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Picornavirus-Specific CD4$^+$ T Lymphocytes Possessing Cytolytic Activity Confer Protection in the Absence of Prophylactic Antibodies†

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Picornaviruses are a family of positive-strand RNA viruses that are responsible for a variety of devastating human and animal diseases. An attenuated strain of mengovirus (vMC$_{24}$) is serologically indistinguishable from the lethal murine wild-type mengovirus and encephalomyocarditis virus (EMCV). Immunogen-specific stimulation of vMC$_{24}$-immune splenocytes in vitro demonstrates preferential activation of CD4$^+$ lymphocytes. vMC$_{24}$-immune splenocytes adoptively transferred to naive recipients conferred protection against lethal EMCV challenge. Immune splenocytes, expanded in vitro, were >92% CD4$^+$ T lymphocytes. Interestingly, adoptive transfer of these expanded cells engendered protection against lethal challenge. In vivo depletion of CD4$^+$ T lymphocytes prior to lethal challenge abrogated survival of transfer recipients, confirming that CD4$^+$ T lymphocytes were essential for protection. Subsequent rechallenge of vMC$_{24}$-immune splenocyte recipients with a greater EMCV dose elicited serum neutralizing antibody titers paralleling the high titers observed in vMC$_{24}$-immunized mice. Unexpectedly, an augmented humoral response was absent in vMC$_{24}$-specific CD4$^+$ T-cell recipients after the secondary challenge. Moreover, comparably low serum neutralizing antibody titers failed to protect passive transfer recipients when correspondingly challenged. vMC$_{24}$-immune splenocytes expanded in vitro (>94% CD4$^+$) lysed vMC$_{24}$-infected A20.J target cells. The ability to transfer protection with primed CD4$^+$ T cells, in the absence of primed B lymphocytes or immune sera, is novel for picornaviral infections. Consequently, mechanisms such as CD4$^+$ cytolytic T-lymphocyte activity are implicated in mediating protection.

Picornaviruses are some of the smallest positive-strand RNA viruses known, and yet they constitute a notably large family of medically and agriculturally important pathogens. Members of this highly virulent family include poliovirus, human rhinovirus, foot-and-mouth disease virus, and encephalomyocarditis virus (EMCV). On the basis of virion structure and numerous biological criteria (42), the family is divided into five genera: enteroviruses, hepatoviruses, rhinoviruses, aphthoviruses, and cardioviruses. According to the current picornavirus paradigm, prophylaxis against infection is afforded by serum neutralizing antibodies (29, 30, 33). Antigenic determinants map to protruding surface loops of one or more of the outermost surface capsid proteins, VP1, VP2, or VP3 (3, 29, 32, 51). Existing picornavirus vaccines (29, 30), in addition to current strategies utilizing recombinant-attenuated and protein-subunit vaccine designs (32, 35), are designed to elicit a protective neutralizing antibody response to capsid determinants within the vaccinated host. Indeed, serum neutralizing titers are utilized to evaluate host immune status to a particular picornaviral pathogen.

Mice are highly susceptible to cardiovirus infections (11, 36), resulting in acute neurotropic disease producing rapid and lethal meningoencephalomyelitis (19, 52). The ability to protect mice against cardiovirus-induced disease by the elicitation or passive transfer of neutralizing antibodies is well documented (5, 16, 31, 40). Mice immunized with an attenuated strain of mengovirus (vMC$_{24}$) elicit high serum neutralizing antibody titers and are protected from lethal EMCV challenge (13). The dramatic attenuation of mengovirus by a truncation in the 5′-noncoding-region poly(C) tract preserves complete integrity of all virally encoded proteins (14), allowing in vivo exposure of structural and nonstructural proteins that may elicit an immune response.

The vast majority of investigations examining cardiovirus cell-mediated immunity have focused on elucidating the potential immunopathologic role that T cells may contribute toward demyelination. T-cell subset depletion of BALB/c mice with anti-CD4 or anti-CD8 antibodies prior to EMCV infection ameliorated clinical disease and reduced the frequency of demyelination (47). T-cell subset depletion following infection failed to abrogate disease progression (50). Mice rendered CD4-deficient prior to Theiler's murine encephalomyelitis virus (TMEV) infection failed to produce neutralizing antibodies and consequently were unable to clear virus from the central nervous system and died from overwhelming encephalitis (39, 53).

Characterization of T cells involved in a protective immune response to picornaviral infections is notably lacking. A recent study indicated that CD4$^+$ T lymphocytes are essential in providing help to B lymphocytes for generation of neutralizing antibodies in TMEV-infected mice (6). Passively transferred anti-TMEV antibody allowed survival of CD4$^+$-cell-depleted mice during the early phase of disease. However, virus control was less efficient in these animals during the latter stages of disease, suggesting that CD4$^+$ T lymphocytes have additional protective functions.

The present study examined an effector role of CD4$^+$ T cells in conferring protection to Mengo virus or EMCV infection. Naive mice were adoptively transferred with vMC$_{24}$-immune

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† This study is dedicated to the memory of Heidi M. Hotchkiss.

splenocytes that were expanded in vitro with UV-inactivated immunogen and recombinant interleukin-2 (rIL-2). These cultured splenocytes were >92% $CD4^+$ and exhibited T-cell-specific defense based on survival of recipients challenged with a lethal dose of EMCV. This protection was rescinded in recipients depleted of $CD4^+$ T lymphocytes prior to challenge. Surprisingly, recipient survival occurred in the absence of prophylactic levels of serum neutralizing antibodies. Additionally, these cultured immune splenocytes demonstrated cytolytic capability by killing vMC_{24}-infected A20.J B-lymphoma target cells. The ability to mediate protection with primed $CD4^+$ T cells is novel for any picornaviral infection, challenging the picornavirus paradigm of antibody-mediated protection from lethal disease. The expression of cytolytic activity represents a potentially important effector function of the transferred cell population, such as $CD4^+$ cytolytic T-lymphocyte (CTL) activity. One possible explanation for the lack of neutralizing antibodies might be in vivo cytolysis directed toward B lymphocytes, functioning as major histocompatibility complex (MHC) class II^+ antigen-presenting cells or harboring viral infection and suppressing a conspicuous humoral response.

MATERIALS AND METHODS

Mice. Six- to 10-week-old BALB/c ($H\text{-}2^d$) mice were obtained from the colony maintained at the Department of Animal Health and Biomedical Sciences (University of Wisconsin—Madison). Mice were handled according to the University of Wisconsin—Madison Research Animal Resource Center guidelines.

Virus stocks. All cardioviruses, EMCV strain Rueckert, intact 5′-noncoding-region poly(C) tract wild-type mengovirus (vMCwt), vMC_{24} [a partially truncated 5′-noncoding-region poly(C) tract-attenuated mengovirus strain] (referred to as pM16 in earlier publications by others [13, 14]), and a completely truncated noncoding-region poly(C) tract-attenuated mengovirus strain (vMC_0), were kindly provided by Ann C. Palmenberg, University of Wisconsin—Madison. Poliovirus (Sabin 1) was a kind gift from Ann G. Mosser, University of Wisconsin—Madison. Stocks were typically provided at 10^7- to 10^{10} PFU/ml in clarified HeLa cell lysates or as sucrose-purified viral preparations.

