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Alpha beta-crystallin expression and presentation following infection with murine gammaherpesvirus 68

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Abstract

Alpha beta-crystallin (CRYAB) is a small heat shock protein that can function as a molecular chaperone and has protective effects for cells undergoing a variety of stressors. Surprisingly, CRYAB has been identified as one of the dominant autoantigens in multiple sclerosis. It has been suggested that autoimmune mediated destruction of this small heat shock protein may limit its protective effects, thereby exacerbating inflammation and cellular damage during multiple sclerosis. It is not altogether clear how autoimmunity against CRYAB might develop, or whether there are environmental factors which might facilitate the presentation of this autoantigen to CD4⁺ T lymphocytes. In the present study, we utilized an animal model of an Epstein Barr Virus (EBV)-like infection, murine gammaherpesvirus 68 (HV-68), to question whether such a virus could modulate the expression of CRYAB by antigen presenting cells. Following exposure to HV-68 and several other stimuli, in vitro secretion of CRYAB and subsequent intracellular accumulation were observed in cultured macrophages and dendritic cells. Following infection of mice with this virus, it was possible to track CRYAB expression in the spleen and in antigen presenting cell subpopulations, as well as its secretion into the blood. Mice immunized with human CRYAB mounted a significant immune response against this heat shock protein. Further, dendritic cells that were exposed to HV-68 could stimulate CD4⁺ T cells from CRYAB immunized mice to secrete interferon gamma. Taken together these studies are consistent with the notion of a gammaherpesvirus-induced CRYAB response in professional antigen presenting cells in this mouse model.

Keywords

Alpha beta-crystallin; autoimmunity; gammaherpesvirus; multiple sclerosis

Introduction

Alpha beta-crystallin (CRYAB) is one member of the small heat shock protein superfamily [1], whose induction can occur in a variety of cells [2], especially under conditions of endogenous and exogenous stress [3–6]. Intracellular CRYAB can function as a molecular chaperone, while extracellular CRYAB has been associated with protective effects [7–10]. Therapeutic use of exogenously administered, recombinant CRYAB has been proposed for central nervous system diseases such as multiple sclerosis [10], injuries such as spinal cord contusions [8], and stroke [7]. Support for such systemic use comes from observations that mice genetically deficient for CRYAB were more susceptible to induced CNS inflammation,

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and that exogenous administration of this protein reduced ongoing experimental autoimmune encephalomyelitis (EAE) in normal mice [9]. The mechanism of protection for therapeutically administered CRYAB appears to be linked to its ability to bind proteins important for the inflammatory process [11–13], and to influence regulatory cell activity [14,15].

These protective effects of CRYAB take on additional significance when considering this protein as one of the dominant autoantigens in multiple sclerosis [16–18]. Inducing an immune response against CRYAB in animal models does not appear to be encephalitogenic [19–21]. However, the presence of such autoimmunity in patients might be able to limit the protective effects of CRYAB [22], which accumulates at sites of brain lesions in multiple sclerosis [14]. These observations have been used to support the notion that the autoimmune response against CRYAB does not directly cause demyelination, but that the autoimmune-mediated destruction of CRYAB limits its protective effect thereby exacerbating multiple sclerosis [9,22].

How autoimmunity against CRYAB might develop in multiple sclerosis patients is not altogether clear. Since this small heat shock protein can be expressed by a variety of cells [2], and in response to a variety of insults, merely its production would not seem sufficient for inducing substantial autoimmunity. Rather, identification of professional antigen presenting cells involved in the initiation of this process, along with environmental factors which might augment the presentation of CRYAB antigens to auto-reactive CD4⁺ T lymphocytes need to be considered. Surprisingly, few studies have investigated the expression of CRYAB following exposure to environmental stimuli in professional antigen presenting cells [23].

One study demonstrated that upon infection with Epstein Barr Virus (EBV), some human B cells can begin to express CRYAB [23]. However the expression of CRYAB protein by other professional antigen presenting cells was not investigated. In the present study, we utilized an animal model of an EBV-like infection to question whether such a virus could modulate the expression of CRYAB by antigen presenting cells. Infection with murine gammaherpesvirus 68 (HV-68) has been utilized as a rodent model for investigating the pathophysiology of herpesvirus infections due to its homology and similarities with Epstein–Barr virus (EBV), Kaposi sarcoma virus and human herpesvirus-8 [24–26]. Intranasal or intragastric inoculation of rodents with HV-68 results in an acute, productive infection of lung or intestinal epithelial cells, respectively, followed by the dissemination of the virus to peripheral organs [27]. B lymphocytes [28–30], macrophages [31], and dendritic cells [32,33] become latently infected soon after inoculation. Levels of latent virus in the spleens of infected animals peak 15 days post-infection, coinciding with a marked splenomegaly [27,28,34] due to an unregulated expansion of leukocyte populations [28,35].

