Original Article

DOI: 10.5582/bst.2019.01197

Human cytomegalovirus IE2 protein regulates macrophage-mediated immune escape by upregulating GRB2 expression in UL122 genetically modified mice

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Summary **Although cytomegalovirus (HCMV) infection is asymptomatic in healthy individuals, the virus can remain latent for many years due to its ability to evade host immune surveillance. However, reactivation of HCMV can lead to life-threatening disease. Recent studies have shown that HCMV infection mediates immune escape by regulating macrophage activity, although the role of the HCMV-encoded IE2 protein is unclear. A ul122 transgenic mouse model was created to stably expresses the IE2 protein, and the proportion of M1 and M2 macrophage populations in their spleen and bone marrow was compared to that in wild-type controls. In addition, the phagocytic function of the macrophages was evaluated in terms of neutral red dye uptake. Spleen and bone marrow macrophages in IE2-expressing mice were mainly of the M2 phenotype and displayed enhanced phagocytic function compared to that in control mice. The relative levels of expression of macrophage-related GRB2 and of IL-4, IFN-γ, IL-13, and TNF-α were also analyzed in the spleen and bone marrow of the two groups. The IE2-expressing mice had increased expression of GRB2 and increased expression of the M2-related cytokines IL-4 and IL-13. Taken together, the current results suggest that HCMV IE2 polarizes the host macrophages to the M2 type via a GRB2/IL-4-related pathway, which enables long-term survival of the virus in the host.**

Keywords: IE2, GRB2, macrophage polarization, immune escape

1. Introduction

Human cytomegalovirus (HCMV), also known as human herpes virus 5 (HHV5), belongs to the beta herpesvirus subfamily of the Herpesviridae subfamily and has a double-stranded DNA genome of ~240 kb that encodes more than 200 proteins (*1,2*). A large portion of the global population is infected with HCMV, with serum infection rates of 40-60% in developed countries and nearly 100% in developing countries (*3*). Although HCMV triggers a host immune response that inhibits viral replication and lowers the viral load below the threshold of clinical detection, it cannot completely eliminate the virus (*4*). HCMV has evolved multiple strategies to evade the host immune surveillance and survive, resulting in lifelong latent infection (*5*). Although infection in healthy adults is usually asymptomatic or mild, it is often associated with the development of chronic inflammatory diseases and even cancer (*6*). There are even reports of HCMV proteins and nucleic acids in breast (*7*), colon (*8*) prostate (*9*), and mucoepidermoid salivary gland (*10*) cancers, as well as glioblastoma (*11*) tissues. In addition, reactivation of the virus during an immuno-deficient state can result in severe clinical disease or even death (*12,13*).

The pathological changes associated with HCMV infection are largely mediated by infected monocytes. Due to diverse surface receptors and the high level of phagocytic activity, monocytes are often used

Released online in J-STAGE as advance publication December 23, 2019.

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by the virus as a conduit to enter other cells (*14*). In order to circumvent its short life cycle and inability to support its own replication, HCMV induces monocyte differentiation into long-lived macrophages that allow viral gene expression and replication (*15,16*). Macrophages are classified as the pro-inflammatory M1 type and anti-inflammatory M2 type that are induced by different cytokines (*17*). HCMV can replicate, survive for long durations in the host cell, and spread by regulating the polarization of macrophages. M1 macrophages facilitate establishment of HCMV infection and transmission, which induce acute inflammation and immune cell recruitment (*18*). M2 type macrophages, in contrast, promote chronic infection by producing anti-inflammatory mediators that facilitate long-term tissue distribution of the virus and that are activated under specific conditions (*16*). In addition, phagocytosis by macrophages is a common strategy used by viruses to evade host immune cells (*19*). Recent transcriptome studies have shown that both M1- and M2-related genes are expressed after HCMV infection of monocytes/macrophages, although the former is more significantly upregulated, resulting in M1 polarization (*20*).

