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Abnormal T Cell Receptor Signal Transduction of CD4 Th Cells in X-Linked Lymphoproliferative Syndrome

Hiroyuki Nakamura,* Jodi Zarycki,* John L. Sullivan,† and Jae U. Jung‡

The molecular basis of X-linked lymphoproliferative (XLP) disease has been attributed to mutations in the signaling lymphocytic activation molecule-associated protein (SAP), an src homology 2 domain-containing intracellular signaling molecule known to interact with the lymphocyte-activating surface receptors signaling lymphocytic activation molecule and 2B4. To investigate the effect of SAP defects on TCR signal transduction, herpesvirus saimiri-immortalized CD4 Th cells from XLP patients and normal healthy individuals were examined for their response to TCR stimulation. CD4 T cells of XLP patients displayed elevated levels of tyrosine phosphorylation compared with CD4 T cells from healthy individuals. In addition, downstream serine/threonine kinases are constitutively active in CD4 T cells of XLP patients. In contrast, TCR-mediated activation of Akt, c-Jun-NH2-terminal kinases, and extracellular signal-regulated kinases in XLP CD4 T cells was transient and rapidly diminished when compared with that in control CD4 T cells. Consequently, XLP CD4 T cells exhibited severe defects in up-regulation of IL-2 and IFN-γ cytokine production upon TCR stimulation and in MLRs. Finally, SAP specifically interacted with a 75-kDa tyrosine-phosphorylated protein upon TCR stimulation. These results demonstrate that CD4 T cells from XLP patients exhibit aberrant TCR signal transduction and that the defect in SAP function is likely responsible for this phenotype. The Journal of Immunology, 2001, 167:2657–2665.

Epstein-Barr virus is a human γ-1 herpesvirus that infects most people in early life. Infection after early childhood frequently results in infectious mononucleosis (IM), a systemic illness that is caused by the proliferation of EBV-infected B lymphocytes and unusually strong NK and virus-specific T lymphocyte responses (1). EBV has been shown to be associated with various diseases in humans (2–4). These include Burkitt’s lymphoma, nasopharyngeal carcinoma, Hodgkin’s Disease, and T cell lymphomas (5–13).

A rare disease called X-linked lymphoproliferative disease (XLP), or Duncan’s disease, is an inherited syndrome characterized by uncontrolled EBV infection leading to severe or fulminant IM, acquired agammaglobulinemia, and malignant lymphoma (14). It has been suggested that the inability of the immune system of XLP patients to control EBV-infected B cells is likely due to defects of Th cells, CTls, and NK cells (15–17). The gene defective in XLP has recently been identified, both by positional cloning and functional cloning approaches, and has been designated src homology 2 (SH2) domain protein 1A, Duncan’s disease SH2 protein, or signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) (18–20). SAP is a small protein of 128 residues that consists of a single SH2 domain with a short carboxyl-terminal region (18–20). It has been shown to bind the cytoplasmic tail of SLAM and 2B4, cell surface receptors of the CD2 superfamily, and this interaction has been shown to be crucial to their signal transduction activity. SLAM is expressed on T and B lymphocytes, mediates homotypic binding, and promotes T cell costimulation, proliferation, and production of Th1 cytokines (21–24). During EBV infection, SLAM-SLAM interactions at the interface between EBV-infected B cells and T cells may promote the development of EBV-specific Th responses. The cytoplasmic region of SLAM contains three tyrosine-based motifs. Surprisingly, SAP binds to one of these motifs irrespective of the phosphorylation state of this site, and this binding promotes signaling by preventing recruitment of the SH2-containing protein tyrosine phosphatase (SHP-2) (20). In XLP patients, SAP deficiency and subsequent recruitment of SHP-2 may impair SLAM signal transduction, leading to reduced IFN-γ production by Th cells (20, 25, 26).

Another receptor of the CD2 superfamily, 2B4, contains cytoplasmic motifs similar to those found in SLAM. It is expressed on T and NK cells, and the engagement of 2B4 on NK cells has been demonstrated to promote spontaneous cytotoxicity and to augment secretion of IFN-γ (27–30). Recent studies have demonstrated that the cytoplasmic region of 2B4 also interacts SAP (31). In fact, ligation of the 2B4 ligand on NK cells from a XLP patients failed to initiate cytotoxicity, implying that the defect in SAP function may contribute to the pathogenesis of XLP syndrome by reducing NK cell lysis of EBV-infected B cells (31–36).

