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Cutting Edge: Defective NK Cell Activation in X-Linked Lymphoproliferative Disease[1]

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X-linked lymphoproliferative disease (XLP) is characterized by a selective immune deficiency to EBV. The molecular basis of XLP has been attributed to mutations of signaling lymphocytic activation molecule-associated protein, an intracellular molecule known to associate with the lymphocyte-activating surface receptors SLAM and 2B4. We have identified a single nucleotide mutation in SLAM-associated protein that affects the NK cell function of males carrying the mutated gene. In contrast to normal controls, both NK and lymphokine-activated killer cell cytotoxicity was significantly reduced in two XLP patients. In addition to decreased baseline cytotoxicity, ligation of 2B4 significantly augmented NK lytic function in normal controls but failed to enhance the cytotoxicity of NK cells from XLP patients. These findings suggest that association of SAP with 2B4 is necessary for optimal NK/lymphokine-activated killer cytotoxicity and imply that alterations in SAP/2B4 signaling contribute to the immune dysfunction observed in XLP. *The Journal of Immunology*, 2000, 165: 3549–3553.

X-linked lymphoproliferative disease (XLP)[3] is characterized by a selective immune deficiency to EBV (1, 2). In XLP patients, severe illness, including fulminant and fatal infectious mononucleosis, manifests following primary infection with EBV, and the overall disease prognosis is extremely poor. The specific immune defects, which manifest following exposure to EBV, include an inability to generate EBV-specific Abs (3), defective Ig class switching (4), and abnormal NK and T cell-mediated cytotoxicity (5–9). The nature of these defects suggests a global dysfunction in cell-mediated immunity.

The genetic basis of XLP has been attributed to mutations in signaling lymphocytic activation molecule (SLAM) associated protein (SAP) (10–12), an intracellular molecule expressed in T cells and consisting almost entirely of a single, noncatalytic, Src-homology 2 domain. SAP has been shown to block recruitment of SHP-2 phosphatase by competitively binding to tyrosine recognition motifs of SLAM and 2B4, costimulatory molecules expressed on the surface of T/B cells and T/NK cells, respectively (10, 13). The engagement of 2B4 on NK cells has previously been demonstrated to promote spontaneous cytotoxicity and augment secretion of IFN-γ (14–17). Because SAP has been shown to interact with 2B4 in vitro (13), we sought to determine whether mutations in SAP might affect normal signaling mediated through ligation of 2B4 in patients with XLP.

We have sequenced a novel mutation in SAP from autopsy specimens obtained from two maternally related cousins clinically diagnosed with XLP. Based on the molecular structure of the SAP-SLAM interaction (18, 19), this mutation ($Arg^{55}Leu$ in exon 2) is predicted to disrupt binding between the Src-homology 2 domain of SAP and the cytoplasmic domain of SLAM. It is likely that this mutation also interferes with SAP/2B4 binding because 2B4 and SLAM share strong amino acid homology, including careful conservation of the tyrosine recognition motifs contained within their cytoplasmic domains (16, 20). We have genotypically identified two additional related males from the extended family who carry the same mutation. Both affected males were healthy, and neither had clinical or serologic (EBV viral capsid Ag IgG negative) evidence of prior EBV infection. In this report, we describe the abnormal function of NK and lymphokine-activated killer (LAK) cells obtained from these boys. We further show that, in contrast to cells obtained from healthy controls, the cytotoxic activity of NK cells obtained from XLP patients cannot be enhanced through 2B4 ligation. These findings suggest that the interaction of SAP with 2B4 is required for optimal NK/LAK cell immunity, that SAP Arg^{55} is essential for MHC-unrestricted cytotoxic function, and that alterations to this pathway contribute to the defective immunity described in XLP.

