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A Small Peptide (CEL-1000) Derived from the β-Chain of the Human Major Histocompatibility Complex Class II Molecule Induces Complete Protection against Malaria in an Antigen-Independent Manner

Yupin Charoenvit,[1] Gary T. Brice,[1] David Bacon,[1] Victoria Majam,[1,2] Jackie Williams,[3] Esteban Abot,[1,2] Harini Ganeshan,[1,2] Martha Sedegah,[1] Denise L. Doolan,[1,4] Daniel J. Carucci,[1] and Daniel H. Zimmerman[5]*

Malaria Program, Naval Medical Research Center,[1] and Department of Entomology, Communicable Diseases and Immunology, Walter Reed Army Institute of Research,[3] Silver Spring, Maryland 20910-7500; Henry M. Jackson Foundation, Rockville, Maryland 20852[2]; Department of Molecular Microbiology and Immunology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205[4]; and CEL-SCI Corporation, Vienna, Virginia 22182[5]

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CEL-1000 (DGQEEKAGVVSTGLIGGG) is a novel potential preventative and therapeutic agent. We report that CEL-1000 confers a high degree of protection against *Plasmodium* sporozoite challenge in a murine model of malaria, as shown by the total absence of blood stage infection following challenge with 100 sporozoites (100% protection) and by a substantial reduction (400-fold) of liver stage parasite RNA following challenge with 50,000 sporozoites. CEL-1000 protection was demonstrated in A/J (*H-2*^{a}) and C3H/HeJ (*H-2*^{k}) mice but not in BALB/c (*H-2*^{d}) or CAF1 (A/J × BALB/c F_1 hybrid) mice. In CEL-1000-treated and protected mice, high levels of gamma interferon (IFN-γ) in serum and elevated frequencies of hepatic and splenic $CD4^+$ IFN-γ-positive T cells were detected 24 h after administration of an additional dose of CEL-1000. Treatment of A/J mice that received CEL-1000 with antibodies against IFN-γ just prior to challenge abolished the protection, and a similar treatment with antibodies against $CD4^+$ T cells partially reduced the level of protection, while treatment with control antibodies or antibodies specific for interleukin-12 (IL-12), $CD8^+$ T cells, or NK cells had no effect. Our data establish that the protection induced by CEL-1000 is dependent on IFN-γ and is partially dependent on $CD4^+$ T cells but is independent of $CD8^+$ T cells, NK cells, and IL-12 at the effector phase and does not induce a detectable antibody response.

Malaria remains a major cause of mortality and morbidity in tropical and subtropical areas of the world, with approximately 300 million people considered at risk of infection. Although several clinical malaria vaccines have been developed and are being evaluated for their protective efficacies (for a review, see reference 19), prophylaxis and treatment with antimalarial drugs remain the only options for effective malaria control. In recent years, drug-resistant parasites have emerged and are now widespread, presenting serious problems for malaria control. Therefore, new and effective antimalarial interventions, both drugs and vaccines, are needed. It was previously demonstrated (3) that immunization with linear synthetic peptides containing B- and T-cell epitopes derived from the *Plasmodium yoelii* 17-kDa hepatocyte-erythrocyte protein (PyHEP17) protected A/J and CD1 mice but not BALB/c or C57BL/6 mice against sporozoite challenge, and this protection was dependent on $CD4^+$ T cells and gamma interferon (IFN-γ) but not on $CD8^+$ T cells. The protected mice displayed a Th1-type antibody profile (immunoglobulin G2a [IgG2a]), while the nonprotected mice displayed a predominant Th2-type antibody profile (IgG1).

The ligand epitope antigen presentation system technology came out of our efforts to direct the immune response toward a Th1 or Th2 orientation for a synthetic peptide vaccine. Immunization of mice with a *Mycobacterium* antigen conjugated to various T-cell binding ligands (TCBLs) induced either Th1- or Th2-type antibody responses to the native epitope of the 38-kDa protein of *Mycobacterium tuberculosis*, depending upon the TCBL used (26). The TCBL peptides have been shown to enhance the immunogenicities of antigenic epitopes, activate T cells, and direct the immune response to either a Th1-type antibody by use of a peptide (referred to as peptide J) from β-2 microglobulin or a Th2-type antibody by use of a peptide (referred to as peptide G) from the β chain of the major histocompatibility complex (MHC) class II (MHC-II) molecule and the immunogens (20, 26, 27). In other studies with the herpes simplex virus (HSV) model, heteroconjugate vaccines containing a T-cell epitope from HSV type 1 (HSV-1) glycoprotein D and peptide J as the TCBLs have been shown to elicit Th1-type responses and protection against HSV-1 challenge (5, 20). More recently, using a modified G called derG (the peptide that has now been renamed CEL-1000), we observed that, in contrast to the TCBL peptide G, CEL-1000 induced a human immunodeficiency virus Gag protein Th1-type antibody profile (IgG2a), with very low levels of antibodies to peptide J or derG (D. H. Zimmerman et al., unpublished

* Corresponding author. Mailing address: CEL-SCI Corporation, 8229 Boone Blvd., Suite 802, Vienna, VA 22182. Phone: (703) 506-9460. Fax: (703) 506-9417. E-mail: Dzimmerman@cel-sci.com.

observations). Since peptide J and CEL-1000 conjugated to immunogenic peptides have been shown to induce specific Th1-type antibody responses and protection in other disease models, the ability of peptide J or CEL-1000 peptide to enhance PyHEP17-mediated protection was evaluated.

Here we report that treatment of A/J mice with a suboptimal dose (5 μg) of conjugated CEL-1000–HEP17 peptide in TiterMax adjuvant induced higher, borderline significantly different levels of protection against challenge with 100 *P. yoelii* sporozoites than treatment with HEP17 alone ($P = 0.0698$). Unexpectedly, we observed that treatment with CEL-1000, a TCBL control peptide, protected 100% of the mice against parasite challenge in the absence of malaria parasite antigen; and this protection was significantly higher than that induced by the HEP17 peptide ($P = 0.0031$). In subsequent experiments we observed that treatment with only 5 μg of CEL-1000 protected 100% of A/J mice against challenge with 5,000 sporozoites, a dose that was 50-fold higher than the minimum infectivity dose (100 sporozoites); we also observed that treatment with CEL-1000 protected C3H/HeJ mice against sporozoite challenge. The finding that CEL-1000 protected A/J mice in the absence of malaria parasite antigen is astonishing. It is not clear why this peptide is protective. To our knowledge, there is no report indicating that CEL-1000 contains a sequence homologous to those of any malaria parasite antigens. This is the first report indicating that a peptide derived from the β chain of human MHC-II is protective against malaria. This finding prompts us to further evaluate the protective effect of CEL-1000 in relation to the potency, duration of protection, stage specificity of protection, and possible mechanisms associated with this protection.