Murine cell lines. All cell lines are the $H\text{-}2^d$ haplotype and were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum (FCS) (herein referred as RPMI 1640–10% FCS). P815 (ATCC TIB-64) is an MHC class I^+ mastocytoma cell line. RAW264.7 (ATCC TIB-71) is an MHC class I^+ monocyte-macrophage cell line. A20.J (ATCC TIB-208) is an MHC class I^+/II^+ B-lymphoma cell line and is surface immunoglobulin G positive (IgG^+). J774A.1 (ATCC TIB-67) is an MHC class I^+ monocyte-macrophage cell line.

Immunization. Mice were immunized intraperitoneally (i.p.) with 10^6 PFU of vMC_{24} in 1.0 ml of phosphate-buffered saline (PBS). At 4 weeks postinoculation, immune sera and primed splenocytes were obtained from donor animals. Control vMC_{24}-inoculated mice were challenged i.p. with 10^6 PFU of EMCV to ensure efficacy of immunization.

Lymphocyte proliferation assay. The T-cell proliferation assay is composed of two phases, antigen-specific activation resulting in up-regulated IL-2 receptor expression followed by IL-2-dependent expansion of activated cells (7). Single-cell suspensions of splenocytes were prepared by gently teasing the organ through 100-mesh grid wire in chilled RPMI 1640 without serum. Viable cells were isolated by using Lymphoprep (Nycomed Pharma AS, Oslo, Norway), followed by three washes in PBS. Cells resuspended in RPMI 1640 supplemented with 2% heat-inactivated syngeneic normal mouse serum were cultured at 2.5×10^5 to 5.0×10^5 per well in 96-well, U-bottom microwell plates (Costar 3799; Costar Corp., Cambridge, Mass.) in 50 μl. Cultures were stimulated with 50 μl of UV-inactivated virus or concanavalin A (2.5 μg/ml, final concentration) diluted in RPMI 1640 without serum. Optimal virus concentrations are shown for individual experiments. Cultures were incubated at 37°C for 72 h in a humidified atmosphere of 5% CO_2. Culture plates were slightly agitated to suspend the cell pellet, and 0.1 ml of RPMI–10% FCS (Sigma) was added to each well. Following resuspension, 0.1-ml aliquots were transferred to wells containing 0.1 ml of RPMI-10% FCS and 100 U of human rIL-2 (Hoffmann-La Roche, Inc., Nutley, N.J.) per ml. The plates were incubated for an additional 48 h, with pulsing with 1.0 μCi of [^{3}H]thymidine (DuPont, Wilmington, Del.) during the final 10 h. Cultures were harvested onto glass filter paper, and [^{3}H]thymidine incorporation was determined by liquid scintillation spectrometry. All assays were performed in triplicate, and the results were expressed as the means (in counts per minute) ± standard deviations.

Antibodies. Hybridomas producing monoclonal antibodies directed against Thy-1.2 (ATCC TIB-107, rat IgG2b), CD4 (ATCC TIB-207, rat IgG2b), and CD8 (ATCC TIB-210, rat IgG2b) were obtained from the American Type Culture Collection. Hybridomas were amplified in vitro, harvested, injected i.p. into pristane-primed *nu/nu* BALB/c mice, and grown to produce ascites. Collected and pooled ascites were treated with Lipid Clearing Solution (Clinetics, Tustin, Calif.) according to the manufacturer's instructions, heat inactivated, filter sterilized, and stored at −70°C.

Antibody plus complement-mediated cytotoxicity. In vitro depletion of T-lymphocyte subsets was achieved by treating splenocytes (5×10^7/ml) with the appropriate monoclonal antibody (ascites diluted 1:100 with RPMI 1640 containing 0.3% bovine serum albumin [BSA]) for 1 h on ice and then incubating the mixture in a 1:10 dilution of rabbit complement (Low-Tox-M; Cederlane Laboratory, Hornby, Ontario, Canada) for 1 h at 37°C. Cell viability postdepletion was determined by trypan blue exclusion. Flow cytometric analysis was performed on pre- and postdepleted cells to determine the efficacy of the procedure. On the basis of flow cytometric analysis, two cycles of anti-CD4 plus complement were required to achieve >98% depletion of the $CD4^+$ T-cell subpopulation.

Cell culture. Bulk culturing of vMC_{24}-primed splenocytes with UV-inactivated virus parallels the lymphocyte proliferation assay described above. Initially, 20 ml of the cell-virus suspension was cultured in 75-cm^2 flasks (Costar 3275) for 72 h, and then 20 ml of RPMI 1640–10% FCS was added. The flasks were gently mixed, and the contents were divided into additional flasks. Subsequently, 20 ml of RPMI–10% FCS with human rIL-2 (100 U/ml) was added to each flask and incubated for an additional 6 to 8 days. Viable cells were collected (Lymphoprep) and utilized in adoptive transfer protocols or CTL assays. A sample of the cultured cells was phenotypically characterized by flow cytometric analysis. Poliovirus-specific cells were generated by a similar culturing protocol by using poliovirus-inoculated donor mouse splenocytes and UV-inactivated poliovirus.

Flow cytometric analysis. Viable cells were assessed by flow cytometry for the percentage of cells possessing the murine surface markers Thy1.2, CD4, CD8, α/β-T-cell receptor (TCR), γ/δ-TCR, CD45R, and mouse IgG. All monoclonal antibodies and antisera were primary conjugates obtained commercially (Becton-Dickinson Immunocytometry Systems, San Jose, Calif., or PharMingen, San Diego, CA). The following reagents were used: rat anti-mouse Thy1.2 fluorescein isothiocyanate (FITC) (IgG2b), rat anti-mouse CD4 PE (IgG2b), rat anti-mouse CD8 FITC (IgG2a), hamster anti-mouse α/β-TCR FITC (IgG), hamster anti-mouse γ/δ-TCR phycoerythrin (PE) (IgG), rat anti-mouse CD45R PE (IgG2a), and goat anti-mouse IgG FITC (GAMIg antisera). Washed cells were resuspended in PBS containing 1% BSA, 0.2% sodium azide, and propidium iodide (1 μg/ml), which allowed for cell viability discrimination. Flow cytometric analysis was performed by using a FACScan (Becton Dickinson) and Lysys II software (Becton Dickinson).

Adoptive transfer and lethal EMCV challenge. All naive mice inoculated with 10^3 PFU of EMCV i.p. died from cardiovirus-like disease ($n = 30$). A dose of 10^4 PFU of EMCV in 1.0 ml of PBS i.p. was given as a challenge to ensure lethality. Adoptive transfer was performed by retroorbital plexus injection of approximately 5×10^7 cells, either vMC_{24}-immune splenocytes or $CD4^+$-cultured vMC_{24}-immune splenocytes, suspended in 500 μl of chilled PBS. Naive recipient mice were lightly anesthetized with methoxyflurane during the procedure. Control mice received an equivalent number of nonimmune splenocytes. Four hours after transfer, recipient mice were lethally challenged with either EMCV or wild-type mengovirus (data not shown) and monitored for disease symptoms and mortality. To determine the neutralizing antibody levels in surviving adoptive transfer recipients, animals were anesthetized and bled via the retroorbital plexus 15 to 20 days following challenge. Neutralizing titers were determined by microneutralization assay against vMC_{24} or EMCV, depending on the specific virus inoculation (13). Additionally, some surviving recipients were exposed to a second challenge (superchallenge) of 10^7 PFU of EMCV and were reassessed for serum neutralizing antibody.