Growth factor receptor-bound protein 2 (GRB2) is an adaptor protein associated with activated epidermal growth factor receptors (EGFRs), and GRB2 recruits accessory proteins in various receptor-mediated signaling pathways (*21*). It is up-regulated after HCMV infection, and it promotes viral replication and spread (*22*) and also affects the polarization, phagocytic function, proliferation, and migration of macrophages (*23-25*). HCMV genes are expressed in a temporal sequence and are accordingly divided into immediate early (IE), early (E), and late (L) genes (*26*). The two key regulators in HCMV replication are UL123 (IE1) and UL122 (IE2), which are encoded by the IE gene. They are expressed at high levels within hours after viral entry, and their re-expression is vital to HCMV reactivation (*27*). IE2 is essential for efficient viral replication, but since HCMV mutants defective in IE1 expression display a severe growth defect, IE2 is considered to be the main promoter of the E and L transcripts (*28*).

The aims of this study were to determine the effects of HCMV IE2 on macrophage polarization and function and to explore the role of GRB2. Due to the strict species-specificity of HCMV infection, previous studies were conducted using *in vitro* models. A ul122 transgenic mouse model was created to stably expresses IE2 in order to elucidate the mechanism of IE2 action *in vivo*.

2. Materials and Methods

2.1. *Animals*

The eukaryotic expression vector pAV.ExBi-CMV-IE2-IRES-eGFP initiated by the CMV promoter was constructed and then microinjected into a fertilized egg to ultimately obtain an F0 generation ul122 transgenic mouse model. Four ul122 mice (2 males and 2 females) stably expressing IE2 were obtained from the Laboratory of Pathogen Biology of Qingdao University and mated with four randomly selected C57BL/6 wild-type breeding mice under SPF conditions. The progeny were genotyped with PCR, and 20-24 week-old male ul122-positive and wild-type mice (15 per group) were selected for study. All animal experiments were approved by the Animal Experiments Committee of Qingdao University.

2.2. *Isolation of spleen cells*

Spleen immune cells were isolated as previously described (*29*), with some minor modifications. Briefly, the spleen was washed twice with PBS, minced with surgical scissors and then homogenized with a sterile 20-mL syringe plunger against a 75-μm stainless steel mesh in 2 mL of RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA). The homogenate was then filtered through another 75-μm mesh to obtain a single cell suspension, which was centrifuged at 60 × g for 1 min to remove any remaining debris. The cells were pelleted at 480 × g for 8 min and then suspended in red blood cell lysis buffer (Solarbio, Beijing, China) and incubated at room temperature for 5 min to lyse red blood cells. After addition of 1 mL of FBS, the cell pellets were centrifuged at 480 × g for 8 min at 8°C and washed twice with PBS.

2.3. *Isolation of bone marrow cells*

Bone marrow cells were isolated as previously described (*30*), with minor modifications. The mice were sacrificed by cervical dislocation and soaked in 75% alcohol for about 5 min. The femur and tibia were disarticulated, and the muscles and fascia were removed in a Petri dish containing PBS. The bones were washed with PBS and immersed in RPMI-1640 medium (HyClone, Logan, UT, USA) on ice. The ends of the bones were cut with scissors, and the medullary cavity was flushed with 5 mL of RPMI 1640 using a 25-gauge needle into a fresh Petri dish. The marrow was fragmented and filtered through a 75-μm stainless steel mesh to obtain a single cell suspension.

2.4. *DNA extraction and PCR*

DNA was extracted from the tails of mice using the CWBIO Universal Genomic DNA Kit (lot: 50223) according to the manufacturer's instructions. The primers used for amplifying the HCMV IE2 gene are listed in Table 1. The reaction conditions were as follows: pre-denaturation at 95°C for 5 min followed by

Table 1. The primers used for PCR and qPCR

Gene	Forward (5'-3')	Reverse (5'-3')
PCR		
IE2	CCGCAAGAAGAAGAGCAAACG	CACCTGGTGCATACTGGGAAT
qPCR		
GRB2	CGGGACATAGAACAGATGCCAC	TGAAGTCTCCTCTGCGAAAGCC
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IFN-γ	GCCACGGCACAGTCATTGA	TGCTGATGGCCTGATTGTCTT
IL-13	TGAGCAACATCACACAAGACC	GGCCTTGCGGTTACAGAGG
TNF-α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG
IE2	CCGCAAGAAGAAGAGCAAACG	CACCTGGTGCATACTGGGAAT

34 cycles of 95°C for 30 sec, 60°C for 35 sec, and 72°C for 35 sec, and final extension at 72°C for 10 min. The PCR products were identified using restriction enzyme digestion and sequence analysis.

