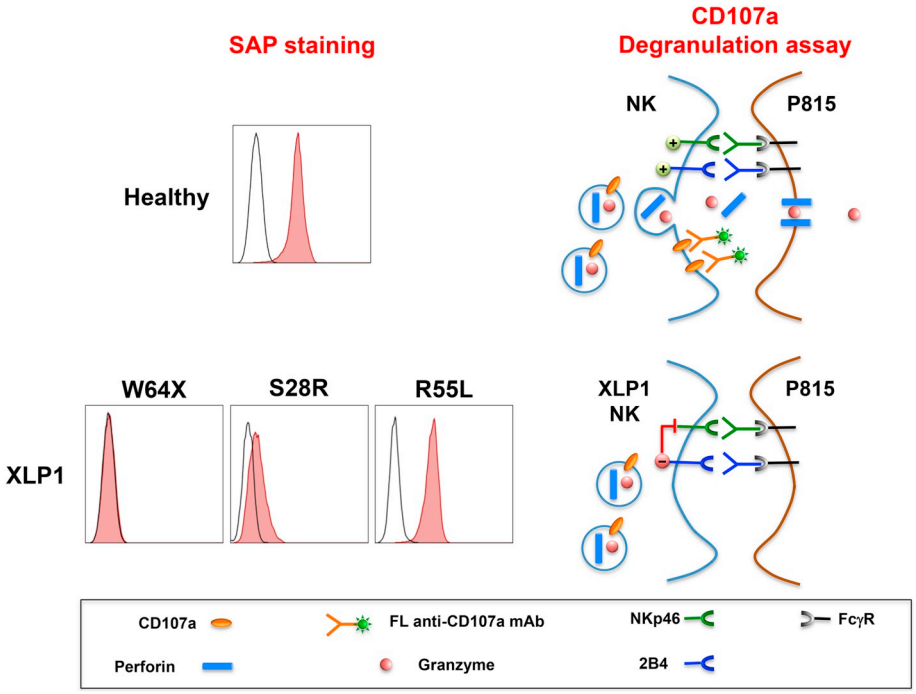

Fig. 1. XLP1 diagnosis by combined phenotypic and functional cytofluorimetric analysis.
(Left) Intra-cytoplasmic SAP staining in NK cells from healthy control (top) and three XLP1 patients (bottom) carrying different *SH2D1A* mutations, showing either absent (W64X), weak (S28R) or normal (R55L) expression. NK cells were stained with 1C9 anti-SAP mAb (red profiles). Empty profiles represent the isotype matched negative control. **(Right)** 2B4 functional evaluation by CD107a degranulation assay. The immunological synapses between effector (NK) and target ($Fc\gamma R^+$ P815) cells are depicted. NK cells were stained with a fluorescent (FL) anti-CD107a mAb. In healthy NK cells, the mAb-mediated engagement of both NKp46 (a member of NCR) and 2B4 induces synergistic degranulation (top). On the contrary, in XLP1 NK cells, 2B4 engagement exerts inhibitory function that blocks NCR-mediated degranulation, independently on the type of SAP mutation (bottom).

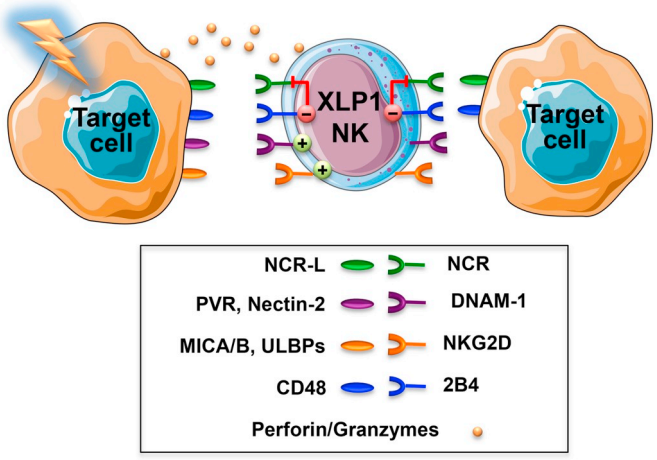

Fig. 2. Inhibitory 2B4 in XLP1 NK cells does not affect NKG2D and DNAM-1 activating pathways.
In XLP1 NK cells, 2B4, upon engagement with CD48, impairs the function of ITAM-dependent activating receptors such as NCR, while does not affect the activity of the NKG2D and DNAM-1 signaling pathways, which are devoid of ITAM. Thus, $CD48^+$ target cells can be susceptible to lysis only if they express sufficient amounts of NKG2D-L and/or DNAM-1-L.

direct gene mutation analysis.

