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Listeria monocytogenes-Infected Hepatocytes Are Targets of Major Histocompatibility Complex Class Ib-Restricted Antilisterial Cytotoxic T Lymphocytes

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Subclinical infection of BALB/c mice with the intracellular bacterial pathogen *Listeria monocytogenes* results in the development of protective antilisterial immunity. *L. monocytogenes* can infect hepatocytes, and antilisterial cytotoxic T lymphocytes (CTL) lyse *Listeria*-infected hepatocytes in a major histocompatibility complex (MHC) class Ia-restricted manner. It remained to be determined whether *L. monocytogenes*-infected hepatocytes are susceptible to MHC class Ib-restricted cytolysis. In this study, we showed that hepatocytes express MHC class Ib molecule Qa-1^b mRNA and protein. We further showed that *Listeria*-infected hepatocytes are susceptible to MHC class Ib-restricted cytolysis, since C57BL/6-derived *Listeria*-infected hepatocytes were lysed by BALB/c-derived antilisterial CTL. These results establish that *Listeria*-infected hepatocytes are susceptible to cytolysis by MHC class Ib restricted *Listeria*-specific CTL.

Injection of BALB/c mice with a sublethal dose of virulent *Listeria monocytogenes* leads to an initial wave of unhindered intracellular replication of the microorganism within the first 24 to 48 h (16). The spleen and liver are the principal target organs for the experimental infection. Hepatocytes are the principal cell type harboring the replicating bacterial pathogen within the liver (8, 18). By 72 h following injection, the numbers of CFU in these organs begin to decline, and by 6 days following injection, *L. monocytogenes* CFU typically are not detectable (14, 15). The decline and eventual clearance of *L. monocytogenes* have been attributed to stimulation of *Listeria*-specific CD4$^+$ and CD8$^+$ T-cell subsets (11, 16). Adoptive transfer studies have shown that the *Listeria*-specific CD4$^+$ T-cell subset mediates delayed-type hypersensitivity reactivity while the *Listeria*-specific CD8$^+$ T-cell subset is responsible for protective immunity against a lethal challenge dose of virulent *L. monocytogenes* (1, 2).

In vitro studies have shown that the CD8$^+$ T-cell subset is cytolytic, and targets cells such as J774 macrophage-like cells, bone marrow-derived macrophages, and fibroblasts that have been infected with viable, hemolysin-secreting strains of *L. monocytogenes* are lysed by *Listeria*-immune CD8$^+$ T cells (3). In addition, gamma interferon is released from *Listeria*-specific CD8$^+$ T cells only following interaction with target cells infected with viable, hemolysin-secreting strains of *L. monocytogenes* (7). Target cells infected with non-hemolysin-secreting strains of *L. monocytogenes* or pulsed with nonviable bacteria such as heat-killed *L. monocytogenes* are not lysed by CD8$^+$ *Listeria*-immune cells (4). The CD8$^+$ T-cell subset is major histocompatibility complex (MHC) class I restricted, and both classical (MHC class Ia) and nonclassical (MHC class Ib) elements have been described as restriction molecules (3). In BALB/c mice, MHC class Ia responses are restricted to H2-K^d-presented peptides. MHC class Ib molecules that are restricting elements for antilisterial cytotoxic T lymphocytes (CTL) include Qa-1^b, which is T region encoded (6), and M3, which is M region encoded (12, 17).

A role for MHC class Ib-restricted CD8$^+$ T cells in antilisterial immunity is indicated from recent studies which showed that short-lived antilisterial protection can be adoptively transferred to MHC class Ia-disparate, MHC class Ib-syngeneic recipients (10, 13). MHC class Ib-restricted CTL appear to be biologically relevant, since MHC class Ib-restricted CTL activity can be detected in vivo when CFU are declining and the disappearance of effector function of this population correlates with clearance of *L. monocytogenes* from the immunized animals (5). It has been clearly established that *L. monocytogenes*-infected macrophages present MHC class Ia- and Ib-associated peptide targets to antilisterial CTL (3). It has recently been established that *Listeria*-infected hepatocytes are susceptible to MHC class Ia-restricted lysis by *Listeria*-specific CD8$^+$ T cells obtained from mice immunized 11 days previously (9). This same CD8$^+$ T-cell population failed to exhibit MHC class Ib-restricted cytolytic activity. This finding suggests that *Listeria*-infected hepatocytes may not present MHC class Ib-associated targets to *Listeria*-specific CTL. The study presented in this report was initiated to address specifically whether hepatocytes express MHC class Ib molecules and to determine if *Listeria*-infected hepatocytes are susceptible to MHC class Ib-restricted cytolysis by utilizing a population of *Listeria*-specific effector cells with known MHC class Ib-restricted CTL activity (3). The data presented show that hepatocytes express MHC class Ib molecules and that *L. monocytogenes*-infected hepatocytes are lysed by MHC class Ib-restricted *Listeria*-specific CTL.