Despite extensive studies of SAP function in SLAM and 2B4 signal transduction, little is known about the role of SAP in TCR-mediated signal transduction. We hypothesize that the inability of the immune system to control EBV-infected B lymphocytes is partly due to defects in Th cell responses (15, 20, 37). To investigate this hypothesis, we generated continuously growing CD4 Th
cells of XLP patients using herpesvirus saimiri (HVS). HVS has been shown to transform primary human T lymphocytes to continuous growth, while maintaining the original phenotype and the functional properties (38–43). Using these cell lines, we demonstrated that CD4 T cells from XLP patients exhibited defects in TCR signaling, as evidenced by the inability to produce cytokines. These findings suggest that, in addition to SLAM and 2B4 signal transduction, SAP is involved in the TCR signal transduction pathway and that alteration of the TCR pathway may potentially contribute to the defective EBV-specific immunity in XLP patients.

Materials and Methods

Cell culture and transfection

HVS-transformed cells were grown with RPMI 1640 medium supplemented with 20% FBS (Life Technologies, Rockville, MD), and COS-1 cells were grown in DMEM (Life Technologies) supplemented with 10% FBS. A lipofectAMINE PLUS (Life Technologies) transfection procedure was used for transient expression in COS-1 cells.

In vitro immortalization of primary lymphocytes

PBMC were isolated from 10 ml heparinized blood specimens from healthy volunteers and two individuals with XLP from a previously well-characterized kindred (44) by centrifugation through lymphocyte separation medium (Organon Teknika, Malvern, PA), followed by washing in RPMI 1640 culture medium. PBMC from each individual were individually washed, resuspended in RPMI 1640, and then distributed in 1 ml volumes containing ~106 cells into 12-well tissue culture plates. Cells were infected at a multiplicity of infection ranging from 1 to 5 with 1 ml purified HVS viral stocks or with 1 ml EBV-containing supernatant from B95-8 cells. Cells were maintained in RPMI 1640 growth medium, which was changed every 3–4 days. Immortalization or cell death was assessed microscopically.

RNA extraction and RT-PCR

Total RNA from HVS-transformed cells was isolated using TRIzol Reagent (Life Technologies) according to the manufacturer’s instructions. Approximately 5 μg total RNA was reverse-transcribed by SuperScript II RNase H− reverse transcriptase (Life Technologies) in a 20 μl reaction mixture with random hexamers for 50 min at 42°C. As a control, cDNA synthesis was performed without the reverse transcriptase. One microliter of the same cDNA preparation was used for PCR amplification in a 50-μl volume of final reaction mixture with 0.5 μM specific primers (5’ primer, GCCGCTCTGATGACGGCATCTCC; and 3’ primer, ATGTTAAGACCGTTTCAGGCAGACATC-3’). Ten microliters of PCR mixtures were electrophoresed through 2% agarose gel. Expected RNA size from the PCR amplification was 630 bp.

Plasmid constructions

To make 6× histidine-tagged SAP cDNA and its mutant form, SAP cDNA was amplified by using following primers: 5’-GGCGAATTCCGGCCGACCATGGACGGCATCTCC-3’ and 5’-CGCTCTGATGACCCGACCATCTCC-3’. Each PCR product was cloned into the EcoRI and XbaI cloning sites of pET11a/Myc-His A expression vector (Invitrogen, Carlsbad, CA). The EcoRI/XbaI fragment containing the His-tagged SAP sequence was cloned into pLPXCl vector (CLONTECH Laboratories, Palo Alto, CA). All PCR-amplified DNA fragments were completely sequenced to verify the presence of the correct sequence and the absence of any other changes.