Passive transfer and lethal EMCV challenge. Neutralizing serum antibody titers were determined by microneutralization assay and expressed as the reciprocal of the highest dilution affording complete protection of a HeLa cell monolayer. Pooled sera from exsanguinated BALB/c mice immunized 2 weeks previously with 10^7 PFU of vMC_{24} had a titer of 2,048. Naive recipient mice were injected i.p. or intravenously with 0.25 to 1.0 ml of serum with or without PBS plus 0.1% BSA 24 h prior to i.p. EMCV challenge. In some experiments, immune serum samples were diluted with PBS–0.1% BSA to achieve antibody titers matching those of surviving adoptive transfer recipients. The neutralizing antibody levels in passive transfer recipients were determined from sera obtained 4 h prior to EMCV challenge. Naive control and passive transfer recipients were challenged with 10^4 or 10^7 PFU of EMCV. Animals were monitored for disease symptoms and mortality for 28 days postchallenge.

In vivo T-lymphocyte depletion. T-lymphocyte subsets were depleted in vivo by using antibody therapy (46). Briefly, 1 mg of anti-CD4 (hybridoma TIB-207: GK1.5) or anti-CD8 (hybridoma TIB-210: 2.43) monoclonal antibody, or both, was administered i.p. 2 days before virus challenge and on the day of virus challenge (day 0). Antibody therapy resulted in ≥98% depletion of specific T-lymphocyte subsets on day 0 in naive and adoptive transfer recipients as determined by flow cytometric analysis (data not shown). T-lymphocyte-depleted mice were subsequently lethally challenged with 10^4 PFU of EMCV i.p. on day 0. Control mice receiving antibody therapy did not exhibit ill effects upon cursory examination.

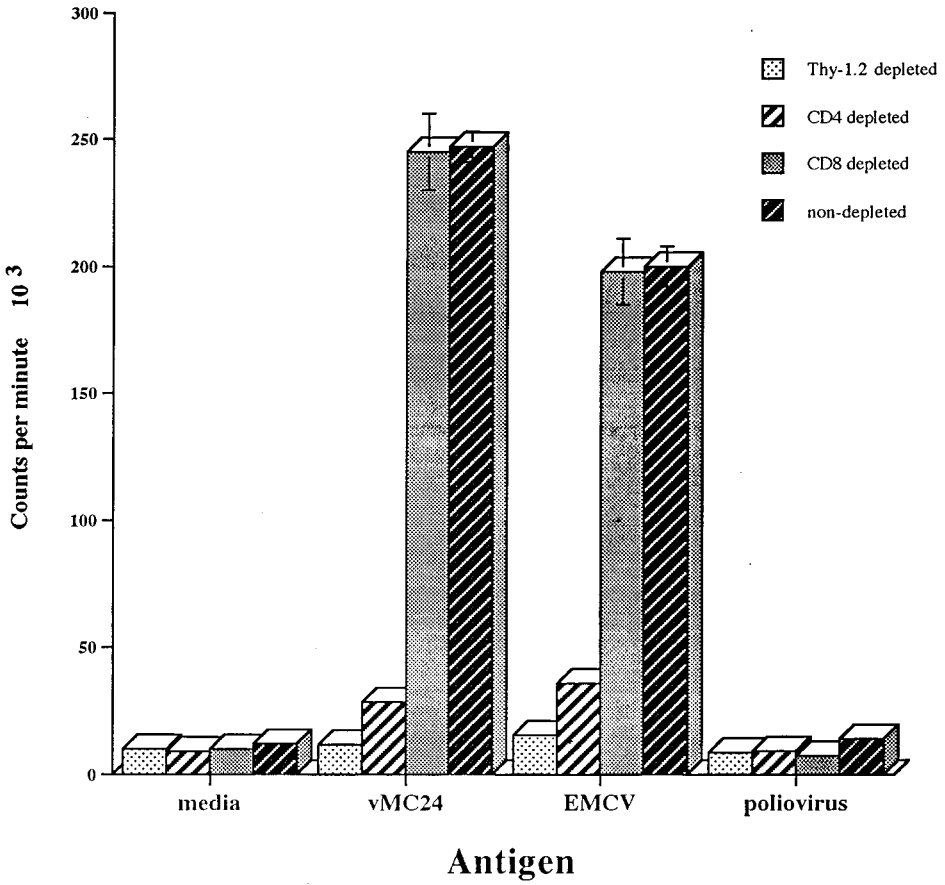

FIG. 1. Specificity of proliferative response by vMC_{24}-immune splenocytes. UV-inactivated virus was cultured with vMC_{24}-immune splenocytes for 5 days as described in Materials and Methods. Optimal concentrations for vMC_{24}, EMCV, and poliovirus were 10^7, 10^8, and 10^8 PFU/ml, respectively. In some experiments, in vitro cytotoxic depletions (see Materials and Methods) of either $CD4^+$, $CD8^+$, or $Thy1.2^+$ lymphocytes were done prior to culturing and proliferative assessment. The background response to HeLa cell lysates was similar to that induced by poliovirus ($\leq 15 \times 10^{-3}$ cpm). The data are the averages for four experiments; error bars indicate standard deviations.

Preparation of CTL target cells. A20.J cells resuspended in fresh RPMI 1640–10% FCS were infected with vMC_{24} at a multiplicity of infection of 10 PFU per cell 24 h preceding the CTL assay. On the day of the assay, 2×10^6 cells were washed and resuspended in 0.1 ml of medium. Cells were labeled by the addition of 100 μCi of $Na^{51}CrO_4$ (stock concentration, 1 mCi/ml) and incubated for 90 min at 37°C in 5% CO_2. These ^{51}Cr-labeled targets were washed three times with PBS, resuspended in 1.0 ml of medium, and counted. Control target cells were uninfected A20.J cells and were labeled in the same manner as the infected targets.

CTL assay. vMC_{24}-immune splenocytes were cultured with UV-inactivated vMC_{24} and 5% human rIL-2 (50 U/ml, final concentration) as described in the bulk-culturing protocol. Following 12 days of culturing, viable cells were harvested and used as CTL effectors. Effectors were determined to be >94% $CD4^+$ and >96% α/β-TCR^+ by flow cytometric analysis. Twofold serial dilutions of effector cells were made in triplicate. ^{51}Cr-labeled target cells (10^4 cells per 0.1 ml) were added to each well of 96-well U-bottom microwell plates (Costar), resulting in final effector-to-target cell ratios of 40:1, 20:1, 10:1, and 5:1. The spontaneous release of radioactivity from labeled cells was obtained by culturing the target cells with medium alone in six wells. The maximum release of radioactivity was determined by lysing the target cells with 2% sodium dodecyl sulfate (SDS). The plates were spun at $200 \times g$ for 2 min and incubated for 5 h at 37°C in 5% CO_2. Following incubation, the plates were centrifuged at $800 \times g$ for 4 min, and 100 μl of the culture supernatant was assessed for ^{51}Cr release in a gamma counter (1272 CLINIGAMMA; LBK-Wallac Turku, Finland). Mean values were calculated for the replicate wells, and the results are expressed as the percent killing according to the formula [(experimental counts − spontaneous counts)/(maximum counts − spontaneous counts)] × 100. The mean spontaneous release for virally infected and uninfected controls ranged between 10 and 20% of the maximum release of radioactivity.

