INFECTION AND IMMUNITY, Jan. 1995, p. 259–263
0019-9567/95/$04.00+0
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Vol. 63, No. 1

Promotion of Cytotoxic T-Cell Generation in Mixed Leukocyte Culture by Phosphatidylinositol-Specific Phospholipase C from *Bacillus thuringiensis*

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Received 20 June 1994/Returned for modification 7 September 1994/Accepted 14 October 1994

Phosphatidylinositol-specific phospholipase C (PIPLC) from *Bacillus thuringiensis*, which cleaves phosphatidylinositol or glycosylphosphatidylinositol on the external cell surface to generate a second messenger for intracellular signal transduction (S. Rahman et al., FEBS Lett. 303:193–196, 1992), was found to preferentially promote the generation of alloantigen-specific cytotoxic T lymphocytes in mixed leukocyte culture. PIPLC affected an early stage of cytotoxic T-lymphocyte activation in culture, and there was no evidence of any soluble cellular mediators of this PIPLC action. PIPLC neither enhanced overall cell proliferation nor noticeably promoted interleukin-2 and -4 production in mixed leukocyte culture. The relative population size of Ly-2$^+$ T cells was increased, however, in a late mixed leukocyte culture with PIPLC. In addition, PIPLC enhanced an anti-CD3 monoclonal antibody-induced early increase in $[Ca^{2+}]_i$. These results suggest a new parasite (bacterium)-oriented mechanism for enhancing antigen-driven host cytotoxic T-lymphocyte immunity which does not include promotion of interleukin-2 production.

A number of cell membrane proteins, including Thy-1 (16, 25), Ly-6A/E (34), LFA-3 (3), and Qa-2 (33), are known to be membrane anchored with glycosylphosphatidylinositol (GPI) on cells in the immune system. It has been suggested that these molecules mediate cell-to-cell adhesion and intracellular signal transduction (5, 11–13, 17, 24, 26, 32), but their role in the immune system is not known. GPI or phosphatidylinositol (PI) is specifically cleaved by PI-specific phospholipase C (PIPLC), which is known to be produced by several bacteria such as *Staphylococcus aureus* (2), *Bacillus cereus* (10), *Bacillus thuringiensis* (37), *Clostridium novyi* (38), and *Listeria monocytogenes* (18). Cleavage of GPI by PIPLC may eliminate GPI-anchored cell interaction molecules required for lymphocyte responses to mitogens (28, 36). However, the cleavage of GPI or PI by PIPLC has also been found to generate inositol phosphates or inositol phosphate glycan-linked protein and diradylglycerol as candidates for second signal messengers (1, 35). The potential modulatory action of bacterial PIPLC on the immune system by this mechanism has not yet been adequately tested.

Various kinds of bacterial products, including outer membrane lipopolysaccharide (8, 23) and cell wall-derived muramyl dipeptide (39), are known to activate B cells polyclonally (8, 20) and to accelerate antigen-specific helper T cell responses for antibody production (8, 21, 23, 39) and delayed-type hypersensitivity (27, 39). These bacterial products stimulate antigen-presenting macrophages and dendritic cells to produce interleukin-1 (IL-1), which in turn activates helper T cells (8, 14, 30). Few reports, however, have demonstrated an ability of these bacterial products to potentiate IL-2-dependent (4), antigen-specific cytotoxic T-lymphocyte (CTL) immunity; lipopolysaccharide-activated B-cell blasts work as both target (31) and stimulator (6) cells of antigen-specific CTL. Recently, we showed that PIPLC promotes receptor (CD3)-mediated early signals for T-cell activation, e.g., protein tyrosine phosphorylation and c-*fos* transcription (29). In this study, we have examined the potential regulatory action of *B. thuringiensis* PIPLC on CTL immunity. The results demonstrate a novel immunomodulatory action of the bacterial PIPLC to preferentially accelerate an early stage of antigen-driven CTL generation, which does not include promotion of IL-2 production.

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MATERIALS AND METHODS

Animals and cell preparation. C57BL/6 (*H-2^b*) and BALB/c (*H-2^d*) strains of mice, 6 to 8 weeks old, which were bred in the Institute for Laboratory Animal Research, Nagoya University School of Medicine, were used as the source of spleen cells and thymic T lymphocytes. Single cell suspensions in Eagle's minimal essential medium were prepared as described previously (22).