Cell stimulation and lysis

For Ab stimulation, 2 × 106 cells were resuspended in 200 μl complete medium. After equilibration to 37°C for 10 min, the cells were stimulated with purified anti-CD3 mAb (OKT3; M-450 CD3 Dynabeads; Dynal Biotech, Great Neck, NY) for the indicated duration and subsequently transferred to dry ice. In some cases, 2 × 106 cells were stimulated with 20 ng/ml 12-0-tetradecanoylphorbol-13-acetate (TPA) for 6 or 12 h. Cells were lysed in ice-cold lysis buffer (0.15 M NaCl, 1% Nonidet P-40, 50 mM HEPES buffer (pH 7.5), 2 mM Na3VO4, 10 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 2 mM PMSF). After incubation in ice for 30 min, lysates were precleared by centrifugation at 4°C for 15 min and used for immunoblotting, immunoprecipitation, or kinase assays.

Immunoprecipitation, immunoblotting, and kinase assays

Lysates were precleared with protein A-Sepharose (Pierce, Rockford, IL) on ice for 30 min and then mixed with the appropriate Ab and protein A-Sepharose for 12 h at 4°C. Immunocomplexes were recovered by centrifugation and washed three times with lysis buffer. Precleared lysates or immunocomplexes were subjected to SDS-PAGE analysis. Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were blocked in TBST and 10% nonfat milk, incubated with the indicated primary Abs and HRP-conjugated secondary Abs, and subjected to ECL (SuperSignal; Pierce).

Sources of commercial Abs are as follows: anti-CD3 (6B10.2), anti-ζ-associated protein 70 (ZAP70) (LR), anti-CD2 (C-15), anti-APC (FL-128), anti-SLAM (N-19), anti-INK1 (C-17), and anti-glycogen synthase kinase (GSK)-3β (0011-A) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); HRP-conjugated anti-phosphotyrosine (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY); and anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182), anti-p38 MAPK, anti-phospho-stress-activated protein kinase/JNK (Thr183/Tyr185), anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-GSK-3α and -β (Ser9/13) were obtained from Cell Signaling Technology (Beverly, MA). Akt kinase assays were performed with the Akt kinase assay kit (Cell Signaling Technology) according to the manufacturer’s instructions.

Measurements of secreted cytokines

A total of 1 × 106 of the indicated cells were cultured in 24-well tissue culture plates with or without anti-CD3 Ab in 1 ml RPMI 1640 supplemented with 20% FBS. The supernatants were harvested 24 h later, and the cytokine production was measured by ELISA kits (BD Pharmingen, San Diego, CA).

Metabolic labeling and pulse-chase analysis

Cos-1 cells were cultured to 70–80% confluence in 10-cm-diameter culture dishes. After 48 h of transfection with pEFi-wild-type (wt) SAP or pEFi-SAP Q99P, cells were rinsed twice with PBS, washed once with labeling medium (RPMI 1640 minus methionine and cysteine; Life Technologies), and starved for 1 h by incubation in the labeling medium. After starvation, 100 μCi [35S]methionine and [35S]cysteine (New England Nuclear, Boston, MA) was added to the labeling medium, and the cells were incubated at 37°C for 2 h. Following removal of the labeling medium, cells were cultured in DMEM supplemented with 10% FBS and chased for 1, 3, 6, or 9 h. Cells were harvested and lysed with the lysis buffer.

Flow cytometric analysis

A total of 5 × 105 cells were washed with PBS containing 2% FCS and incubated with FITC-conjugated or PE-conjugated mAbs for 30 min at 4°C. After washing, each sample was fixed with 2% paraformaldehyde solution, and flow cytometric analysis was performed with a FACScan (BD Biosciences, Mountain View, CA). Sources of commercial Abs are as follows: RPA-2.10 Ab for CD2, H1T3a Ab for CD3, RPA-T4 Ab for CD4, PE-FITC-antibodies for CD8 and HLA-ABC; C1.7.1 Ab for CD5, B159 Ab for CD56, DX2 Ab for CD95, T10B9.1A-31 Ab for TCRαβ, RPA-2.10 Ab for CD2, HIT3a Ab for CD3, RPA-T4 Ab for CD4, H1T3a Ab for CD8, H1T9 Ab for CD19, B16H Ab for CD30, H130 Ab for CD45, B159 Ab for CD56, DX2 Ab for CD95, T10B9.1A-31 Ab for TCRβ, and A12 Ab for SLAM were obtained from BD Pharmingen; L243 Ab for HLA-DR was obtained from BD Biosciences; and C1.7.1 Ab for CD5 from Immunotech (Westbrook, ME).