MATERIALS AND METHODS

Mice. Four- to 5-week-old inbred female A/J (*H-2*^{a}), BALB/c (*H-2*^{d}), CAF1 (F_1 hybrid between A/J and BALB/c), and C3H/HeJ (*H-2*^{k}) mice (The Jackson Laboratory, Bar Harbor, Maine) and outbred CD1 mice (Charles River Laboratory, Wilmington, Mass.) were used. The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (15). All animal studies were performed with the approval of the Navy Medical Research Command Institutional Animal Care and Use Committee.

Parasites. *P. yoelii* (nonlethal strain 17XNL, clone 1.1) and *P. berghei* (lethal strain ANKA) were maintained by alternating passage of the parasites in *Anopheles stephensi* mosquitoes and outbred CD1 mice. Sporozoites isolated from the salivary glands of infected mosquitoes were used in the challenge studies to determine the blood stage parasitemia. Sporozoites isolated from the thoraxes of infected mosquitoes by the discontinuous gradient technique (17) were used in the challenge studies to determine the liver stage parasite burden.

Peptides. The five peptides used in this study included HEP17, a 25-amino-acid peptide (SFPMNEESPLGFSPEEMEAVASKFR) containing protective B and T epitopes from the *P. yoelii* hepatocyte-erythrocyte 17-kDa protein (3); J, a 16-amino-acid TCBL peptide (DLLKNGERIEKVEGGG) from human MHC-I β-2 microglobulin (5, 18, 20, 26, 27); CEL-1000, an 18-amino-acid TCBL peptide (DGQEEKAGVVSTGLIGGG) from the second domain of the β chain of the human MHC-II molecule (2, 5, 9, 20, 26, 27); J-HEP17, a peptide containing amino acid sequences from the J and HEP17 peptides (DLLKNGERIEKVEGGG-SFPMNEESPLGFSPEEMEAVASKFR); and CEL-1000–HEP17, a peptide containing amino acid sequences from the CEL-1000 and HEP17 peptides (DGQEEKAGVVSTGLIGGG-SFPMNEESPLGFSPEEMEAVASKFR). All peptides were synthesized by Biosource International (Hopkinville, Mass.) or UCB (Atlanta, Ga.) by the 9-fluorenylmethoxy carbonyl method and were purified by high-pressure liquid chromatography (>95% purity). These peptides were used for immunization or treatment studies. The HEP17 peptide was also used in an enzyme-linked immunosorbent assay (ELISA) for analysis of serum HEP17-specific antibodies.

Antibodies. The antibodies used for the in vivo depletion study included purified rat Ig control antibodies (Rockland Company, Gilbertsville, Pa.); anti-$CD4^+$ monoclonal antibody (MAb) GK1.5, rat IgG2a (from a hybridoma cell line; catalog no. TIB207; American Type Culture Collection, Manassas, Va.); anti-$CD8^+$ MAb 2.43, mouse IgG2a (from a hybridoma cell line; catalog no. TIB210; American Type Culture Collection); anti-IFN-γ MAb XMG-6, rat IgG1 (from a hybridoma cell line; provided by F. Finkelman, University of Cincinnati Medical Center, Cincinnati, Ohio); anti-interleukin-12 (anti-IL-12) MAb C17.8, rat IgG2a (from a hybridoma cell line; provided by M. Wysocka and G. Trinchieri, Wistar Institute, Philadelphia, Pa.); and anti-NK cell antibody, rabbit anti-*N*-asialo GM1 sera (Wako Bioproducts, Richmond, Va.). Ascitic fluids were produced by Harlan Bioproducts for Science (Indianapolis, Ind.), using hybridoma cell lines secreting the MAbs listed above. The MAbs were purified by 50% ammonium sulfate precipitation, and the final antibody concentrations were determined by measurement of the optical density.

Protection of A/J mice immunized with HEP17 peptide conjugated to J or CEL-1000 peptide. A study was designed to determine the adjuvant effect of J or CEL-1000 on the enhancement of protective immune responses of the *P. yoelii* peptide (HEP17) in a murine model of malaria. Briefly, groups of 10 A/J mice were subcutaneously immunized at the base of the tail, two times at 3-week intervals, with 5 or 25 μg of peptides in the presence of TiterMax adjuvant. TiterMax adjuvant-immunized mice and malaria-naïve mice served as adjuvant controls and infectivity controls, respectively. At 10 days after the second immunization, sera were collected from all mice for HEP17-specific antibody analysis. At 2 weeks after the last immunization, all mice were challenged by intravenous injection in the tail vein of 100 infectious *P. yoelii* sporozoites (the minimum infectivity dose) suspended in 200 μl of medium 199 containing 2% normal mouse serum. Parasitemia (blood stage infection) levels were determined by microscopic examination of 200 oil-immersion fields of Giemsa-stained thin smears of blood obtained from mice at 7 and 14 days postchallenge. Mice with negative blood smears through 14 days postchallenge were considered protected.

Malaria parasite-specific antibody analysis. Pooled sera obtained 10 days after the second immunization from mice immunized with J-HEP17, CEL-1000–HEP17, and HEP17 were analyzed by ELISA for HEP17-specific antibody levels, as described previously (3), by using 1 μg of the HEP17 peptide per ml as the test antigen and the J or CEL-1000 peptide as the control antigen.

ELISA analysis of serum for IFN-γ. Mice that received two immunizations of conjugated or unconjugated peptide and that were protected against sporozoite challenged were boosted again at 4 weeks after the second immunization with the same dose of peptide used in the earlier immunizations. Twenty-four hours later these mice were exsanguinated. Serum collected from the mice in each group were pooled and analyzed in duplicate for circulating IFN-γ by a standard ELISA with cytokine ELISA kits (Endogen, Woburn, Mass.) and by the protocol described by the manufacturer. The IFN-γ concentration was calculated by interpolation from a standard curve based on recombinant IFN-γ dilutions run in parallel in the same plate and read on an automated micro-ELISA reader (MR5000; Dynatech) equipped with the manufacturer's software.

