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Self HSP60 peptide serves as an immunogenic carrier for a CTL epitope against persistence of murine cytomegalovirus in the salivary gland

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Abstract

Murine cytomegalovirus (MCMV) infection is associated with persistence of virus in the salivary glands, despite relatively rapid clearance of virus from the spleen. An effective immunization against MCMV should prevent such viral persistence. We previously reported that a peptide (p458) from the sequence of the 60 kDa heat shock protein (HSP60) molecule in a conjugate vaccine can provide T cell help for the induction of protecting antibody against bacterial capsular polysaccharides. We now report that the p458 peptide as a carrier peptide can also enhance the immunogenicity of a dominant CTL epitope of the MCMV pp89 antigen—89pep. We synthesized a linear combined peptide: chimeric p458-89pep. We immunized young BALB/c mice and challenged them with MCMV. We found that the p458-89pep chimeric peptide was more effective than the 89pep in inducing 89pep-specific IFNγ secretion and specific CTL activity. Moreover, the p458-89pep chimeric peptide induced sustained IFNγ secretion in the salivary gland specific to 89pep and only this immunization was associated with clearance of virus from the salivary gland. These results suggest that a peptide epitope of HSP60 may be advantageous as a T cell carrier peptide in the induction of specific T cell immunity against infectious agents.

Keywords: MCMV; HSP60; IE-1

1. Introduction

Human cytomegalovirus (HCMV) is a ubiquitous double-stranded DNA virus from the betaherpesvirus group; it is endemic in all human populations. In North America, HCMV infects about 50% of the population outside of urban centers and up to 90% of the population within cities [1]. HCMV disease presents two major medical problems: first, it is the most common congenital viral infection, causing birth defects including mal-development of the central nervous system; up to 25% of asymptomatic infected infants will develop neurologic sequelae. Second, HCMV becomes re-activated in immunocompromised patients.

A self-limiting acute phase of viral infection, persistent and latent phases normally characterize the pathogenesis of HCMV infection in the immunocompetent host [2–4]. The clinical outcome of HCMV infection is determined by the ability of infected individuals to mount protective humoral and T cell mediated immune responses [2,3,5]. In immunocompromised hosts, including persons with HIV infection, cancer patients and allograft recipients, primary HCMV infection or reactivation of a latent virus results in multi-organ HCMV disease, associated with high rates of morbidity and mortality. These grave clinical consequences emphasize the need for effective HCMV vaccines to prevent not only primary infection but also to limit or prevent reactivation.

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Murine cytomegalovirus (MCMV) is a betaherpesvirus, a natural pathogen of mice, and resembles HCMV in physical structure and in pathogenesis [6]. In the absence of a better animal model for HCMV, MCMV has been used as a model for developing and studying HCMV vaccines. The nonstructural, immediate-early protein pp89 of MCMV is the first viral protein synthesized after infection and has a regulatory function in viral gene expression. Despite its localization in the nucleus of infected cells, pp89 is also one of the dominant antigens recognized by MCMV-specific CTLs. The amino acid sequence YPHFMPTNL of pp89 (MCMV; Smith strain) constitutes an immunodominant T cell epitope recognized in association with H-2L^d (MCMV pp89 ie1-CTL epitope) [7]. This epitope will be referred as 89pep.

The presence of adequate T cell help is important for the construction of potent vaccines. Vaccines that induce both helper T cells and CTLs may be more effective that those that induce CTLs only. Indeed, the importance of cooperation between CD4$^+$ and CD8$^+$ T cells is emphasized in the therapeutic vaccination against chronic viral infection [8,9]. In the present study, we used p458, a peptide derived from the 60-kDa heat shock protein (HSP60) [10], to help augment the response of BALB/c mice to the MCMV pp89 CTL epitope, 89pep. The impetus for this immunization approach was our earlier observation that the p458 peptide was effective in vaccinating BALB/c mice against lethal pneumococcal challenge; if p458 could provide effective T cell help for protective antibody to a bacterium, might it also be able to provide help for cellular immunity to MCMV viral infection?

We directed our attention to HSP60 as a source of T cell epitopes because HSP60 belongs to a family of chaperone molecules highly conserved throughout evolution; a similar HSP60 molecule is present in all cells, prokaryotes and eukaryotes. Apparently, no cell can exist without the ability to express HSP60 [11]. Mammalian HSP60 is highly homologous to the bacterial cognates, showing about 50% amino acid identity [12]. Thus, HSP60 is shared by the host and its parasites, and is immunogenic, cross-reactive, and universally expressed in inflammation. Furthermore, HSP60 is well recognized by the immune system [10,13] and is a part of the set of self-molecules for which autoimmunity naturally exists; HSP60 is a member of the immunologic homunculus [14]. Heat shock, IFNγ, bacterial or viral infection, and inflammation, all result in the presentation of endogenous HSP60 epitopes on MHC class II molecules leading to the activation of HSP60-specific T cells, even in healthy individuals [15–17]. Thus, T cell reactivity to a self-epitope, in this case p458, in a conjugate vaccine can be mobilized to induce help for resistance to a lethal infection [10].

In this report, we compared the effects of vaccination with 89pep alone or by 89pep together with T-helper epitopes p458 or the TTp30 peptide of tetanus toxoid (TT), on (i) resistance to MCMV and, (ii) on the immune response to the MCMV epitope 89pep elicited in the spleen and salivary gland.

2. Materials and methods

2.1. Mice

BALB/c female mice were purchased from Harlan Olac (Bicester, UK). Mice were maintained under specific pathogen free conditions and were allowed to adjust to the facility for 1 week before any experiments were performed. For the pathogenesis experiments, mice were used at 6–8 weeks of age and for the immunization experiments, mice were used at 3 weeks of age. The mouse experiments were approved by and performed according to the guidelines of the Ben Gurion University Faculty of Health Sciences Animal Safety Committee.