RESULTS

In vitro proliferative response of vMC_{24}-primed splenocytes to cognate immunogen. The genetically engineered attenuated strain of mengovirus (vMC_{24}) is both serologically and antigenically indistinguishable from the lethal wild-type virus despite a partial truncation in the 5′-noncoding poly(C) tract. Mice immunized with vMC_{24} are resistant to lethal EMCV challenge and possess high levels of neutralizing antibody titers (13). Since neutralizing antibodies are an accepted paradigm for protection in picornavirus immunity (3–5), and viral infections exemplifying a protective humoral response also require T-cell involvement (16, 50), we examined the ability of vMC_{24}-immune splenocytes to proliferate to the cognate immunogen as well as the serologically identical EMCV (42). vMC_{24}-immune splenocytes proliferated in response to vMC_{24} and EMCV but not to the distantly related poliovirus (Fig. 1), while nonimmune splenocytes showed no response to any of the viral antigens tested (data not shown). The optimal concentration for each inactivated virus was determined (data not shown) and subsequently used in all T-cell proliferation assays.

Recognition of vMC_{24} by the T-cell subpopulation(s) was determined by proliferation assay following in vitro cytotoxic depletion of specific subsets. Thy1.2 and CD4 depletion, but not the depletion of the CD8 subset, significantly reduced the

TABLE 1. Survival of lethally challenged mice

Group no.	Treatment prior to challenge[a]	No. of mice surviving to day 28[b]
1	None	0/6
2	vMC_{24} immunization	6/6
3	Adoptive transfer of naive splenocytes[c]	0/6
4	Adoptive transfer of vMC_{24}-immune splenocytes	6/6
5	Rechallenge of group 4 with 10^7 PFU of EMCV	5/6
6	Adoptive transfer of poliovirus-specific $CD4^+$ lymphocytes	0/6
7	Adoptive transfer of vMC_{24}-specific $CD4^+$ lymphocytes	6/6
8	Rechallenge of group 7 with 10^7 PFU of EMCV	6/6

[a] All mice (six in each group) were challenged with 10^4 PFU of EMCV i.p. unless otherwise stated.
[b] Day 28 was the final time point of the experiment.
[c] Naive mice received 5×10^7 spleen cells as described in Materials and Methods.

proliferative response of vMC_{24}-immune splenocytes to the immunogen. Similar results were seen when EMCV was used as the antigen (Fig. 1). Thus, the proliferative response of vMC_{24}-immune splenocytes to these inactivated cardioviruses is achieved primarily by $CD4^+$ T cells.

Survival of vMC_{24}-immune splenocytes adoptive transfer recipients following lethal EMCV challenge. Given that mice immunized with vMC_{24} are resistant to lethal EMCV challenge, we examined the ability of adoptively transferred vMC_{24}-immune splenocytes to confer protection. Approximately one spleen equivalent of cells (5×10^7) was transferred via retroorbital plexus injection to naive syngeneic recipients. Recipients of vMC_{24}-immune splenocytes consistently survived lethal EMCV challenge (Table 1). The response of control mice receiving an equivalent number of nonimmune splenocytes, paralleled the disease progression and death of naive mice subsequent to EMCV challenge. Lethally challenged vMC_{24}-immune splenocyte recipients were subsequently rechallenged with 10^7 PFU of EMCV. All but one of the rechallenged mice survived the highly virulent rechallenge, demonstrating that survival was the result of a potent immunological response.

Adoptive transfer of $CD4^+$ vMC_{24}-immune lymphocytes confers immunologic protection. Since $CD4^+$ T lymphocytes are the predominant splenocyte population proliferating in vitro (Fig. 1), we questioned whether bulk-cultured vMC_{24}-immune splenocytes would generate a primarily $CD4^+$ T-cell population, and if so, whether these expanded cells could confer protection in recipient mice. To generate virus-specific $CD4^+$ T cells, splenocytes from vMC_{24}-immunized or poliovirus-inoculated mice were cultured with respective antigens for 10 to 12 days until rested, as indicated by reduced [^{3}H]thymidine uptake and the loss of T-lymphoblast morphology. Viable cells were isolated and washed prior to adoptive transfer. To characterize the population of cells adoptively transferred, flow cytometric analysis was performed on freshly isolated

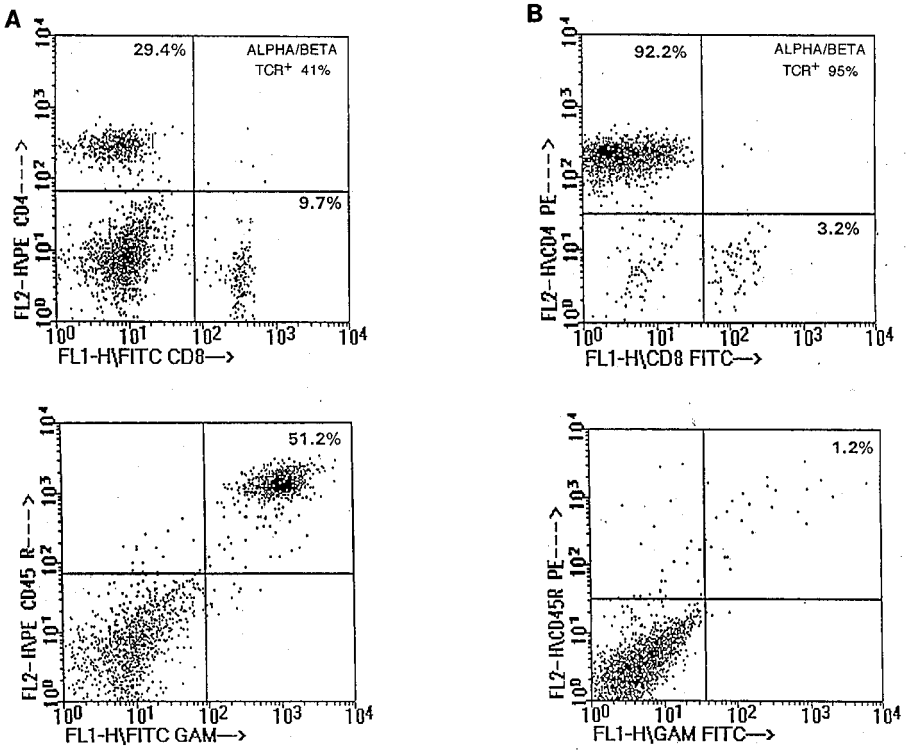

FIG. 2. Flow cytometric profiles of pre (A)- and post (B)-cultured vMC_{24}-immune splenocytes. (A) Freshly isolated vMC_{24}-immune splenocytes were characterized by two-color cytometric analysis using FITC- and PE-conjugated monoclonal antibodies to cell surface molecules. The profiles include CD8-FITC, CD4-PE, FITC–α/β-TCR, PE–γ/δ-TCR, FITC–goat anti-mouse Ig (GAM), and PE-CD45R. (B) vMC_{24}-immune splenocytes were cultured as described in Materials and Methods for 10 to 12 days and subjected to analysis as described for panel A. The percentage of viable cells positive for α/β-TCR is indicated in the upper right quadrant of the CD4/CD8 profiles. Each profile is divided into quadrants, some of which indicate numerical percentage of cells located in that quadrant. Cells were gated as live or dead by propidium iodide exclusion. The height of forward light detection (FL-H) for the respective antibodies is indicated on the *x* and *y* axes.