Following exposure to HV-68, Vesicular Stomatitis Virus (VSV), Herpes Simplex Virus-1 (HSV) or LPS, *in vitro* secretion of CRYAB or intracellular accumulation were observed in cultured macrophages and dendritic cells. Following infection of mice with HV-68, it was possible to track CRYAB mRNA and protein expression in the spleen, as well as its secretion into the blood. The percentage of myeloid-derived bone marrow cells which expressed intra-cellular CRYAB also increased following infection with HV-68. Of interest was the fact that increased intracellular CRYAB was observed during a time frame when viral latency had been established. Mice immunized with human CRYAB mounted a significant immune response against this heat shock protein. Although CRYAB has been identified as an autoantigen in multiple sclerosis, mice mounting an immune response against this small heat shock protein showed no evidence of neurological symptoms. Further, co-cultures of HV-68 infected dendritic cells with CD4⁺ T cells from CRYAB

immunized mice resulted in increased secretion of interferon gamma when compared to control co-cultures. Taken together these studies are consistent with the notion of a gammaherpesvirus-induced CRYAB response in professional antigen presenting cells in this mouse model.

Materials and methods

Animals

Six- to 8-week-old female SJL mice (18–22 g) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the vivarium in filter top cages containing sterile bedding. After arrival, mice were quarantined for at least 5 days, and fed chow and water ad libitum. All animal experiments were in compliance with protocols approved by the University of North Carolina at Charlotte Animal Care and Use Committee.

Viruses and lipopolysaccharide

Murine gammaherpesvirus-68 (HV-68; ATCC # VR-1465) stocks were prepared by infecting baby hamster kidney cells (BHK-21; American Type Culture Collection # CCL-10) at a low multiplicity of infection, followed by preparation of cellular lysates, as described previously [36–38]. Replicating HV-68 in viral stocks was quantified using 1:3 serial dilution of the cell lysates on BALB/3T12-3 cell (ATCC # CCL-164) monolayers. Dilutions of lysates from non-infected BHK-21 cells were used in some experiments to control for any non-specific effects.

Vesicular Stomatitis Virus (VSV, Indiana strain, ATCC #VR-1238) stocks were prepared as described previously [39]. Briefly, monolayer cultures of BHK-21 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Cellgro, Manassas, VA) supplemented with 9% fetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY). To produce VSV, cells were infected with virus at a multiplicity of infection of 0.05 plaque-forming units per cell in MegaVir HyQSFM4 serum-free medium (Hyclone, Thermo Scientific, Waltham, MA) and incubated for 24–48 h at 34 °C.

Herpes Simplex Virus-1 (HSV) stocks were prepared as described previously [40]. Briefly, HSV viral stocks were prepared by infecting monolayer cultures of Vero cells (ATCC #CCL-81) with HSV-1 (MacIntyre strain from a patient with encephalitis; ATCC, VR-539) at a low multiplicity of infection of 0.05 plaque-forming units per cell and incubated for 48 hours at 34 °C in SFM4MegaVir protein-free medium (Thermo Scientific Hyclone, Waltham, MA). Cells were removed with trypsin and pulse sonicated (Vibra Cell; Sonics & Materials Inc., Newtown, CT) to release intact virions. The sonicated material was centrifuged to remove unwanted cellular debris and the viral titers in the cell-free supernatant was quantified using a standard plaque assay of serial dilutions of HSV-1 on Vero cells at 34 °C.

Lipopolysaccharide (LPS) was purchased (Sigma Chemical Co., St. Louis, MO), and used at the indicated concentrations.

Cultured bone marrow-derived macrophages and dendritic cells

Bone marrow-derived macrophages and dendritic cells were isolated and characterized as previously described [36–38]. Cells were incubated in 24-well plates with HV-68 (virus to cell ratio 0.1:1 to 10:1), VSV (ratio 1:1), HSV-1 (ratio 1:1), or LPS (10 ng/ml). At the indicated times, cells were isolated for RNA extraction. Cells from duplicate cultures were harvested and lysed using 0.3% SDS followed by Western blot analyses. Supernates from

duplicate cultures were also removed for ELISA analyses. In some experiments, lysates from non-infected BHK-21 cells were added to control for non-specific effects.

HV-68 infection of mice and isolation of cells and tissue

Groups of mice were anesthetized with isoflurane and mock treated by intranasal instillation of saline or infected intranasally with 6×10^4 plaque forming units of HV-68. At the indicated times post infection, sera was taken from animals to determine CRYAB protein levels using an ELISA. In addition, at the indicated times post-infection, mice were euthanized and spleens and bone marrow removed. Splenic tissue was weighed and used to isolate RNA or for Western blot analyses as described next. For tissue homogenates, spleens were weighed, homogenized in 0.5 ml of PBS containing a cocktail of protease inhibitors (1 mM phenyl-methylsulfonylfluoride, 1 mM iodoacetamide, 1 ug/ml Pepstatin A in DMSO containing 0.1% b-mercaptoethanol). Single cell suspensions were also made from spleens by passing tissue through a 30-gauge wire mesh. Cells were then used for Western blot analyses or isolation of splenic subpopulations. Bone marrow cells were isolated as previously described, and used for FACS analyses described next.