2.2. MCMV

The Smith strain of MCMV was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Highly virulent salivary gland-passaged MCMV stocks were prepared as a 10% (wt/vol) homogenate of salivary gland from day 14-infected BALB/c in DMEM-10% FCS. Homogenates were clarified by low speed centrifugation, DMSO was added to final concentration of 10%, and virus stocks were stored in aliquots at −70 °C until use [18].

MCMV titers in these salivary gland suspension (SGS) stocks were determined by a quantitative plaque assay [19]. Briefly, confluent monolayers of secondary mouse embryo fibroblasts (MEF) were prepared in 24 well plates. Serial 10-fold dilutions of SGS containing MCMV were prepared in DMEM supplemented with 2% FCS. The growth medium from each well in MEF plates was aspirated, and duplicate wells were inoculated with 0.2 ml of diluted SGS. After an adsorption period of 1 h at 37 °C, monolayers were overlayed with 0.8 ml of growth medium containing 0.75% carboxymethyl cellulose (CMC), incubated for 5 days at 37 °C in a humidified 5% CO_2 incubator, fixed in PBS-10% formaldehyde and stained with Crystal Violet to visualize virus plaques. Titers were expressed as $\log_{10}$ pfu/0.1 g tissue. Thorough this study we used virus stocks containing 1.75×10^8 pfu/0.1 g of tissue.

2.3. Infection with MCMV and virus titers in target organs

To study the course of MCMV infection in naive or immunized BALB/c mice, mice were inoculated intraperitoneally (i.p.) with 5×10^4 pfu of stock virus in 0.2 ml PBS. Mice were sacrificed at different time points, spleens and salivary glands (pooled three mice per group at each time point) were removed and 10% (wt/vol) homogenates were prepared as previously described [18]. Samples were stored at −70 °C until infectious virus titrations were performed on primary cultures of MEF.

2.4. Preparation of DNA and amplification by PCR

DNA was extracted from naive and infected spleens and salivary gland using QiAmp Tissue Kit (QIAGEN Inc., Chatsworth, CA, USA), according to appropriate QiAmp protocols. DNA oligonucleotide primers were synthesized according to the published sequence of MCMV gB gene [20]. The sequence of gB sense strand primer was based on the cDNA sequence number 2416–2443 (5′-AAG-CAG-CAC-ATC-CGC-ACC-CTG-AGC-GCC-3′) and the antisense number 2745–2772 (5′-CCA-GGC-GCT-CCC-GGC-GGC-CCG-CTC-TCG-3′). This gB gene primer pair amplifying a 356 bp segment was found the most sensitive in previous studies [18]. For gene amplification, 1 μg of DNA sample was added to the reaction mixture containing 200 μM each dNTP, 100 pmol each primer, 1.0 mM $MgS0_4$, 10 mM KCI, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCI (pH 8.8), 0.1% Triton X-100 and 2U of vent polymerase (Biolabs) in a total reaction volume of 50 μl each. Samples were amplified for 30 cycles in an automated thermal cycler (Perkin Elmer Cetus, USA). Each cycle entailed denaturation at 94 °C for 60 s, annealing at 68 °C for 90 s and primer extension at 72 °C for 120 s. PCR products were electrophoretically separated on 1.5% agarose gel, stained with ethidium bromide, and photographed. The lower limit of detection for this method under the experimental conditions was 5 fg of viral DNA corresponding to about 20 copies of the MCMV genome [18].

2.5. Peptides

Peptides were prepared in the Weizmann Institute of Science (Rehovot, Israel), and in Albert Einstein College of Medicine (New York, USA). The purity of the peptides was ascertained by analytical reversed-phase HPLC and amino acid (aa) analysis. The sequences of the six peptides synthesized are: (1) 89pep (MCMV pp89 ie1-CTL epitope [7])-YPHFHPTNL; (2) p458 (the active peptide derived from mouse HSP60, [10])-NEDQKIGIEIIKRALKI; (3) p458-89pep (combined)-NEDQKIGIEIIKRALKIYPHFHPTNL; (4) negative control for p458 (the p431 peptide of the mycobacterial HSP60, 442val-deleted)-EGDEATGANIKVALEA; (5) control-89pep (combined)-EGDEATGANIKVALEAYPHFHPTNL; and (6) TTp30-89pep (combined)-FNNFTVSFWLRVPKVSASHLEYPHFMPTNL. The p30 of TT (aa 947–967) [21] is now being used as a carrier peptide in various vaccines [22,23]. The mycobacterial p431 peptide (442val-deleted) was used as a negative control peptide since it is homologous in sequence to mammalian p458, but did not elicit a $CD4^+$-dependent immune response against itself or p458.

2.6. Immunization and challenge of mice with MCMV

The immunizing dose of each peptide was equimolar to 15 μg of p458 [10]. All peptides were emulsified in incomplete Freund's adjuvant (IFA), and the volumes for intrafootpad (i.f.p.) and subcutaneous (s.c.) injections were 50 and 100 μl, respectively. Two different protocols were used. To study the immune response of mice to the chimeric peptide (p458-89pep), groups of 6-week-old to 8-week-old mice were immunized once into the hind footpad with peptides emulsified in IFA. Ten days later several mice were sacrificed, and organs were harvested for IFNγ and IL-4 assays. To study the protective efficacy of the combined peptide, groups of 3-week-old mice were immunized and boosted according to the following protocol: mice were immunized i.f.p. on day (−24), and boosted s.c. two weeks later on day (−10). Ten days later (day 0), mice were challenged IP with 5×10^4 pfu of MCMV. Mice were sacrificed on days 14, 21, and 28 after challenge, and organs were harvested for virus titrations, PCR, cytotoxic T cell and cytokine assays.

2.7. Preparation of spleen and salivary gland mononuclear cell cultures

Spleen pulp was extruded from the capsule in a non-tissue culture Petri dish in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10 mM HEPES and 5% FCS (base-RPMI). Spleen cell suspensions were passaged through a cell strainer, washed once, treated 2 min with ACK lysing buffer (0.15 M NH_4Cl, 0.01 $KHCO_3$; 2 ml/spleen) for elimination of erythrocytes, and washed twice in base-medium. Splenocytes were resuspended in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10 mM HEPES, 5×10^{-5} M β-mercaptoethanol and 10% FCS (complete RPMI) in a final concentration of 5×10^6 cells/ml.