Intracellular cytokine analysis. Spleen and liver cells were obtained from some of the protected mice immunized with the conjugated and the unconjugated peptides ($n = 3$ mice per group) and that were boosted again at 4 weeks after the second immunization. Mice immunized with TiterMax adjuvant alone served as negative controls. At 24 h after the last immunization, the livers and spleens were removed from the mice for use in this assay. These organs were homogenized in phosphate-buffered saline (PBS) in a homogenizer (Tissue Sieve; Bellco Glass Inc., Vineland, N.J.), and 5×10^5 spleen or liver cells from the tested or control mice were aliquoted into 96-well U-bottom plates and washed with cold PBS. Cells were stained with labeled antibodies to the surfaces of CD8 cells (fluorescein isothiocyanate [FITC]), CD4 cells (phycoerythrin [PE]), or NK cells (allophycocyanin; Pharmingen, San Diego, Calif.) for 20 min on ice. The cells were washed twice and permeabilized in 100 μl of Cytofix/Cytoperm buffer (Pharmingen) and then stained intracellularly for cytokines with PE or FITC antibodies conjugated to IL-2, IL-4, IL-5, IL-10, IFN-γ, and tumor necrosis factor alpha (Pharmingen), according to the directions of the manufacturer. For each sample, 80,000 to 200,000 events collected with a four-color FACSCALIBUR instrument (Becton Dickinson, San Jose, Calif.) were analyzed with Cellquest software (Becton Dickinson). All assays were performed in 96-well plates.

Protection of mice treated with CEL-1000 peptide in TiterMax adjuvant. To determine the protective efficacy of CEL-1000, groups of 10 A/J mice were subcutaneously injected once or twice with 1.25 to 25 μg of CEL-1000 in TiterMax adjuvant. Mice that received adjuvant alone or malaria-naïve mice served as negative controls. At 2 weeks after administration of the last dose of CEL-1000,

the mice were challenged with 100 *P. yoelii* sporozoites and parasitemia levels were determined at 7 and 14 days postchallenge.

To determine the kinetics and maintenance of protection, the mice were challenged with 100 *P. yoelii* sporozoites at 1, 2, or 4 weeks after the administration of one or two doses of 5 μg of CEL-1000 in TiterMax adjuvant.

To determine the protective effect of CEL-1000 in different mouse strains, groups of 10 mice of different strains (A/J, BALB/c, CAF1, and C3H/HeJ) were subcutaneously injected twice at 3-week intervals with 25 μg of CEL-1000 in TiterMax adjuvant and were challenged 2 weeks later with 100 *P. yoelii* sporozoites. Mice that received TiterMax adjuvant alone and naïve mice served as negative controls.

In some experiments mice treated twice at 3-week intervals with 5 μg of CEL-1000 were challenged with 100 *P. yoelii*-infected erythrocytes (RBCs) to determine the stage specificity of protection or with 100 *P. berghei* sporozoites to determine the species specificity of protection. Since our previous infection studies indicated that malaria-naïve mice that received an intravenous injection of 100 *P. yoelii*-infected RBCs or 100 *P. berghei* sporozoites developed parasitemia within 2 to 10 days postinjection, we determined daily the parasitemia levels in mice treated with CEL-1000 and challenged with infected RBCs or mice treated with CEL-1000 and challenged with *P. berghei* sporozoites, beginning on day 2 and continuing to day 10 postchallenge. Mice that were blood smear negative through 14 days after *P. yoelii* sporozoite challenge or mice that were blood smear negative through 10 days after *P. yoelii*-infected RBC or *P. berghei* sporozoite challenge were considered protected.

Evaluation of liver stage parasite burden. (i) Preparation of liver RNA for real-time quantitative PCR (RTQ-PCR). For the evaluation of the liver stage parasite burden, liver cells isolated from mice that received two injections of 5 μg of CEL-1000 in TiterMax adjuvant were used for TaqMan analysis (25). Livers harvested from mice at 42 h after challenge with 50,000 sporozoites were placed in 15 ml of Trizol reagents (Life Technologies, Gaithersburg, Md.) and homogenized for 90 s at full power with a TH polytron homogenizer (Omni International, Warrenton, Va.). Liver homogenates were aliquoted and stored at −80°C until further RNA isolation could be performed. Total RNA was purified by the standard protocol specified for Trizol by the manufacturer (Invitrogen Life Technologies, Carlsbad, Calif.). The purified total RNA was resuspended in 200 μl of 1× lysis buffer (catalog no. 4305895; Applied Biosystems, Foster City, Calif.) and loaded into a 96-well plate (Invitrogen). Total RNA was further purified with an ABI Prism 6700 automated nucleic acid workstation equipped with an RNA purification tray plate (part no. 4305673; Applied Biosystems. RNA samples were eluted with 200 μl of nucleic acid purification elution solution (catalog no. 4305893; Applied Biosystems).

(ii) Liver parasite burden assay (RTQ-PCR). The master mixture for RTQ-PCR that was prepared contained 2× One-Step RT-PCR master mixture (Applied Biosystems), 100 nM VIC-labeled rodent GAPDH-specific probe (Applied Biosystems, Foster City, Calif.), 200 nM 6-carboxyfluorescein-labeled *P. yoelii* 18S rRNA-specific probe, 80 nM rodent GAPDH-specific forward primer (Applied Biosystems), 20 nM rodent GAPDH-specific reverse primer (Applied Biosystems), and 300 nM each *P. yoelii* 18S rRNA-specific forward primer (primer Py685f) and reverse primer (primer Py782R). The ABI Prism 6700 automated nucleic acid workstation (Applied Biosystems) was programmed to dispense 18 μl of each purified RNA sample into 22 μl of the complete master mixture, and the plates were sealed with optical heat seal covers (part no. 43D7726; Applied Biosystems). Each sample and control was tested in triplicate. Real-time fluorescence detection of the PCR products was performed with an ABI 7700 detector (Applied Biosystems) by using the following thermocycling conditions: 42°C for 30 min to synthesize cDNA, 95°C for 10 min to inactivate the reverse transcriptase and activate the DNA polymerase, and 50 cycles at 95°C for 15 s and 60°C for 1 min. Standard curves were generated for both the rodent GAPDH and *P. yoelii* 18S RNA, and the quantitative parasite burden data were calculated as outlined previously (25). The liver stage parasite burdens are expressed as the mean ± standard error of the mean ratio between *P. yoelii* 18S RNA plasmid equivalents and mouse GAPDH plasmid equivalents obtained from the tested and control mice ($n = 3$).

Mechanism of CEL-1000-induced protection. To identify the specific cell subsets and cytokines involved in CEL-1000-induced protection, groups of 10 A/J mice were treated twice at 3-week intervals with 5 μg of CEL-1000 in TiterMax adjuvant. These mice were then treated with purified rat Ig control antibodies (rat Igs) or with specific antibodies (anti-$CD4^+$ T cells, MAb GK1.5; anti-$CD8^+$ T cells, MAb 2.43; anti-IFN-γ, MAb XMG 6; anti-NK cells, anti-asialo GM1), as described previously (3). For anti-IL-12 treatment, mice received two intraperitoneal injections of 1 mg of MAb C17.8 (anti-IL-12) in 0.5 ml of PBS 12 h before and 3 h after sporozoite challenge. All mice were challenged 14 days after administration of the second dose of CEL-1000, and the level of parasitemia was determined as described above. To confirm the efficacy of cell subset depletion, at 1 day before sporozoite challenge, blood samples were collected from each group of mice and analyzed by FACScan analysis (Becton Dickinson) for determination of the percentage of each cell type. The depletion efficiencies were >97% for $CD4^+$, $CD8^+$, and NK cells.