Materials and Methods

Cell culture

PBMC were isolated by Ficoll density gradient centrifugation. Cells were resuspended in complete medium and incubated in culture flasks at 37°C

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[3] Abbreviations used in this paper: XLP, X-linked lymphoproliferative disease; SLAM, signaling lymphocytic activation molecule; SAP, SLAM-associated protein; LAK, lymphokine-activated killer; CRA, chromium release assays; ADCC, Ab-dependent cellular cytotoxicity.

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for 4 h to deplete monocytes. The nonadherent fraction was used as effector cells in cytotoxicity assays. The cell lines used were K562, Raji, NK-92ci, and MV4;11 (Dr. K. Schultz, University of British Columbia, Vancouver British Columbia, Canada). Primary lymphocyte subsets were purified with immunomagnetic cell separation kits (Miltenyi Biotec, Auburn, CA) according to the manufacturer's specifications. The study was approved by the University of British Columbia Clinical Research Ethics Board, and informed consent was obtained from all subjects before the collection of blood.

Flow cytometry and Abs

Samples were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson). Briefly, 200,000 cells were analyzed after staining with appropriate Abs: anti-CD3-FITC, anti-CD56-PE, anti-CD48, or isotype controls (PharMingen, Mississauga, Ontario, Canada). The C1.7 Ab was purchased from Biodesign International (Kennebunkport, ME).

Chromium release assays (CRA)

CRA were performed using standard protocols. Effector cells were either nonadherent PBMC or LAK cells, and target cells were ^{51}Cr-labeled K562, MV4;11, or Raji. Plates were incubated for 6–8 h at 37°C. Supernatants (100 μl/well) were collected and γ radiation was measured. Spontaneous release was determined by incubation of target cells in medium alone, and maximum release was determined by the addition of 100 μl of 5% Triton X-100 detergent to target cell suspension. Specific lysis (%) was calculated according to the formula: 100 (experimental release − spontaneous release)/(maximum release − spontaneous release). For 2B4 augmentation assays, mAb (isotype control or C1.7) was added directly to the plated samples at a final concentration of 0.2 μg/ml.

LAK cell generation

Nonadherent PBMCs were plated at 2×10^6/ml in six-well plates in complete medium with 1000 IU/ml recombinant human IL-2 (PharMingen). The supernatant (nonadherent cells and media) was removed on day 3 and replaced with 2 ml of media, 2 ml of medium, and 500 IU/ml of IL-2. Every 3 days, for 12 days, media was replaced accordingly. On day 14, adherent cells were harvested for use in cytotoxicity assays.

Expression analysis

Cells were lysed in Trizol (Life Technologies, Grand Island, NY) and RNA was isolated according to the manufacturer's instructions. SAP was amplified by PCR from oligo(dT) cDNA using the forward primer 5′-GAG GAA TTC AGG CCA TGG ACG CAG TGG C-3′ and reverse primer 5′-AAG CCGCTC GAG TTT TAT TTT TCT TCA TG-3′. The PCR parameters were 95°C × 30 s, 60°C × 30 s, and 72°C × 1 min for 30 cycles. DNA from PBMC was purified using the PureGene DNA isolation Kit (Gentra Systems, Minneapolis, MN). Amplification of DNA from exon 2 of SAP was performed using SAP exon 2 forward primer sequence 5′-CAA TGA CAC CAT ATA CGT GT-3′ and SAP exon 2 reverse primer sequence 5′-GCT TCC TTA ATG ATC CAT GA-3′.

Results

NK cytotoxicity is defective in males carrying a mutation of SAP

Nonadherent PBMC derived from healthy controls and two XLP patients were assessed for their ability to lyse K562 cells in standard CRA. In accordance with previous reports (5, 9), NK cells obtained from XLP patients effected significantly less cytotoxicity against target cells than NK cells obtained from healthy controls (Fig. 1; $p < 0.005$ at all E:T ratios, Student's *t* test). To determine whether equivalent numbers of NK cells were present in each assay, we immunophenotyped the effector cells to determine the proportion of CD3-CD56$^+$ cells. The numbers of NK cells present in the effector cell fraction from healthy control and XLP patients ranged from 7 to 19 and 5 to 7%, respectively (data not shown). However, the vast majority of healthy controls had between 7 and 10% NK cells in their effector PBMC, suggesting that XLP patients have only a modest decrease in numbers of NK cells and that this decrease in numbers alone cannot account for the large decrease in cytotoxicity. Nevertheless, because the proportion of NK cells

