Title: B Cell-activating Factor Regulates the Survival of B Lymphocytes Infected
with Human Cytomegalovirus

Author names and affiliations:

Haiyan Xu¹, Panpan Dong², Xuyi Ma¹, Dan Song², Dong Xue¹, Renfang Xu¹, Hao
Lu¹, Xiaozhou He¹

¹Urology Department, Third Affiliated Hospital of Soochow University, Changzhou 213003,
China

²Soochow University, Suzhou 215007, China

Corresponding author:

Haiyan Xu, Third Affiliated Hospital of Soochow University,
xuhaiyan09@163.com,
Tel: 86-519-68870351, Fax: 86-519-86621235

Present address: Rm209, No.11 Building, #185 Juqian St. Changzhou 213003, China

Highlights

1. BAFF expression was rather upregulated and high BAFF-R expression sustained on B cells infected with HCMV during the 7-day observation *in vitro*.
2. Enhancement of BAFF/BAFF-R signaling transduction prolonged the survival time of B cells infected with HCMV.
3. BAFF/BAFF-R signal crosstalk with HCMV/TLR9 transduction, and the stimulation ability of HCMV DNA to TLR9 is weaker than TLR9 agonist.

Keywords:

BAFF; HCMV; B lymphocytes

Abbreviations:

APC: allophycocyanin

BAFF: B cell activating factor belonging to TNF superfamily

BAFF-R: BAFF receptor

BCMA: B cell maturation antigen

FCS: fetal calf serum

FCM: flow cytometry

FITC: fluorescein isothiocyanate

HCMV: human cytomegalovirus

HFL-1: human embryonic lung fibroblast-1

ODN: oligodeoxynucleotide

LD₅₀: half lethal dose

PBS: phosphate buffer saline

PE: phycoerythrin

RTx: renal transplantation

TACI: TNFR homology transmembrane activator and calcium modulator and
cyclophilin ligand interactor

TCID₅₀: tissue culture infection dose 50

Abstract

Background: Previous studies have suggested that B lymphocytes can be polyclonally activated by human cytomegalovirus (HCMV), and individuals infected by HCMV exhibit characteristic features of an autoimmunity disease. B cell-activating factor (BAFF) plays important roles in the survival and differentiation of B cells; however, few studies have examined the potential role of BAFF on B cells infected by HCMV.

Methods: HCMV virus strain (HCMV AD-169) was concentrated by normal methods and used to infect microbead-purified tonsil CD19⁺ B cells. Cells and supernatants were collected at the 1st, 3rd, 5th, and 7th day of co-culture, respectively. Cellular phenotypes, including expression of BAFF and its cognate receptors (BAFF-R, TACI, and BCMA) were detected by flow cytometry (FCM); cells apoptosis rates were also examined by FCM; and IgG titers in supernatants was detected by ELISA. In parallel, neutralizing anti-BAFF-R antibody was applied to observe the effect of BAFF/BAFF-R signaling on apoptosis and the IgG secretion ability of B cells stimulated by HCMV.

Results: LogTCID₅₀ of 3rd and 4th generation of HCMV was -3.54 and -3.28, respectively. FCM results showed that the purity of CD19⁺ B cells was >98%. BAFF-R was highly expressed and upregulated on HCMV-infected B cells (93.5%–99.3%), compared with B cells prior to HCMV infection and uninfected group; while BAFF-R expression gradually decreased with time and to the lowest level at 5th day (81%) in the control medium-only group. In contrast, expression of TACI and BCMA gradually increased during culture in both HCMV-infected and medium-only control B cells. Furthermore, the apoptosis rate of HCMV-infected and medium-only control B cells did not vary significantly during culture, but IgG secretion ability of HCMV-infected B cells significantly increased over time while no changes were observed with the medium-only control. Importantly, the apoptosis rate of B cells significantly increased when BAFF/BAFF-R signal was blocked prior to HCMV infection ($P < 0.05$), although no significant changes of IgG levels were observed ($P > 0.05$). **Conclusions:** BAFF-R was consistently expressed on B cells

infected by HCMV. Enhancement of BAFF/BAFF-R signaling decreased the apoptosis rate and extended the survival of B cells.