PIPLC. PIPLC was prepared from culture supernatants of *B. thuringiensis* as described before (9, 37). This highly purified PIPLC gave a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was protease free; all activity of the preparation to modulate receptor-mediated signal transduction was eliminated by passage through an anti-PIPLC antibody-conjugated affinity column (29). Cells (5×10^6/ml) were treated with 10 to 300 mU of PIPLC per ml for 30 min, and mixed leukocyte culture (MLC) was done in the presence or absence of PIPLC. This treatment was effective for specifically removing GPI-anchored Thy-1 from >80% of murine T lymphocytes when 30 mU of PIPLC or more per ml was used (25).

MLC and CTL assay. MLC was done with responder BALB/c spleen cells (10^7 cells per well) and X-ray-irradiated (2,000 R) stimulator C57BL/6 (allogeneic MLC) or BALB/c (syngeneic MLC) spleen cells (5×10^6 cells per well) in a 24-well culture plate with 2 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, 200 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10^{-5} M 2-mercaptoethanol (FCS-RPMI) per well. These cells were incubated at 37°C in an atmosphere of 5% CO_2 in air for 3 to 15 days. The cytotoxicity developed in MLC was assayed as described previously (19) against ^{51}Cr-labeled EL-4 (*H-2^b*), P1.HTR (*H-2^d*), and YAC-1 (*H-2^a*) tumor cells as targets. Each assay was done at different effector/target cell ratios, and percentages of specific ^{51}Cr release were calculated.

Assay of cell proliferation and IL-2 and IL-4 production. MLC was done for 1 to 9 days in a 96-well culture plate at 1/10 scale of MLC for the CTL assay. [^{3}H]thymidine (37 kBq per well) was added for the last 4 h of each MLC. After the cells were harvested on filter paper, the radioactivity (counts per minute) was determined by liquid scintillation counting. IL-2 and IL-4 activity was assayed by determining the ability of the culture supernatants obtained from 3- to 9-day MLC to stimulate proliferation of an IL-2- and IL-4-dependent cell line, CTLL-2, as described before (40). Briefly, CTLL-2 cells (5×10^3 per 100 μl per

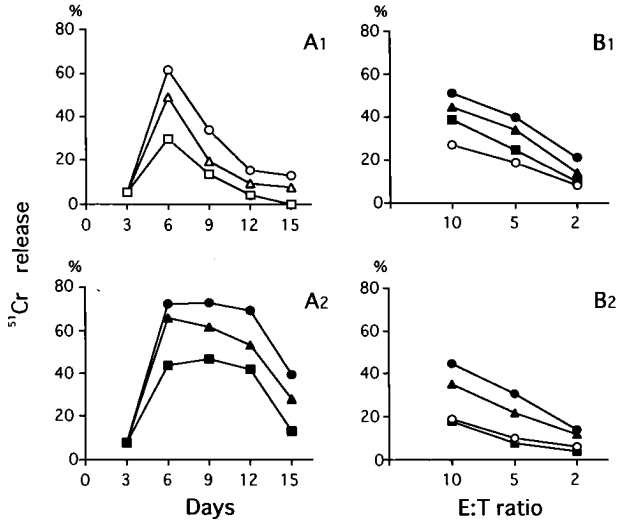

FIG. 1. PIPLC promotes development of cytotoxicity in MLC. (A) Allogeneic (BALB/c anti-C57BL/6) MLC was performed for 3 to 15 days with cells treated (A2) with 150 mU of PIPLC per ml or untreated cells (A1). For treatment with PIPLC, responder and stimulator cells were first treated with PIPLC at 37°C for 30 min separately and then were mixed for culture in the presence of PIPLC. Cells were harvested on the indicated days, and cytotoxicity was measured against EL-4 cells (*H-2*^{b}) by a ^{51}Cr-release assay at effector/target cell (E:T) ratios of 10 (circles), 5 (triangles), and 2 (squares). (B) Allogeneic MLC was done for 9 (B1) and 12 (B2) days with cells untreated (open circles) or treated with 10 (closed squares), 30 (closed triangles), or 300 (closed circle) mU of PIPLC per ml. Cells harvested on the last day of culture were assayed for cytotoxicity against EL-4 at the indicated effector/target cell ratios. Each point here and in Fig. 2 to 4 shows the mean of triplicate assays, and the standard deviation was less than 10% in every determination. Shown are representatives of six experiments with consistent results.