Results

Characterization of SAP mutation in XLP patients

Full-length SAP cDNA of two XLP siblings (Huami and Weljo) was amplified from PBMC by RT-PCR, followed by DNA sequence analysis. PBMC from two healthy volunteers (JJ and SC), Jurkat T lymphocytes, and BJAB B lymphocytes were used as controls. The full-length SAP gene was detected in PBMC of both XLP patients as well as PBMC of healthy individuals and Jurkat T cells, whereas it was not detected in BJAB B lymphocytes (Fig. 1 and data not shown). DNA sequence analysis revealed that the SAP genes of both XLP patients harbored a missense mutation at nucleotide 382 (A→C), which changed glutamate at residue 99 to proline. SAP genes from the PBMC of JJ and SC were found to have wt sequences (data not shown).
A 6× His-tagged wt SAP and SAP Q99P mutant were expressed in COS-1 cells using two different promoters, elongation factor 1 (EF1) promoter and CMV early promoter. An immunoblot assay with an anti-His Ab showed that the steady-state level of the SAP Q99P mutant expression was markedly lower than that of wt SAP (Fig. 2A). To investigate whether the low level of expression was attributable to protein stability, COS-1 cells were transfected with an expression vector containing the wt SAP or SAP Q99P mutant metabolically labeled with $^{[35}S] $methionine for 2 h and chased for 1, 3, 6, and 9 h. Cell were lysed by 1% Nonidet P-40 detergent and, $^{[35}S $labeled SAP protein was precipitated with an anti-His Ab. This showed that the half-life of the SAP Q99P mutant was drastically reduced compared with that of wt SAP (Fig. 2B). The wt SAP protein was relatively stable with an approximate half-life of 9 h in culture (Fig. 2B). In contrast, the SAP Q99P mutant was rapidly degraded and was undetectable after 3 h of chase (Fig. 2B). These results indicate that the Q99P mutation of SAP drastically reduces the protein’s stability.

In vitro immortalization of primary lymphocytes from XLP patients with EBV and HVS

Human B and T lymphocytes are efficiently immortalized to cytokine-independent, permanent cell growth by infection with EBV and HVS, respectively (38, 45). In addition, HVS-immortalized lymphocytes have been shown to maintain their original phenotypes and functional properties (38–43). Therefore, this in vitro immortalization was used to generate continuously growing, autologous B and T lymphocytes of XLP patients. Lymphocytes of two healthy individuals (JJ and SC) were also included as controls. EBV and HVS were separately added to aliquots of unstimulated PBMCs from Huami and Weljo, XLP patients, and JJ and SC, healthy individuals. Months after infection, continuously growing IL-2-independent B and T cells were established. No significant difference in growth rate was observed between XLP and control lymphocytes after immortalization with EBV or HVS. To examine SAP expression, equal amounts of proteins from HVS-immortalized T cells were used for immunoblot assay with an anti-SAP Ab. Jurkat T cells were included as controls. This showed that SAP protein was readily detected in Jurkat T cells and the HVS-transformed JJ and SC T cells, whereas it was not detected in HVS-transformed Huami and Weljo T cells (Fig. 3).

Flow cytometric analysis showed that HVS-transformed control JJ and SC cells and HVS-transformed XLP Huami and Weljo T cells were CD3$^+$CD4$^+$CD8$^-$ T cells, suggesting that these cells are a population of Th cells. There was no significant difference of surface expression of CD2, CD3, CD4, CD25, CD45, CD86, CD95, TCRaβ, HLA-DR, SLAM, and 2B4 lymphocyte Ags other than CD30 (data not shown). A large population of the Huami and Weljo T cells displayed higher surface expression of CD30 than control JJ and SC T cells (data not shown). In addition, no apparent difference of surface Ag expressions, including IgM, CD19, CD23, CD80, CD86, HLA-DR, and SLAM, was also observed between EBV-transformed Huami B cells and EBV-transformed JJ and SC B cells (data not shown).