2.5. *Real-time PCR assay*

Total RNA was extracted from the spleen and bone marrow using an RNA isolation kit (TIANGEN, Shanghai, China) and reverse transcribed using a reverse transcription kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. The cDNA transcripts (5 μL) were mixed with 15 μL of a PCR mixture (FastStart Essential DNA Green Master) and run for 40 cycles in a BIO-Rad iQ™5 Instrument. The primers are listed in Table 1. The mRNA levels were normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method.

2.6. *Western blotting*

Proteins were extracted from the spleen and quantified using the BCA method. Equal amounts of protein per sample were resolved with 10% SDS-PAGE and transferred to a 0.45-mm nitrocellulose membrane (EMD Millipore, Billerica, MA, USA) for 1 h. The membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated overnight with the specific primary antibodies [diluted 1:1,000 in 5% skim milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.15% Tween-20)] against GRB2 (ab32037; Abcam), β-Actin (bsm-33036M; Bioss Bioscience), and IE2 (MAB8131; Millipore). The membranes were washed and separately placed in secondary antibodies. The membranes of GRB2 and β-actin were placed in anti-mouse IgG-HRP (abs20001; Absin Bioscience), and the membrane of IE2 was placed in anti-rabbit IgG-HRP (abs20002; Absin Bioscience) (both diluted 1:2,000 in 5% skim milk in TBST) for 2 h at room temperature. After membranes were washed three times with TBST at room temperature, positive bands were developed using the SuperSignal West Pico Trial kit (Thermo Fisher Scientific, Inc.) and detected with the VilberLourmat imaging system (VilberLourmat, Marne-la-Vallée, France).

2.7. *Phagocytosis assay*

Neutral red uptake by macrophages was measured using the method described by Long *et al*. (*31*) with some modifications. Briefly, the macrophages were seeded on 96-well plates at a density of 1×10^6 cells/well and incubated for 4 h at 37°C in a 5% CO_2 atmosphere. The medium was discarded, and then cells were washed with PBS, and 100 μL of 0.1% filtered neutral red dye was added to each well. The cells were incubated with the dye for 2 h. The un-phagocytized neutral red was rinsed off with PBS, and then 200 μL of cell lysis buffer (acetic acid/ethanol = 1:1, mL/mL) was added to each well. The cells were incubated overnight with the lysis buffer at 4°C, and the OD at 570 nm was measured.

2.8. *Flow cytometry analysis*

All antibodies and buffers were from Biolegend (San Diego, CA). The cells were suspended in the staining buffer at a density of 1×10^7 cells/mL and incubated with TruStainfcX™ (anti-mouse cd16/32) to block non-specific Fc staining. One-hundred-microliter aliquots were dispensed in different tubes and incubated with FITC anti-mouse/human CD11b, PerCP/Cyanine5.5 anti-mouse F4/80, APC anti-mouse CD80, PE anti-mouse CD206 (MMR), APC Armenian Hamster IgG isotype control, PE Rat IgG2a, and κ isotype control as appropriate at room temperature for 30 min. The reaction was stopped by adding 2 mL of staining buffer per tube, and the cells were pelleted at 350 g for 5 min. After fixation with 0.5 mL of fixation buffer at room temperature for 20 min, the cells were again pelleted and re-suspended in 2 mL of intracellular staining perm wash buffer. After centrifugation at 350 g for 5 min, the previous step was repeated twice. The cells were then re-suspended in 0.1 mL of intracellular staining perm wash buffer, and the appropriate amount of anti-CD206 antibody or the corresponding control was added. The cells were incubated at room temperature for 30 min, diluted with 2 mL of intracellular staining perm wash buffer, and pelleted. The supernatant was discarded, 0.1 mL of cell staining buffer was added, and the stained cells were analyzed in the Cyto-FLex flow cytometer

(Beckman Coulter). The results were analyzed with the software FlowJo Version 10 (TreeStar).

2.9. *Statistical analysis*

All images were formatted for optimal presentation using Adobe Illustrator CS4 (Adobe Systems, Inc., San Jose, CA, USA). The Student's *t*-test was used to compare two groups, and one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test was used to compare multiple groups. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. *Verification of UL122 genetically modified mice*

The progeny of the transgenic and wild-type mice were genotyped with PCR. The genotyping results are shown in Figure 1 and in the supplementary results (supplementary Figure S1. *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=50*). Based on the presence or absence of the *UL122* gene, the mice were divided into a control group and an experimental group.