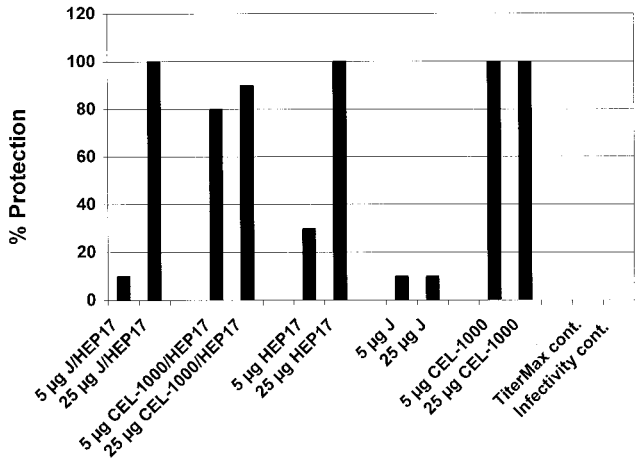

FIG. 1. Protective efficacies of peptides against *P. yoelii* infection in A/J mice immunized with conjugated peptides (J-HEP17 and CEL-1000–HEP17) or unconjugated peptides (HEP17, J, and CEL-1000). Mice ($n = 10$ mice per group) were immunized with 5 or 25 μg of each peptide in TiterMax adjuvant twice at 3-week intervals and were challenged 2 weeks later with 100 sporozoites. The levels of parasitemia were then determined as described in Materials and Methods. Mice that received TiterMax adjuvant alone and malaria-naïve mice served as negative controls (cont.). Protection was defined as negative blood smear results through 14 days postchallenge.

Statistical analysis. The differences in the levels of protection among groups was analyzed by the chi-square test (uncorrected) or Fisher's exact test (two tailed) if the expected cell value was less than 5 (Epi Info Version 6.04b; Centers for Disease Control and Prevention, Atlanta, Ga.). The differences in the levels of parasite RNA among groups were analyzed by Student's unpaired *t* test of log-transformed data. The differences in the frequencies of cell subsets producing IFN-γ among the groups were analyzed by Student's unpaired *t* test.

RESULTS

Protection induced by HEP17 peptide conjugated to J or CEL-1000 peptide. Initially, we evaluated the effect of conjugation of either the J or CEL-1000 peptide to a PyHEP17 peptide (representing defined B- and T-cell epitopes) on protection against *P. yoelii* sporozoite challenge. The results shown in Fig. 1 indicate that A/J mice ($n = 10$ mice per group) that received a high dose (25 μg) of J-HEP17, CEL-1000–HEP17, or unconjugated HEP17 in TiterMax adjuvant had similar levels of protection (90 to 100%). Mice that received a suboptimal dose (5 μg) of CEL-1000–HEP17 had a significantly higher level of protection than mice that received J-HEP17 ($P = 0.0017$) but not a significantly higher level of protection than mice that received HEP17 alone ($P = 0.0697$). Mice immunized with J-HEP17, CEL-1000–HEP17, and HEP17 developed HEP17-specific antibodies; but none of the mice immunized with J or CEL-1000 developed anti-HEP17, anti-J, or anti-CEL-1000 antibodies (data not shown). Unexpectedly, we observed 100% protection in mice that received 5 or 25 μg of CEL-1000 without malaria parasite antigen. The finding that CEL-1000 is protective against sporozoite-induced malaria prompted us to further evaluate the prophylactic effect of this

TABLE 1. Potency of CE1-1000 in A/J mice[a]

Expt no. and antigen dose (μg) or treatment	No. of sporozoites used for challenge	No. of mice protected/no. tested	% Protection	*P* value[b]
Expt 1				
1.25	100	6/10	60	0.0190763
2.5	100	8/10	80	0.0016537
5	100	10/10	100	0.0000523
TiterMax adjuvant (control)	10	1/10	10	
Expt 2				
5	100	10/10	100	0.0000077
5	1,000	10/10	100	0.0000077
5	5,000	10/10	100	0.0000077
TiterMax adjuvant (control)	100	0/10	0	

[a] Mice were injected twice at 3-week intervals with various doses of CEL-1000 in TiterMax adjuvant and were challenged 2 weeks later with different numbers of *P. yoelii* sporozoites, as indicated. Protection was defined as negative blood smear results through 14 days postchallenge.
[b] A *P* value ≤0.05 (chi-square test) compared to the result for the relevant control was considered significant.

compound in the murine model of malaria in relation to the potency, duration of protection, and the species and stage specificities of protection and the possible mechanisms associated with this protection.

Evaluation of potency of CEL-1000. To evaluate the potency of CEL-1000, A/J mice (n = 10/group) that received two injections of 1.25 to 5 μg of CEL-1000 in TiterMax adjuvant were challenged with 100 to 5,000 sporozoites, followed by determination of the parasitemia level. The results in Table 1 indicate that two injections of as little as 1.25 μg of CEL-1000 in TiterMax adjuvant protected 60% of the mice against challenge with 100 sporozoites (the minimum infectivity dose), and two injections of 5 μg of CEL-1000 protected 100% of the mice against challenge with up to 5,000 sporozoites. These data demonstrate that CEL-1000 is a very potent agent for prophylaxis against *P. yoelii* sporozoite-induced infection.