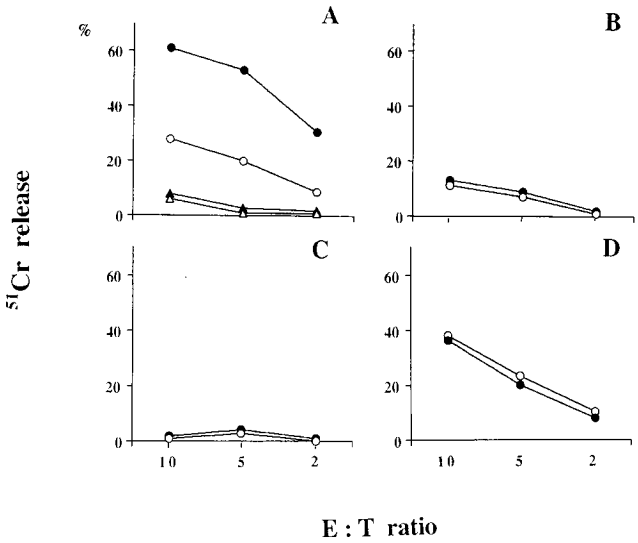

FIG. 2. The promotion by PIPLC of development of cytotoxicity in MLC is alloantigen specific and occurs during MLC. (A-C) Allogeneic MLC (BALB/c anti-C57BL/6 [circles]) and syngeneic MLC (BALB/c anti-BALB/c [triangles]) were performed with cells treated (closed) with PIPLC (150 mU/ml) or untreated (open) as described in the legend to Fig. 1. Cells were harvested on day 9 of culture, and cytotoxicity was measured against EL-4 (*H-2*^{b} [A]), P1.HTR (*H-2*^{d} [B]), and YAC-1 (*H-2*^{a} [C]) cells at the effector/target cell (E:T) ratios indicated. (D) Allogeneic MLC was done in the absence of PIPLC, and cells harvested on day 9 were assayed for cytotoxicity against EL-4. Both effector and target cells were treated (closed symbols) with PIPLC (150 mU/ml) at 37°C for 30 min separately before the cytotoxicity assay or were left untreated (open symbols). Shown is a representative of two experiments.

well) were cultured for 24 h for [^{3}H]thymidine uptake assay (counts per minute) in FCS-RPMI containing 50% culture supernatant. In a preliminary study, we confirmed that the concentration of IL-2 added related almost linearly to the counts per minute up to 100,000 cpm for serially diluted standard IL-2 solution (supernatant of concanavalin A-stimulated rat spleen cell culture) (not shown).

Laser flow cytometry. Cells (10^6 cells per sample) suspended in minimal essential medium were stained with fluorescein isothiocyanate-labeled anti-Ly-2 and phycoerythrin-labeled anti-L3T4 monoclonal antibody (Becton Dickinson, Mountain View, Calif.) and were analyzed with a laser flow cytometer (EPICS Profile; Coulter Electronics, Hialeah, Fla.).

Measurement of $[Ca^{2+}]_i$. Thymic T lymphocytes were incubated in buffer A (140 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM KH_2PO_4, 10 mM glucose, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4]; 2×10^7 cells per ml) containing 3 to 6 mM Fura-2 AM (Molecular Probes Inc., Eugene, Ore.) for 1 h at 37°C and then were washed twice with minimal essential medium and resuspended in buffer A (5, 15). The Fura-2-loaded lymphocytes (10^6 cells), suspended in 500 μl of prewarmed (37°C) buffer A, were applied to a Ca^{2+} analyzer (model CAF-100; JASCO Ltd., Tokyo, Japan) and stimulated with agonists under constant stirring (26). Fluoresence at 340 and 380 nm was recorded continuously to measure the ratio of 340/380 nm before and after stimulation with anti-CD3 (145-2C11; ascites) monoclonal antibody (15) and anti-hamster immunoglobulin G rabbit antibody (MBL, Nagoya, Japan) that cross-links anti-CD3 monoclonal antibody (hamster immunoglobulin G) and after lysis of cells with 0.2% Triton X-100 in the presence or absence of 3 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. $[Ca^{2+}]_i$ was calculated according to the formula described in reference 7.