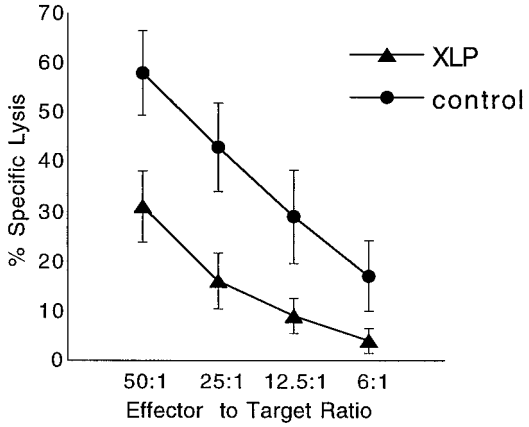

FIGURE 1. NK cytotoxic function is diminished in males carrying a mutation of SAP. Nonadherent PBMC obtained from healthy controls (n = 8, ●) and XLP males (n = 2, ▲) were assayed for lytic activity against ^{51}Cr-labeled K562 cells. All assays were performed in triplicate and on three separate occasions. Each data point is the mean value of the repeated experiments and the error bars refer to the SD generated from the three independent assays.

present within the effector cell population varied considerably among normal controls, we could not conclude with certainty that the reduced cytotoxicity was due to an intrinsic NK defect and not to fewer absolute numbers of effector cells. Therefore, we sought to characterize the cytotoxic function of LAK cells generated from PBMC, reasoning that this lymphocyte subset would represent a more homogenous population of effector cells.

LAK cell cytotoxicity is reduced in XLP

We generated long-term cultured LAK cells by incubating nonadherent PBMC from two XLP patients and healthy controls (including an unaffected sibling) with recombinant IL-2. LAK cultures generated from XLP patients and controls were assessed for their capacity to mediate lysis of the NK-resistant cell line, Raji (Fig. 2*a*). Equivalent numbers of cultured LAK cells from the XLP patients effected significantly lower killing than those generated from healthy controls, indicating that LAK cells generated from XLP patients possess a profound defect in cytotoxic function. To determine whether the effector cells established from XLP patients and controls were phenotypically similar, LAK cells were stained for surface expression of CD3 and CD56 (Fig. 2, *b* and *c*). Interestingly, LAK cells obtained from XLP patients yielded substantially fewer CD3$^-$CD56$^+$ cells than healthy controls, suggesting a defect in generating this particular subset of LAK effector cells. Furthermore, because the proportion of CD3$^+$CD56$^+$ cytokine-induced killer cells was similar in both groups, and because this population of cells normally exhibits potent cytotoxicity (21, 22), the inability of these effectors to kill Raji targets implies that these cells may be similarly affected by mutations in SAP.

SAP is expressed in NK and LAK cells

Because the cytotoxic function of both NK and LAK cells was deficient in the XLP patients, we sought to establish whether SAP is normally expressed in these lymphocyte subsets to correlate aberrant function with the Arg^{55}Leu mutation. Expression of SAP mRNA was detected by RT-PCR in an NK-derived cell line, NK-92ci (23), IL-2 established LAK cells from XLP patients or healthy controls and primary NK cells (Fig. 3). This observation suggests that the mutant form of SAP, which is expressed in NK and LAK cells, might account for their defective cytotoxic function.