Key words: Human cytomegalovirus (HCMV); B cell-activating factor (BAFF); apoptosis; B cells

1. Introduction

B cell-activating factor belonging to TNF superfamily (*BAFF*, also known as BLys, THANK, or zTNF4) is mainly expressed by innate immune cells, such as monocytes, macrophages, and dendritic cells. Receptors for BAFF, specifically BAFF receptor (BAFF-R), B cell maturation antigen (BCMA), and TNFR homology transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), are mainly expressed on B lymphocytes. Upon ligation, *BAFF* receptors, especially BAFF-R, can induce important effects on the development, survival, and differentiation of B lymphocytes [1]. Abnormality in the *BAFF* signaling is a major predisposition risk factor for systemic lupus erythematosus, Sjogren's syndrome, and rheumatoid arthritis [2].

Human cytomegalovirus (HCMV) is a common pathogen that belongs to the Herpesviridae family. HCMV is the most common virus impacting renal transplant (RTx) recipients, and not only is HCMV closely correlated with complications post-transplantation, but it also reduces the long-term outcome of infected patients, [3,4]. Enhancement of *BAFF* signaling is also tightly correlated with renal allograft antibody-mediated rejection [5].

Our previous studies showed that *BAFF* signaling increased in RTx recipients who were complicated with HCMV infection, meanwhile, the level of anti-HCMV pp65-IgG and total IgG were also significantly increased [6]. Those results suggested that HCMV may induce or enhance the activation of *BAFF* in RTx recipients; however, there have been very few related studies since.

In this present *in vitro* study, we examined the expression profile shifts of *BAFF* and its receptors; moreover, the effects of *BAFF* signaling on B cells in the presence of HCMV infection were investigated.

2. Materials and methods

2.1 Virus and cells

Human cytomegalovirus strain (HCMV AD-169) was purchased from Virus Institute of Wuhan University (Wuhan, China), concentrated, and quantified

according to conventional methods. Human embryonic lung fibroblast (HFL)-1 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), cultured, and passaged according to conventional methods. Excisional fresh tonsil tissues were provided by Third Affiliated Hospital of Soochow University, permitted by Hospital Ethics Committee.

2.2 Purification of CD19⁺ B lymphocytes

Tonsil tissues were sheared and completely ground in the stainless steel filter mesh (200), then rinsed with phosphate buffer saline (PBS). Cell suspensions were centrifuged and precipitation was collected, and washed with PBS 3 times. Then, 1×10^5 cells were stained with phycoerythrin (PE)-Cy5.5-anti-human CD19 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), and detected by flow cytometry (BD FACSCanto II). CD19⁺ B lymphocytes were purified by sorting with positive microbeads (Miltenyi Biotec), according to manufacturer's instructions.

2.3 Preparation and quantification of HCMV AD-169

The brief steps of virus enrichment are as follows: HFL-1 cells were cultured in F12K medium (Gibco, Grand Island, NY, USA) with 10% fetal calf serum (FCS, Gibco) until cells were confluent, then medium was discarded and cells were washed with PBS twice. HCMV AD-169 virus was added to infect HFL-1 cells and cultured for 2 hours at 37°C. Then virus was then discarded, F12K medium with 2% FCS was added, and cells cultured in incubator with 5% CO₂ at 37°C. When cells lesion enlarged up to 80% (within 4–6 days), cells were collected, repeatedly subjected to free-thaw cycles of –80°C and 25°C to induce cell lysis. Cell supernatants were collected and stored at –80°C after centrifugation at 3000 rpm for 10 minutes.

Tissue culture infection dose 50 (TCID₅₀) was detected by Reed-Muench method. $\text{LogTCID}_{50} = \log(\text{dilutions of above 50\% of death}) + \text{half lethal dose (LD}_{50}) \times \text{dilutions}$; $\text{LD}_{50} = \log(\text{above 50\% of death} - 50\%) / (\text{above 50\% of death} - \text{below 50\% of death})$.

2.4 *In vitro* stimulation of B lymphocytes by HCMV

Approximately 5×10^6 B cells/well were cultured in 6-well plates, stimulated by diluted HCMV AD-169 virus with 10 times RPMI 1640 medium and cultured in a

humid 37°C incubator with 5% CO₂. The plates were shaken gently every 15 minutes for two hours. Then, 1 ml RPMI 1640 medium supplemented with 20% FCS and 2% penicillin/Streptomycin (Gibco) was added into the culture system, gently mixed and cultured for 1, 3, 5, 7 days, respectively.