Total RNA isolation and synthesis of cDNA

RNA was isolated using the Trizol method (Invitrogen; Carlsbad, CA), as described by the manufacturer. RNA concentrations were determined with a Gene Spec III spectrophotometer (Naka Instruments, Japan). For cDNA synthesis, one ug of RNA was reverse-transcribed in the presence of random hexamers (50 ng/ul), 10 mM dNTPs, 2.5 mM $MgCl_2$ using ImProm-II reverse transcriptase (Promega, Madison, WI) in the buffer supplied by the manufacturer. cDNA was precipitated with one-tenth volume of 3 M sodium acetate (pH 5.2) and 3 volumes of EtOH, and resuspended in 50 ul of nuclease-free H_2O . In some experiments, duplicate reactions were performed without the addition of reverse transcriptase to demonstrate the absence of any contaminating DNA.

Semiquantitative PCR and DNA product analysis

mRNA transcript levels of CRYAB and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were examined by PCR using the synthesized cDNA. Then, 100 ng of the cDNA was combined with 2.5 U of Taq polymerase (Promega), 0.2 mM each dNTP, 25 pmol of each primer and PCR buffer containing 2.5 mM $MgCl_2$ as provided by the manufacturer. Samples were cycled using 95° denaturation for 35 s, 60 °C annealing for 75 s and 72 °C extension for 90 s, with the first 3 cycles using extended denaturation, annealing and extension times. PCR was for 30 cycles with the extension time of the last cycle for 5 min at 72 °C. Forty percent of each amplified PCR product was electrophoresed on an ethidium bromide-stained agarose gel and photographed under UV illumination.

PCR primer sets were designed using IDT SciTools and purchased from IDT (Integrated DNA Technologies, Coralville, IA). Primer sets used for amplification are as follows:

CRYAB (accession no. NM_009964.2; 426 bp - spans exons 1 to 3):

Forward: 5'-GAG AGC ACC TGT TGG AGT CT - 3'

Reverse: 5'-TGC GGC GAC AGC AGG CTT CT - 3'

G3PDH (accession no. NM_008084.2; 346 bp - spans exons 5 to 7):

Forward: 5'-CCA TCA CCA TCT TCC AGG AGC GAG - 3'

Reverse: 5'-CAC AGT CTT CTG GGT GGC AGT GAT - 3'

Enzyme-linked immunosorbent assay (ELISA) to quantify CRYAB secretion

Sera from animals or culture supernates were isolated as described here and used in an ELISA to quantify CRYAB secretion. Capture ELISAs were constructed using an affinity purified goat anti-CRYAB antibody (R&D system multiple sclerosis, Minneapolis, MN) and an affinity purified, biotinylated rabbit anti-CRYAB antibody (Novus Biological, Littleton, CO). Briefly, goat anti-CRYAB antibody (20 ug/ml) was coated onto microtiter wells (Costar, Bloomington, MN) in 100 μ L of 0.1 M carbonate buffer (pH 8.3) overnight at 4°C. After washing, plates were blocked with 2% BSA in PBS, and dilutions of sera or culture supernates were added and incubated overnight at 4 °C. After washing, the biotinylated rabbit anti-CRYAB antibody was added, and bound antibody detected by the addition of a TMB substrate (Surmodics, Eden Prairie, MN). The enzymatic reactions were stopped by addition of 1 M sulfuric acid and absorbances were read at 405 nm. Dilutions of recombinant, human CRYAB (U.S. Biological, Inc., Swampscott, MA) were used to generate a standard curve for determination of experimental values.

Isolation of splenic cell subpopulations

Single cells suspensions from spleens were made as described above. Splenic subpopulations were isolated by sorting on a FACSaria II (BD BioScience, San Diego, CA). Briefly, aliquots of cells were incubated on ice for 45 minutes with the appropriate fluorochrome-conjugated antibodies, including eFluor 450-anti-B220, Alexa Fluor 488-anti-CD3, eFluor 450-anti-CD11b, or Alexa Fluor 488-anti-CD11c (eBioScience). Then, 200 000 cells were sorted for each of the stained populations, pelleted at 1000 $\times$ g, and resuspended in SDS sample buffer for use in Western blotting analyses.

FACS analyses of bone marrow-derived cells

Bone marrow cells were isolated as described above, washed, and resuspended in PBS containing 10% non-immune rabbit serum (Invitrogen, Camarillo, CA). Cells were incubated with eFluor 450-anti-CD11b antibody (eBioscience) plus DyLight 488 anti-CRYAB antibody (Novus Biologicals, Littleton, CO), followed by staining separate aliquots with PE-anti-Ly6G antibody or PE-anti-Ly6C antibody (eBioScience). These triple stained cells were then washed, resuspended, and sorted using a FACSaria II (BD Biosciences). To control for non-specific staining, irrelevant antibodies conjugated with the appropriate fluorochrome (eBioScience or Novus Biologicals) were used to define quadrants.