RESULTS AND DISCUSSION

We first asked if and how treatment of cells with bacterial PIPLC would change the extent of generation of CTL in MLC. The result is shown in Fig. 1. In normal control allogeneic (BALB/c anti-C57BL/6) MLC, cytotoxicity was low by day 3, attained a peak on day 6, and then subsided to the basal level on day 15 (Fig. 1A1). In MLC of cells treated with 150 mU of PIPLC per ml, however, a higher level of cytotoxicity developed on day 6 than in a control MLC; this cytotoxicity was sustained at a high level until day 12 and then declined slowly, but it still remained above the control level on day 15 (Fig. 1A2). The effect of PIPLC was dependent on its concentration; 30 mU of PIPLC or more per ml, which was active in cleaving GPI-anchored Thy-1 from thymocytes (25), was definitively effective, whereas the effect of 10 mU of PIPLC per ml was marginal (Fig. 1B). This effect of PIPLC was not seen in syngeneic (BALB/c anti-BALB/c) MLC (Fig. 2A, triangles). Moreover, the PIPLC-enhanced cytotoxicity in allogeneic MLC was alloantigen specific, killing *H-2*^{b} EL-4 (Fig. 2A, circles), but not *H-2*^{d} P1.HTR (Fig. 2B) cells. In addition, cytotoxicity against YAC-1 as the target of NK cells was not enhanced by PIPLC (Fig. 2C). These results show that PIPLC accelerates the generation of alloantigen (*H-2*^{b})-specific CTL in MLC. On the other hand, treatment with PIPLC of effector CTL and target cells for a cytotoxicity assay was ineffective in modulating the cytotoxicity (Fig. 2D). This confirmed that the PIPLC-mediated augmentation of cytotoxicity took place during MLC. In addition, these results show that partial elimination of GPI-anchored adhesion molecules by PIPLC did not interrupt cell interaction for either CTL generation or CTL-mediated target cell killing.

Both previous treatment of responder cells with PIPLC before MLC and the addition of PIPLC to MLC at the start of culture were effective in enhancing CTL generation in MLC, although a combination of both was a little more effective than either alone (Fig. 3A). Because the PIPLC effect was not obvious by day 3 of MLC with preadded PIPLC, the action of PIPLC added on day 3 of MLC was compared with that of PIPLC added on day 0. As shown in Fig. 3B, the latter, but not the former, was definitely effective in enhancing cytotoxicity measured on days 12 and 15 of MLC, although the former was partially effective on days 6 and 12. All of these results sug-

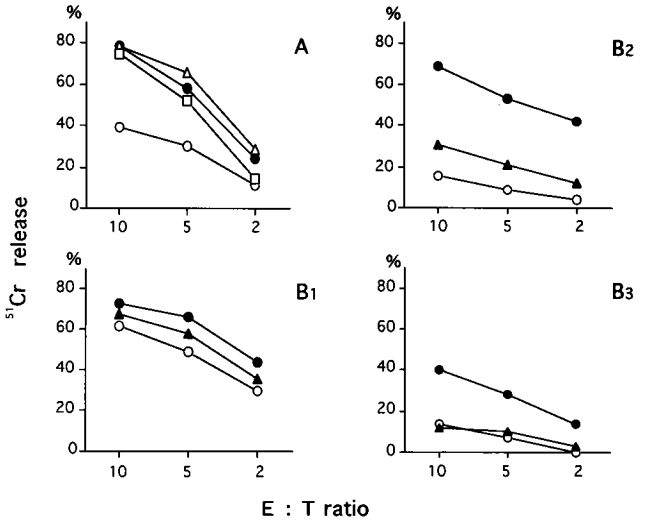

FIG. 3. PIPLC primarily affects the early stage of MLC for promotion of development of cytotoxicity. Allogeneic MLC was done under the following conditions. (A) Responder cells were treated with PIPLC (300 mU/ml) for 30 min at 37°C or were left untreated and were then washed. MLC was performed by mixing the PIPLC-treated (triangles, closed circles) and untreated (squares, open circles) responder cells with allogeneic stimulator cells for MLC in the presence (triangles, squares) or absence (closed circles, open circles) of freshly added PIPLC (300 mU/ml). (B) PIPLC was added to allogeneic MLC on day 0 (closed circles) or 3 (closed triangles) of culture. Open circle, no PIPLC addition (control). Cells were harvested on days 6 (B1), 12 (A and B2), and 15 (B3) of MLC, and cytotoxicity was measured against EL-4 cells. Shown is a representative of three experiments. E:T, effector/target cells.