Equal volume of B cells in completed RPMI 1640 medium (with 20% FCS and 2% penicillin-streptomycin (Gibco)) without virus were set as control groups (named medium-only group). Meanwhile, 5 µg/mL CpG ODN 2006 and its negative controls, 2.5 µg/mL CpG ODN 4084F, 50 µg/mL poly(I:C) (Invivogen, France), and 200 ng/mL rhBAFF (Peprotech, USA), were set as controls for HCMV virus. The working concentrations were based on previously published studies.

At the each time-point, cells and supernatants were collected, respectively, for phenotype detection, apoptosis analysis, and IgG titers tests. Each reagent was set in three repeated wells and each experiment was repeated at least three times.

2.5 BAFF stimulation and BAFF-R blockade

To elucidate the effects of *BAFF*/BAFF-R signal transduction on the biological behavior of B cells, *BAFF* stimulation group and BAFF-R blockade groups were also established. 10 µg/mL anti-BAFF-R neutralizing antibody (R&D system, MN, USA) was added into cell culture system, before or after 2 hours of HCMV infection.

After 5 days culture, cells and supernatants were collected, respectively. Experiments were repeated for three times at least. ODN2006 and its negative controls, medium-only, HCMV, and anti-BAFF-R Abs alone treatment groups were set simultaneously.

2.6 Cell phenotypes detection by flow cytometry (FCM)

Purity of B cells, expression of *BAFF* and *BAFF* receptors on B cells were examined by FCM. Appropriate amount of PE-Cy5-anti-CD19 antibody, PE-anti-CD38, fluorescein isothiocyanate (FITC)-anti-IgD, allophycocyanin (APC)-anti-BAFF, anti-BAFF-R, anti-TACI, anti-BCMA antibody (Miltenyi Biotech) were added into each tube with 10⁵ B cells, for 1 hour at 4°C. Then cells were washed with PBS twice, 400 µl sheath liquid were added into cells precipitation and detected by FCM.

2.7 Cells apoptosis rates

PE-Annexin V apoptosis detection kit (BD Pharmingen, Franklin Lakes, NJ, USA) was used to analyze apoptosis rate. Detailed procedures were operated according to manufacturer's instructions. Briefly, cells were washed with cold PBS twice and resuspended with $1\times$ binding buffer to adjust the cell density to $1 \times 10^6/\text{mL}$. Next, 5 μl PE-Annexin V and 5 μl 7-AAD were added into 100 μl cell suspension per tube, mixed gently and incubated for 15 mins at room temperature; 400 μl $1\times$ binding buffer were added into each tube and detected by flow cytometry.

2.8 IgG concentration in cell culture supernatant

IgG levels in cell culture supernatants were examined by Human IgG total Ready-SET-Go Immunosorbent (eBioscience, San Diego, CA, USA) according to manufacturer's instructions.

2.9 Statistical analysis

Data was analyzed using SPSS version 17.0 statistical software package (SPSS, Chicago, IL, USA). One-way ANOVA or Student's *t*-test method was used for analysis. Statistical significance was defined as *P* values less than 0.05. All *P* values were two-sided.

3. Results

3.1 Toxicity of HCMV AD-169

In this present study, logTCID₅₀ of 3rd and 4th generation of HCMV AD-169 was -3.54 and -3.28 , respectively. These two generations of virus were used to infect human B cells *in vitro*.

3.2 Expression of BAFF and its receptors on B cells after HCMV infection

After MACS purification, CD19⁺ B cells (> 98% purity) were used in the subsequent experiments.

Phenotypic detection results suggested that *BAFF* expression was not detected in resting B cells, but was rather upregulated after HCMV stimulation (Fig. 1A). Compared with the medium-only control group, HCMV-infected B cells had high BAFF-R expression which was maintained during the 7-day observation

(93.5%–99.3%, Fig. 1B). However, BAFF-R expression on B cells without HCMV infection gradually downregulated, and was at the lowest level at 5th day (near 81%). Contrastingly, the expression patterns of TACI and BCMA were similar between the HCMV-infected and medium-only control groups, from low level to moderate/high level along with prolonged culture time (Fig. 1C, 1D).