Western blot analyses of CRYAB expression

Immunoblot analyses were performed as described previously by our laboratory [41]. Briefly, tissue or cells isolated as described here were normalized by weight or cell number, respectively, prior to homogenization in 0.3% SDS buffer. Following electrophoresis on 10% polyacrylamide gels, blots were incubated with a polyclonal anti-CRYAB antibody (Abcam, Cambridge, MA). Bound antibody was detected using an HRP-conjugated anti-mouse antibody (Cell Signaling, Danvers, MA). To ensure equal loading, some blots were stripped and re-probed using an anti-actin antibody (eBioscience).

Immunization of mice with human CRYAB

Groups of mice were immunized intraperitoneally with 25 ug of recombinant, human CRYAB (US Biological) emulsified in Freund's adjuvant. Control mice received 25 ug of keyhole limpet hemocyanin (KLH, Calbiochem, La Jolla, CA) emulsified in Freund's adjuvant. Mice were observed daily and were scored for the development of any symptomology using the following scale: 0, no evidence of disease; 1, limp tail or hind limb weakness; 2, limp tail and hind limb weakness; 3, hind limb paralysis; 4, moribund.

Enzyme-linked immunosorbent assay (ELISA) to quantify CRYAB antibody titers

Following immunization of mice with CRYAB as described above, sera was collected at the indicated times and an ELISA performed to quantify the presence of anti-CRYAB antibodies. For this ELISA, microtiter plates (Costar, Tewksbury, MA) were coated with 200 ng/well of CRYAB (US Biological) in 100 μ L of 0.1 M carbonate buffer (pH 8.3) overnight at 4°C. Wells were then blocked with 2% BSA in PBS. After washing, serial dilutions of sera were incubated in wells for 2 hours at room temperature. After washing off unbound material, an HRP-conjugated anti-mouse antibody (Cell Signaling, Danvers, MA) was added for two hours. Following washing, plates were incubated with TMB substrate (Surmodics, Eden Prairie, MN) at room temperature. The enzymatic reactions were stopped by addition of 1 M sulfuric acid and absorbances were read at 405 nm. Endpoint titers were defined as the last serum dilution with an absorbance double that of animals immunized in a similar manner with KLH.

In vitro antigen re-stimulation assay

At 30 days post-immunization, mice were euthanized and spleens removed. Splenic CD4⁺ T lymphocytes were isolated from each CryaB immunized animal and co-cultured in the presence of varying ratios of dendritic cells (APC) that were uninfected or had been previously exposed to HV-68 or VSV *in vitro*. After 72 hours of co-culture, supernates were taken and interferon gamma (IFN- γ) was quantified by ELISA (R&D Systems, Minneapolis, MN).

Statistics

For statistical analysis, data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Analyses were performed using one-way analysis of variance (ANOVA) and analyzed post-test using Tukey's Multiple Comparison Test. Mean values are presented in figures $\pm$ the Standard Error of the Mean (SEM). Results marked with an (*) were determined to be statistically significant at $p < 0.05$.

Results

CRYAB mRNA and protein expression following *in vitro* exposure to HV-68

To begin to understand the relationship between CRYAB expression and gammaherpesvirus infection, we questioned whether HV-68 could affect the expression of this heat shock protein *in vitro*. Bone marrow derived macrophages and dendritic cells were cultured with HV-68 at varying ratios of virus to cell, and for varying periods of time. Cultured macrophages (Figure 1A) and dendritic cells (Figure 1B) showed constitutive expression of the mRNA encoding CRYAB, and exposure to HV-68 resulted in increased expression of this mRNA at 1 and 10 HV-68/cell ratios after 4 and 8 hours of culture (Figures 1A and B).

Western blot analyses were performed to detect intracellular CRYAB in cultured macrophages (Figure 1C) and dendritic cells (Figure 1D). Constitutive intracellular CRYAB protein expression decreased as soon as 1 hour following exposure of either cell population to HV-68. Some intracellular accumulation of this protein was subsequently observed after 8–24 hours' post infection.

ELISA analyses demonstrated secretion of CRYAB from culture macrophages (Figure 1E) and dendritic cells (Figure 1F) within 1 hour following exposure to HV-68. We concluded from these studies that CRYAB mRNA and intracellular protein are constitutively expressed in bone marrow derived macrophages and dendritic cells, possibly as a consequence of their isolation and culture. Following exposure to HV-68, CRYAB was rapidly secreted into

culture supernates, which likely accounted for the disappearance of the pre-formed, intracellular protein.