MATERIALS AND METHODS

Bacteria. *L. monocytogenes* 10403 serotype 1 was originally obtained from the American Type Culture Collection (Rockville, Md.). Virulence was maintained by repeated passage in BALB/c mice.

Mice and immunization. Six-week-old female BALB/c (*H-2^d*) and C57BL/6 (*H-2^b*) mice were purchased from Bantin-Kingman (Fremont, Calif.). Eight-

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week-old mice were immunized with approximately 0.1 50% lethal dose of viable *L. monocytogenes* in 0.2 ml injected via the tail vein.

Cell lines and reagents. The J774 cell line was maintained by culture in Dulbecco modified Eagle medium (DMEM; antibiotic free; Gibco, Grand Island, N.Y.) supplemented with nonessential amino acids (Gibco) and 5% fetal calf serum (FCS; Tissue Culture Biologicals, Tulare, Calif.).

Bone marrow macrophage cultures. Bone marrow was obtained from femurs of mice of the above-listed strains and cultured in 100- by 20-mm plates (Falcon) at 2×10^5 cells/ml (2×10^6 cells/plate) in DMEM containing 10 μg of gentamicin (Sigma, St. Louis, Mo.) per ml, 20% FCS, 30% L-cell supernatant (as a source of colony-stimulating factor 1) at 37°C in an atmosphere of humidified air with 7.5% CO_2. After 6 days, the bone marrow macrophages (BM-MAC) were isolated and used as target cells.

Splenocyte culture. Spleen cells from mice immunized 6 days prior with *L. monocytogenes* were stimulated in culture with 1.0 μg of concanavalin A (Sigma) per ml in RPMI 1640 (Gibco) containing 100 U of penicillin (Sigma) per ml, 100 μg of streptomycin (Sigma) per ml, 5% FCS, and 5×10^{-5} M 2-mercaptoethanol (Sigma). A total of 10^8 cells in 50 ml were cultured in 75-cm^2 flasks for 72 h at 37°C in an atmosphere of humidified air with 7.5% CO_2. Following culture, the recovered cells were used in assays of CTL activity.

Preparation of hepatocytes. C57BL/6 (Jackson Laboratory, Bar Harbor, Maine)-derived hepatocytes were prepared following perfusion of mouse livers by a two-step technique as described previously (9). Livers of mice were perfused with calcium- and magnesium-free Hanks balanced salt solution (BioWhittaker, Walkersville, Md.) containing 0.5 mM EGTA (pH 7.3) followed by Leibovitz's L-15 medium (BioWhittaker) containing 100 U of collagenase (type A; Boehringer Mannheim, Indianapolis, Ind.) per ml. The perfused livers were teased apart, and the resultant cell suspension was centrifuged twice at $30 \times g$ for 4 min each at 4°C. The purified cell population obtained in the final cell pellet was composed of greater than 96% hepatocytes. F4/80-positive mononuclear phagocytes and N-418-positive dendritic cells represented 1.5 and 0% of the population, respectively. Hepatocytes were cultured in HEPES-buffered RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 10^{-7} M recombinant human insulin (Humulin R; Eli Lilly and Co., Indianapolis, Ind.), and 10% heat-inactivated FCS.