TABLE 2. Neutralizing serum antibody titers in EMCV-challenged adoptive transfer recipients[a]

Group no.	Transferred cells	Neutralizing serum antibody titer post-EMCV challenge with[b]:	
		10^4 PFU	10^7 PFU
1	None (vMC_{24}-immunized mice)	>1,024	>1,024
2	vMC_{24}-immunized splenocytes[c]	64, 64, 128, 128, 256, 256	512, >1,024, >1,024, 1,024, 512[d]
3	Cultured vMC_{24}-specific $CD4^+$ splenocytes[e]	16, 16, 16, 32, 32, 32	16, 16, 16, 32, 32, 32

[a] Naive mice received 5×10^7 to 1×10^8 splenocytes via retroorbital plexus infusion.
[b] Titers for individual mice (six in each group) are expressed as the reciprocal of the highest dilution affording complete protection of HeLa cell monolayers. For group 1, the result was the same for all mice. Mice were initially challenged with 10^4 PFU of EMCV and subsequently rechallenged with 10^7 PFU of EMCV.
[c] 10^7 PFU of vMC_{24} inoculated i.p.
[d] One animal died post-EMCV challenge; the death was not related to cardiovirus.
[e] vMC_{24}-immune splenocytes cultured for an initial 3 days in the presence of UV-inactivated virus and 1% normal mouse serum followed by 8 days of culture with 10% FCS and 50 U of rIL-2 per ml.

vMC_{24}-immune splenocytes and postcultured cells. The majority of freshly isolated vMC_{24}-immune splenocytes were B cells (>50% CD45R–mouse IgG, doubly positive), with fewer than 29% $CD4^+$ (Fig. 2). In contrast, 92% $CD4^+$ T cells and 1.2% B cells were present following culture with inactivated immunogen.

Mice receiving vMC_{24}-specific bulk-cultured $CD4^+$ T cells consistently survived lethal challenge, while recipients of poliovirus-specific $CD4^+$ T cells rapidly developed disease and died from EMCV challenge (Table 1, groups 6 and 7). Again, lethally challenged vMC_{24}-specific $CD4^+$ recipients were subsequently rechallenged with 10^7 PFU of EMCV. All rechallenged mice survived, indicating that survival could be effected by virus-primed T lymphocytes in the absence of antigen-specific humoral constituents. In some experiments, adoptive transfer recipients were lethally challenged with a virulent dose of wild-type Mengo virus. Recipient survival of Mengo virus-challenged animals paralleled that observed in EMCV-challenged recipients (data not shown).

Postchallenge serum neutralizing antibody titers in surviving adoptive transfer recipients. Neutralizing antibody titers were determined by the microneutralization assay of samples obtained 3 weeks following EMCV challenge. Sera from vMC_{24}-immune mice had neutralizing titers of >1,024, while the titers for recipients receiving vMC_{24}-immune splenocytes were 64 to 256. Surprisingly, the recipients of vMC_{24}-specific bulk-cultured $CD4^+$ T cells elicited neutralizing antibody titers of only 16 to 32 (Table 2).

Transfer recipients surviving initial challenge were rechallenged with 10^7 PFU of EMCV and again assessed for serum neutralizing antibody titers. Antibody titers remained identical to the initial postchallenge values except for recipients of vMC_{24}-immune splenocytes (Table 2). Unexpectedly, the vMC_{24}-specific bulk-cultured $CD4^+$ T-cell recipients survived the secondary superchallenge of 10^7 PFU of EMCV and maintained the relatively low antibody titers of 16 to 32 observed following initial challenge. A single animal in the vMC_{24}-immune splenocyte recipient group died during the course of superchallenge, although death was not attributed to cardiovirus disease.

Passive transfer of neutralizing antibodies. As a consequence of the low neutralizing antibody titers following initial and secondary EMCV challenge in vMC_{24}-specific bulk-cultured $CD4^+$ T-cell recipients (Table 2), we determined the prophylactic potential of vMC_{24}-immune serum dilutions to passively transfer protection against an EMCV superchallenge. Table 3 shows neutralizing antibody titers of serum samples obtained from passive transfer recipients 4 h preceding challenge. Following challenge, transfer recipients possessing neutralizing titers of ≥128 were protected and displayed no disease symptoms. Recipients possessing neutralizing titers <128 became diseased and died, indicating that low levels of neutralizing antibodies alone were unable to protect against such a virulent challenge.

Modulation of bulk-culture effector cell phenotype. To determine whether the predominant $CD4^+$ T-lymphocyte phenotype observed following bulk culturing was the consequence of culturing parameters, vMC_{24}-immune splenocytes were cultured as described in Materials and Methods, with modifications in the concentration of human rIL-2 and the infectious status of vMC_{24}. Splenocytes were cultured in either 25 or 100 U of rIL-2 per ml with UV-inactivated or live vMC_{24}. Following 8 to 10 days of culturing, viable cells were harvested and phenotypically profiled by flow cytometric analysis analogous to the analysis of bulk-cultured splenocytes, for which the results are characterized in Fig. 2. Cultures were analyzed several days earlier relative to the bulk cultures utilized in the adoptive transfer procedures for Fig. 2, because of reduced cell viability resulting from vMC_{24}-induced cytopathology. Immune splenocytes cultured with live vMC_{24} exhibited a dramatic increase in the percentage of $CD8^+$ T lymphocytes compared with cultures with UV-inactivated virus (Fig. 3). Additionally, an in-

TABLE 3. Passive transfer of vMC_{24}-immune sera and EMCV superchallenge[a]

Type and amt (μl) of serum transferred	Neutralizing antibody titer[b]	Recipient survival (days)[c]
None	UN	5, 5, 5, 5, 5, 6
PBS–0.1 BSA-treated, 250	UN	5
Nonimmune, 1,000	UN	5, 5, 5, 5, 6, 6
Immune		
15	32	5
31	64	5
63	64–128	9, 10, 10, 28, 28, 28[d]
125	256	28
250	512	28
500	1,024	28
1,000	>1,024	28

[a] 10^7 PFU of EMCV inoculated i.p.
[b] Titer expressed as the reciprocal of the highest dilution affording complete protection of HeLa cell monolayers. UN, undetected.
[c] Days survival post-EMCV challenge for each of six mice in a group (final time evaluated, day 28); a single result indicated that the results were the same for all six mice.
[d] In some experiments, animals in this group became diseased and then recovered.