CRYAB protein expression following *in vitro* exposure to LPS, VSV or HSV-1

For comparison, we conducted similar *in vitro* studies to question whether different stimuli could affect CRYAB expression. When cultured macrophages or dendritic cells were exposed to LPS, VSV, or HSV-1 for varying periods of time, similar results were observed. Western blot analyses demonstrated that each stimulus resulted in the reduction of constitutive levels of intracellular CRYAB as early as 2 hours' post-exposure, with accumulation of this protein by 24 hours (Figure 2). These results mirrored those observed following exposure to HV-68 (Figure 1C and D). We concluded from these studies that the effects of HV-68 and these various stimuli on CRYAB expression and secretion were qualitatively similar.

CRYAB mRNA and protein expression following *in vivo* HV-68 infection

Following infection of mice with HV-68, CRYAB mRNA expression increased in splenic tissue as early as day 6 post-infection (Figure 3A). This virus-induced increase in message was accompanied by increases in intracellular CRYAB protein expression by splenocytes which persisted up to 30 days post-infection (Figure 3B). Conversely, increases in the secretion of CRYAB into the sera of infected mice could only be observed during the first few days following HV-68 infection (Figure 3C). We concluded from these studies that HV-68 infection could stimulate the release of CRYAB systemically during acute HV-68 infection. As mononucleosis began to develop (approximately day 10), intracellular CRYAB levels increased and persisted during the establishment of viral latency (day 30).

HV-68-induced CRYAB protein expression in splenic and bone marrow cell subpopulations

To define which cell subpopulations were induced by HV-68 infection to upregulate intracellular CRYAB protein expression, CD11b⁺, CD11c⁺, CD3⁺, and B220⁺ splenocytes were isolated. Western blot analyses on each of these cell subpopulations showed that CD11b⁺ and B220⁺ cells could be induced to express the highest levels of this heat shock protein (Figure 4A). Such increases occurred at 10 to 15 days' post-infection. We concluded from these studies that the two antigen presenting cell populations that were most effectively induced to express CRYAB following HV-68 infection were cells of the myeloid and B lymphocyte lineages.

To further address the ability of myeloid-derived antigen presenting cells to express CRYAB, we used intracellular fluorescence staining to follow the percentages of CD11b⁺ Ly6C⁺ and Ly6G⁺ cells that expressed this protein. Significant increases in the percentage of CD11b⁺ Ly6C⁺ (Figure 4B) and CD11b⁺ Ly6G⁺ (Figure 4C) were observed at 10–30 and 10–15 days post-infection, respectively. These studies support the notion that myeloid-derived antigen presenting cells are induced during the establishment of HV-68 latency to express intracellular CRYAB. These studies also support the notion that myeloid-derived antigen presenting cells are induced to express intracellular CRYAB during the establishment of HV-68 latency.

Mice immunized with human CRYAB mount a significant antibody response, but have no detectable CNS disease

Groups of SJL mice ($n = 4$) were immunized with KLH or with recombinant human CRYAB, and at days 15 and 30 post immunization sera was taken to determine antibody titers against this heat shock protein. Using an ELISA, it was possible to demonstrate IgG

antibody titers approaching 1:10 000 in CRYAB immunized mice (Figure 5A). To control for non-specific binding of immunoglobulin to wells coated with this heat shock protein (12), similar assays were performed on sera from mice immunized with KLH. Taken together, these studies demonstrate that human CRYAB was highly immunogenic in SJL mice.

During the immunization period, mice in both the KLH and CRYAB immunization groups were screened for the appearance of clinical symptoms consistent with the development of EAE. Animals in each immunization group showed no such symptoms (clinical scores 0.0 ± 0.0). We concluded from these studies that human CRYAB was immunogenic in SJL mice, but that the host response against this heat shock protein induced no detectable CNS disease.

Co-culturing CD4+ T lymphocytes from CRYAB immunized mice with dendritic cells infected *in vitro* with HV-68 show augmented interferon gamma production

Varying ratios of non-infected, VSV-infected, or HV-68 infected dendritic cells were co-cultured with CD4+ T lymphocytes isolated from CRYAB immunized animals to question whether viral infection had any effect on T cell activation. At a ratio of 1:1, HV-68 infected dendritic cells were able to significantly augment the ability of CD4+ T cells from CRYAB immunized mice to secrete interferon gamma when compared to co-cultures containing non-infected or VSV-infected dendritic cells (Figure 5B). In similar studies, no significant increase in interferon gamma secretion was observed using co-cultures of CD4+ T cells from unimmunized animals with HV-infected dendritic cells when compared to co-cultures with uninfected dendritic cells (data not shown). We concluded from these studies that HV-68 infected antigen presenting cells can augment T helper cell activation when these cells are isolated from CRYAB immunized mice. Cultured dendritic cells exposed to HV-68 do induce CRYAB secretion (Figure 1F) followed by its intracellular accumulation (Figure 1D), however it was not clear from these studies whether the augmentation in cytokine secretion observed (Figure 5B) was solely antigen specific or involved other mechanisms.