Enhanced tyrosine phosphorylation of XLP CD4$^+$ T cells upon TCR stimulation

The biochemical event subsequent to TCR stimulation is the induction of tyrosine phosphorylation of a number of cellular proteins (46–49). We examined the effects of the SAP mutation on cellular tyrosine phosphorylation upon TCR stimulation. After anti-CD3 Ab stimulation of HVS-transformed JJ and SC CD4 T cells and HVS-transformed Huami and Weljo CD4 T cells, the course of tyrosine phosphorylation induction was observed by immunoblot assay with an anti-phosphotyrosine Ab (Fig. 4). Stimulation with an anti-CD3 Ab rapidly induced cellular tyrosine phosphorylation in all cell lines (Fig. 4). However, the level of tyrosine phosphorylation after anti-CD3 Ab stimulation was not only higher but also more prolonged in Huami and Weljo CD4 T cells than in JJ and SC CD4 T cells (Fig. 4). Specifically, proteins of 25, 35, 60, 70, 120, and 160 kDa in Huami and Weljo CD4 T displayed an enhanced...
level of tyrosine phosphorylation when compared with those in JJ and SC cells (Fig. 4). In addition, the basal level of tyrosine phosphorylation was slightly enhanced in the Huami and Weljo CD4 T cells compared with the JJ and SC CD4 T cells (Fig. 4). These results demonstrate that tyrosine phosphorylation upon TCR stimulation is more extensive in HVS-transformed Huami and Weljo CD4 T cells than that in HVS-transformed JJ and SC CD4 T cells.

Enhanced tyrosine phosphorylation of CD3 ζ-chain, ZAP70, and Cbl in XLP CD4+ T cells

To delineate the enhanced tyrosine phosphorylation of XLP CD4 T cells, we further examined the level of tyrosine phosphorylation of several cellular proteins, including CD3ζ, ZAP70, and Cbl. These signaling molecules are extensively tyrosine phosphorylated upon TCR stimulation, and this modification is important for their signal-transducing activity in TCR signal transduction pathway. After stimulation with an anti-CD3 Ab, precleared lysates of HVS-transformed JJ and Huami CD4 T cells were used for immunoprecipitation with anti-CD3ζ, anti-ZAP70, and anti-Cbl Abs, followed by immunoblot assay with an anti-phosphotyrosine Ab. Upon TCR stimulation, tyrosine phosphorylation of CD3ζ, ZAP70, and Cbl proteins were induced in both JJ and Huami T cells (Fig. 5A). However, as seen with overall tyrosine phosphorylation, basal and TCR-induced tyrosine phosphorylation of CD3ζ, ZAP70, and Cbl was significantly higher in Huami CD4 T cells than in JJ CD4 T cells (Fig. 5A). These results demonstrate that XLP CD4 T cells have an enhanced level of basal and TCR-induced tyrosine phosphorylation of CD3ζ, ZAP70, and Cbl.

Previous reports have demonstrated that SAP interacts with several tyrosine-phosphorylated cellular proteins including Dok1, SLAM, Ku70, and 2B4 (20, 31, 50). To further investigate the role of SAP in the TCR signal transduction pathway, we examined whether SAP interacted with these cellular proteins upon TCR stimulation. After the stimulation with an anti-CD3 Ab, precleared lysates of HVS-transformed JJ CD4 T cells and HVS-transformed Huami CD4 T cells were used for immunoprecipitation with an anti-SAP Ab, followed by immunoblot assay with an anti-phosphotyrosine Ab. This experiment demonstrated that the SAP protein strongly interacted with a 75-kDa tyrosine-phosphorylated protein upon TCR stimulation (Fig. 5B). However, extensive immunoblot analyses revealed that the associated protein was not Dok1, ZAP70, SLAM, 2B4, SHP-2, Ku70, Ku86, or p85 phosphatidylinositol 3-kinase (data not shown). This suggests that an additional cellular protein that remains to be identified may be important for SAP function in TCR-mediated signal transduction.