Serum cytokine levels and frequencies of cell subsets in peptide-immunized and protected mice. Since previous studies (3) established that PyHEP17 peptide-induced protection is dependent on $CD4^+$ T cells and IFN-γ, we investigated whether the protection induced by these conjugated and unconjugated peptides was mediated by similar immune mechanisms. Accordingly, we measured serum IFN-γ levels in the immunized and protected mice by ELISA and the frequencies of $CD4^+$ IFN-γ-positive (IFN-γ^+) T cells in livers and spleens by staining for intracellular cytokines. The immunized and protected mice were boosted again with the same dose of peptide used in the previous immunization, and sera collected 24 h later from each group of mice were pooled and analyzed by ELISA, as described in Materials and Methods. The results in Fig. 2 show that sera pooled from CEL-1000–HEP17-immunized and protected mice (5-μg dose, n = 5; 25-μg dose, n = 9), HEP17-immunized and protected mice (5-μg dose, n = 3; 25-μg dose, n = 10), and CEL-1000-immunized and protected mice (5 or 25 μg dose, n = 10) had different serum IFN-γ levels, with the highest levels detected in CEL-1000-immunized and protected mice. Intracellular staining of liver cells isolated from immunized and protected mice (n = 3 mice per group) 24 h after the booster injection showed higher frequencies of $CD4^+$ IFN-γ^+ T cells in the immunized and protected mice that received the high dose of CEL-1000–HEP17 or the HEP17 peptide and the mice that received 5 or 25 μg of CEL-1000 than the frequencies in mice that received TiterMax adjuvant (n = 2 mice per group), with the highest frequency detected in CEL-1000-immunized and protected mice (Fig. 3). Because of the small sample sizes (n = 3) and the low frequencies of $CD4^+$ IFN-γ^+ cells, the only comparison that can be made is between the TiterMax adjuvant-treated control group and the CEL-1000-treated group (P = 0.0719, Student's t test, two tailed). Slight increases in the frequencies of splenic $CD4^+$ IFN-γ^+ T cells and $CD4^-$ IFN-γ^+ T cells were also observed (data not shown). These findings establish that immunization of mice with CEL-1000 in TiterMax adjuvant induces higher levels of serum IFN-γ and higher frequencies of hepatic and splenic $CD4^+$ IFN-γ^+ T cells, as well as higher levels of protection, compared to those achieved by immunization with conjugated CEL-1000–HEP17 or unconjugated HEP17 peptide. The data are consistent with the roles of

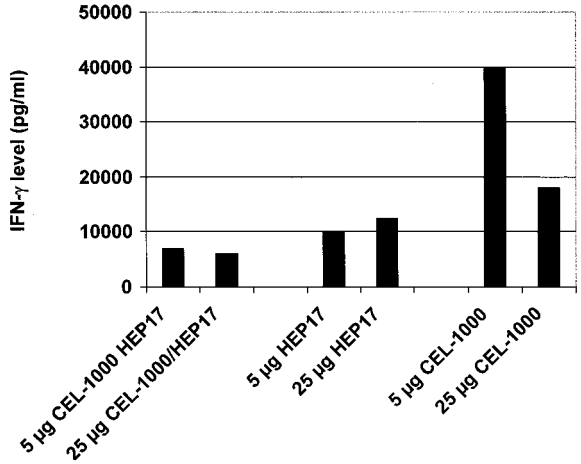

FIG. 2. Serum IFN-γ levels in immunized and protected mice. At 4 weeks after the second immunization, the immunized and protected mice (5 μg of CEL-1000–HEP17, n = 8; 25 μg of CEL-1000–HEP17, n = 9; 5 μg of HEP17, n = 3; 25 μg of HEP17, n = 10; 5 or 25 μg of CEL-1000, n = 10 for each dose group) were boosted again with the same dose of each peptide used in the previous immunization. Serum samples were collected from the mice 24 h later. Sera from mice in each group were pooled and analyzed by ELISA to determine IFN-γ levels, as described in Materials and Methods.

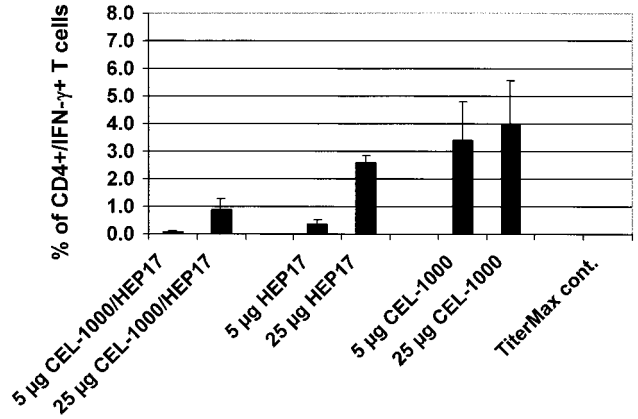

FIG. 3. Frequencies of $CD4^+$ IFN-γ^+ T cells in the livers of immunized and protected mice. At 4 weeks after the second immunization, the immunized and protected mice ($n = 3$ mice per group) were boosted again with the same dose of immunogens used in the previous immunization. Mice that received TiterMax adjuvant alone served as controls (cont.). Liver cells harvested from individual mice 24 h later were analyzed by intracellular cytokine staining, as described in Materials and Methods. The results are reported as the group mean ± standard error of frequencies of $CD4^+$ IFN-γ^+ T cells.

IFN-γ and $CD4^+$ IFN-γ^+ T cells in CEL-1000-induced protection in the murine model of *P. yoelii* malaria.

Kinetics of induction and maintenance of CEL-1000-induced protection. Since CEL-1000 was capable of protecting mice against sporozoite challenge, we next evaluated the kinetics of induction and maintenance of protection in A/J mice. Mice injected with one or two doses of 5 μg of CEL-1000 (at 3-week intervals) were challenged with 100 *P. yoelii* sporozoites at 1, 2, or 4 weeks after administration of the last dose of CEL-1000. Mice treated with TiterMax adjuvant alone served as controls. The results in Fig. 4 demonstrate that as early as 1 week after treatment, 40% of the mice that received a single dose of CEL-1000 and 90% of the mice that received two doses

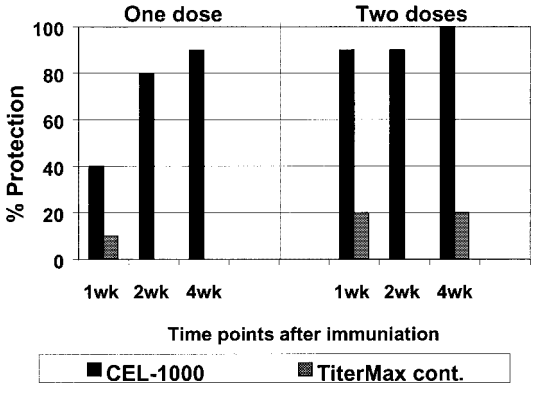

FIG. 4. Kinetics and duration of protection in A/J mice treated with CEL-1000 peptide before challenge. Mice ($n = 10$ mice per group) that received one or two injections of 5 μg of CEL-1000 in TiterMax adjuvant at 3-week intervals and mice that received TiterMax adjuvant alone were challenged with 100 *P. yoelii* sporozoites at 1, 2, or 4 weeks after the last injection, followed by determination of parasitemia levels, as described in Materials and Methods. Protection was defined as negative blood smear results through 14 days postchallenge.

of CEL-1000 were protected. The level of protection increased with time after CEL-1000 treatment (greater protection at week 2 compared to that at week 1) and was maintained for at least 4 weeks, with protection levels of 90 and 100% in mice that received one and two doses of CEL-1000, respectively. A small number ($n = 5$) of protected and available mice were rechallenged 4 months later after they were given an additional booster injection, and 80% of these mice remained protected (data not shown). These data suggest that the protection against *P. yoelii* sporozoite challenge induced by CEL-1000 is long lasting.