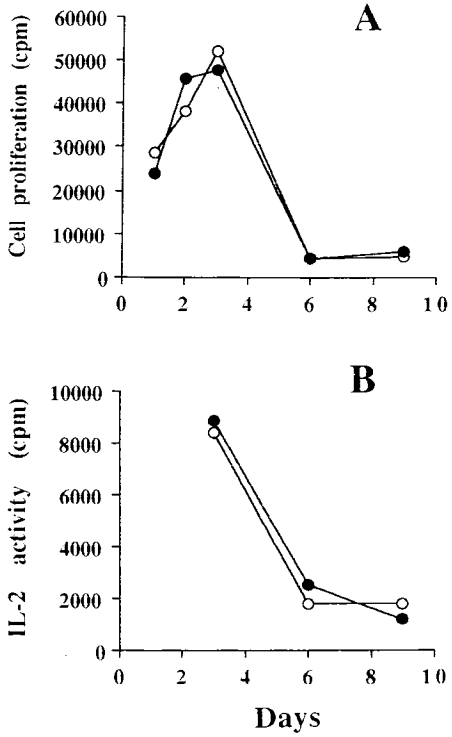

FIG. 5. PIPLC neither enhances overall cell proliferation nor promotes IL-2 production in MLC. Allogeneic MLC was done in the presence (30, 150, or 300 mU/ml) or absence of PIPLC. The levels of cell proliferation (A) and IL-2 activity (B) in MLC with (solid circles) or without (open circles) PIPLC were assayed on the indicated days. Only data from MLC with added PIPLC at 150 mU/ml are shown, because none of the three different concentrations of PIPLC showed a significant effect. Each point shows the mean of triplicate assays, and the standard deviation was less than 20% in every determination. Shown is a representative of three experiments.

gested that the action of PIPLC or responder cells at the initial stage of MLC was crucial for the enhancement of CTL generation.

The enhanced development of CTL activity by PIPLC might be mediated by accelerated cytokine production. Two steps of MLC were performed by exchanging cells and culture supernatants at the end of the primary MLC for a secondary MLC. As shown in Fig. 4, however, no such soluble mediator activity was demonstrable in culture supernatants of the primary MLC with PIPLC. In addition, the result showed that PIPLC which might remain in the supernatant of the primary MLC was not active on cells in the day 7 MLC for enhanced generation of CTL (cf. closed and open triangles).

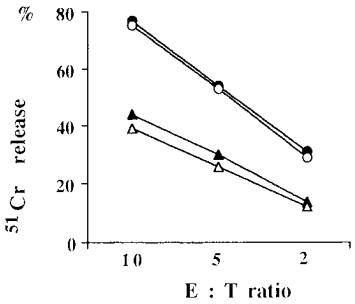

FIG. 4. Evidence of failure to demonstrate soluble cellular mediators of the PIPLC action. Two steps of allogeneic MLC were performed. For the primary MLC, responder spleen cells were mixed with stimulator cells in the presence (MLC-A) or absence (MLC-B) of PIPLC (300 mU/ml), and the mixture was incubated for 7 days. Cells and culture supernatants were obtained separately from MLC-A and MLC-B at the end of the primary MLC; the secondary MLC was carried out by mixing MLC-A cells with MLC-A (closed circles) or MLC-B (open circles) supernatant or by mixing MLC-B cells with MLC-A (closed triangles) or MLC-B (open triangles) supernatant. Cells were harvested on day 5 of the secondary MLC, and cytotoxicity was measured against EL-4 cells. Shown is a representative of two experiments. E:T, effector/target cells.