Salivary gland cell suspensions were prepared by initially cutting the salivary glands into small fragments (<2 mm) in a non-tissue culture Petri dish. Fragments were treated with base-medium containing 1mg of collagenase-dispase (Roche Diagnostics, Germany)/ml and 50 μg of DNase I (Boehringer Mannheim, Germany)/ml. After 1 h incubation in 37 °C, cells were resuspended in 45% Percoll (Sigma Chemical Co., Israel), overlayed on 66% Percoll and centrifuged at $800 \times g$ for 25 min. Mononuclear cells collected at the interphase, were counted and resuspended in complete-RPMI to a final concentration of 5×10^6 cells/ml.

2.8. IFNγ and IL-4 ELISA assays

Mononuclear cell cultures from spleens and salivary gland were prepared as described above. Cell suspensions were divided into 24 well plates (5×10^6 cells/well) and were stimulated in vitro with either 10 μg/ml of 89pep or p458 or with 5 μg/ml Concanavalin-A (Con-A). Cells were incubated for 72 h (with or w/o stimulation) at 37 °C in a humidified 5% CO2 incubator. After incubation supernatants were collected, and IFNγ and IL-4 levels were measured using indirect ELISA according to Pharmin-

gen cytokine ELISA protocol (Pharmingen, San Diego, CA).

2.9. FACS analysis of cell phenotypes and intracellular IFNγ

For phenotypic analysis, spleen and salivary gland mononuclear cells of MCMV-infected and naive mice were cultured as described above and were stained for CD8 and CD4, IFNγ and IL-5 using directly-labeled antibodies (PharMingen, San Diego, CA). Intracellular cell staining (ICCS) for IFNγ, IL-4 and IL-5 was performed using PharMingen's Cytofix/Cytoperm Plus kit with GolgiPlug (containing Brefeldin A) according to the manufacturer's instructions. Briefly, GolgiPlug was added to the 8-h incubated immune cell cultures (established as described above, with or w/o peptide stimulation). After the additional 6 h of incubation in the incubator cells (minimum 10^6 per sample) were harvested, washed in PBS supplemented with 2% FBS and 0.09% sodium azide, and incubated in 50 μl of FC blocker, labeled with anti-CD4 and anti-CD8 surface markers. Then cells were fixed, permeabilized and treated with anti-IFNγ, anti-IL-4 or anti-IL-5 antibodies for intracellular cytokine detection. Stained cells were immediately analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, Mansfield, MA) and 50,000–100,000 events/sample were acquired and analyzed with CellQuest software.

2.10. Cytotoxic T cell assay

The cytotoxic activity against the MCMV 89pep was assessed in a 4-h cytotoxic assay using the CytoTox 96 non-radioactive, colorimetric-based kit (Promega, Madison, WI), according to manufacturer instructions. This assay is based on the quantitative measurement of lactate dehydrogenase, a stable cytosolic enzyme that is released upon cell lysis. Spleen cell suspensions from immunized mice, prepared as described above, were re-stimulated in vitro for 6 days with 89pep (10 μg/ml) and rhIL-2 (25 IU/ml) from day 2. Target cells for the lysis assay were P815 cells (mastocytoma, H-2^d). P815 were either non-pulsed or pulsed with 89pep (1 μg/ml) for 2 h and then washed before incubation with effector cells. In all experiments shown, the spontaneous release was less than 25% of maximal release. Each point in a lysis assay represents the average of triplicate values. The range of the triplicates was within 5% of their mean.

3. Results

3.1. Natural history of MCMV dissemination in spleen and salivary gland

MCMV infection is characterized by different kinetics and viral loads in different organs ([6] and BR, unpublished results). BALB/c mice, 6–8 weeks old, were injected i.p. with 5×10^4 pfu of MCMV. Mice were sacrificed on days 1, 3, 7, 14 and 28 after infection, and spleens and salivary glands were assayed for infectious virus and MCMV DNA. Fig. 1 shows a typical pattern of MCMV replication in spleen and salivary gland. Virus replication peaked in the spleen on day 3 after infection, and slowly declined thereafter (Fig. 1A). By day 14, no infectious virus could be recovered from this organ. To detect MCMV DNA in infected organs, we used a sensitive PCR using a gB gene primer pair that amplifies a 356 bp segment [18]. Viral DNA was detected in the spleen as early as day 1 after infection, peaked on day 3 and by day 14 no DNA could be detected (Fig. 1B).

In the salivary gland, virus appeared on day 7. Virus replication in this organ steadily increased, peaking by day 14 (3×10^8 pfu/0.1 g tissue, Fig. 1A). A moderate decline in virus titers ensued, and at day 28, 1.5×10^6 pfu/0.1 g tissue were still recoverable from the salivary gland. No infectious virus could be detected in the SG (and in any other organ) by day 42 post challenge (data not shown and [24]) The detection of viral DNA was associated with the presence of infectious virus. DNA increased from day 7–14. Large amounts of viral DNA could still be detected on day 28 after infection (Fig. 1B). On this background of viral dissemination, replication, splenic clearance and salivary gland persistence, we evaluated the efficacy of immunization with the p458-89pep chimeric peptide. We also studied MCMV load in lungs after