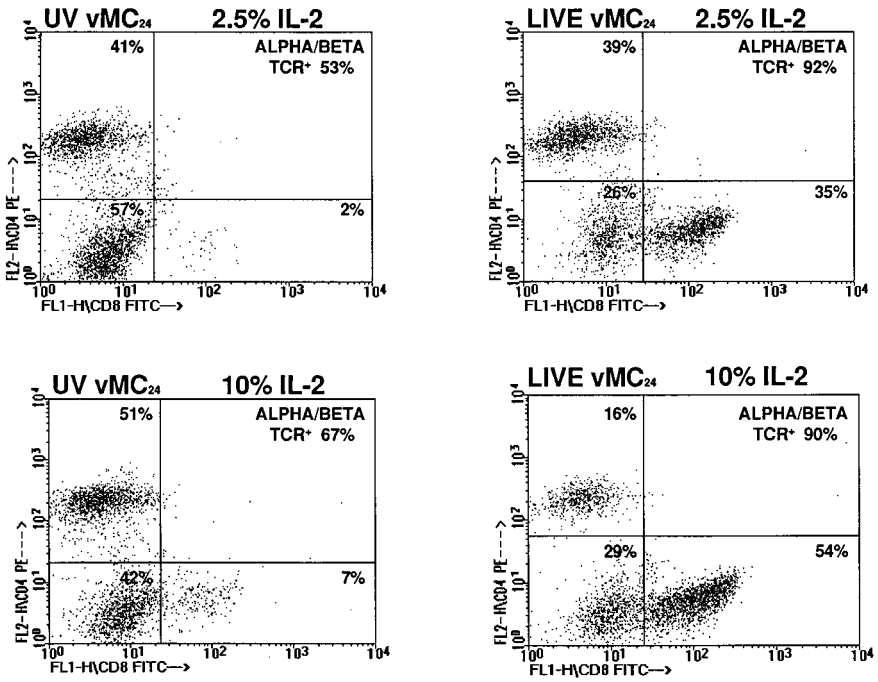

FIG. 3. Ratio modulation of CD4/CD8 phenotype of bulk-cultured vMC_{24}-immune splenocytes. vMC_{24}-immune splenocytes were cultured as described in Materials and Methods with variations in two of the protocol parameters. (i) The rIL-2 concentration was adjusted to either 25 U/ml (2.5%) or 100 U/ml (10%) (final concentration). (ii) vMC_{24} was added to the culture as either UV-inactivated or live virus. Phenotypic profiles were assessed by flow cytometric analysis, as described in the legend to Fig. 2, following 8 to 10 days of culturing. The earlier time of 8 to 10 days for cytometric analysis was selected because of the decline in the number of viable cells in cultures with live vMC_{24}.

crease in the concentration of rIL-2 also augmented the percentage of $CD8^+$ T lymphocytes in cultures with either UV-inactivated or live virus. The seemingly predominant α/β-TCR^+ cell population observed in the live vMC_{24} cultures is likely artifactual and possibly due to the loss of α/β-TCR^- splenocytes from viral infection.

In vivo depletion of adoptively transferred effector cells. To evaluate the possibility that cells other than $CD4^+$ T lymphocytes generated by the bulk-culturing protocol effected protection in lethally challenged adoptive transfer recipients, we performed in vivo depletion studies. Adoptive transfer recipients were depleted of specific T-lymphocyte subsets by antibody therapy prior to lethal EMCV challenge. Naive mice depleted of either $CD4^+$ or $CD8^+$ or both T-lymphocyte subsets failed to abrogate fatal disease progression following challenge (Table 4). Recipients of vMC_{24}-immune splenocytes depleted of $CD8^+$ T lymphocytes survived lethal challenge, whereas in vivo depletion of $CD4^+$ T lymphocytes rescinded protection. A similar pattern of protection was observed in recipients of bulk-cultured vMC_{24}-specific splenocytes (>92% $CD4^+$ T lymphocytes). Transfer recipients succumbed to EMCV-induced cardiovirus disease when depleted of $CD4^+$ T lymphocytes prior to challenge. Conversely, depletion of $CD8^+$ T lymphocytes did not encumber protection, demonstrating most convincingly that vMC_{24}-specific $CD4^+$ T lymphocytes generated in vitro are capable of orchestrating a protective immune response.

Cytolytic activity of bulk-cultured vMC_{24}-specific splenocytes. The data presented above strongly suggest that bulk-

TABLE 4. Survival of lethally challenged T-lymphocyte-depleted mice

Group no.	Treatment prior to challenge[a]	No. of mice surviving to day 28[b]
1	None	0/6
2	$CD4^+$ T-lymphocyte depletion	0/6
3	$CD8^+$ T-lymphocyte depletion	0/6
4	$CD4^+$ and $CD8^+$ T-lymphocyte depletion	0/6
5	Adoptive transfer of vMC_{24}-immune splenocytes[c]	6/6
6	Adoptive transfer of vMC_{24}-immune splenocytes followed by $CD4^+$ T-lymphocyte depletion	0/6
7	Adoptive transfer of vMC_{24}-Immune splenocytes followed by $CD8^+$ T-lymphocyte depletion	5/6
8	Adoptive transfer of vMC_{24}-specific $CD4^+$ lymphocytes	5/6
9	Adoptive transfer of vMC_{24}-specific $CD4^+$ lymphocytes followed by $CD4^+$ T-lymphocyte depletion	0/6
10	Adoptive transfer of vMC_{24}-specific $CD4^+$ lymphocytes followed by $CD8^+$ T-lymphocyte depletion	5/6

[a] All mice (six mice in each group) were challenged with 10^4 PFU of EMCV i.p. In vivo T-lymphocyte depletions were performed as described in Materials and Methods. Effectual depletion was assessed in representative mice from each group by flow cytometric analysis.

[b] Day 28 was the final time point of the experiment.

[c] Naive mice received 5×10^7 spleen cells as described in Materials and Methods.

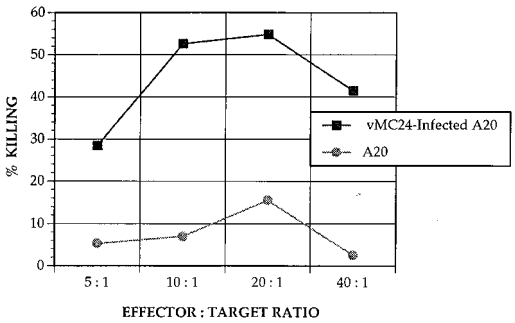

FIG. 4. Cytotoxic activity of bulk-cultured vMC_{24}-immune splenocytes. Bulk-cultured vMC_{24}-immune splenocytes were used as effector cells at various ratios in CTL assays with ^{51}Cr-labeled or vMC_{24}-infected A20.J cells as described in Materials and Methods. Effector cells were >94% $CD4^+$ and >96% α/β-TCR^+ by flow cytometric analysis. The data are representative of four experiments.

cultured vMC_{24}-specific $CD4^+$ T lymphocytes mediate protection via a mechanism(s) other than provisional T-cell helper activity to B lymphocytes for eliciting an effectual humoral response. Additionally, the notable lack of an augmented humoral response in superchallenged bulk-cultured vMC_{24}-specific $CD4^+$ T-lymphocyte recipients (Table 2) suggested that protection may be afforded through CTL activity, possibly directed toward antigen-harboring B lymphocytes and suppressing neutralizing antibody production.

A prerequisite for evaluating potential cytolytic activity is a suitable target cell. Four *H-2*^{d} haplotype murine cell lines were surveyed for sustaining a productive vMC_{24} viral infection. Our results indicate that all murine cell lines examined (A20.J, RAW 264.7, J774, and P815) were capable of supporting productive infections of the three mengovirus strains (vMwt, vMC_{24}, and vMC_0) evaluated (data not shown). Additionally, preliminary studies demonstrated that all evaluated cell lines supported an EMCV infection but quickly succumbed to overwhelming virus cytopathology (data not shown). A20.J is a B-lymphoma cell line that is both MHC class I^+ and class II^+ and was the most appropriate target cell for testing our hypothesis in vitro.