CFU reduction assay. Target cells (in 1.0 ml of antibiotic-free tissue culture medium [HEPES-buffered RPMI 1640 medium supplemented with 1 mM sodium pyruvate and 10^{-7} M recombinant human insulin for hepatocytes or DMEM supplemented with nonessential amino acids and 5 or 10% FCS for J774 cells or BM-MAC, respectively]) were deposited at 1×10^5 to 2×10^5/well, in 24-well tissue culture plates 18 h prior to performance of the assay (3). The target cell monolayers were infected with *L. monocytogenes* (obtained from a log-phase culture) at a multiplicity of infection (MOI) of 2 to 5 for J774 cells or 10 to 20 for BM-MAC and hepatocytes. After 60 min, the monolayers were washed three times with sterile phosphate-buffered saline (37°C) and covered with 0.5 ml of the appropriate tissue culture medium containing gentamicin sulfate at 80 μg/ml (J774 cells) or 40 μg/ml (BM-MAC or hepatocytes). Effector cells, obtained following culture stimulation with concanavalin A, were added (at an effector-to-target cell ratio [E/T] of 30:1) in 0.5 ml of the appropriate tissue culture medium, in triplicate, 3 to 4 h after initiation of the infection. The assays were terminated 4 to 5 h later, and the number of intracellular bacteria remaining in each well was determined. Specifically, the medium was aspirated and replaced with 1 ml of distilled water. Five minutes later, dilutions of the cell lysates were plated onto brain heart infusion agar (Difco, Detroit, Mich.) plates, which were incubated for 24 h at 37°C, and the numbers of CFU were determined. Data are presented as percent CFU reduction, calculated with the formula [1 − (CFU in target monolayers incubated with effector cells)/(CFU in target monolayers incubated without effector cells)] × 100 (see Fig. 2 and 3).

Metabolic labeling and immunoprecipitation. Cells (2×10^6 to 4.5×10^6, or 50×10^6 for freshly prepared splenocytes) were starved in methionine-free RPMI 1640 (ICN, Irvine, Calif.) at 37°C for 30 min and then labeled with 0.2 mCi of Tran^{35}S-label (ICN, Irvine, Calif.) per ml for 30 min. After being washed twice with cold phosphate-buffered saline, the cells were lysed in 0.5 ml of lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl [pH 7.5], 0.14 M NaCl, 5 mM iodoacetamide, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1 U of the trypsin inhibitor aprotinin per ml). The lysates were clarified by centrifugation, and an aliquot of each of the supernatants was assayed for activity by liquid scintillation. Equivalent amounts of lysates were adjusted to 0.8 ml and precleared with fixed *Staphylococcus aureus* (Sigma) preadsorbed with normal rabbit serum. Qa-1^b was collected by sequential incubations with a Qa-1^b cytoplasmic tail-specific antiserum and protein A-Sepharose beads (Pierce, Rockford, Ill.). Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% gel and visualized with a PhosphorImager (Molecular Dynamics, Seal Beach, Calif.).

RNase protection assay. A 1.2-kb *Bam*HI-*Sac*I fragment containing exons 1 to 3 of the *Qa-1^b* gene was subcloned into pGEM3z (Promega, Madison, Wis.). The resulting plasmid was linearized with *Eco*RV and used as a template to generate the antisense RNA probe containing exon 3 of *Qa-1^b*. The β-actin DNA template was provided by I. Stroynowski (University of Texas Southwestern Medical Center). The [^{32}P]CTP-labeled RNA probes were synthesized with a riboprobe in vitro transcription system (Promega) and purified on denaturing acrylamide gels. Total RNA was isolated with RNA STAT-60 (Tel-Test "B", Friendwood, Tex.). RNA probes and 20 μg of RNA were hybridized at 42°C for 16 h and then digested with RNase A (10 μg/ml) and RNase T1 (60 U/ml) (both from Boehringer Mannheim) at 35°C for 45 min. The protected RNA fragments were analyzed by SDS-PAGE on a 6% denaturing acrylamide gel.