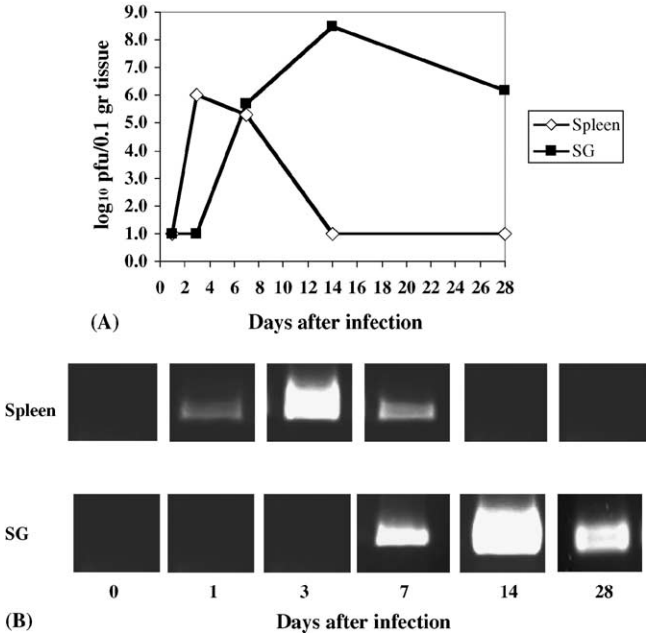

Fig. 1. Kinetics of MCMV infection in spleen and salivary gland (SG) of BALB/c mice. Mice were challenged i.p. with 5×10^4 pfu of MCMV. (A) Infectious virus titers in spleen and salivary gland were measured at different time points after infection (calculated as $\log_{10}$ pfu/0.1 g tissue). The data represent the average of five experiments. (B) PCR amplification of the 356 bp product of MCMV gB DNA in spleen and salivary gland at different time points after infection. Results are from one representative experiment of three performed.

challenge of naive 6–8-week-old mice; MCMV load (pfu) maximized on day 7 and disappeared by day 14 (data not shown). Thus, in our model we concentrated on the salivary gland because it is considered as the major site for viral persistence of MCMV in mice (Fig. 1 and [2,3,5,6]).

3.2. Immunization with p458-89pep suppresses MCMV persistence in the salivary gland

89pep is the H-2L^d-restricted YPHFMPTNL epitope of MCMV-pp89 [7]. We synthesized chimeric p458-89pep and compared its protective efficacy against MCMV to that induced by 89pep alone or by negative control-89pep (see Section 2 for list and sequences of the peptides). p458 is a MHC class II-restricted peptide derived from murine HSP60 (aa 458–474) and capable of inducing CD4$^+$ T responses in BALB/c mice [25]. The mycobacterial HSP60431-447 aa peptide (with a val deletion at position 442) did not elicit an immune response to itself or to p458, and thus served as a negative control peptide for immunization; control-89pep.

To investigate whether immunization with the different peptides would decrease MCMV replication in salivary glands, 3-week-old BALB/c mice were immunized twice with IFA alone, 89pep, p458-89pep or control-89pep (Fig. 2). Peptides for vaccination were emulsified in IFA. Ten days after the last immunization, mice were challenged i.p. with 5×10^4 pfu of MCMV (day 0). Mice were sacrificed on days 14, 21 and 28 after challenge, and infectious virus titers and MCMV-DNA were measured by plaque and PCR assays in the salivary glands. As shown in Fig. 2B, no effect of immu-