Bulk-cultured vMC_{24}-specific splenocytes were >94% $CD4^+$ and >96% α/β-TCR^+ by flow cytometry and were utilized as CTL effector cells against vMC_{24}-infected ^{51}Cr-labeled A20.J target cells. Bulk-cultured effector cells exhibited lytic activity toward virally infected target cells at all effector-target cell ratios examined (Fig. 4). Virally infected target cells were lysed, with various degrees of efficiency, ranging from 28 to 55%. Noninfected control targets were minimally affected, with lysis of only 4 to 15%. Further CD4-enrichment procedures utilizing negative-selection murine CD4-enrichment columns and positive-selection flow cytometric cell sorting resulted in only minimal increases in $CD4^+$ T-cell purity (2 to 4%) with a 50 to 75% reduction in cell yield. Postenriched $CD4^+$ T cells exhibited CTL activity toward pMC_{24}-infected A20 targets (data not shown) comparable to that of bulk-cultured vMC_{24}-specific splenocytes, further substantiating the in vitro cytolytic potential that $CD4^+$ cells possess.

DISCUSSION

To our knowledge, this is the first study demonstrating that picornavirus-specific $CD4^+$ T cells mediate protection against virus challenge in the natural host. T-cell-specific protection is achieved in the absence of prophylactic levels of serum neutralizing antibodies and contrasts the long-standing picornavirus paradigm that host protection is afforded by neutralizing antibodies (5, 16, 31, 40). Adoptive transfer of vMC_{24}-specific bulk-cultured $CD4^+$ T cells to naive mice protects against EMCV-induced lethal encephalomyelitis. Depletion of $CD4^+$ T cells in these transfer recipients rescinded protection, indicating that $CD4^+$ T cells were essential to the transfer of protection. Although low levels of serum neutralizing antibodies were detected in recipients following challenge, the passive transfer of antibody at such low titers did not prevent lethal disease. The lack of humoral protection in $CD4^+$ T-cell recipients was unanticipated. We expected virus-specific $CD4^+$ T cells to mediate protection through helper/inducer functions, stimulating antigen-specific B cells for induction of protective levels of antibodies (12). Moreover, vMC_{24}-specific bulk-cultured $CD4^+$ T cells exhibited in vitro cytolytic activity by lysing vMC_{24}-infected A20.J target cells, suggesting that in vivo protection may be afforded through $CD4^+$ CTL effector functions.

Mengovirus and EMCV are members of a single cardiovirus serotype and are serologically indistinguishable by immune sera (42). We demonstrated prophylaxis against lethal EMCV challenge in recipients with passively transferred high titers of vMC_{24}-immune sera, further substantiating the commonality of B-cell antigenic sites between mengovirus and EMCV. This is not surprising in view of the high degree of nucleotide identity and the predicted amino acid homologies between these two cardioviruses, such as the 95% amino acid identity in the VP1 capsid region (36). Moreover, the existence of common $CD4^+$ T-cell epitopes is supported by the in vitro proliferation of vMC_{24}-primed $CD4^+$ T splenocytes to inactivated EMCV and the survival of vMC_{24}-specific bulk-cultured $CD4^+$ T-cell recipients against EMCV challenge. Reduced proliferation of vMC_{24}-primed splenocytes depleted of $CD4^+$ but not $CD8^+$ T-cell subsets indicates that $CD4^+$ T cells are the predominant in vitro population that responds to inactivated virus. Although the preferential activation of $CD4^+$ T cells has been reported for other picornaviruses (2, 4, 8, 45, 50), no study has addressed the potential protection virus-primed $CD4^+$ T cells can endow via adoptive transfer. In our study, the distantly related poliovirus did not stimulate a proliferative response from vMC_{24}-primed splenocytes, confirming the lack of cross-reactive T-cell determinants between cardio- and enteroviruses reported by others (4, 17). The lack of cross-reactivity is anticipated since members of the enterovirus genera, such as poliovirus, are serotypically unrelated to cardioviruses (42) and share only 33% nucleotide identity for capsid region sequences (36).

vMC_{24}-immune splenocytes represent a heterogeneous population of primed immune effector cells capable of preventing the onset of fatal EMCV-induced encephalomyelitis, as evident by the survival of lethally challenged vMC_{24}-immune splenocyte recipients. Several explanations involving primed B and/or T cells for the protection observed in these transfer recipients are possible. The parameter that we used to score protection, survival or lethality, does not address the precise mechanism(s) involved. The protective levels of neutralizing antibodies seen in vMC_{24}-immune splenocyte recipients after initial EMCV challenge (titers ranging from 64 to 256) may have resulted from antigen-specific activation of donor vMC_{24}-primed B cells. Flow cytometric data indicated that the majority of these vMC_{24}-immune splenocytes were B cells, which are likely to require T-cell help for antibody production (16, 50). Subsequent superchallenge of these same recipients with 10^7 PFU of EMCV resulted in an augmented humoral response paralleling the antibody titers exhibited by vMC_{24}-immunized mice (>1,024). Provisional help was provided by recipient naive T cells and/or donor vMC_{24}-primed T cells, since in vivo

depletion of $CD4^+$ T cells abrogated protection. Additionally, vMC_{24}-primed T cells may provide help to naive B cells, fashioning a kinetically hastened adaptive humoral response. However, the lack of protective antibody production by recipients of vMC_{24}-specific bulk-cultured $CD4^+$ T cells following challenge (titer of ≤ 32) suggests that cultured $CD4^+$ T cells do not afford protection to naive B cells through conventional helper functions.

The virus-specific $CD4^+$ T cells generated by culturing were $>92\%$ $CD4^+$ by flow cytometric analysis. We chose not to restimulate and further enrich for $CD4^+$ T cells since others have previously reported that long-term-cultured T cells display aberrant homing and functional characteristics (10). Bulk-cultured vMC_{24}-specific $CD4^+$ T-cell recipients were protected from the initial lethal EMCV challenge and the subsequent superchallenge, whereas poliovirus-specific T-cell recipients were unable to ameliorate the onset of fatal encephalomyelitis, indicating a lack of cross-protection. In vivo depletion of $CD4^+$ T cells in vMC_{24}-specific bulk-cultured T-cell recipients prior to lethal challenge demonstrated that $CD4^+$ T cells are mandatory in mediating protection (Table 4). Low titers of neutralizing antibodies were detected following initial challenge (titers ranging from 16 to 32) and remained unchanged after superchallenge. Passive antibody transfer experiments confirmed these antibody titers to be insufficient to protect against such potent challenge, signifying that T-cell-specific protection occurred in the absence of prophylactic levels of serum neutralizing antibodies. Thus, $CD4^+$ T-cell-mediated protection is rendered by a mechanism(s) other than provisional help in eliciting protective antibodies.

CTL responses have been demonstrated in a number of picornavirus models. BALB/c mice inoculated with a Mahoney strain of poliovirus exhibited $CD8^+$ CTL activity (26). DBA/2 mice infected with TMEV demonstrated MHC class I-restricted in vitro cytolysis toward viral antigen-expressing P815 targets (41). Indeed, a number of studies have demonstrated the existence of CTL activity in mengovirus (20)- and TMEV (27, 41)-infected mice. A possible scenario for protection in vMC_{24}-specific $CD4^+$ T-cell recipient mice might be the rapid induction of an EMCV-specific $CD8^+$ CTL response preventing fatal viremia. Our data does not support such a CTL mechanism, since in vivo depletion of $CD8^+$ T cells prior to challenge does not abrogate protection in these recipients.