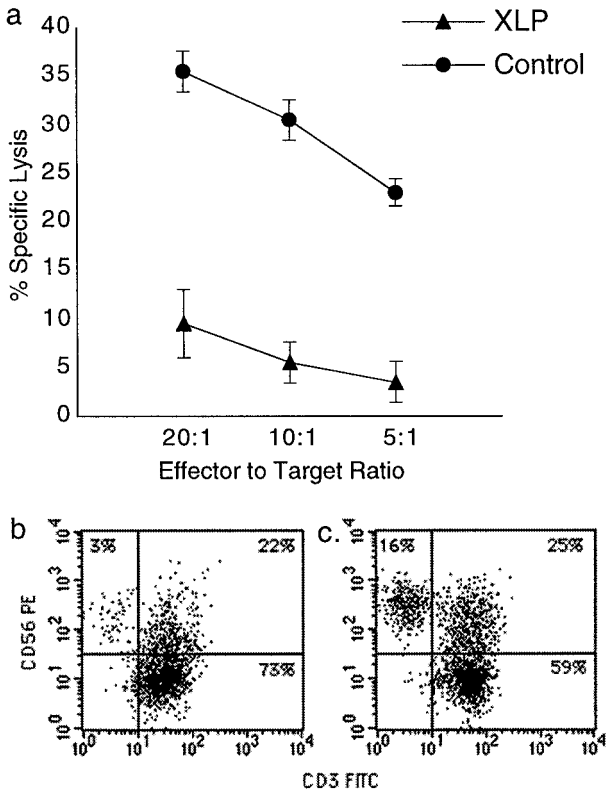

FIGURE 2. LAK cell function and immunophenotype in males carrying a mutation of SAP. *a*, LAK cells cultured from the PBMC of two XLP males (▲) or healthy controls (●) were tested for lytic function against ^{51}Cr -labeled Raji cells. Assays were performed three times, and each result represents the average of the three experiments. Error bars refer to the SD generated from the repeated assays. LAK cells derived from an XLP patient (*b*) and a healthy control (*c*) were stained for CD3 (FITC) and CD56 (PE) expression.

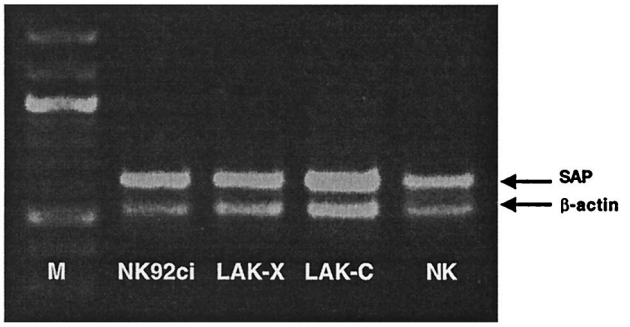

FIGURE 3. Expression of SAP in NK and LAK cells. NK cells were purified by positive and negative selection methods using Abs conjugated to magnetic beads (Miltenyi-Biotec). cDNA from NK92ci cell line (NK92ci), XLP LAK cells (LAK-X), control LAK cells (LAK-C), and primary NK cells (NK) were subjected to PCR using primers specific for SAP cDNA (expected PCR product size = 630 bp).