3.2. *Effect of IE2 on macrophage count and phagocytic function*

Macrophages play a key role in the antiviral immune response (*32*). To determine the effect of IE2 on the relative proportion and phagocytic function of macrophages, macrophages from the experimental group and control group were compared. There were no significant differences in the total number of bone marrow macrophages (BMs) and spleen macrophages (SPMs) in the two groups (Figures 2A-2C). The production of macrophages is related to the production of single cells per mouse. Neutral red absorption was used to detect the phagocytic activity of the macrophages, and significantly higher phagocytic activity was noted in both spleen (Figure 2D, $p < 0.01$) and bone marrow (Figure 2E, $p < 0.05$) macrophages in IE2-expressing mice compared to that in the controls. Taken together, these findings indicate that IE2 enhances the phagocytic function of macrophages.

3.3. *IE2 induces M2 polarization*

Immuno-typing of the SPMs and BMs revealed a significant increase in the proportion of M2-type SPMs in IE2-expressing mice compared to that in the controls, with a concomitant decrease in M1-type macrophages (Figures 3A and 3B). Thus, IE2 induces the polarization of macrophages to the anti-inflammatory M2 phenotype (Figures 3C-3E).

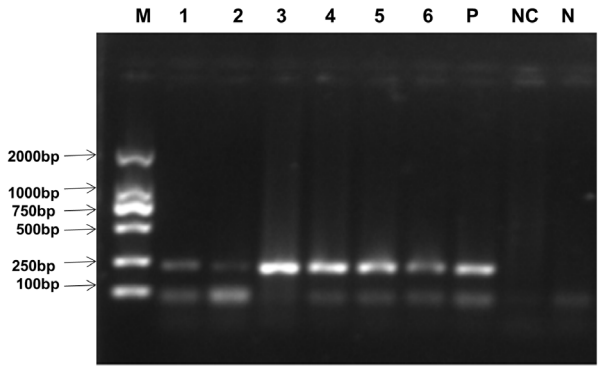

Figure 1. Identification of UL122-positive and -negative mice with PCR. Lanes 3-6 are positive and Lanes 1 and 2 negative for UL122. Lanes P, NC, and N are the positive control, water, and negative control, respectively. PCR product size = 229 bp.

3.4. *IE2 upregulates GRB2 protein in UL122 genetically modified mice*

HCMV infection is known to upregulate GRB2 in human cells (*22*). Consistent with this, levels of GRB2 mRNA (Figures 4A and 4B) and protein (Figure 4C and 4D) were significantly up-regulated in the spleen and bone marrow of the transgenic mice compared to that in the wild-type control group.

3.5. *Expression of mRNA of the polarization-related cytokines IL-4, IFN-γ, IL-13, and TNF-α*

To further confirm the polarizing effect of IE2, the levels of expression of IL-4, IFN-γ, IL-13, and TNF-α mRNA were evaluated in the spleen and bone marrow of the mice. While IFN-γ and TNF-α activate M1 macrophages, IL-4 and IL-13 are associated with M2 polarization (*33*). As shown in Figure 5, IL-4 and IL-13 were both up-regulated in the IE2-expressing mice compared to the control group, with a significant increase in the level of IL-4.

4. Discussion

HCMV has developed several ways to evade the immune system, such as being phagocytosed by macrophages. As the first target of HCMV infection, they are essential for viral persistence and spread (*34-37*). M1 and M2 macrophages exhibit distinct phenotypes and functions. M1 is the classic activated type, which functions in immune surveillance by secreting pro-inflammatory cytokines and chemokines and by presenting antigens to T cells. M2 is an alternately activated type with weak antigen-presenting ability, and it plays an important role in immune tolerance by secreting inhibitory cytokines (*38*). HCMV infection polarizes a macrophage subpopulation to the M1 type, which enables their migration from the blood into the tissues, thereby