Discussion

The ability of professional antigen presenting cells to express CRYAB following interaction with HV-68 has several implications for the proposed notion that the protective effects of CRYAB expression can be reduced by autoimmune-mediated destruction in multiple sclerosis. First, we show that dendritic cells, macrophages, and B lymphocytes modulate expression of CRYAB following exposure to HV-68. We routinely found that the constitutive expression of intracellular CRYAB in cultured bone marrow derived macrophages and dendritic cells was quite high (Figure 1C and D), and may reflect the stimuli that these cells are exposed to (e.g. adherence to plastic and/or conditioned media containing colony stimulating factors). Regardless, exposure to HV-68 induced these cultured cells to secrete CRYAB into the medium within 1 hour (Figure 1E and F).

This acute time frame suggests that the CRYAB secreted was a result of preformed intracellular protein released into the supernates. This possibility is supported by the finding that intracellular stores of CRYAB were reduced as secretion into the medium occurred (Figure 1). Results from the *in vivo* studies were qualitatively different. Although detectable, constitutive expression of CRYAB mRNA (Figure 3A) and intracellular (Figure 3B) protein could be observed in spleens as was expected [23], HV-68 infection resulted in a significant increase in mRNA and intracellular CRYAB accumulation. This intracellular accumulation could be detected in professional antigen presenting cells in the spleens, as we routinely observed CD11b+, CD11c+ and B220+ cells upregulating expression of intracellular CRYAB (Figure 4A), as well as CD11b + Ly6C+ and CD11b + Ly6G+ myeloid cells

(Figures 4B and C). Together these results (Figures 3 and 4) clearly demonstrate an increased expression of intracellular CRYAB protein following HV-68 infection *in vivo*.

Second, few studies have focused on the ability of infectious agents to modulate expression of CRYAB [42,43]. Because this small heat shock protein can be induced by a variety of endogenous and exogenous stressors [3–6], it was not surprising that we found viruses (HV-68, HSV-1, or VSV) and a TLR-4 agonist (LPS) could modulate intracellular expression (Figures 1C, D, and 2) and extracellular secretion (Figures 1E and F) of CRYAB in macrophage and dendritic cell cultures. Based on these *in vitro* studies, it would be difficult to make a case that HV-68 is unique in its ability to modulate expression of CRYAB. However, it should be noted that in HV-68 infected mice, accumulation of CRYAB mRNA and intracellular protein could be observed at days 15 to 30 post-infection.

At this point in the life cycle of the virus, latency has been established [44] in B lymphocytes and other antigen presenting cells [24,31–33,35], with few infectious virions remaining [36,37,44,45]. Mechanisms by which the presence of a latent gammaherpesvirus might stimulate intracellular CRYAB accumulation in antigen presenting cells are presently not clear. It has been noted that EBV infection of cultured B lymphocytes results in the upregulation of CRYAB [23], however comprehensive studies have not been performed to determine the extent of CRYAB expression in human antigen presenting cells harboring latent EBV *in vivo*. Furthermore, no studies have been performed to investigate the expression of CRYAB in antigen presenting cells during reactivation from latency to maintain the persistent phenotype for the life of the host. Future studies will be required to dissect the relationship between a life-long infection with gammaherpes-viruses and expression of this small heat shock protein in professional antigen presenting cells.

Third, mechanisms responsible for the induction of autoimmune responses against CRYAB are not clear. Although detectable autoantibodies against CRYAB have been suggested to exist in the normal population [18], it has also been suggested that CRYAB can bind non-specifically to immunoglobulin with high affinity [12]. Such technical difficulties make it difficult to discern the existence of a specific autoantibody response against CRYAB in multiple sclerosis patients. However autoimmunity mediated by T lymphocytes has been observed by several groups, supporting the notion of an autoimmune response against this heat shock protein [16–18,46–48]. Because a variety of stressors [3–6] and stimuli (e.g. Figure 2) can modulate CRYAB expression, its mere presence is unlikely to be sufficient to induce autoimmunity.

Other factors must contribute to developing a response against this heat shock protein, however it is not clear what these might be. It is also not clear whether autoimmunity against CRYAB in multiple sclerosis patients develops first in the periphery, followed by the extravasation of reactive T cells into the CNS. Such mechanisms have been suggested for T cells reactive to myelin autoantigens [49]. Alternatively, it has been suggested that T cells auto-reactive for CRYAB occur naturally in humans, but not other mammals [18,23]. Local production of CRYAB in the CNS by innate immune cells could then recruit and activate these auto-reactive T cells, provoking a local, destructive response [15].