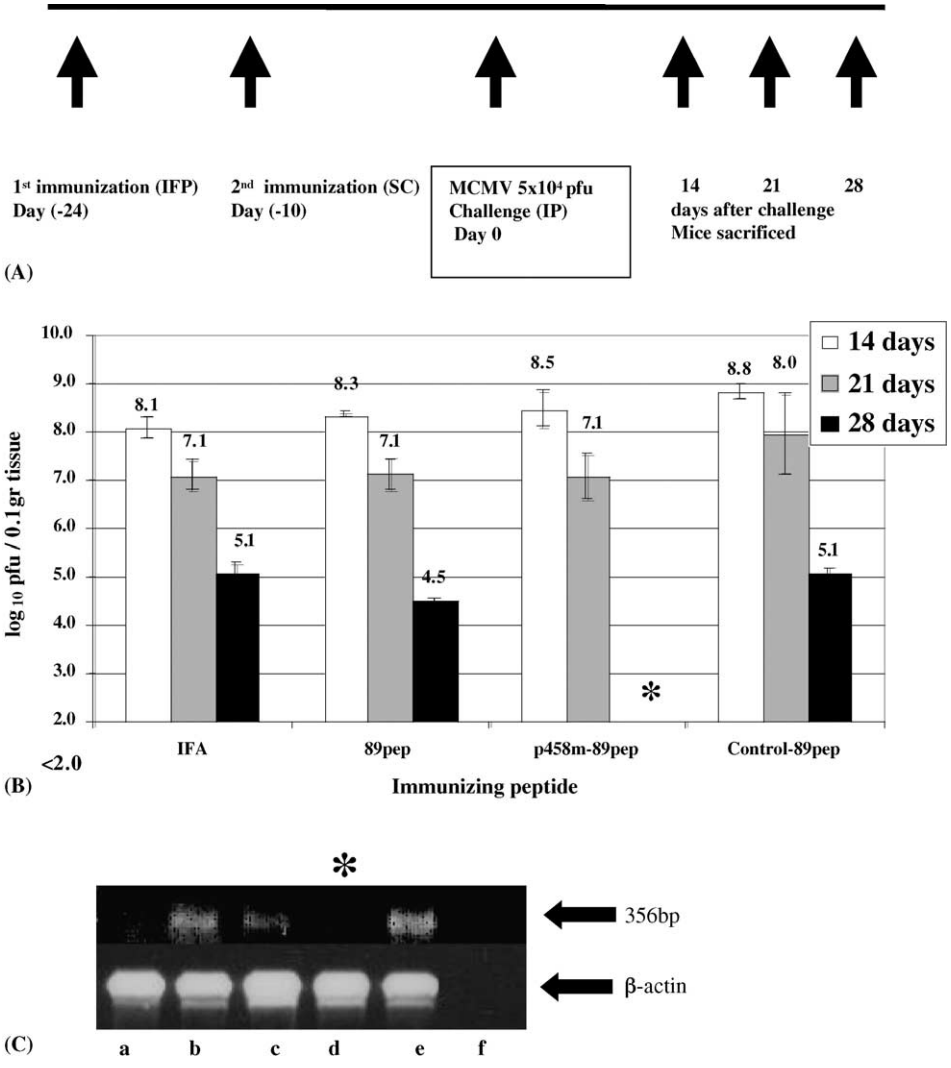

Fig. 2. The effectiveness of the p458-89pep vaccine. (A) The experimental design. Three-week-old, female BALB/c mice were immunized (i.f.p.) with various peptides, and were boosted (s.c.). Two weeks later, the mice were challenged (i.p.) with 5×10^4 pfu MCMV, day 0. Three mice from each group were sacrificed on days 14, 21 and 28 after challenge. (B) Infectious MCMV titers in salivary gland 14, 21 and 28 days after challenge. Data represent the average titer (±S.E.) of the salivary glands of three individual mice of each group. In salivary glands of mice immunized with p458-89pep, virus titers on day 28 (asterisks) were below detection (i.e. $<2 \log_{10}$ pfu/0.1 g tissue). (C) PCR amplification of the 356 bp product of MCMV gB. Template DNA was extracted from salivary glands of immunized mice on day 28 after MCMV challenge. Immunization with: (a) IFA only without challenge; (b) IFA only; (c) 89pep; (d) p458-89pep; (e) control-89pep; (f) PCR mix without template DNA (negative PCR control). Results are from 1 representative experiment of 2 performed.

nization with any of the peptides could be demonstrated on days 14 and 21 after virus challenge; on day 14, virus titers ranged from 8.1 to 8.8 $\log_{10}$ pfu/0.1 g, and on day 21 ranged from 7.1 to 8.0 $\log_{10}$ pfu/0.1 g.

On day 28, however, MCMV was not detectable in the salivary glands of the p458-89pep-immunized mice (<2 $\log_{10}$ pfu/0.1 g). Immunization with 89pep alone showed a marginal advantage compared to IFA-immunized mice (Fig. 2B); the viral load was 4.5 and 5.1 $\log_{10}$ pfu/0.1 g, respectively. Immunization with control-89pep did not affect the viral load. Other experiments were performed with the same experimental design in which TTp30-89pep was used; immunization with TTp30-89pep did not affect viral load on days 14 and 21, reduced viral load on day 28 by two fold, on average, but failed to eliminate infectious virus on day 28. To further evaluate the virus suppression induced by the p458-89pep immunization, we used a sensitive viral gB PCR to detect viral DNA. We previously showed that 1 pfu is the equivalent of approximately 1500 viral genomes [24]. Yet, on day 28, even this assay failed to reveal any gB PCR product in salivary glands of mice immunized with p458-89pep (Fig. 2C, lane d). Therefore, only immunization with the p458-89pep led to the elimination of detectable MCMV from the salivary gland, on day 28.

3.3. IFNγ secretion by 89pep-specific T cells following infection and vaccination

It is well established that clearance of MCMV during acute infection depends primarily on Th1 IFNγ secretion and protective CTL responses [6,7]. We tested whether IFNγ secretion was stimulated by 89pep from spleen and salivary gland cell cultures of MCMV-challenged mice. Cell cultures were prepared on days 1, 3, 7, 14, 21 and 28 after infection, plated for 3 d with or without 89pep, and IFNγ secretion was measured. In the absence of 89pep stimulation, secretion of IFNγ was detected only in spleen cultures from days 1 and 3 after infection. This result probably reflects NK activity in the early stages of infection. When 89pep was added to the cultures, IFNγ secretion in spleen and salivary glands was correlated with the kinetics of viral replication in these organs (Figs. 1A and 3). It is noteworthy that no significant IL-4 secretion was detected in the culture supernatants; however, the cells were capable of secreting IL-4 along with other cytokines in response to stimulation with Con-A (data not shown).

We investigated whether immunization with p458-89pep induced 89pep-specific IFNγ secretion. Mice received a single immunization with the following peptides: p458-89pep, 89pep, p458, control-89pep or TTp30-89pep. TTp30 is a MHC class II-restricted peptide capable of inducing vigorous $CD4^+$ T responses and IFNγ production in BALB/c mice and used as a universal adjuvant [21,25]. In addition, a non-vaccinated group was infected with MCMV. Ten days after immunization with the different peptides or infection, mice were sacrificed, and spleen cell and salivary gland cultures

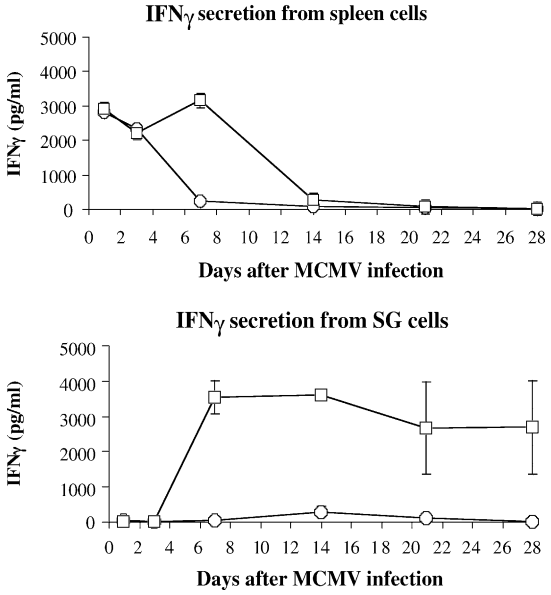

Fig. 3. IFNγ secretion from spleen and salivary gland (SG) cell cultures of MCMV infected mice. Spleen cell and fractionated salivary gland mononuclear cell cultures were prepared on different days after virus infection as described in methods. IFNγ secretion in supernatant was measured by ELISA after 3 days culture with (squares) or without (circles) 89pep stimulation (10 μg/ml) in vitro. Data represent the average (±S.E.) of three experiments.