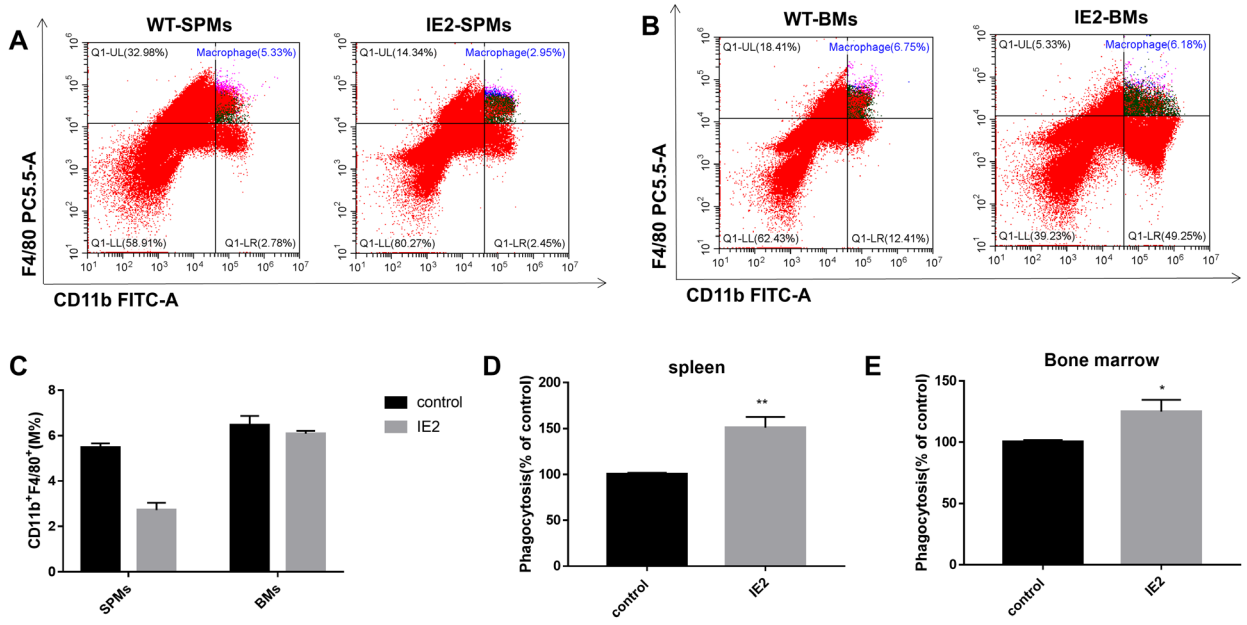

Figure 2. Effect of IE2 on macrophage count and phagocytic function. Representative FACS plots of total **(A)** SPMs and **(B)** BMs in the control group and IE2 mice. Bar graphs showing the number of macrophages **(C)** in the spleen and bone marrow. The phagocytic activity of **(D)** SPMs and **(E)** BMs in IE2 mice was higher than that in the control group. Data are expressed as the mean ± SEM, $n = 15$. $*p < 0.05$, $**p < 0.01$ *vs*. NC.