Ligation of 2B4 augments NK cytotoxicity

Most clinically defined cases of XLP studied so far have been associated with mutations of SAP (24). Because SAP has been shown to associate with the intracellular domain of human 2B4 (13), an activating molecule expressed on human NK cells (16, 17, 20), we assessed the consequence of 2B4 ligation on NK-mediated cytotoxicity. Using C1.7, a mAb with specificity for the human 2B4 Ag (25), we first determined the effect of 2B4 ligation on NK cytotoxicity using nonadherent PBMC derived from healthy volunteers as effector cells. Although 2B4 is expressed by several leukocyte populations, including $CD8^+$ T cells, the ligation of 2B4 induces effector functions and biochemical changes solely in NK cells (26). Therefore, augmentation of cytotoxicity by the addition of mAbC1.7 reflects the specific activity of NK cells and not other cytotoxic lymphocytes. Fig. 4*a* demonstrates the effect of adding mAbC1.7 to a standard NK cell CRA. Addition of mAbC1.7 (to a final concentration of 0.2 μg/ml) significantly increased specific lysis by healthy control-derived NK cells, whereas the addition of an isotype-matched Ab (directed against CD57) failed to influence cytotoxicity. The augmentation effect reflects enhanced spontaneous cytotoxicity and not Ab-dependent cellular cytotoxicity (ADCC) as K562 cells do not express 2B4 (Fig. 4*d*). To further confirm this result, we reassessed this phenomenon using an NK effector cell line, NK-92ci (23), which also expresses the 2B4 molecule (Fig. 4*c*). Addition of mAbC1.7 reproducibly augmented NK-92ci-mediated cytotoxicity against the myeloid leukemic cell line, MV4;11 (27), whereas addition of an isotype-matched Ab directed against another highly expressed surface molecule on NK-92ci (CD28) failed to enhance killing (Fig. 4*b*). Again, the possibility that ADCC may have confounded the enhancing effects of mAbC1.7 was ruled out as NK-92ci does not express CD16 (Fig. 6*d*). These results, taken together, indicate that ligation of 2B4 by mAbC1.7 specifically augments killing by non-HLA restricted effector cells via a spontaneous cytotoxic mechanism.

Ligation of 2B4 fails to augment NK cytotoxicity in XLP patients

We subsequently determined the ability of 2B4 ligation to augment NK cytotoxicity in PBMC obtained from XLP patients. Nonadherent PBMCs from healthy controls (n = 7) and XLP (n = 2) patients were repeatedly tested for their ability to lyse ^{51}Cr-labeled K562 target cells in the presence or absence of mAbC1.7. The addition of mAbC1.7 significantly augmented the specific lysis of NK cells obtained from healthy controls. However, mAbC1.7 ligation of 2B4 failed to augment the lytic activity of NK cells obtained from XLP patients (Fig. 6). Therefore, aside from exhibiting a reduced basal level of cytotoxicity, NK cells from XLP patients are unresponsive to 2B4-mediated enhancement of cytotoxicity.

Augmentation of NK lysis by 2B4 ligation is dependent on target cell expression of CD48

Because the natural ligand for 2B4 is CD48 (16, 17, 20), we next investigated whether the augmentation of NK cytotoxic function by ligation of 2B4 is influenced by the use of target cells that express CD48. We repeated the cytotoxicity assays using as effector cells NK-92ci but, rather than using CD48-negative target cells such as MV4:11 or K562, we used the CD48-positive cell line, Raji (Fig. 5, *a–c*). We reasoned that physiological ligation of 2B4 by endogenously expressed CD48 might conceal the effects of exogenous ligation by mAbC1.7. As predicted, ligation of 2B4 failed to augment NK-92ci cytotoxicity against Raji cells (Fig. 5*e*).

Discussion

In this report, we describe the consequences that a single nucleotide mutation of SAP has on the cytotoxic function of NK cells. We examined NK cells because of their potential role in controlling early viral infection and because of previous reports of NK cell dysfunction in XLP patients (5, 8, 9). NK cell lytic function was significantly decreased in two males carrying an $Arg^{55}Leu$ mutation in SAP. Moreover, LAK cells generated from the XLP