XLP1 patients are also characterized by a peculiar absence of iNKT cells ($CD3^+$, $TCRV\alpha24^+$ and $TCRV\beta11^+$) in PB [35] and by a reduced T cell susceptibility to restimulation-induced cell death (RICD) [36–38]. However, iNKT cells are rare also in healthy individuals (0.08% of PB T cells); thus it may be difficult to discriminate between disease and personal trait. Furthermore, the analysis of RICD, although useful to better characterize the patient' disease, is unsuitable as routine screening assay.

1.3. Crosstalk between 2B4 and NK cell activating pathways in XLP1

NK cells are equipped with a set of activating receptors involved in natural cytotoxicity and cytokine production. Receptor engagement by specific ligands, usually up-regulated on stressed cells, is required for the delivery of activating signals to NK cells [39], which become capable to destroy cells undergoing viral infection or neoplastic transformation. Major activating receptors include the NCR (i.e. NKp46, NKp30 and NKp44), NKG2D and DNAM-1 [6,40,41]. The NCR cell surface ligands identified so far include BAT3 and B7-H6 (NKp30-L) and an isoform of mixed-lineage leukemia-5 (MLL5) (NKp44-L). Moreover, microbial and soluble ligands have also been described [42–45]. NKG2D and DNAM-1 receptors, are also expressed by $CD8^+$ T cells and recognize MICA/B and ULBPs, Poliovirus receptor (PVR, CD155) and Nectin-2 (CD112), respectively [17,18]. An efficient NK cell activation is also mediated by CD16 ($Fc\gamma$RIIA), responsible for ADCC [46], and by the activating isoforms of KIR (aKIR) [20,47]. The function of activating receptors is supported by co-receptors including 2B4, NTB-A and NKp80 that recognizes the activation-induced C-type lectin (AICL) [48].

This highly redundant system of activating receptors/co-receptors is coupled to various signaling molecules and ensures the appropriate functional interaction of NK cells with several different targets [49]. Efficient stimulation of resting NK cells requires synergy among activating molecules [50]. For example, in healthy individuals 2B4 and NCR co-operate in eliminating cells infected by EBV, a γ-herpes virus that targets and sequesters itself in B cells [11,51]. 2B4 engagement by up-regulated CD48 on B-EBV cells, causes tyrosine phosphorylation of ITSMs present in its cytoplasmic tail and recruitment of SAP, which transduces Fyn-dependent activating signals [15]. When SAP is absent or dysfunctional as in XLP1, 2B4 associates with protein tyrosine phosphatases (SHP1, SHP2 and SHIP-1) and delivers strong inhibitory signals blocking instead of sustaining killing of infected cells [10,52,53]. Interestingly, it has been recently shown that inhibitory 2B4 differentially affects the various triggering receptors (Fig. 2) [54]. Indeed, it exclusively acts on receptors such as NKp46, NKp30, NKp44, CD16 and aKIR transducing via ITAM ($YxxL/I\text{-}X_{6-8}\text{-}YXXL/I$)-bearing molecules (e.g. CD3ζ, $Fc\varepsilon R\gamma$ and DAP12). In contrast, NKG2D, which associates with DAP10 (a signaling subunit characterized by YINM sequence), and DNAM-1, which directly transduces signal via immunoreceptor tyrosine tail (ITT)–like motifs, were exempt from 2B4-mediated inhibition. However, although maintaining their functional capability, these receptors lack the 2B4-mediated support, as demonstrated by studies on signal transduction pathways involved in the