were prepared and stimulated in vitro for 3 d with 89pep or p458. Fig. 4 shows that spleen cells derived from mice immunized with p458-89pep and re-stimulated in vitro with 89pep secreted significantly higher ($p < 0.05$) levels of IFNγ compared to mice immunized with 89pep, p458, control-89pep or IFA-only. 89pep-restimulated splenocytes from p458-89pep-immunzed mice secreted significantly higher ($p < 0.05$) levels of IFNγ compared to the same but non-re-stimulated splenocytes (Fig. 4). Thus, immunization with p458-89pep induced specific and significantly enhanced IFNγ secretion. In these experiments we also tested the TTp30-89pep. Immunization with TTp30-89pep followed by 89pep re-stimulation induced IFNγ levels similar to those of mice immunized with p458-

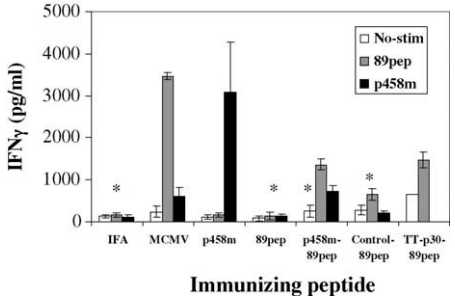

Fig. 4. IFNγ levels from spleen cell cultures after vaccination with p458-89pep. Spleen cell cultures were prepared 10 days after vaccination with the various peptides or after challenge of naive mice with MCMV. A control group received IFA without any peptide. IFNγ secretion in supernatants was measured by ELISA after 3 days stimulation in vitro with p458, 89pep (10 μg/ml), or without stimulation (no-stim). Data represent the average of six experiments (±S.E.). * $p \leq 0.05$ compared to p458-89pep, two-tailed *t*-test.

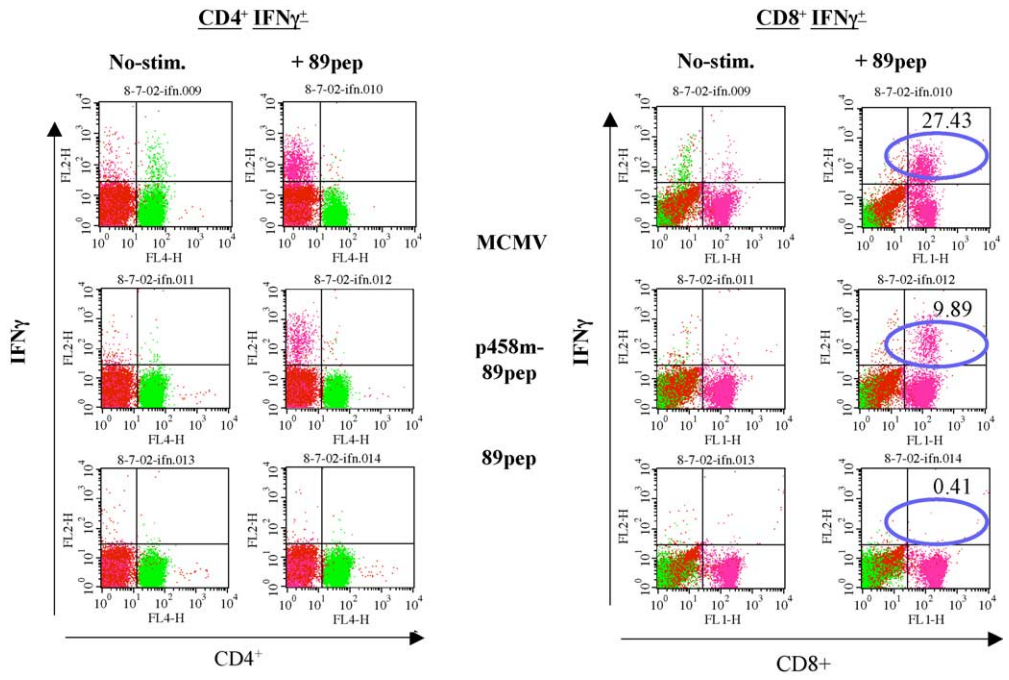

Fig. 5. IFNγ-positive spleen cells after vaccination with p458-89pep. Spleen-cell cultures were prepared 7 days after vaccination with various peptides, or after challenge of naive mice with MCMV. After 5 days of stimulation in vitro with 89pep or without stimulation (no-stim), the cells were stained for CD4 or CD8 markers and for IFNγ. Numbers (27.43, 9.89 and 0.41) are percentage of IFNγ$^+$ cells in total CD8$^+$ cells. Results are from 1 representative experiment of 2 performed.

89pep (Fig. 4). The highest levels of 89pep-specific IFNγ secretion were obtained in spleen cell cultures from mice infected with virus (Fig. 4). This high IFNγ secretion by spleen cells from MCMV-infected mice, after in vitro stimulation with 89pep, indicates the dominance of this epitope in the response to MCMV. No 89pep-specific IFNγ was detected in salivary gland cell cultures after immunization with the different peptides (data not shown). Thus, infection of the salivary gland with MCMV appeared to be needed for recruitment to the organ of 89pep-specific IFNγ producing cells (Figs. 1A and 3).

The response of spleen cell cultures to stimulation with p458 induced high levels of IFNγ in mice immunized with p458 or p458-89pep, but not in other groups; this indicates that the responses were immunologically specific (Fig. 4). No significant IL-4 secretion after either immunization was detected; nonetheless the cells were capable of secreting IL-4 after stimulation with Con-A. IL-4 levels measured after Con-A stimulation in vitro were 242, 146, 184 and 317 pg/ml for IFA-only, 89pep, p458, and p458-89pep, respectively. Taken together, these results imply that the protection induced by p458-89pep was associated with elevation in MCMV-specific IFNγ production.

3.4. Immunization with p458-89pep induces 89pep-specific IFNγ$^+$CD8$^+$ T cells and CTL activity

We characterized the nature of cells secreting the IFNγ by flow cytometry. Mice were immunized once with the different peptides and an additional group was infected with MCMV. Seven days later, spleens were removed and cell suspensions were cultured for 5 days with or without 89pep. Immunization with p458-89pep followed by 5 days of re-stimulation with 89pep induced IFNγ$^+$CD8$^+$ T cells and no IFNγ$^+$CD4$^+$ T cells (Fig. 5); very few IFNγ$^+$CD8$^+$ T cells were detected after immunization and re-stimulation with 89pep alone (Fig. 5). Infection with MCMV and re-stimulation with 89pep induced the highest percentage of IFNγ$^+$CD8$^+$ T cells (Fig. 5). Staining was specific to IFNγ since no CD8$^+$IL-4$^+$ or CD8$^+$IL-5$^+$ cells were observed (data not shown).