Protection against *P. yoelii* blood stage parasite challenge. To evaluate the protective efficacy of CEL-1000 against blood stage parasite challenge, mice that received two injections of 5 μg of CEL-1000 in TiterMax adjuvant at 3-week intervals were challenged at 2 weeks after the second injection with 100 *P. yoelii*-infected RBCs. Mice that received TiterMax adjuvant alone served as controls. None of the CEL-1000-treated mice or TiterMax adjuvant-treated control mice were protected against challenge with infected RBCs (data not shown). This is in contrast to the results of sporozoite challenge, in which protection was observed (Fig. 1 and 4). These results suggest that the protection induced by CEL-1000 is not directed against the blood stage parasites.

Protection against *P. berghei* sporozoite challenge. In the experiment evaluating protection against *P. berghei* sporozoite challenge, we treated groups of 10 A/J mice with two injections of 5 μg of CEL-1000 at 3-week intervals and challenged the mice 2 weeks later with 100 *P. berghei* (lethal strain ANKA) sporozoites. Only 2 of 10 (20%) of these mice were protected, whereas none of the TiterMax adjuvant-treated control mice (0 of 10) or the infectivity control mice (0 of 10) were protected (data not shown). These results indicate that with this administration regimen, CEL-1000 confers some degree of protection against challenge with sporozoites from a second species of murine malaria parasites. The lower level of protection against *P. berghei* sporozoite-induced malaria compared to that against *P. yoelii* sporozoite-induced malaria may be because *P. berghei* (lethal strain ANKA) is more virulent than *P. yoelii* (nonlethal strain 17XNL). Normally, mice challenged with 100 *P. berghei* sporozoites exhibit blood stage parasitemia within 2 to 10 days and progress to death within 14 days, but in this study parasitemia and not death was used as an end point of the study (per Institutional Animal Care and Use Committee approval). In contrast, mice challenged with 100 *P. yoelii* 17XNL sporozoites exhibited parasitemia later, within 5 to 14 days, and self-cured within 21 days.

Evaluation of liver stage parasite burden. To determine whether the protection induced by CEL-1000 is directed against the liver stage parasites, we injected A/J mice with two doses of 5 μg of CEL-1000 in TiterMax adjuvant and challenged the mice 2 weeks later with 50,000 *P. yoelii* sporozoites. Mice that received TiterMax adjuvant alone or untreated mice served as negative controls. The results in Fig. 5 demonstrate that treatment of mice with CEL-1000 prior to challenge significantly inhibited the liver stage parasite burden ($P = 0.01$), as determined by TaqMan RTQ-PCR analysis for parasite RNA in the mouse livers. It should be noted that a greater than 400-fold reduction in the level of parasite RNA was observed in mice treated with CEL-1000 before challenge compared to

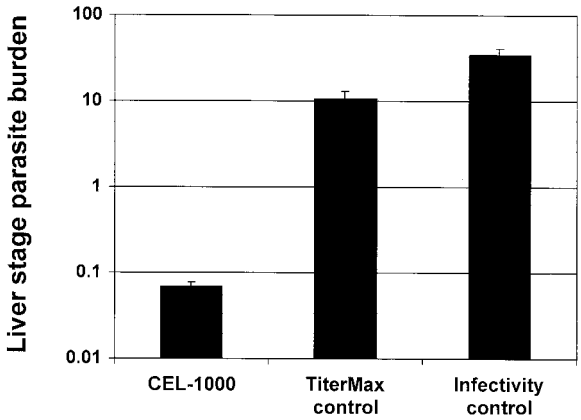

FIG. 5. *P. yoelii* liver stage parasite burden in CEL-1000-treated and challenged mice. Mice were treated with two injections of 5 μg of CEL-1000 in TiterMax adjuvant ($n = 5$). Mice that received TiterMax adjuvant alone ($n = 5$) or malaria-naïve mice ($n = 3$) served as controls. At 2 weeks after the second injection, the mice were challenged with 50,000 sporozoites and their livers were isolated 42 h later for analysis of the liver stage parasite burden, as described in Materials and Methods. The liver stage parasite burden was expressed as the group mean ± standard error of the mean of a ratio between amplified parasite RNA plasmid equivalents and amplified mouse GAPDH plasmid equivalents.

the levels in the TiterMax adjuvant-treated control mice or the infectivity control mice. These data establish that the protection induced by CEL-1000 is directed against the liver stage of the parasite life cycle.

Mechanisms of CEL-1000-induced protection. Next we attempted to elucidate the immune mechanisms responsible for CEL-1000-induced protection. Since both IFN-γ and $CD4^+$ T cells have been implicated in malaria parasite peptide-induced protection against sporozoite challenge (2, 21, 22), the mechanisms of CEL-1000-induced protection were evaluated by the in vivo depletion of various cell subsets and cytokines from CEL-1000-treated mice just prior to sporozoite challenge. The results in Table 2 demonstrate that the mice that received CEL-1000 in TiterMax adjuvant were completely protected against parasite challenge, whereas the control mice treated with TiterMax adjuvant alone were not protected. Treatment of mice that received CEL-1000 with antibodies to IFN-γ prior to challenge abolished the protection ($P = 0.0000077$), whereas 100% protection was achieved in mice that received CEL-1000 and rat Ig control antibodies. Depletion of $CD4^+$ T cells partially reduced (30%) the level of protection ($P = 0.001$). However, depletion of either $CD8^+$ T cells or NK cells or treatment with antibodies to IL-12 did not alter the protection levels. These data suggest that the protection against *P. yoelii* malaria delivered by CEL-1000 is dependent on IFN-γ and is partially dependent on $CD4^+$ T cells but is independent of $CD8^+$ T cells, NK cells, or IL-12 at the effector phase of the responses, which is consistent with a Th1 response.