Altered activation of ERK1/2, JNK, p38, and Akt activities in XLP CD4+ T cells upon TCR stimulation

Downstream of the early activation events, three major subfamilies of MAPKs, ERKs, JNKs, and p38 kinase are activated upon TCR stimulation (51–56). In addition, Akt/protein kinase B and GSK, which are pleotropic protein serine/threonine kinases, are implicated in a variety of cellular functions such as survival, metabolism, transcription, and translation (57–62). Specific phosphorylations of these kinases have been shown to be critical for their kinase activation (63, 64). To examine the effect of the SAP mutation on the downstream serine/threonine kinase activity, in situ kinase activities were monitored after TCR stimulation by immunoblot analysis with phospho-specific Abs that react only with the activated forms of these kinases. Upon anti-CD3 Ab stimulation, phosphorylation of ERK1/2, p38, and JNK were markedly increased in JJ, Huami, and Weljo CD4 T cells (Fig. 6A). However,
a significant level of phosphorylation of ERK1/2, p38, and JNK was detected in Huami and Weljo T cells without TCR stimulation, whereas it was not detected in JJ T cells (Fig. 6A). Conversely, TCR-induced phosphorylation of ERK1/2 and JNK rapidly declined after 5 min of stimulation in Huami and Weljo T cells, whereas it was prolonged until 30 min of stimulation in JJ T cells (Fig. 6A). This indicates that these serine/threonine kinases in HVS-transformed XLP CD4 T cells are transiently activated upon TCR stimulation.

Akt kinase of Huami and Weljo T cells also exhibited a pronounced difference from that of JJ T cells. Without TCR stimulation, Akt had a significantly higher level of phosphorylation in Huami and Weljo CD4 T cells compared with JJ T cells (Fig. 6A). However, as seen with ERK1/2, p38, and JNK, TCR-mediated phosphorylation of Akt kinase rapidly and drastically declined in Huami and Weljo T cells (Fig. 6A). Consistently, enzymatic activity of Akt in Huami T cells was constitutively active before stimulation, but it rapidly declined by 15 min of TCR stimulation (Fig. 6B). In contrast, the enzymatic activity of Akt in JJ T cells was not active without TCR stimulation, and it was strongly activated for a long period time upon TCR stimulation (Fig. 6B). Unlike Akt, no significant difference in the activation of GSK-3α and -3β kinases was observed between JJ and Huami cells (Fig. 6A). These results demonstrate that, despite their constitutive activation, TCR-induced activation of ERK1/2, p38, JNK, and Akt kinases is transient and rapidly declines in HVS-transformed XLP CD4 T cells. In contrast, when these cells were treated with the TPA stimulation that bypasses TCR signal transduction pathway, ERK1/2 and p38 kinase exhibited a similar level of activation in both HVS-transformed JJ and Huami CD4 T cells (Fig. 7). These results indicate that defect in SAP function is specifically responsible for the abnormal TCR signal transduction.

**Defects of cytokine production of XLP CD4 T cells upon SLAM and TCR stimulation**

Triggering of SLAM and TCR on CD4+ T cells has been shown to strongly induce IFN-γ production (21). In addition, Akt kinase has been shown to provide the costimulatory signal for up-regulation of IL-2 and IFN-γ production of CD4 Th cells (65). To examine the effect of the SAP defect on cytokine production, 1 × 10^5 HVS-transformed Huami, Weljo, and JJ CD4 T cells were engaged with an anti-SLAM Ab overnight, and IFN-γ production was assessed by ELISA. Although Huami and Weljo CD4 T cells had a high level of basal IFN-γ production, these cells showed little or no up-regulation of IFN-γ production upon anti-SLAM stimulation (Fig. 8A and data not shown). In contrast, JJ CD4 T cells exhibited a low level of IFN-γ production without stimulation, and a significant up-regulation of IFN-γ production was detected upon anti-SLAM stimulation (Fig. 8A). All three cells displayed an equivalent level of SLAM surface expression (data not shown).