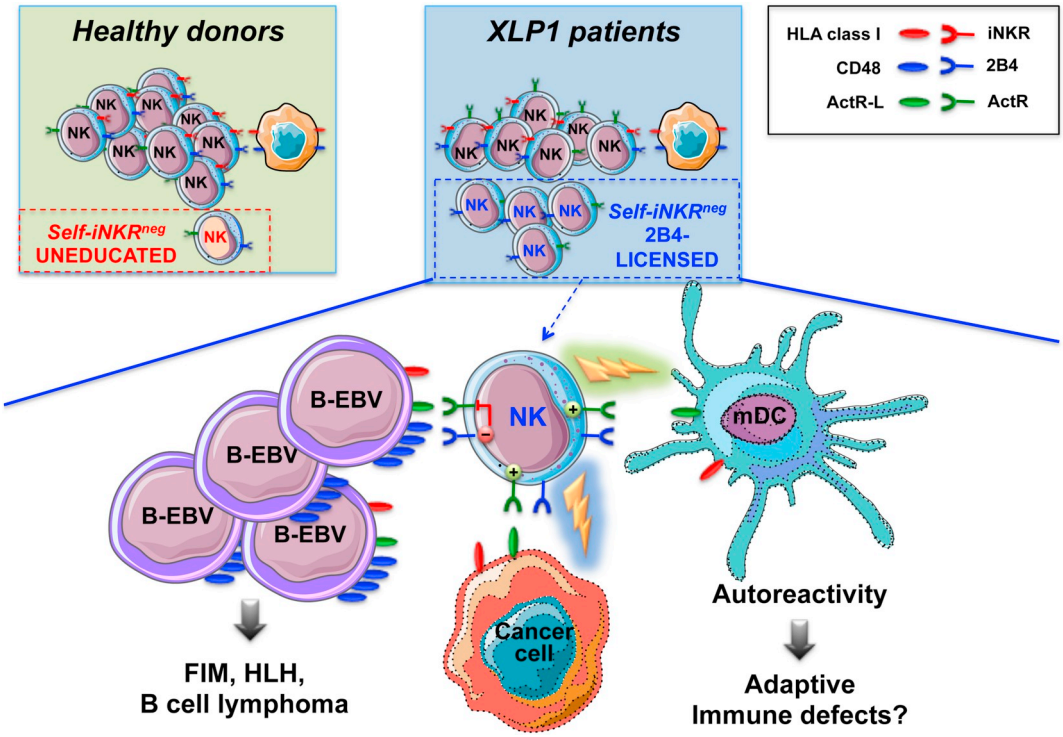

Fig. 3. XLP1 patients present unconventionally educated self-iNKR^{neg} NK cells.
In healthy individuals, the few self-iNKR^{neg} NK cells (red marked) are "uneducated" and thus anergic. In XLP1 patients, self-iNKR^{neg} NK cells (blue marked) are well represented and functionally active, being licensed by 2B4-CD48 interactions. Self-iNKR^{neg} XLP1 NK cells are unable to kill CD48^{high} EBV infected B cells. On the contrary, via various activating receptor/ligand interactions, they efficiently kill CD48^{neg} tumor cells as well as CD48^{neg} autologous mature dendritic cells (mDC), which are not protected by HLA class I expression. Autoreactivity against mDC may cause a defective antigen presentation contributing to impairment of adaptive immune responses.
ActR, activating receptor(s); ActR-L, ligand(s) of activating receptor(s); FIM, fulminant infectious mononucleosis; HLH, Hemophagocytic lymphohistiocytosis.

cooperative activation. In normal NK cells, NKG2D synergizes with 2B4 through complementary phosphorylation of SLP-76 at Tyr^{128} and Tyr^{113}, respectively [15,55]. Vav1 binds to both phospho-tyrosine residues resulting in a highly efficient activation of nuclear factor-κB (NF-κB), a key transcription factor inducing NK effector functions. Optimal activation of NF-κB also requires phosphorylation of the p65 subunit at Ser^{536} by PI3K-Akt pathway, primarily mediated by the engagement of NKG2D and DNAM-1 [56]. In XLP1 NK cells, no synergistic Vav1 phosphorylation occurs upon NKG2D and 2B4 co-engagement, while NKG2D-dependent phosphorylation of Vav1 and Akt is preserved.

The in-depth study of the interplay among SFRs and the different triggering receptors provided new insights in the immune defect typical of XLP1 patients. Indeed, XLP1 NK cells are unable to kill CD48^{+} targets devoid of NKG2D and DNAM-1 ligands such as B-EBV and lymphoma B cells. On the contrary, target cells expressing sufficient amounts of NKG2D and/or DNAM-1 specific ligands remain susceptible to lysis through the preferential usage of these receptors (Fig. 2) [54]. These findings may be also extended to T lymphocytes, whose TCR function, paradigmatic model of ITAM-mediated signaling, is inhibited by 2B4 and NTB-A [57], whereas the activity of NKG2D and DNAM-1 could be un-affected.