Protection in mice with different genetic backgrounds. Studies on the protective effect of CEL-1000 in mice with different genetic backgrounds indicated that CEL-1000 had a protective effect in A/J ($H\text{-}2^a$) and C3H/HeJ ($H\text{-}2^k$) mice, with A/J mice protected at higher levels than C3H/HeJ mice. However, CEL-1000 had no protective effect in BALB/c ($H\text{-}2^d$) and CAF1 mice (CAF1 is an F_1 hybrid of A/J and BALB/c mice) (Table 3). This genetic restriction of protection induced by CEL-1000 was similar to that induced by two malaria parasite peptides that also protected A/J mice but not BALB/c mice against sporozoite challenge (3, 23). Sera collected from A/J, BALB/c, CAF1, and C3H/HeJ mice 10 days after the second injection of 25 μg of CEL-1000 were analyzed by ELISA against CEL-1000 peptide and by immunofluorescence assay against mouse liver cells and were negative by both assays, suggesting that treatment of mice with CEL-1000 by this administration regimen does not induce antibodies to MHC-II molecules (autoantibodies).

DISCUSSION

CEL-1000 is an 18-amino-acid TCBL peptide containing amino acid sequences derived from the β2 chain of human MHC-II, except that the glutamine at the N terminus is replaced by glutamic acid. Initially, we evaluated the capacities of two TCBL peptides (J and CEL-1000) to enhance the protection offered by a malaria parasite peptide (HEP17) in a murine model of malaria. Two immunizations with a high dose (25 μg) of conjugated J-HEP17, conjugated CEL-1000–HEP17, or unconjugated HEP17 peptide in TiterMax adjuvant induced similar levels of protection (90 to 100%). However, two immunizations with a suboptimal dose (5 μg) of CEL-1000–HEP17

TABLE 2. Depletion of IFN-γ eliminates protection from mice[a] that received CEL-1000 in TiterMax adjuvant and depletion of $CD4^+$ T cells partially reduces protection level

Treatment	No. of mice protected/no. tested	% Protection	*P* value[b]
CEL-1000, undepleted	10/10	100	0.0000077
Control TiterMax adjuvant	0/10	0	
Anti-CD4 T cells	7/10	70	0.0010320
Anti-CD8 T cells	10/10	100	
Anti-NK cells	10/10	100	
Anti-IFN-γ	0/10	0	0.0000077
Anti-IL-12	0/10	100	
Rat Ig control	10/10	100	

[a] Mice that received CEL-1000 were treated with specific reagents (test and control antibodies), as described in Materials and Methods. Depletion efficiency was determined by fluorescence-activated cell sorter analysis and was >97% for all cell types ($CD4^+$, $CD8^+$, and NK cells). Protection was defined as negative blood smear results through 14 days postchallenge.

[b] A *P* value ≤0.05 (chi-square test) compared to the result for the relevant control was considered significant.

TABLE 3. Protection against *P. yoelii* sporozoite challenge in different mouse strains following treatment with CEL-1000 in TiterMax adjuvant[a]

Expt no. and mouse strain	Treatment or group	No. of mice protected/no. tested	% Protection	*P* value[b]
Expt 1				
A/J (*H-2*^{a})	CEL-1000, TiterMax adjuvant	10/10	100	0.0000077
	TiterMax adjuvant (control)	0/10	0	
	Infectivity control	0/10	0	
BALB/c (*H-2*^{d})	CEL-1000, TiterMax adjuvant	1/10	10	
	TiterMax adjuvant (control)	1/10	10	
	Infectivity control	0/10	0	
Expt 2				
A/J (*H-2*^{a})	CEL-1000, TiterMax adjuvant	8/10	80	0.0002607
	TiterMax adjuvant (control)	0/10	0	
	Infectivity control	0/10	0	
BALB/c (*H-2*^{d})	CEL-1000, TiterMax adjuvant	0/10	0	
	TiterMax adjuvant (control)	1/10	10	
	Infectivity control	0/10	0	
CAF1 (F_1 hybrid)	CEL-1000, TiterMax adjuvant	0/10	0	
	TiterMax adjuvant (control)	1/10	10	
	Infectivity control	0/10	0	
C3H/HeJ (*H-2*^{k})	CEL-1000, TiterMax adjuvant	4/10	40	0.0327271
	TiterMax adjuvant (control)	0/9	0	
	Infectivity control	0/10	0	

[a] Mice were injected twice at 3-week intervals with 25 μg of CEL-1000 in TiterMax adjuvant and were challenged with 100 *P. yoeliii* sporozoites 2 weeks after the second injection. Mice that received TiterMax adjuvant alone and malaria-naïve mice served as negative controls. Parasitemia was determined as described in Materials and Methods. Protection was defined as negative blood smear results through 14 days postchallenge.

[b] A *P* value ≤0.05 (chi-square test) compared to the results for the TiterMax adjuvant-treated control was considered significant.

induced higher levels of protection (80%) compared to those achieved with two immunizations of HEP17 (30%), J-HEP17 peptide (10%), and the TiterMax adjuvant control (0%). These results suggest that enhanced protection can be observed with suboptimal doses of CEL-1000–HEP17, but this level of protection was not significantly different from that induced by HEP17 alone ($P = 0.0698$). Unexpectedly, CEL-1000 yielded 100% protection when it was administered with TiterMax adjuvant without any malaria antigen. It is not known why CEL-1000 is more protective in mice than CEL-1000–HEP17. Two possible explanations based on our observations are as follows. (i) Mice treated with CEL-1000 and then challenged produced higher levels of IFN-γ than mice immunized with CEL-1000–HEP17 and then challenged (Fig. 1), and IFN-γ is absolutely required for protection against sporozoite challenge (3, 4, 22, 23). (ii) Mice that received CEL-1000 alone developed only a Th1 response, as shown by the high levels of IFN-γ produced in their sera with no detectable antibodies to CEL-1000, while mice that received CEL-1000–HEP17 developed HEP17-specific antibodies and IFN-γ, indicative of both Th2 and Th1 responses. The Th2 portion of the response may downregulate the Th1 response (IFN-γ production), thereby diminishing protection.

P. yoelii sporozoites are highly infectious. Injection of 100 sporozoites intravenously consistently infected 100% of A/J mice within 14 days. The findings that two injections of as low as 1.25 μg of CEL-1000 in TiterMax adjuvant could protect 60% of these mice against challenge with 100 *P. yoelii* sporozoites and that two injections of only 5 μg of CEL-1000 could protect 100% of the mice against challenge with up to 5,000 sporozoites clearly demonstrate that CEL-1000 has a potent prophylactic ability in the *P. yoelii* model of malaria. CEL-1000 at the same doses was also able to confer some degree of protection against a second species of organisms causing malaria, the more virulent species *P. berghei* (lethal strain ANKA), although the protection conferred was not as strong as that conferred against *P yoelii* (a nonlethal strain). This regimen of treatment (two doses of 5 μg of the CEL-1000 peptide at 3-week intervals) may not be adequate, and it is possible that a higher dose of CEL-1000 may be required to induce complete protection against *P. berghei*, a highly virulent species causing malaria. It should be noted that in our studies parasitemia and not death was used as the end point of the assay.