3.3 HCMV infection does not affect apoptosis of B cells

FACS results indicated that with prolonged incubation, the apoptosis rates of B cells were similar between HCMV-infected B cells or medium-only control group (Fig. 2).

3.4 Activation state of B cells during HCMV infection

FACS results suggested that, once infected by HCMV, B cells were activated quickly and differentiated into antibody-secreting plasma cells. Before B cells were added into wells, most B cells are primary B cells (CD19^{hi}CD38^{lo}IgD⁺), while at day 1st over 50% of B cells were activated (CD19^{hi}CD38^{lo}IgD⁺ >50%), and at 3rd about 50% of plasma cells (CD19^{lo/-}CD38^{hi}IgD⁺) could be detected (Table 1).

3.5 IgG secretion ability of B cells after infected by HCMV

As shown in Fig 3, HCMV-infected B cells secreted significantly higher levels of total IgG from 3rd day to 7th day of culture, as compared to medium-only control cells (Fig. 3).

3.6 IgG secretion ability of B cells after infected by CpG ODN and poly (I:C)

HCMV is a type of DNA virus, thus synthetic CpG ODN (Class B) and its negative controls were added as control groups in order to identify the changes of IgG secretion ability of B cells after stimulation by HCMV. Meanwhile, synthetic RNA (poly (I:C)) group and rhBAFF stimulation group were also established.

Results suggested that, compared with medium-only group, ODN 2006 (TLR9 agonist) could significantly increase the IgG secretion ability of B cells from 3rd day, but the addition of ODN 2006 negative control (ODN 2006-C) group had no impact on IgG secretion. Likewise, no significant changes of IgG concentration in culture supernatant was observed in ODN 4084-F (TLR9 antagonist) or poly (I:C)-treated

group. However, rhBAFF could significantly increase the ability of IgG secretion by B cells and the effects were greatly enhanced over time (Fig. 4).

3.7 Effects of BAFF/BAFF-R signaling blockade on apoptosis rate of B cells infected by HCMV

Pre-treatment with neutralizing Abs to BAFF-R could significantly upregulate the apoptosis rate of B cells, compared with groups pretreated with HCMV then neutralizing Abs to BAFF-R (<anti-BAFF-R first + HCMV> vs <HCMV first + anti-BAFF-R>: 34.43 ± 5.06 vs 25.70 ± 0.06 , $P < 0.01$). Additionally, similar results were obtained from B cells pretreated with neutralizing Abs to BAFF-R then ODN2006 treatment, compared with ODN2006-treated first then neutralizing Abs-treated group (<anti-BAFF-R first + CpG ODN2006> vs <CpG ODN2006 first + anti-BAFF-R>: 24.53 ± 5.06 vs 14.63 ± 6.11 , $P < 0.01$).

Compared with anti-BAFF-R Abs treatment alone, the apoptosis rate of B cells which were pretreated with ODN2006 then neutralizing Abs to BAFF-R afterwards significantly decreased ($P < 0.05$). However, the apoptosis rate of B cells that were first treated with anti-BAFF-R Abs then with HCMV afterwards significantly increased, ($P < 0.05$).

3.8 Effects of BAFF/BAFF-R signaling blockade on IgG secretion of B cells infected by HCMV

ELISA results showed that, the sequence of treatment with neutralizing Abs to BAFF-R and HCMV infection did not significantly affect the IgG secretion ability of B cells. However, IgG levels significantly increased in HCMV infection groups regardless of BAFF-R blockade compared with medium-only group. On the other hand, pretreatment with neutralizing Abs to BAFF-R then with ODN2006 treatment could significantly downregulate IgG secretion by B cells, compared with ODN2006 first then neutralizing antibodies or ODN treatment alone group.

4. Discussion

Previous studies have confirmed that active HCMV infection induces immune dysfunction in hosts, [7,8]. Various autoantibodies have long been discovered in

systemic HCMV infection patients, and autoimmune-like immune disorder is the most common phenomenon, which suggests that polyclonal B cells are activated in HCMV hosts, [9,10].