Next, we tested the action of PIPLC on cell proliferation and production of IL-2 in MLC, which had been shown to be crucial for the generation of CTL (4). In contrast to the noted action of PIPLC on CTL generation, 30 to 300 mU of PIPLC per ml neither enhanced overall cell proliferation (Fig. 5A) nor promoted IL-2 and IL-4 production (Fig. 5B) noticeably in MLC. These results, together with the results presented in Fig. 4, showed that the demonstrated PIPLC action on CTL was not through promotion of IL-2 production. Correspondingly, analysis by laser flow cytometry of 5,000 cells from late (day 15) MLC demonstrated a relative increase in the number of Ly-2$^+$ T cells in MLC added with PIPLC (85%) compared with that in control MLC (57%), whereas the relative number of L3T4$^+$ T cells in the former MLC (12%) was only marginally more than that in a control MLC (9%). These results support the conclusion that PIPLC preferentially affected Ly-2$^+$ CTL lineage cells to promote their antigen-driven activation, with little involvement of activation and proliferation of the L3T4$^+$ T cells that should produce IL-2 and IL-4. However, the latter does not necessarily mean that the CTL generation in PIPLC-treated MLC is totally independent of IL-2 because IL-2 was actually produced in both PIPLC-treated and untreated MLC at the same levels (Fig. 5). It appears, therefore, that PIPLC works to either supplement or potentiate the known IL-2 action for CTL generation.

Bacterial PIPLC is known to cleave extracellular GPI or PI

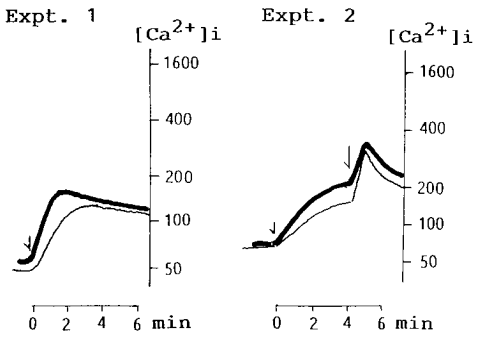

FIG. 6. Acceleration of anti-CD3 monoclonal antibody-induced $[Ca^{2+}]_i$ rise in thymic T lymphocytes by PIPLC. Thymic T lymphocytes loaded with Fura-2, which were previously treated (thick lines) with PIPLC (150 mU/ml for 30 min) or were untreated (thin lines), were stimulated by anti-CD3 (1:100, short arrow) (experiment 1) or anti-CD3 (1:500, short arrow) plus cross-linking anti-immunoglobulin (long arrow) (experiment 2). The $[Ca^{2+}]_i$ level was measured continuously after stimulation. Shown is a representative of three experiments.

to produce diradylglycerol at the outer surface of the plasma membrane, and the diradylglycerol produced on the external cell surface turns to the internal surface to activate protein kinase C (1, 35). As expected, cell surface-acting bacterial PIPLC barely increased $[Ca^{2+}]_i$ by itself (data not shown), probably because it did not produce inositol phosphates after cleavage of PI inside the cell. Interestingly, however, it accelerated the rise of $[Ca^{2+}]_i$ induced by cross-linkage of T-cell receptor (CD3 in this study) (Fig. 6). This suggests that the cleavage of PI or GPI on the outer surface of lymphocytes provides a mechanism to modulate the T-cell receptor-mediated signal for a $[Ca^{2+}]_i$ increase, possibly through the action of the internally translocated diradylglycerol. Synergism of the actions of PIPLC and antireceptor antibody was also observed previously for induction of protein tyrosine phosphorylation and c-*fos* transcription (29). Taken together, it is suggested that PIPLC provides a costimulatory signal to CTL and CTL precursors which might cooperate with the T-cell receptor-mediated or IL-2-mediated signal for CTL generation.

Whatever the underlying molecular mechanism is, our data suggest a novel parasite (bacterium)-oriented pathway of selective enhancement of the antigen-driven host CTL immunity and a role of GPI or PI on the external cell surface in signaling lymphocytes responding to microbial products. The question of whether any or all bacteria known to produce PIPLC would modulate CTL generation in response to conventional antigens possessing CTL epitopes as a normal defense mechanism, however, remains to be answered.

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