RESULTS AND DISCUSSION

Listeria-immune CD8$^+$ T cells lyse *L. monocytogenes*-infected J774 macrophage-like cells, BM-MAC, and fibroblast target cells (3). A consistent observation is that only target cells infected with viable, hemolysin-secreting strains of *L. monocytogenes* are susceptible to CD8$^+$ CTL-mediated cytolysis. Thus, the escape of *L. monocytogenes* from the phagosome to the cytoplasm is an important step in the development of the MHC class I-associated peptide target. It was recently established that the requirements for the development of MHC class I peptide targets are similar for *L. monocytogenes*-infected hepatocytes and *L. monocytogenes*-infected macrophages (9). That is, hepatocytes infected with viable, listeriolysin O (LLO)-secreting *L. monocytogenes* are lysed by CD8$^+$ antilisterial CTL, while hepatocytes infected with avirulent, non-LLO-secreting *L. monocytogenes* or with the unrelated bacterial pathogen *Salmonella typhimurium* are not susceptible to cytolysis. In addition, this report established that *L. monocytogenes*-infected hepatocytes present MHC class I-associated *Listeria*-derived peptides to *Listeria*-specific CTL in what appears to be only a MHC class Ia-restricted manner; MHC class Ib-restricted activity was not detected.

It is possible that hepatocytes do not express MHC class Ib molecules. This would account for the report which showed that *L. monocytogenes*-infected hepatocytes were susceptible to only MHC class Ia-restricted cytolysis. However, it seems unlikely that hepatocytes do not express MHC class Ib molecules, since freshly isolated liver cells as well as hepatomas express MHC class Ib elements (19). We hypothesized that if Qa-1^b was present on the cell surface of the hepatocytes, this MHC class Ib molecule would be a likely target for MHC class Ib-restricted antilisterial CTL, since we recently identified Qa-1^b-restricted CTL as a component of the antilisterial CTL population (6). To determine if hepatocytes express Qa-1^b molecules, an RNase protection assay using a probe specific for exon 3 of *Qa-1^b* mRNA was performed. The data presented in the left panel of Fig. 1 show that both the liver tissue and freshly isolated hepatocytes express *Qa-1^b* mRNA. Further, the expression of Qa-1^b protein in hepatocytes was confirmed by metabolic labeling and immunoprecipitation (Fig. 1, right panel). A rabbit anti-Qa-1^b cytoplasmic tail-specific antiserum which could specifically precipitate Qa-1^b molecules from Qa-1^b-transfected L cells (L-g37) and C57BL/6 spleen cells also precipitated a protein of the same size from hepatocytes. Normal rabbit serum did not react with this protein. These data indicate that hepatocytes express the MHC class Ib molecule Qa-1^b.

We hypothesized that hepatocytes, having been shown to express Qa-1^b molecules (Fig. 1), would be susceptible to MHC class Ib-restricted cytolysis. To determine if *L. monocytogenes*-infected hepatocytes present MHC class Ib-associated targets to *Listeria*-specific CTL, C57BL/6-derived hepatocytes were infected with *L. monocytogenes*. Following infection of these target cells, BALB/c-derived antilisterial CTL (a population of effector cells that we have previously reported to possess both MHC class Ia- and Ib-restricted CTL activities [3]) were added, and effector function was assessed in a standard CFU reduction assay. Although C57BL/6 (*H-2^b*) and BALB/c (*H-2^d*) are disparate for MHC class Ia alleles, these strains share many MHC class Ib alleles, including *Qa-1^b*. The data presented in Fig. 2 show that *L. monocytogenes*-infected