Indeed, the mouse hepatitis virus strain JHM (JHMV) model (49) suggests that transferred $CD4^+$ T cells induce recipient $CD8^+$ CTL activity. JHMV infection is similar to cardiovirus infections in several ways; (i) both are neurotropic in rodents, (ii) infections result in acute encephalomyelitis, and (iii) demyelination results from lysis of oligodendrocytes. A striking analogy to our data is presented in a recent investigation of the antiviral T-cell response to JHMV in rats (25). JHMV-specific $CD4^+$ T-cell lines protected adoptive transfer recipients from lethal infection without eliciting neutralizing antibodies. Interestingly, these investigators indicate that preliminary (unpublished) data suggested that most of the JHMV-specific $CD4^+$ T cells possessed CTL activity. A study investigating heat-shock protein induction in picornavirus-infected cardiocytes (22) detected virus-specific $CD4^+$ CTLs in coxsackievirus B3-infected mice. Recently, a report of $CD4^+$ CTL-mediated antibody suppression (44) supports a $CD4^+$ CTL model in which virus-specific B cells, capable of antibody production and antigen presentation, are killed.

We examined several murine cell lines for their ability to support productive vMC_{24} infection. Our data indicated that the MHC class I^+/II^+ B-lymphoma cell line A20.J, in addition to macrophage/monocyte cell lines RAW264.7 and J774, supports productive vMC_{24} infections. Cultured vMC_{24}-specific splenocytes ($>94\%$ $CD4^+$) exhibited significant in vitro cytolysis toward vMC_{24}-infected A20.J targets, even at the lowest effector-to-target cell ratio (5:1) evaluated (Fig. 4). The severe cytopathic effect caused by EMCV infection precluded the use of EMCV-infected targets in the cytotoxicity assay (unpublished observation). This technical limitation with cardiovirus-infected targets has been reported by others (41). A model in which $CD4^+$ CTLs mediate antibody suppression correlates both the protective capability of vMC_{24}-specific $CD4^+$ T cells in the absence of protective neutralizing antibody titers and the cytolytic activity toward infected B cells.

The existence of $CD4^+$ CTLs has been described for a number of host-pathogen systems, although the cytolytic mechanism remains controversial (23). In one study, several $CD4^+$ T-cell clones were established from human donors immunized with live vaccinia virus (28) and demonstrated cytolytic activity against vaccinia virus-infected targets. In another study of humans, peripheral blood mononuclear cells from attenuated dengue virus-immune donors required 5 days of in vitro culturing prior to exhibiting $CD4^+$ CTL ability (54). Borna disease virus-specific $CD4^+$ T cells, known to lyse Borna disease virus-infected astrocytes (38), either prevented or exacerbated clinical Borna disease in Borna disease virus-infected rats (37). Central to these studies and our own work is that $CD4^+$ CTLs were activated in vitro by culturing primed donor cells with inactivated virus or noninfectious virus preparations. We, and others (28), concede that $CD4^+$ CTLs may be preferentially selected by virtue of the in vitro-culturing protocol that utilizes a noninfectious immunogen as the stimulus. Interestingly, when we generated effector cells with live vMC_{24} and varied concentrations of rIL-2, profound changes in the $CD4^+/CD8^+$ phenotypic ratios were observed. Effector cells cultured with live virus and 10% rIL-2 were predominantly $CD8^+$ (Fig. 3) and were also capable of in vitro cytolytic activity toward vMC_{24}-infected A20.J targets (unpublished observation).

Of paramount importance to our hypothesis, that virus-specific $CD4^+$ T-cells mediate protection and suppress neutralizing antibody production, was the demonstration that vMC_{24}-specific bulk-cultured $CD4^+$ T cells possessed cytolytic function. We elected to employ A20.J cells as the potential CTL targets on the basis of a number of criteria. A20.J cells are both MHC class I^+ and II^+, thus allowing recognition by $CD8^+$ and $CD4^+$ CTL effector cells (24, 34). Demonstrated cytolysis of A20.J targets by vMC_{24}-specific bulk-cultured $CD4^+$ T cells would further substantiate an in vivo model in which cardiovirus-infected B cells are killed, suppressing a notable humoral response. Additionally, recent studies indicate that $CD4^+$ T-cell-mediated killing of MHC class II^+ antigen-presenting cells is dependent on the proliferative status of the target (15, 18). Normal resting B cells are not killed, whereas lipopolysaccharide-stimulated B-cell blasts and A20 cells are efficiently lysed by $CD4^+$ CTL effector cells. Furthermore, ligation of the Fas antigen on target cells by $CD4^+$ effector cells appears to be the predominant mechanism for induction of apoptotic target cell lysis (48), as high levels of Fas expression in A20 and activated B cells correlate with efficient target lysis. We are currently investigating the mechanism of in vitro lysis and have preliminary data that suggest that vMC_{24}-specific bulk-cultured $CD4^+$ T cells kill infected A20 targets in a Fas-dependent fashion (unpublished observation).

Previous studies indicate that an amelioration of EMCV-induced disease results from in vivo depletion of $CD4^+$ T cells. However, our data appear diametric to the results of these earlier studies, but reconciliation of this difference may be attributed to experimental protocols with alternative EMCV

variants and mouse strains. In our study, T-cell-subset-depleted BALB/c mice were infected with 10^4 PFU of EMCV strain Rueckert. Earlier T-cell depletion investigations involved BALB/c mice infected with 60 PFU of EMCV strain M2 H4 (47) or A/J mice infected with 100 PFU of EMCV strain E (1). In addition, clarification may come from studies in which resistance or susceptibility of mice correlates with the induction of a T-cell helper 1 response in disease resolution and a T-cell helper 2 response in lethal infection (9, 21, 43). This ability to alter the course of disease suggests that mice have the potential to develop both protective and fatal responses. Thus, in vivo $CD4^+$ T-cell depletions prior to EMCV inoculation may eliminate immunopathology-mediating $CD4^+$ T cells as well as those $CD4^+$ T cells that would potentiate a protective response. In earlier studies, survival of $CD4^+$-depleted mice following EMCV infection may be due to a compensatory response of the remaining immune system. In our study, transferred vMC_{24}-specific bulk-cultured $CD4^+$ T cells represent an expanded population of $CD4^+$ T cells that mediated protection and not pathology.

In summary, we present novel evidence suggesting that picornavirus-specific $CD4^+$ T cells defend against lethal challenge in the absence of prophylactic titers of serum neutralizing antibodies. The mechanism appears to deviate from the long-standing picornavirus paradigm of the necessity of humoral involvement. The ability of $CD4^+$ T cells to prevent disease may reflect the compensatory capacity of the natural immune response to subvert a fatal viremia in the absence of neutralizing antibodies, possibly suppressing a conspicuous humoral response through virus-specific $CD4^+$ cytolytic functions. Furthermore, this initial study warrants additional exploration of immune effector mechanisms, other than neutralizing antibodies, that may provide protection.

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