To further investigate an effect of the SAP mutation on TCR signal transduction, 1 × 10^5 HVS-transformed Huami, Weljo, and JJ CD4 T cells were stimulated with anti-CD3 Ab overnight, and IFN-γ and IL-2 cytokine production was assessed by ELISA. As seen with an anti-SLAM stimulation, control JJ CD4+ T cells showed a drastic increase in IFN-γ and IL-2 production upon anti-CD3 stimulation, whereas Huami and Weljo CD4+ T cells did not up-regulate IFN-γ and IL-2 cytokine production under the same conditions (Fig. 8B). All three cells displayed an equivalent level of CD3 surface expression (data not shown). These results demonstrate that XLP CD4+ T cells exhibit defects in the up-regulation of cytokine production upon SLAM and TCR stimulations.
Defects of cytokine production in XLP CD4+ T cells upon MLR

A MLR induces drastic activation of target cells, which induces a significant level of cytokine production (66, 67). To further examine T cell responsiveness, we examined the MLR response of HVS-transformed control CD4 T cells and HVS-transformed XLP CD4 T cells with autologous or heterologous B lymphocytes by measuring IL-2 and IFN-γ cytokine production. After 24 h of mixing 1 × 10^6 autologous B cells and T cells, IFN-γ and IL-2 production was measured by ELISA. JJ CD4 T cells produced significant amounts of IL-2 and IFN-γ upon syngenic MLR, whereas Huami CD4 T cells showed little or no up-regulation of IL-2 and IFN-γ production under the same conditions (Fig. 9). To further examine T cell responsiveness, JJ T cells were mixed with Huami B cells, or Huami T cells were mixed with JJ B cells overnight. Allogenic MLR response was assessed by measuring IFN-γ and IL-2 production with ELISA. These experiments also showed that Huami CD4 T cells did not induce IL-2 and IFN-γ production upon allogenic MLR, whereas JJ CD4 T cells produced significant amounts of IL-2 and IFN-γ as they did upon syngenic MLR (Fig. 9). These results demonstrate that, besides abnormal SLAM and TCR signal transduction, XLP CD4+ T cells also exhibit severe defects in MLR.

Discussion

A rare disease called XLP, or Duncan’s disease, is an inherited syndrome characterized by uncontrolled EBV infection leading to severe or fulminant IM, acquired agammaglobulinemia, and malignant lymphoma (14). The molecular basis of XLP disease has been attributed to the mutations of SAP gene. In addition, the pathogenesis of the XLP syndrome has been suggested to be due to a defect of SAP function in NK cell lysis of EBV-infected B cells. In this report, we demonstrate that CD4 T cells from XLP patients exhibit an aberrant TCR signal transduction and that defects of SAP function are likely responsible for this phenotype. Furthermore, an abnormal TCR signal transduction pathway in XLP CD4 T cells results in inability to up-regulate cytokine production in response to several stimuli. These results suggest that, in addition to the lack of NK cell lysis, defects of CD4 Th cell responses may also contribute to abnormal immune responses in the control of EBV-infected B lymphocytes in XLP patients.
Two independent experiments. Huami and JJ CD4 T cells were engaged with 1/H11003 SH2 domain with a short carboxyl-terminal region (18). Defects of MLR-mediated up-regulation of IFN-γ and IL-2 production in XLP CD4 T cells. A total of 1 × 10⁶ HVS-transformed Huami and JJ CD4 T cells were engaged with 1 × 10⁶ syngenic or allogenic EBV-transformed Huami and JJ B cells for 24 h, and production of IFN-γ and IL-2 was assessed by ELISA. Numbers represent the average of two independent experiments.

FIGURE 9. Defects of MLR-mediated up-regulation of IFN-γ and IL-2 production in XLP CD4 T cells. A total of 1 × 10⁶ HVS-transformed Huami and JJ CD4 T cells were engaged with 1 × 10⁶ syngenic or allogenic EBV-transformed Huami and JJ B cells for 24 h, and production of IFN-γ and IL-2 was assessed by ELISA. Numbers represent the average of two independent experiments.

SAP is a small protein of 128 residues that consists of a single SH2 domain with a short carboxyl-terminal region (18–20). A large portion of SAP mutations result in truncation of the produced protein due to either nonsense or frame shift mutation or to gross deletion (18–20). In addition, several identified mutations introduce a change in the most conserved amino acid among SH2 domains. These include R32T, C42W, T53I, E67D, and T68I (18, 70). These mutations have been suggested to impair the SH2 function. Because glutamine at residue 99 is located outside of the SH2 domain, Q99P mutation does not appear to affect the SH2 function. Indeed, x-ray structural study suggests that Q99P mutation likely disrupts hydrophobic and hydrogen bonding interactions that are necessary for stabilizing the βG strand (71). Consistent with this hypothesis, we demonstrate that Q99P mutation of SAP in our XLP patients drastically diminishes its protein stability, demonstrated by the undetectable level of SAP protein in XLP CD4 T cells.