Evaluation of the kinetics and the maintenance of protection indicated that protection could be achieved as early as 1 week after the administration of one or two doses of CEL-1000 (40% of the mice were protected after they received a single dose and 90% were protected after they received two doses). The levels of protection increased with time and reached a peak when the mice were challenged 4 weeks after CEL-1000 treatment (90% were protected after they received a single dose and 100% were protected after they received two doses). The two-dose regimen resulted in a higher level of protection than the single-dose regimen at both early (1 week) and late (4 weeks) times of challenge. The protected mice that received

two doses of CEL-1000 were rechallenged with 100 sporozoites 4 months later, and 80% of these mice did not become infected, suggesting that the protection induced is long lasting.

Treatment with CEL-1000 before challenge with a dose of sporozoites more massive ($n = 50,000$) than that normally used to infect all animals (100 sporozoites) significantly reduced the liver stage parasite burden (>400-fold [$P = 0.01$]) compared to that in the TiterMax adjuvant-treated controls. CEL-1000 conferred no protection against blood stage parasite challenge. Together, these results establish that the protection induced by CEL-1000 is targeted at the liver stage and not at the blood stage.

The finding that CEL-1000 protected A/J mice in the absence of malaria antigens is very much surprising to us. It is not clear why this peptide is protective. To our knowledge, there is no report indicating that CEL-1000 contains an amino acid sequence homologous to amino acid sequences of *P. yoelii* and HSV antigens. This is the first report indicating that a peptide derived from the β chain of human MHC-II effectively offers protection against malaria. We therefore decided to further evaluate the possible mechanisms associated with this protection by performing a depletion study.

Analyses of cell populations in CEL-1000-treated and protected mice demonstrated elevations in the frequencies of hepatic $CD4^+$ IFN-γ^+ T cells but not $CD8^+$ T cells or NK cells. In addition, elevated levels of splenic $CD4^-$ IFN-γ^+ cells but not $CD8^+$ T cells or NK cells were also observed, suggesting that other cell types may contribute in part to the production of IFN-γ responsible for this protection. In vivo depletion of cell subsets and cytokines from CEL-1000-treated mice just prior to challenge with *P. yoelii* sporozoites established that the protection delivered by CEL-1000 was totally dependent on IFN- γ and was partially dependent on $CD4^+$ T cells but was not dependent on $CD8^+$ T cells, NK cells, or IL-12. The exact mechanism of CEL-1000-induced protection is not completely understood at present and may be due in part to the production of nitric oxide as a consequence of the action of IFN-γ. In vitro and in vivo data from other studies indicate that the IFN-γ produced by malaria parasite-specific $CD8^+$ T cells stimulated *Plasmodium*-infected hepatocytes to produce NO to eliminate the liver stage parasites (11, 12).

Treatment of A/J (*H-2*^{a}) or C3H (*H-2*^{k}) mice with CEL-1000 prior to challenge with infectious *P. yoelii* sporozoites, in the absence of malaria antigen, solidly protected the mice against parasite challenge. Interestingly, CEL-1000 was not protective in BALB/c (*H-2*^{d}) or CAF1 (A/J × BALB/c F_1 hybrid) mice, indicating genetic differences in the effects of CEL-1000. Others have shown that BALB/c mice are more susceptible to infections with parasites, such as *Leishmania major*, since they are prone to have Th2-type immune responses (6, 7, 8, 13, 14, 16), and this offers an explanation for the lack of protection in CEL-1000-treated BALB/c mice upon challenge with *P. yoelii* sporozoites.

It appears that the mechanism associated with the protection induced by CEL-1000 in this study is similar to that induced by two malaria parasite peptides, PyHEP17 and PySSP2, which also depend on IFN-γ and $CD4^+$ T cells (3, 23), but is different from that induced by *P. yoelii*-irradiated sporozoites or circumsporozoite protein DNA vaccine, which is dependent on $CD8^+$ T cell or $CD8^+$ T cells plus NK cells for the production of the IFN-γ, IL-12, and nitric oxide that are responsible for this protection (4, 21, 22, 24). In the HSV model, treatment of mice with CEL-1000 confers protection against HSV-1 challenge, and this protection appears to be initiated by IL-12, as indicated by the rapid and prolonged (>5 days) appearance of IL-12 in the sera after CEL-1000 treatment, and on the basis of MAb treatment involves CD4 cells and IFN-γ but not CD8 cells (N. Goel, unpublished observations). IL-12 is a strong inducer of IFN-γ production by CD4 and NK cells. However, IL-12 production could be involved in CEL-1000-mediated protection in the inductive phase and not at the effector or challenge phase, or it may not be involved at all in CEL-1000-mediated protection, which explains the ineffectiveness of anti-IL-12 and anti-NK cell antibodies at the challenge phase in the murine model of *P. yoelii* malaria. The exact mechanism of CEL-1000-induced protection is unknown. The restriction of protection to mouse strains with certain genetic backgrounds is probably due to differences at the MHC-II loci, which are identical in A/J and C3H/HeJ mice (*I-A*^{k}, *I-E*^{k}), which were protected. These loci are totally different from those in the nonprotected BALB/c strain (*I-A*^{d}, *I-E*^{d}). The protection induced by CEL-1000 could also be due to nonspecific stimulation of cytokine production. A/J mice have the ability to produce IL-12 (1, 10), while this ability is defective in BALB/c mice (6, 7, 8, 16); and IL-12 is a potent activator of IFN-γ production (Th1 response). However, these mechanisms are only speculations. Detailed studies to determine the exact mechanisms responsible for CEL-1000-induced protection need to be further elucidated.

In summary, we have demonstrated that treatment with the CEL-1000 peptide prior to challenge with sporozoites from *P. yoelii* protects A/J and C3H/HeJ mice against parasite challenge. This protection is totally dependent on IFN-γ, is partially dependent on $CD4^+$ T cells, but is not dependent on IL-12, $CD8^+$ T cells, or NK cells. We have also demonstrated that CEL-1000 is a very potent prophylactic agent, with only two injections of 5 μg each conferring complete protection against challenge with up to 5,000 sporozoites (500 times the minimum infectivity dose). Moreover, CEL-1000 has also been shown to induce protection against HSV-1 challenge (N. Goel, unpublished), and this protection is also dependent on $CD4^+$ T cells and IFN-γ. Together, these results demonstrate that CEL-1000 has a potential prophylactic capability against malaria and HSV, with the data for HSV also suggesting that it has therapeutic potential. Further evaluation of the capacity of CEL-1000 to protect against other malaria parasite species and other diseases whose protection is mediated by Th1-type immunity is warranted.

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