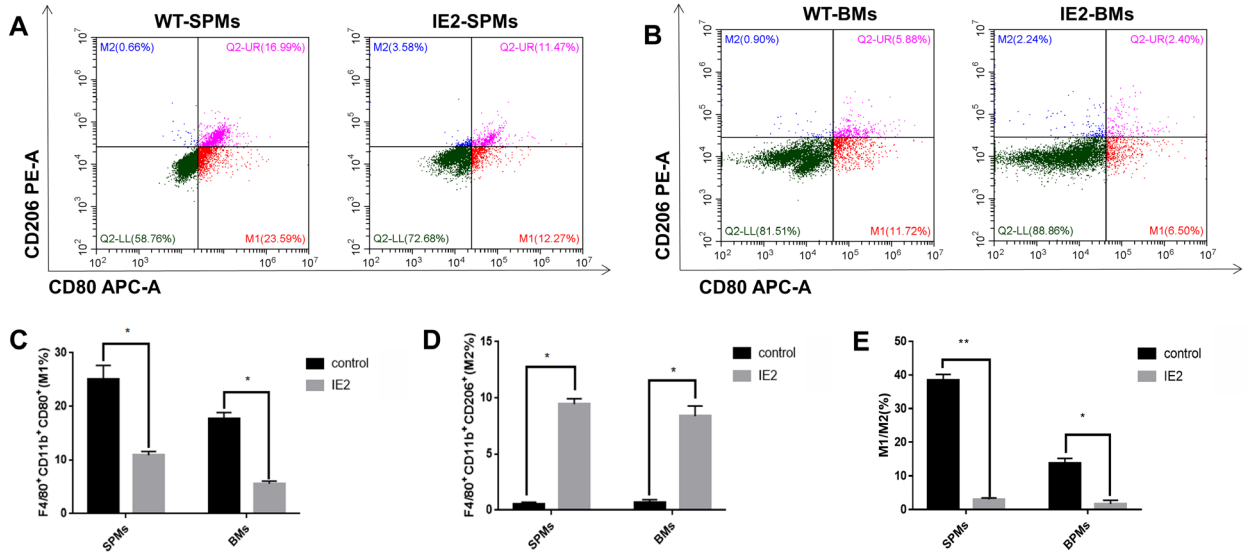

Figure 3. HCMV IE2 leads to M2 polarization in both the spleen and bone marrow. Representative FACS plots of M1 and M2 macrophages in the **(A)** spleen and **(B)** bone marrow. Bar graphs showing the number of **(C)** M1 macrophages and **(D)** M2 macrophages, and **(E)** M1/M2 ratios in the spleen and bone marrow. Data are expressed as the mean ± SEM, $n = 15$. $*p < 0.05$, $**p < 0.01$ *vs*. NC.

promoting viral transmission (*39*). In contrast, M2 macrophages allow long-term incubation of the virus and its reactivation in response to specific stimuli, and these macrophages are highly susceptible to HCMV infection (*16,37*).

Silencing of the IE2-encoding gene inhibits HCMV DNA replication and production of viral progeny, indicating that IE2 plays a major role in HCMV infection (*40*). Due to the high species specificity of HCMV, studies of IE2 have been limited to *in vitro* models of infection. Use of ul122 overcomes species specificity and provides an effective way to study IE2 in macrophage-mediated immune changes. ul122 mice exhibited M2 polarization in their spleen and bone marrow, which is significant since the HCMV is known to evade the host immune response by promoting M2 polarization that enables long-term viral replication, survival, and transmission (*20*). In addition, HCMV also