B lymphocytes activation is common in organ transplant recipients who are complicated with HCMV infection [11]. In RTx recipients with HCMV infection, the incidence of complications can increase even in the absence of clinical manifestation. Moreover, HCMV infection is often associated with ill effects, which can severely impact the long-term outcome of renal allograft and recipients [12]. Thus, B cell activation and its immune injury mechanism induced by HCMV infection remains an attractive research area of focus.

In this present study, we also observed that B cells were activated quickly once infected by HCMV, which were consistent with previous studies. And we explored the association of BAFF/BAFF-R signaling and HCMV infection on B cells *in vitro*. Results first showed that expression of BAFF-R progressively increases on B cells when infected by HCMV. Furthermore, enhancement of BAFF/BAFF-R signaling transduction prolonged the survival time of B cells when HCMV infection was present, which is consistent with the biological function of *BAFF* code.

There is evidence that interactions between some TLRs with virus influence both the immune response and outcome of HCMV infection [13]. HCMV activates host cells *via* multiple TLRs, predominantly those that reside on cell surface or in endosomes. TLR9, localized intracellularly within endolysosomes, recognizes unmethylated CpG dinucleotide motifs located in viral DNA and plays a central role in host defense against viral infection, [14,15,16]. To explore the effect of HCMV/TLR9 signal transduction on B cells, a TLR9 agonist and antagonist were applied. Our results suggested that HCMV/TLR9 pathway transduced signals to influence the biological function of B cells.

Abu-Rish *et al.* reported that TLR9 activation induces expression of membrane-bound *BAFF* on human B cells and leads to increased proliferation in response to both soluble and membrane-bound *BAFF* [17]. In addition, Szili *et al.* found that, during NF- κ B activation induced by CpG ODN, TLR9 –mediated signal

pathway synergized with *BAFF* signal pathway, followed by enhancement of NF- κ B activation, and consequently, B cells proliferated, and cytokine and IgG secretion ability increased [18]. In this present study, similar results were also observed in TLR9 agonist treatment group, as BAFF-R expression on B cells progressively increased, apoptosis rates of B cells significantly decreased and IgG secretion ability significantly increased. However, in addition to the same expression profile of BAFF-R and proliferation ability, increased IgG secretion capacity was not observed in the pretreatment of neutralizing Abs to BAFF-R group. Nonetheless we observed that *BAFF*/BAFF-R signal had some crosstalk with HCMV/TLR9, and the enhancement of *BAFF*/BAFF-R signal could protect B cells from apoptosis followed by HCMV infection. Furthermore, the results also indicated that, the stimulation ability of HCMV DNA to TLR9 is weaker than TLR9 agonist. Detailed analysis of HCMV structure could very well explain the differences.

In the present study, the other two receptors of *BAFF*, TACI and BCMA, were examined at the same time. Very low level of expression and no significant changes were observed. Thus we conferred that expression of these two receptors were not affected by direct or indirect HCMV infection, including treatment with TLR9 agonist and antagonist (data not shown). Our results were not consistent with the results of Katsenelson's, who had found that CpG ODN could up-regulate expression of TACI on B cells *via* TLR9, which contribute to Ig secretion by B cells [19]. We posit that the different sources of B cells in these two studies may explain the contradictory results.

Macrophages can maintain appropriate antigen presentation skills after infection by HCMV, and enhanced inflammatory response [20]. HFL-1 cells are non-specific antigen presentation cells, and our data suggested that both the apoptosis level and IgG secretion ability of B cells co-cultured with HFL-1, which had been infected by HCMV, were affected, but no statistical discrepancy was observed (data not shown). Whether HFL-1 can transform into APCs after infection by HCMV is worth studying.

Poly(I:C), structurally similar to double-stranded RNA, is a natural stimulant of TLR3, which is expressed in the membrane of B cells, macrophages, and dendritic

cells [21]. In the poly(I:C)-treated group, BAFF-R expression on B cells was significantly downregulated and apoptosis rates of B cells was significantly upregulated, while IgG levels did not obviously vary. Thus, although double-stranded RNA would be produced during HCMV replication, the effect on IgG secretion capability cannot be ignored.