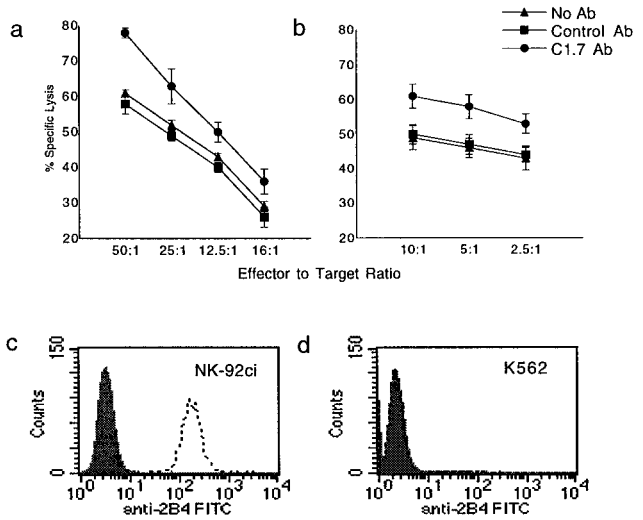

FIGURE 4. Ligation of 2B4 augments cytotoxic function in NK cells from a male carrying a mutation of SAP. *a*, Nonadherent PBMC obtained from a healthy control were tested in two independent assays for cytotoxic function against ^{51}Cr-labeled K562 target cells without Ab (▲), with C1.7 (●), or with an isotype control Ab to CD57 (■). *b*, NK-92ci were tested in three independent assays for cytotoxic function against ^{51}Cr-labeled MV4;11 target cells without Ab (▲) or with 0.2 mg/ml of either C1.7 (●) or an isotype control to CD28 (■). Each result is the average value of the repeated experiments, and the error bars refer to the SD generated from the repeated assays. *c* and *d*, Expression of 2B4 on NK-92ci (*c*) and K562 (*d*) cells using mAbC1.7.

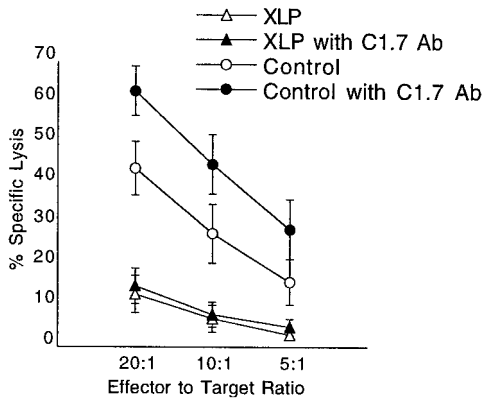

FIGURE 5. Ligation of 2B4 fails to augment cytotoxic function in NK cells from males carrying a mutation of SAP. Nonadherent PBMC from healthy controls (n = 7, ●, ○) or XLP patients (n = 2, ▲, △) were tested in three independent assays for cytotoxicity against ^{51}Cr -labeled K562 cells in the presence (●, ▲) or absence (○, △) of C1.7 Ab (0.2 μg/ml). Each result is the mean value of the repeated experiments, and the error bars refer to the SD generated from the independent assays.

patients displayed markedly diminished cytotoxicity, raising the possibility that LAK dysfunction may also contribute to the immunodeficiency phenotype observed in XLP patients. This finding provides evidence that NK cell function, as defined in terms of LAK cell-mediated cytotoxicity, is significantly diminished in XLP patients independent of absolute effector cell numbers, and that NK cells from XLP patients respond poorly to IL-2-induced LAK cell activation. Moreover, the defect in LAK cell function raises the possibility that XLP may, in the future, be treated by the adoptive transfer of autologous LAK cells that have been modified to express normal SAP.

In addition, we have demonstrated that SAP is expressed in NK and LAK cells derived from healthy controls as well as in LAK cells derived from XLP patients with an Arg^{55}Leu SAP mutation. Therefore, the altered NK/LAK cell function observed in XLP patients correlates with the expression of the Arg^{55}Leu mutant form of the SAP gene and may explain why XLP patients are unable to control infection with a common viral pathogen and maintain immune surveillance against lymphoid malignancies.