We also investigated whether the CD8$^+$IFNγ$^+$ cells induced by the p458-89pep were able to lyse specifically 89pep-loaded target cells. Mice were immunized once and 7 d later, spleens were harvested and re-stimulated with 89pep. Six days later, lytic activity was assayed on P815 (H-2^d) loaded with the 89pep. No lytic activity was observed from the cultures of 89pep-immunized mice, but CTLs induced by p458-89pep lysed the target cells (Fig. 6). Similar to our results with IFNγ production by CD8$^+$ T cells, the 89pep-specific lytic activity induced by MCMV infection was higher than that induced by p458-89pep immunization.

3.5. Salivary gland-specific response after immunization and virus challenge

We found, above, that IFNγ secretion in the salivary gland was virus-specific and depended on MCMV infection

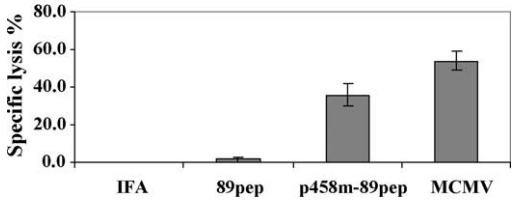

Fig. 6. CTL activity in spleen cell cultures after vaccination with p458-89pep. Spleen-cell cultures were prepared 7 days after immunization with various peptides or after challenge of naive mice with MCMV. A control group received IFA without any peptide. CTL activity was measured after 6 days of stimulation in vitro with 89pep (10 μg/ml). Target cells were P815 pulsed with the 89pep (1 μg/ml). E:T ratio is 25:1. The data represent the average (±S.E.) of 3 different experiments.

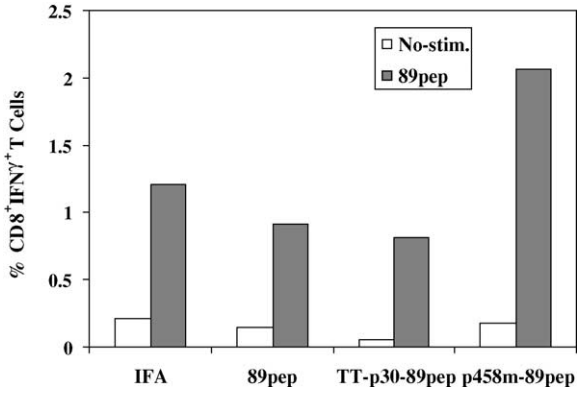

Fig. 7. IFNγ-positive salivary gland mononuclear cells 28 days after MCMV challenge of vaccinated mice. Mice were vaccinated and then challenged with MCMV. Cells were stained for CD8 and for IFNγ. No-stimulation (No-stim) or stimulation with 89pep (89pep) relates to the presence of 89pep during the 8 h incubation with golgi-stop step in the ICCS protocol for IFNγ. Results are from one representative experiment of two performed.

(Figs. 1 and 3). In the present experiment, we monitored IFNγ production in immunized mice 28 days after virus challenge. Staining of mononuclear cells for IFNγ-production was performed immediately after excision of the salivary gland, and stimulation with 89pep for 8 h. The salivary glands of mice immunized with IFA, 89pep or TTp30-89pep and challenged with virus contained infectious virus on day 28 post challenge (Fig. 2). Likewise, $CD8^+IFN\gamma^+$ cells were observed in these day 28-infected salivary glands. In contrast, mice immunized with p458-89pep showed no infectious MCMV in the salivary gland 28 days after infection (Fig. 2). Nevertheless, the number of $CD8^+IFN\gamma^+$ cells was larger than that of the other groups (Fig. 7). This indicates that vaccination with p458-89pep induced a large reservoir of 89pep-specific $CD8^+$ T cells along with termination of salivary gland infection.

4. Discussion

Although HCMV elicits prominent immune responses that control infection, the virus evades the immune system and persistently infects the host [2–4]. The salivary gland is considered to be an organ which HCMV infects abundantly [2–4]. Similarly, MCMV too replicates to high titers in the salivary gland long after it is cleared from systemic organs; virus is still detectable in the salivary gland for months following infection—see Fig. 1 and [6,24,26,27]. Vaccination, one might hope, could provide a more effective immune response that would modulate the natural course of HCMV infection and limit or prevent its reactivation. Here we describe a novel approach based on the use of a T cell epitope derived from self HSP60 in linear sequence with a specific MCMV CTL epitope.

Healthy persons or mice manifest natural T cell reactivity to epitopes of the self HSP60 molecule, and a self HSP60 peptide, p458, was identified as a dominant epitope for this reactivity in BALB/c mice [13]. Previous reports showed that p458 provided T cell help in a conjugate vaccine for an antibody response to the capsular polysaccharides of *Salmonella typhi* and *Streptococcus pneumoniae* type 4 [10,13,25]. Here we extend the use of p458 as a carrier for anti-MCMV immunization. The construct used was a linear chimeric peptide, composed of the amino acid sequence of p458 followed by a MCMV-dominant CTL epitope, and emulsified in IFA. Immunization with the p458-89pep chimeric peptide, followed by MCMV challenge, resulted in clearance of MCMV in the salivary gland on day 28 post infection (Fig. 2). The helper effect of p458 was evident; vaccination with the MCMV epitope, 89pep, alone resulted in a salivary gland viral load similar to that of IFA-treated mice. The effect of the p458 was specific; a peptide in which the p458 was replaced with a control sequence did not induce any protection (Fig. 2).