5. Conclusion

BAFF/BAFF-R signaling crosstalk with HCMV/TLR9 signaling promotes the IgG secretion ability of B lymphocytes; meanwhile, enhancement of *BAFF*/BAFF-R signaling decreases apoptosis rate and promotes survival of B lymphocytes upon HCMV stimulation.

Conflict of interest

All the authors declare that no competing interests exist.

Funding:

This work was supported by the National Natural Science Foundation of China (grant numbers 81273267) and the National Science Foundation of Jiangsu Province (grant numbers BK2011248).

Author contribution

XHY and HXZ designed and coordinated study; DPP, MXY, and SD performed experiments; DPP and MXY analyzed data; XHY, DPP, MXY, XD, XRF, LH, and HXZ contributed to the writing and editing of this manuscript; XHY and DPP wrote the manuscript. All authors read and approved the final manuscript.

References

- [1] F. Mackay, P. Schneider, Cracking the BAFF code, *Nat. Rev. Immunol.* 9 (2009) 491-502.
- [2] F. B. Vincent, E. F. Morand, P. Schneider, F. Mackay, The BAFF/APRIL system in SLE pathogenesis, *Nat. Rev. Rheumatol.* 10 (2014) 365-373.
- [3] C. Puttini, M. Carmellini, G. Garosi, B. Rossetti, M. L. Riccio, G. Tordini, et al., HCMV infection in renal transplant recipients: a retrospective cohort study, *New. Microbiol.* 36 (2013) 363-371.
- [4] C. Martin-Gandul, N. J. Mueller, M. Pascual, O. Manuel, The Impact of Infection on Chronic Allograft Dysfunction and Allograft Survival After Solid Organ Transplantation, *Am. J. Transplant.* 15 (2015) 3024-3040.
- [5] M. Chhabra, T. M. Conlon, K. Saeb-Parsy, G. J. Pettigrew, BAFF and associated TNF superfamily members in renal transplantation: an end to BLySful ignorance, *Transplantation.* 96 (2013) 853-859.
- [6] H. Xu, P. Dong, X. He, X. Ma, D. Xue, Y. Zhang, et al., B-cell-activating factor code and human cytomegalovirus infection in renal transplant recipients, *Microbiol. Immunol.* 58 (2014) 439-448.
- [7] I. J. ten Berge, R. A. van Lier, The interaction between cytomegalovirus and the human immune system, *Immunol. Lett.* 162 (2014) 141-144.
- [8] S. E. Jackson, G. M. Mason, M. R. Wills, Human cytomegalovirus immunity and immune evasion, *Virus. Res.* 157 (2011) 151-160.
- [9] P. Price, S. D. Olver, A. E. Gibbons, G. R. Shellam, B-cell activation following murine cytomegalovirus infection: implications for autoimmunity, *Immunology.* 78 (1993) 14-21.
- [10] L. M. Hutt-Fletcher, N. Balachandran, M. H. Elkins, B cell activation by cytomegalovirus, *J. Exp. Med.* 158 (1983) 2171-2176.
- [11] F. Bruno, C. Fornara, P. Zelini, M. Furione, E. Carrara, L. Scaramuzzi, et al., Follicular helper T-cells and virus-specific antibody response in primary and reactivated human cytomegalovirus infections of the immunocompetent and immunocompromised transplant patients, *J. Gen. Virol.* 97 (2016) 1928-1941.
- [12] Y. V. Smedbraten, S. Sagedal, T. Leivestad, G. Mjoen, K. Osnes, H. Rollag, et al., The impact of early cytomegalovirus infection after kidney transplantation on long-term graft and patient survival, *Clin. Transplant.* 28 (2014) 120-126.
- [13] K. H. Yew, B. Carsten, C. Harrison, Scavenger receptor A1 is required for sensing HCMV by endosomal TLR-3/-9 in monocytic THP-1 cells, *Mol. Immunol.* 47 (2010) 883-893.
- [14] E. Latz, J. Franko, D. T. Golenbock, J. R. Schreiber, Haemophilus influenzae type b-outer membrane protein complex glycoconjugate vaccine induces cytokine production by engaging human toll-like receptor 2 (TLR2) and requires the presence of TLR2 for optimal immunogenicity, *J. Immunol.* 172 (2004) 2431-2438.
- [15] S. A. Vaidya, G. Cheng, Toll-like receptors and innate antiviral responses, *Curr. Opin. Immunol.* 15 (2003) 402-407.
- [16] A. C. Iversen, B. Steinkjer, N. Nilsen, J. Bohnhorst, S. H. Moen, R. Vik, et al., A proviral role for CpG in cytomegalovirus infection, *J. Immunol.* 182 (2009) 5672-5681.
- [17] E. Y. Abu-Rish, Y. Amrani, M. J. Browning, Toll-like receptor 9 activation induces expression of membrane-bound B-cell activating factor (BAFF) on human B cells and leads to increased proliferation in response to both soluble and membrane-bound BAFF, *Rheumatology (Oxford).* 52 (2013) 1190-1201.
- [18] D. Szili, Z. Banko, E. A. Toth, G. Nagy, B. Rojkovich, T. Gati, et al., TGFbeta activated kinase 1