To examine the role of 2B4 in a uniform population of effector cells, we assessed the ability of the NK-derived cell line, NK-92ci, to lyse different target cell lines. NK-92ci effected enhanced lysis only of those targets that lacked expression of the 2B4 ligand, CD48, implying that target cell expression of CD48 conceals the effect of mAbC1.7. In support of this observation, transfection of K562 cells with CD48 reduced their susceptibility to anti-2B4-induced augmentation while enhancing their overall susceptibility to lysis (26). Interestingly, the susceptibility of CD48-transfected K562 cells to lysis could not be entirely accounted for by the transfection, implying that additional factor(s) must contribute to the augmenting effects of mAbC1.7. Moreover, addition of Fc fragments to NK cytotoxicity assays failed to entirely block the effect of 2B4 ligation (26). Although many of the CD48-negative cell lines express Fc receptors on the surface, this phenomenon was not due to ADCC because NK-92ci does not express CD16, and K562 does not express 2B4. An alternative explanation is that myeloid cells, which are primarily CD48 negative (28), may express an additional surface molecule that provides the primary stimulus needed to initiate costimulation through 2B4. The role of ADCC in the clinical phenotype of XLP is unclear. Some investigators report normal ADCC function in XLP patients (8), whereas others suggest that ADCC may be abnormal (6). We are currently investigating the ADCC function of NK cells obtained from these affected boys to determine whether the defect in 2B4

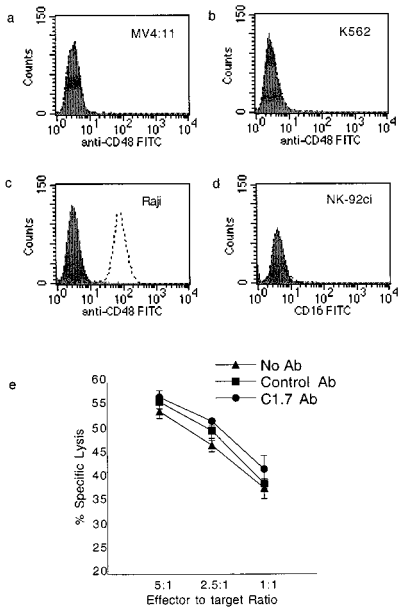

FIGURE 6. Correlation of CD48 expression with 2B4-augmented cytotoxicity. Expression of CD48 on MV4:11 (*a*), K562 (*b*), and Raji (*c*) was assessed by staining cells with FITC-conjugated anti-CD48. Expression of CD16 on NK-92ci was examined by staining cells with FITC-conjugated anti-CD16 (*d*). The NK-92ci cell line was tested for cytotoxic function against a CD48-positive cell line, Raji (*e*) by using ^{51}Cr-labeled targets without Ab (▲) or with 0.2 mg/ml of either C1.7 (●) or an isotype control to CD28 (■). Each result is the average value of two repeated experiments, and the error bars refer to the SD generated from the repeated assays.

augmentation is one of several global defects in NK cell signaling or whether perhaps SAP is involved in ADCC.

Although there is considerable evidence supporting the role of CTL in controlling EBV infection (29–31), less is known about the specific function of NK or LAK cells in controlling either acute or latent EBV infection. NK cells are known both to proliferate during acute EBV infection (32) and to regulate the outgrowth of EBV-transformed B cells in vitro (33, 34), and LAK cells have been useful in the treatment of posttransplantation EBV-associated malignancies (35, 36). Because the major defect in XLP patients is an inability to control EBV infection, our findings provide evidence that NK and/or LAK cells may be vital for protective immunity to EBV.

How might defective NK or LAK cells account for the EBV-selective immunodeficiency seen in XLP patients? CD48, the natural ligand for human 2B4, has been shown to be dramatically up-regulated on B lymphocytes following EBV infection (37). Subsequent interaction of CD48 with 2B4 may potentiate the cytotoxic effects of normal NK or LAK cells on EBV-infected cells, and this interaction may be an essential element in the control of viral replication. Alternatively, NK and/or LAK cells may play a role in regulating the cellular response to viral infection, and functional expression of 2B4 may be critical for maintaining CTL homeostasis following EBV infection. This latter mechanism would account for the uncontrolled lymphoproliferation seen in XLP patients following exposure to EBV.

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