However, it is not clear why such an autoimmune response would selectively develop in multiple sclerosis, and not in a variety of other pathologies in the CNS [50] or peripheral tissues [51], where local or systemic CRYAB expression occurs. In the present study, we demonstrate the ability of professional antigen presenting cells to express CRYAB in response to HV-68 *in vivo* (Figure 3). Further, we show that CD4⁺ T cells from CRYAB immunized mice are stimulated to secrete interferon gamma when cultured with HV-68 infected dendritic cells under conditions where CRYAB is expressed (Figure 5B). Together

these studies demonstrate the ability of a gammaherpesvirus to induce the peripheral expression of this heat shock protein in a manner that may be conducive to its presentation by antigen presenting cells as a possible autoantigen.

Fourth, microbial pathogens have long been implicated as environmental insults that might contribute to the development and/or severity of multiple sclerosis. EBV is a gammaherpesvirus that infects more than 90% of world's population [52]. Several recent reviews summarize the evidence that is accumulating which suggest an association between EBV infection and multiple sclerosis [53–57]. Serological studies point to an almost 100% seropositivity to EBV for patients suffering from multiple sclerosis. There is also a strong correlation with the rise in antibody titers against EBV and the incidence of multiple sclerosis. Further, the risk of multiple sclerosis is very low in individuals not infected with EBV but increases exponentially in the same individuals following infection with this virus. Although the association with multiple sclerosis and EBV gains support, whether the effect of viral infection is a direct or indirect one is not clear [54].

Previous work in our laboratory demonstrated the ability of the EBV-like virus, HV-68 [24–26], to exacerbate EAE [58]. Although infection with this gammaherpesvirus by itself did not induce any detectable CNS disease, animals that had been infected with HV-68 experienced exacerbated EAE when this disease was induced by immunization or cellular transfer [58]. Further, we found that acute or latent HV-68 infection could exacerbate the disease course in two different rodent models of EAE. Mechanisms for this exacerbation were not clear, though later studies demonstrated virus-mediated immune modulation [36,37,59]. Ongoing studies are aimed at defining how HV-68 can modulate the immune response in a manner that facilitates the development of autoimmunity [58], cancers [60], or other inflammatory diseases [61].

A previous study demonstrated the ability of EBV to induce expression of CRYAB in human B lymphocytes [23]. The authors suggested that this viral infection might facilitate triggering autoimmunity against this heat shock protein. However, due to limitations inherent in performing studies with patients, and the fact that most individuals have been previously exposed to EBV (52), definitive experiments to follow viral-induced CRYAB expression *in vivo* following acute infection or latent gammaherpesvirus persistence will be challenging. In the present study we questioned whether infection with the EBV-like virus, HV-68, could induce expression of CRYAB in professional antigen presenting cells using a mouse model of gammaherpesvirus infection. Taken together, our studies demonstrate induced CRYAB expression in spleen tissue (Figure 3), in B220+, CD11b+, and CD11c+ splenocytes (Figure 4A), and in bone marrow-derived myeloid cells (Figure 4B). The potential for HV-68 infected dendritic cells to augment the ability of CD4+ T cells isolated from CRYAB immunized mice (Figure 5A) to secrete interferon gamma was also demonstrated (Figure 5B).

In summary, our results support the notion that an EBV-like infection can induce expression of the small heat shock protein, CRYAB. These studies expand upon the initial report that the presence of EBV can induce CRYAB in human B lymphocytes [23]. Specifically, we could detect secreted, as well as intracellular accumulation of CRYAB, in antigen presenting cells. This finding raises the question of whether extracellular or intracellular CRYAB serves as the predominant source of protein used for processing and presentation of this autoantigen in multiple sclerosis patients. Further, this intracellular accumulation could be observed at 15 to 30 days following HV-68 infection *in vivo*. Because few infectious virions can be detected during this time, the mechanisms by which cells harboring latent HV-68 can persist in their expression of intracellular CRYAB need investigation. Therefore, when considering the role of gammaherpesviruses in exacerbating autoimmunity, the relative

importance of the initial mononucleosis, the establishment of viral latency, and subsequent reactivations of infectious virus from latency will need to be dissected.

Finally, co-culturing CD4⁺ T lymphocytes from CRYAB immunized mice with dendritic cells infected *in vitro* with HV-68 show augmented interferon gamma production. Future studies will be required to dissect whether the mechanisms responsible for such an observation result predominantly from antigen specific or non-antigen specific pathways. Due to the proposed immune dysregulation associated with gammaherpesvirus latency and reactivation over the life of the host [62,63], and due to the wide variation of EBV viral burden between individuals [63–67], mechanisms mediating a virus-exacerbated autoimmune response cannot necessarily be assumed to be wholly autoantigen specific [68]. Regardless, the present work is consistent with the notion of a gammaherpesvirus-induced CRYAB response. This HV-68 model should facilitate future investigations into the possibility of a viral-exacerbated autoimmune response against CRYAB.

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Figure 1.