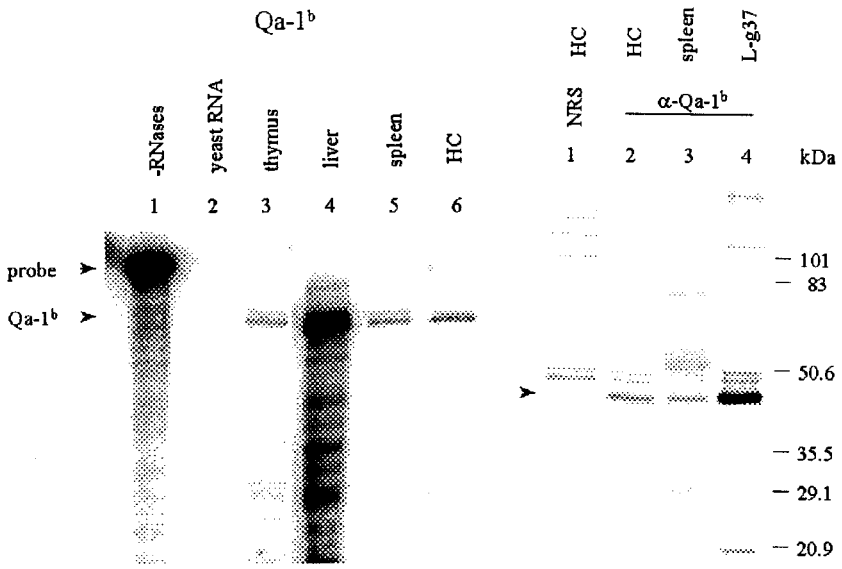

FIG. 1. Hepatocytes express MHC class Ib molecules. (Left) Expression of *Qa-1*^{b} mRNA as detected by RNase protection assay. Total RNA from the thymus (lane 3), liver (lane 4), spleen (lane 5), and hepatocytes (HC) (lane 6) was hybridized with a 360-nucleotide *Qa-1*^{b}-specific probe. After RNase digestion, a 276-nucleotide *Qa-1*^{b}-protected fragment was detected by using 6% denaturing acrylamide gels. RNase-treated (lane 2) and untreated (lane 1) yeast RNA-hybridized probes served as controls. The level of β-actin expression was used to measure the relative RNA content in each lane. (Right) Detection of the Qa-1^b protein by immunoprecipitation. Freshly isolated hepatocytes (lanes 1 and 2), spleen cells (lane 3), and *Qa-1*^{b}-transfected cell line L-g37 (lane 4) were metabolically labeled with ^{35}S and lysed with Nonidet P-40 buffer. The lysates were precipitated with a rabbit anti-Qa-1^b cytoplasmic tail serum (α-Qa-1^b) (lanes 2 to 4). The band corresponding to Qa-1^b is indicated by an arrowhead. Nonimmune rabbit serum (NRS) (lane 1) was used as a negative control.

C57BL/6-derived hepatocytes were lysed by BALB/c-derived antilisterial CTL. The level of the CFU reduction response was reduced compared to cytolysis of *L. monocytogenes*-infected J774 cells (BALB/c in origin); however, the relative magnitude of CFU reduction (60 to 70%) was similar to what is typically observed for MHC class Ib-restricted cytolysis (3). These results show that *L. monocytogenes*-infected hepatocytes are susceptible to MHC class Ib-restricted cytolysis. Although these results do not specifically demonstrate that hepatocytes present a Qa-1^b-associated *L. monocytogenes*-derived epitope(s), the data are consistent with our previous results demonstrating that Qa-1^b-restricted CTL are present in the antilisterial population utilized in these assays of CTL effector function (6).

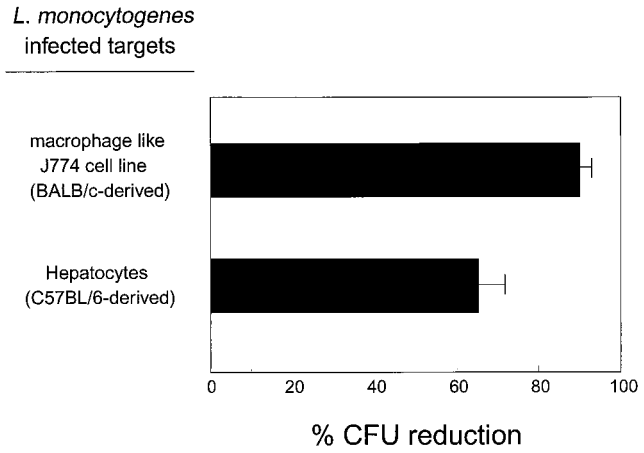

FIG. 2. Hepatocytes are susceptible to MHC class Ib-restricted cytolysis. Cells of the macrophage-like cell line J774 or hepatocytes from C57BL/6 mice were deposited at 10^5/well for use in a CFU reduction assay. These target cells were infected with *L. monocytogenes* at MOIs of 2 to 5 and 10 to 20, respectively. BALB/c-derived antilisterial CTL (E/T, 30:1) were added 3 h following infection; the assay was terminated 5 h later, and the remaining CFU were determined as described in Materials and Methods.