1.4. NK cell education

The NK cell activity is under the control of different types of HLA class I specific inhibitory NK receptors (iNKR): (i) iKIRs specific for epitopes shared by groups of HLA-A, −B, or −C allotypes; (ii) CD94/NKG2A, a heterodimer recognizing non-classical HLA-E; (iii) LILRB1 with a broad HLA class I specificity [20,58]. The iKIR are clonally distributed and, together with CD94/NKG2A and LILRB1, create stochastic but tolerant repertoires of NK cells. In healthy individuals, the NK cells repertoire is selected during maturation in a way that each NK cell expresses at least one iNKR for self-HLA class I molecules (self-iNKR). This phenomenon is termed classical "licensing" or "education" and provides the basis of self-tolerance [59]. In developing NK cells, iNKR are engaged, possibly in trans, by surrounding self-HLA class I expressing cells [60] and transduce permissive signals that allow their phenotypic/functional maturation. Educated NK cells are self-tolerant but "armed", becoming fully responsive to the engagement of activating receptors, exerting strong cytotoxicity against abnormal targets and releasing large amounts of immunostimulatory cytokines. Lack of expression of self-iNKR results in the generation of "uneducated" NK cells that are functionally anergic. Self-iNKR^{neg} NK cells are detectable in small percentages in healthy individuals (Fig. 3). Cytokine stimulation can "rescue" these NK cells, which therefore acquire spontaneous and receptor-mediated cytolytic capabilities [61]. Importantly, they de novo express CD94/NKG2A and/or iKIRs (through epigenetic modification of KIR promoters), thus avoiding autoreactivity [62].

It has recently been shown that XLP1 patients harbor in the peripheral blood a substantial proportion of "theoretically" uneducated self-iNKR^{neg} NK cells. At variance with those detected in healthy donors, these cells are functionally active ("armed"), and maintain their peculiar phenotype upon cytokines stimulation [63]. As occurs for self-iNKR^{pos}, self-iNKR^{neg} XLP1 NK cells are unable to kill hematopoietic targets such as B-EBV cells, which over-express CD48 engaging the inhibitory 2B4, responsible to switch off the function of activating receptors such as NCR (Fig. 3). Conversely, self-iNKR^{neg} NK cells show optimal cytokine release and cytotoxicity against cells lacking CD48. Targets include not only tumor cells but also autologous mature dendritic cells (mDC), which are not protected by the high HLA class I expression levels (Fig. 3). The anomalous autoreactivity of self-iNKR^{neg} XLP1 NK cells against crucial antigen presenting cells (APCs) may result

in defective antigen presentation to T cells and impaired adaptive responses, further exacerbating the patients' immune defect. These observations suggest that, in XLP1 patients, self-iNKR^{neg} NK cells may have been “licensed” by the non-classical, HLA class I-independent inhibitory 2B4/CD48 interaction (occurring either in cis or in trans). This process however does not result in a proper “education” since it generates NK cells with unwanted autoreactivity [63]. A role in NK cell licensing has been proposed also for the homotypic receptor NTB-A [64–67].

The mechanisms leading to augmented NK cell function following classical and non-classical NK education/licensing in healthy donors or XLP1 remain poorly understood. It is of note that, in healthy donors at immature stages of development NK cells lack SAP and the inhibitory SLAM receptors could represent a fail-safe mechanism preventing killing of surrounding CD48^{pos} hematopoietic cells [68]. Regarding the signaling pathways involved, in the absence of SAP both 2B4 and NTB-A, upon ligand recognition, associate with phosphatases. These include SHP-1 that, in a mouse model, has been shown to be required for classical (MHC class I-dependent) NK cells education [69].

2. Conclusion

The study of PID “illuminates” the biology of immune system, especially in the field of antiviral immunity and tumor immunosurveillance [70]. This applies to XLP1 and NK cells that are cytolytic effectors with functions relying on integrated signals mediated by activating and inhibitory receptors/co-receptors.

NK cells represent an important cell compartment to study PID, particularly those characterized by defects in the cytotoxic machinery. In early 2000 we had the evidence that 2B4 and NTB-A, upon engagement with their specific ligands, exerted inhibitory instead of activating function in SAP deficient NK cells from XLP1 patients [5,10]. These inhibitory pathways were responsible of the impaired killing of B cells infected with EBV with severe clinical consequences. Thereafter, new information have been obtained on NK cell biology with the discovery of additional receptor/ligand interactions and the iNKR-mediated education process, which occurs during maturation. Recent studies showed that in XLP1 NK cells the inhibitory 2B4 alters the function of some triggering pathways, while sparing that of others. Thus, the inability of XLP1 NK cells to kill one or another target depends on the type and number of receptor/ligand interactions involved. In addition, in recent years it has been suggested the involvement of inhibitory 2B4 and NTB-A in NK cell education, a phenomenon that may be important in cells that physiologically lack SAP, such as NK cells at immature stages of development or those populating the decidua. In XLP1 patients however, the extension of this alternative education mechanism has negative consequences since it allows the generation of functional NK cells with a defective self-HLA-specific receptor repertoire. These cells are able to kill normal autologous CD48^{neg} cells as mDC, likely exacerbating the patient's immune dysfunction.

All these new data provide novel insight in NK cell biology and further expand our knowledge of XLP1, a congenital immunological derangement that goes beyond the selective inability to control EBV infection.

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