CryaB mRNA expression and intracellular and secreted CryaB protein following *in vitro* exposure to HV-68. Cultured bone marrow-derived macrophages (Panel A) and bone marrow derived dendritic cells (Panel B) were untreated (0) or exposed to HV-68 at the indicated ratios of virus to cells (0–10) and for the indicated periods of time (4–8 h). Cells were removed and semiquantitative RT-PCR was performed to detect CryaB mRNA expression. Results are presented as amplified products electrophoresed on ethidium bromide-stained gels. mRNA expression of the housekeeping gene (G3PDH) was used as a positive control to indicate that similar amounts of cDNA were present in each sample. Western blot analyses were also performed on cell lysates from macrophages (Panel C) and dendritic cells (Panel D) that were cultured without (0) or with HV-68 (ratio 1:1) for the indicated times (1–24 h). To ensure equal loading, the membrane was stripped and re-probed for the control protein, actin (control). ELISA analyses were performed on culture supernates taken from macrophage (Panel E) or dendritic cell (Panel F) cultures to quantify the amount of secreted CryaB. Results are presented as mean values of triplicate determinations. Asterisks indicate a statistically significant difference ($p < 0.05$) when compared to uninfected cultures. As a control, mock infected BHK lysates (BHK) were

added to either cell population. These studies were performed at least twice with similar results.

Figure 2.

Western blot analyses to detect CryaB protein in cell lysates following exposure to LPS, VSV, or HSV-1. Cultured bone marrow-derived macrophages (top panel) and bone marrow derived dendritic cells (bottom panel) were untreated (0) or exposed to LPS (10 ng/ml), VSV (ratio 1:1), or HSV (ratio 1:1) for the indicated periods of time (2–24 hours). Western blot analyses were performed on cell lysates to detect the presence of intracellular CryaB. These studies were performed twice with similar results.

Figure 3.

CryaB mRNA expression and intracellular and secreted CryaB protein following *in vivo* exposure to HV-68. Groups of SJL mice (N = 4 per time point) were uninfected (0) or infected with HV-68. At the indicated number of days post-infection, mice were euthanized and sera and spleens processed for analyses. Panel A shows a representative time course of splenic CryaB mRNA expression using semiquantitative RT-PCR. Results are presented as amplified products electrophoresed on ethidium bromide-stained gels. mRNA expression of the housekeeping gene (G3PDH) was used as a positive control to indicate that similar amounts of cDNA were present in each sample. A mock cDNA reaction containing no reverse transcriptase (no RT) was amplified as a control to demonstrate the absence of contaminating DNA in the cDNA preparations. Panel B shows a representative time course of splenic CryaB protein expression using Western blot analyses. To ensure equal loading, the membrane was stripped and re-probed for the control protein, actin (control). Panel C quantifies the presence of CryaB in sera of mice using an ELISA. Results are presented as

mean values of 4 different mice for each time point. Asterisks indicate a statistically significant difference ($p < 0.05$) when compared to uninfected animals. These studies were performed at least twice with similar results.

Figure 4.

HV-68-induced CryaB protein expression in splenic and bone marrow cell subpopulations. Groups of SJL mice (N = 4 per time point) were untreated (0) or infected with HV-68 and at the indicated days post infection, spleens and bone marrow were removed for isolation of cell subpopulations. Panel A shows a representative time course for intracellular CryaB protein expression in CD11b+, CD11c+, CD3+, and B220+ splenocytes using Western blot analyses. Panel B shows the percentage of CD11b + Ly6C+ bone marrow cells that were positive for intracellular CryaB protein expression in groups of mice at varying times post HV-68 infection. Panel C shows the percentage of CD11b + Ly6G+ bone marrow cells that were positive for intracellular CryaB protein expression in groups of mice at varying times post HV-68 infection. Results are presented as mean values of 4 different mice for each time point. Asterisks indicate a statistically significant difference ($p < 0.05$) when compared to uninfected animals. These studies were performed at least twice with similar results.

Figure 5.

CryaB expression by viral-infected antigen presenting cells (APC) can induce interferon gamma production when cultured with CD4⁺ T lymphocytes. Groups of SJL mice (N = 4) were immunized with KLH or with recombinant human CryaB protein. At the indicated days post-immunization, sera were isolated from each animal, and antibody titers against CryaB were determined using an ELISA (Panel A). Results are presented as mean values of 4 different mice for each time point. At 30 days post-immunization, mice were euthanized and spleens removed. Splenic CD4⁺ T lymphocytes were isolated from each CryaB immunized animal and co-cultured in the presence of varying ratios of dendritic cells (APC) that were non-infected or had been previously exposed to HV-68 or VSV. After 72 hours of co-culture, supernates were taken and interferon gamma (IFN-gamma) was quantified by ELISA (Panel B). Results are presented as means ($\pm$ S.E.) of triplicate determinations. An asterisk indicates a statistically significant difference ($p < 0.05$) when compared to uninfected APCs.