Hepatocytes are inherently fragile in vitro; hence, it was possible that the results presented in Fig. 2 were indicative of the unstable character of this target cell population. To eliminate this possibility, additional studies were conducted to assess the activity of *L. monocytogenes*-immune effector cells and compare it to that of nonimmune effector cells, thus allowing a measure of *Listeria*-specific cytotoxicity. If the infected hepatocytes are highly susceptible to nonspecific CTL activity, then we would expect to see no differences in CFU reduction when comparing immune and nonimmune populations. When the percent specific CFU reduction was utilized in the analysis, the data consistently showed that *L. monocytogenes*-infected C57BL/6-derived hepatocytes were lysed by BALB/c-derived antilisterial CTL (Fig. 3, experiment A). Also presented are results showing the levels of percent specific CFU reduction for cytolysis of *L. monocytogenes*-infected BALB/c-derived BM-MAC (a syngeneic target cell representing the total MHC class I-restricted activity) as well as for *L. monocytogenes*-infected C57BL/6-derived BM-MAC (a positive control for the presence of MHC class Ib-restricted cytolysis). The results of experiment B (Fig. 3) were similar: *L. monocytogenes*-infected hepatocytes were found to be susceptible to MHC class Ib-restricted cytolysis.

The immune response that develops following immunization with *L. monocytogenes* includes MHC class Ia- and Ib-restricted effector cells. MHC class Ib-restricted CTL are present as CFU are declining, and the disappearance of MHC class Ib-restricted CTL effectors correlates with the clearance of *L. monocytogenes* CFU in the spleen (5). *L. monocytogenes*-infected hepatocytes are susceptible to MHC class Ia-restricted cytolysis (9). This study utilized a population of *Listeria*-specific CTL with established MHC class Ib-restricted CTL activity to show further that *L. monocytogenes*-infected hepatocytes are

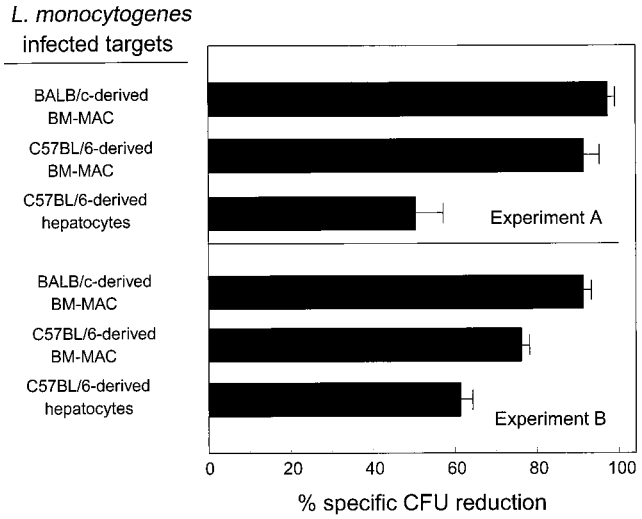

FIG. 3. Hepatocytes are susceptible to *L. monocytogenes*-specific MHC class Ib-restricted cytolysis. BALB/c- or C57BL/6-derived BM-MAC or hepatocytes from C57BL/6 mice were obtained and deposited at 10^5/well for use in a CFU reduction assay. The target cells were infected with *L. monocytogenes* at an MOI of 10 to 20. BALB/c-derived nonimmune effector cells (E/T, 30:1) or *L. monocytogenes*-immune effector cells (E/T, 30:1) were added 3 h following infection; the assay was terminated 5 h later, and the remaining CFU were determined as described in Materials and Methods. Specific CFU reduction was determined by dividing the level of CFU reduction measured in target cell monolayers containing immune cells by the level of CFU reduction measured in target cell monolayers containing nonimmune cells. Nonspecific CFU reduction against *Listeria*-infected BM-MAC was less than 20% in both experiment A and experiment B. Nonspecific CFU reduction against *Listeria*-infected hepatocytes was 42% for experiment A and 37% for experiment B.

also susceptible to MHC class Ib-restricted cytolysis. These findings further support the biological relevance of MHC class Ib-restricted CTL. Due to the relatively less-polymorphic nature of MHC class Ib molecules, this MHC class Ib-restricted CTL response may represent an alternative target for vaccine development.

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