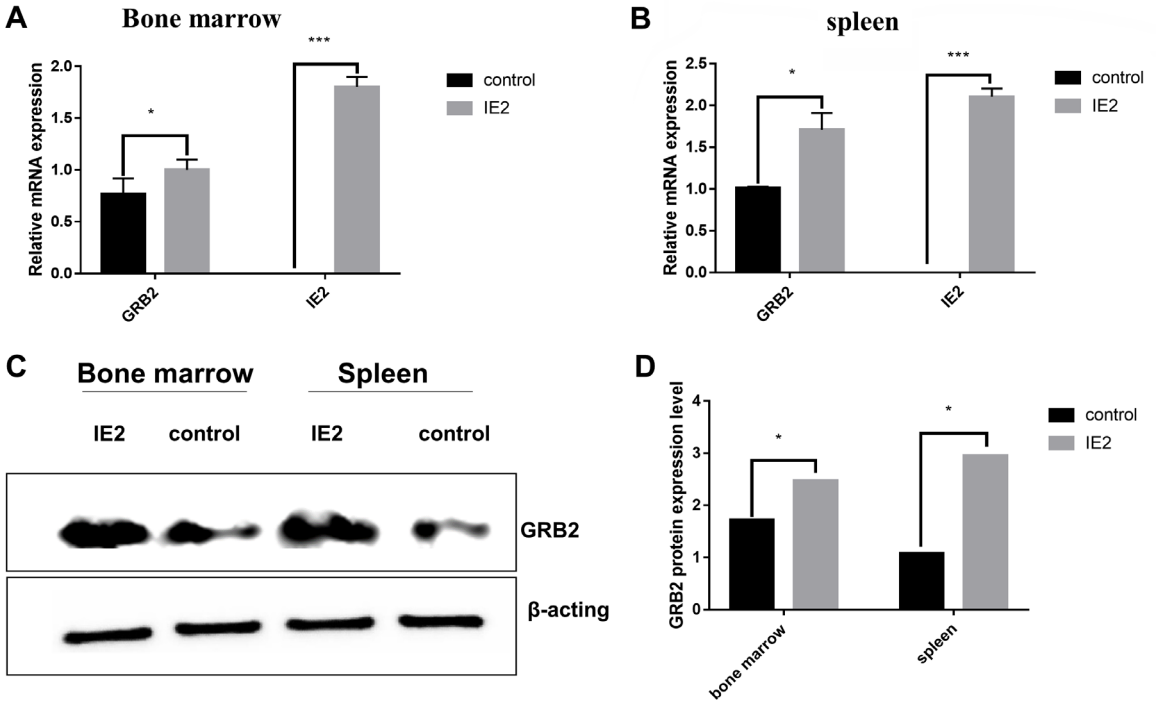

Figure 4. GRB2 is upregulated in IE2-expressing mice. Bar graph showing relative level of grb2 mRNA in the **(A)** spleen and **(B)** bone marrow. **(C)**. Immunoblots showing relative levels of GRB2 protein in IE2 mice. **(D)**. Quantitative analysis of GRB2 protein levels in the IE2 group and control group. Data are expressed as the mean ± SEM, n = 15 mice per group, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs*. control group.

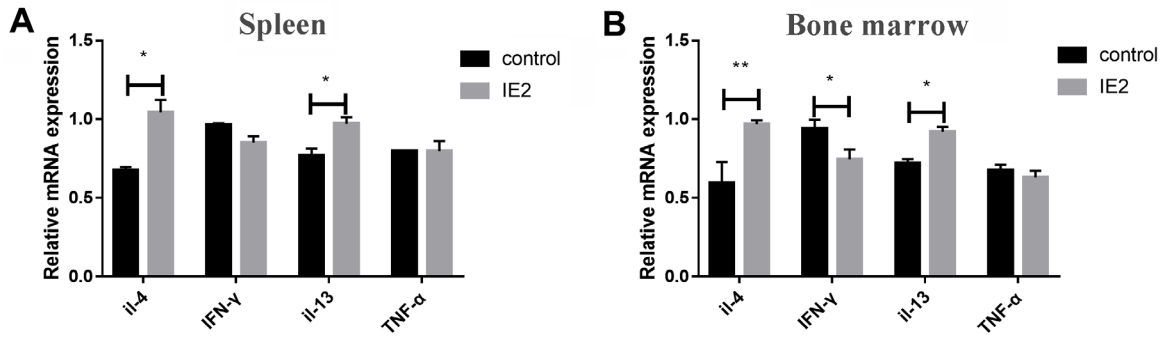

Figure 5. IE2 modulates cytokines associated with macrophage polarization. Bar graphs showing relative levels of IL-4, IFN-γ, IL-13. and TNF-α mRNA in the **(A)** spleen and **(B)** bone marrow of the control group and IE2 mice. The qPCR data are normalized to GAPDH. Data are expressed as the mean ± SEM, n = 15 mice per group, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs*. control group.

interferes with the phagocytic function of macrophages *via* different mechanisms (*41*), and consistent with this increased phagocytic function of macrophages was noted in ul122 mice (Figures 2D and 2E). Taken together, these findings indicate that IE2 facilitates viral transmission and persistence in M2 macrophages, where they can be reactivated under specific conditions.

Previous studies have shown that GRB2 plays an important role in regulating macrophage differentiation, proliferation, phagocytosis, migration, adhesion, and invasion (*21,24,42,43*) and that silencing of Grb2 disrupts phagocytosis (*23*). High levels of GRB2 expression and increased phagocytic activity were noted in the macrophages of IE2 mice, along with increased production of M2 cytokines such as IL-4 and IL-13. The binding of IL-4 to the IL-4 receptor recruits the p85 regulatory subunit of PI3K and GRB2, resulting in M2 polarization (*44,45*). Taken together, these findings indicate that IE2 significantly up-regulates GRB2 *via* increased production of M2-promoting IL-4, although the exact mechanism by which IE2-GRB2 mediates immune escape requires further investigation. The first step in viral infection is contact with the host cell and engulfment, which can enable long-term survival of

the virus in the host. Therefore, IE2 likely participates in long-term HCMV survival and latent infection by promoting GRB2-mediated macrophage polarization.

Acknowledgements

The authors wish to thank the individuals who helped to make this study possible. This work was supported by the National Natural Science Foundation of China (81471958).

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(Received July 27, 2019; Revised September 29, 2019; Accepted October 28, 2019)