IFNγ plays a pivotal role in anti-HCMV and -MCMV responses [28,29]. It was shown that both HCMV and MCMV have inhibitory effects on Ag presentation in the MHC class I pathway that result in a lack of T cell recognition, which could be restored by IFNγ [29–33]. $CD4^+$ T cells and IFNγ are required for clearance of MCMV infection from the salivary gland in a process that requires weeks to months. The inefficiency of salivary gland clearance is due to virus interference with IFNγ-mediated induction of specific MHC class II genes. This impairs Ag presentation to $CD4^+$ T cells, and may contribute to the capacity of MCMV to spread and persist within the infected host [34]. Nevertheless, we found clearance of MCMV from the salivary gland could be induced by vaccination with the p458-89pep chimeric peptide. Clearance correlated with the induction of 89pep-specific IFNγ secretion. Indeed, the p458 peptide provided T cell help for an efficient induction of 89pep-specific IFNγ secretion; vaccination with 89pep alone failed to induce significant IFNγ secretion (Fig. 4). Vaccination with p458-89pep, followed by 5 days of in vitro re-stimulation with 89pep, induced an increase of $CD8^+IFN\gamma^+$ cells. Indeed, none or only few $CD8^+IFN\gamma^+$ were detected after immunization with 89pep alone (Fig. 5). The induction was specific to the presence of intact p458 in the chimeric peptide, since the control peptide fused to the 89pep epitope did not induce IFNγ secretion above background levels (Fig. 4).

Immunological studies in the mouse demonstrate the requirement for T lymphocytes in protection against MCMV infection (reviewed in [3]). The essential role of CTLs in protective immunity to MCMV was shown in studies employing adoptive transfer of $CD8^+$ CTLs to immunosuppressed mice; the T cells conferred protection from lethal MCMV challenge [35]. MCMV IE1-pp89 is one of the dominant antigens recognized by MCMV-specific CTL, and the YPHFMPTNL sequence of pp89 (89pep) constitutes an immunodominant T cell epitope recognized in association with $H\text{-}2^d$ [7,36]. We found that the potency of p458-89pep in clearing MCMV correlated with its ability to induce 89pep-specific CTLs (Fig. 6). Similarly, the inefficiency of immunization with 89pep alone correlated with its inability to induce 89pep-specific CTL (Fig. 6). We also assessed MCMV-specific Abs in sera taken from immunized or non-immunized mice, 28 days after challenge with the MCMV. Our results show no significant differences in levels of MCMV-specific Abs between the different vaccination protocols and as compared to the non-immunized group (data not shown). This indicates that the effect of pre-immunization with the p458-89pep on MCMV persistence was specific to CTL and IFNγ.

89pep-specific IFNγ production and lytic activity in spleens of non-immunized mice challenged with MCMV was higher than that found in p458-89pep-immunized, non-challenged mice (Figs. 4–6). Apparently, the infection was a more effective stimulus in activating the immune system than was immunization with p458-89pep alone. To specifically test the potency of p458, we compared it to the TTp30 T cell peptide epitope. TTp30 is considered as a universal T helper epitope, and has been tested in clinical vaccine trials [21,37]. Compared to TTp30-89pep, the p458-89pep peptide induced similar 89pep-specific IFNγ (Fig. 4). After immunization with p458-89pep and virus challenge, the percentage of $CD8^+IFN\gamma^+$ cells was two-fold that of the TTp30-89pep vaccination (Fig. 7).

We previously compared p458 to TTp30 in providing T cell help for the induction of anti-capsular antibodies and resistance to lethal pneumococcal challenge [25]. Overall, we find that the self p458 peptide of HSP60 can provide T cell help in various types of constructs and that it is at least as potent as foreign effective epitopes ([13,25] and this report). Moreover, two advantages can be noted for p458 compared to the foreign peptide TTp30:

(i) Pre-existing immunity to TT exists in western countries that include TT in their vaccine programs for children. Yet, immunity to self peptides exists in each individual, independent of childhood vaccination. This T cell memory ensures rapid and potent induction of T cell help upon vaccination. In addition, TT-specific T cell memory needs to be boosted every 5–10 years, while the T cell memory for HSP60 epitopes does not need boosting.
(ii) Immunization with the TTp30 carrier induces anti-p30 Abs, while immunization with the p458 carrier does not induce anti-p458 Abs [25]. Moreover, repetitive use of foreign protein carriers such as TT or diphtheria toxoid can result in overall reduced responses [38]. Stress protein-derived peptides are probably involved in any pathogen-induced danger signal [39]. Thus, reduction in the immune response to these peptides upon repeated exposure is unlikely.

Recent advances have enabled the rapid identification of minimal cytotoxic epitopes required to trigger CTL responses. For HCMV, epitope mapping to date has mainly focused on the pp65 antigen but other antigens such as IE1 are starting to be mapped [40]. The first HCMV CTL epitope identified is from the pp65 and restricted to the abundant HLA A*0201 allele [41]. To date, about 25 HCMV $CD8^+$ T cell epitopes identified that originate from pp65 and IE1 and cover HLA alleles that are predominant in the Caucasoid population [40]. The ongoing identification of HCMV epitopes suggest that a sub-unit vaccine based on these epitopes could be developed to protect against HCMV infection and reactivation. Our results indicate that the human p458 equivalent [13] might be considered as T cell carrier for HCMV epitopes in the development of sub-unit vaccines intended to prevent or limit HCMV reactivation.

An important question is whether vaccination with self-peptide p458 might induce an autoimmune disease. Accumulating data from previous publications indicate that the use of HSP60 and/or fragments (e.g. p458) does not induce autoimmunity: (i) p458 has been injected repeatedly into many dozens of mice without ever inducing an autoimmune disease, either when injected alone [42] or when injected as a conjugate [10,13,25] (mice were followed for over a year after p458 vaccination); (ii) in addition, whole HSP60, or its peptides or other HSP60 fragments only protects against autoimmune disease [43–45]. Yet, the question remains to be settled. The mechanism by which p458-89pep vaccination modulated the salivary gland immune response also needs further study. Nevertheless, the results reported here demonstrate that different carrier peptides can influence the site of the immune response, and not only its quality and magnitude.

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