SAP protein functions as a regulator of the signal transduction pathways initiated by at least two distinct surface receptors belonging to the CD2 family: SLAM, which is expressed on T and B cells, and 2B4, which is primarily expressed on NK cells (20, 31). It has been shown that binding of SAP to SLAM prevents an association of SLAM with SHP-2 (20). In addition, this molecular interaction is crucial for transducing activating signals via SLAM to induce up-regulation of cytokine activating signals. In contrast, the signal mediated by 2B4 and SAP interaction affects not only the spontaneous cytotoxicity, but also the NK cell activation induced via CD16 and natural cytotoxicity receptors (32). In this report, we demonstrate that, besides SLAM and 2B4 signal transduction, SAP protein is involved in the TCR signal transduction pathway. The lack of a functional SAP protein in XLP CD4 T cells leads to aberrant TCR signal transduction. Because SAP is involved in a variety of cellular activities, its mutation in XLP patients may induce various defects in host immune effectors, including Th cells, CTLs, and NK cells, which lead to the inability of the immune system to control EBV infection.

The fact that SAP consists of a single SH2 domain suggests that SAP does not independently transduce signals. Instead, SAP recruits signal transduction molecules to regulate cellular signal transduction networks. Dok1, a major tyrosine-phosphorylated protein downstream of numerous signaling molecules (50), has been shown to strongly interact with SAP in a tyrosine phosphorylation-dependent manner (50). Here, we find that upon TCR stimulation, the SAP protein strongly interacts with a 75-kDa tyrosine-phosphorylated protein. Our limited study revealed that the 75-kDa protein associated with SAP upon TCR stimulation is not Dok1, ZAP70, SLAM, 2B4, SHP-2, Ku70, Ku86, or p85 phosphatidylinositol 3-kinase, indicating that an additional cellular protein that remains to be identified is likely involved in SAP-mediated signal transduction. In addition, because SAP interacts with a 75-kDa tyrosine-phosphorylated protein upon TCR stimulation, this interaction likely plays an important role in transducing the TCR signal to elicit cellular activation. Further study including an identification of a 75-kDa protein associated with SAP potentially delineates detailed roles of SAP in the TCR signal transduction pathway. Thus, through interactions with cellular signaling molecules, SAP likely plays pleotropic roles in numerous signal transduction pathways, and this activity may be relevant to the failure of XLP patients to survive following acute EBV infection (20, 31–36, 44, 72).

The host T cell responses are particularly important for controlling herpesvirus infection. XLP patients appear to be normal in their response to childhood infections before they encounter EBV (44, 73). A unique feature of EBV infection is the extraordinary proliferation of activated Ag-specific T cells and the EBV-infected B cells (73). For example, as much as 10% of the total B cell population may be infected in the course of normal infection. Dramatic numbers of responding NK cells and CD4⁺ and CD8⁺ T lymphocytes are typically involved in the normal response to primary EBV infection. Furthermore, the large number of activated effector T cells are associated with the release of cytokines and inflammatory mediators that ordinarily lead to IM. These factors suggest that EBV infection is the foremost trigger for the phenotypic expression of XLP syndrome due to the excessive T cell activation. During EBV infection, SLAM-SLAM and MHC II molecule-TCR interactions at the interface between EBV-infected B cells and CD4 T cells, or CD48–2B4 interaction between EBV-infected B cells and NK cells, may promote the development of EBV-specific Th responses. However, SAP deficiency in XLP patients impairs SLAM and 2B4 signal transduction, leading to reduced IFN-γ production by Th cells and decreased cytotoxicity of NK cells (20, 31–36, 72). In addition, aberrant TCR signal transduction in XLP patients may result in abnormalities of CD4 T cell function and CD8 cytotoxic T cell function, contributing to the inability of the immune system to control EBV infection. Further study of SAP function in lymphocyte signal transduction pathways...
will pave the way for new strategies and the treatment of XLP syndrome.

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References