(TAK1) at the crossroad of B cell receptor and Toll-like receptor 9 signaling pathways in human B cells, PLoS One. 9 (2014) e96381.

[19] N. Katsenelson, S. Kanswal, M. Puig, H. Mostowski, D. Verthelyi, M. Akkoyunlu, Synthetic CpG oligodeoxynucleotides augment BAFF- and APRIL-mediated immunoglobulin secretion, Eur. J. Immunol. 37 (2007) 1785-1795.

[20] C. Bayer, S. Varani, L. Wang, P. Walther, S. Zhou, S. Straschewski, et al., Human cytomegalovirus infection of M1 and M2 macrophages triggers inflammation and autologous T-cell proliferation, J. Virol. 87 (2013) 67-79.

[21] S. Chattopadhyay, G. C. Sen, dsRNA-activation of TLR3 and RLR signaling: gene induction-dependent and independent effects, J. Interferon. Cytokine. Res. 34 (2014) 427-436.

Figure Legends

Figure1. BAFF and its receptors expression on B cells infected by HCMV

Expression of BAFF (A) and its receptors, BAFF-R (B), TACI (C), and BCMA (D), on B cells stimulated by HCMV at 0, 1st, 3rd, 5th, 7th day, compared with medium-only group.

BAFF expression significantly increased since 3rd day, and compared with medium-only group (at 3rd day, *, $P < 0.05$; at 5th and 7th day, **, $P < 0.01$). BAFF-R expression gradually decreased in medium-only, especially in 5th day to reach the trough, while slightly increased from 3rd day in HCMV pulsed group. And at 5th day BAFF-R expression in HCMV stimulation group significantly higher than that in medium-only group ($P < 0.05$). The expression of TACI and BCMA on B cells did not show significant different in HCMV stimulation group and medium-only group.

Figure2. Effect of HCMV infection on apoptosis of B cells

No significant effect of HCMV infection on apoptosis of B cells at 1st, 3rd, 5th, 7th day with or without HCMV infection.

Figure3. HCMV promotes IgG secretion ability of B cells *in vitro*

When B cells were stimulated by HCMV *in vitro*, IgG secretion ability of B cells is significantly increased, and IgG levels in supernatant is significantly elevated and sustained from the 3rd day to 7th day, compared with medium-only group (***: $P < 0.001$).

Figure4. TLR9 and BAFF stimulation promotes secretion of IgG levels *in vitro*

TLR9 agonists (ODN 2006) and antagonist (4084-F), poly(I:C) and rhBAFF were used to stimulate B cells for 7 days, IgG levels in supernatants were detected, and medium-only used as controls. IgG levels were significantly up-regulated in TLR9 agonist (CpG ODN 2006)-treated group and rhBAFF-treated group from 3rd day, while IgG levels remain unchanged. As to ODN2006-treated group, IgG level at 3rd day, 5th day and 7th day significantly increased and sustained compared with medium-only group (***, $P < 0.001$). In the rhBAFF-treated group, IgG levels increase from 3rd to 7th day, with day 7 showing significant increase (***, $P < 0.001$).

Figure5. The effect of sequence of treatment with neutralizing Abs to BAFF-R and HCMV infection on apoptosis rate of B cells

Pretreatment with neutralizing Abs to BAFF-R for two hours, followed by HCMV infection (anti-BAFF-R first +HCMV) significantly increases the apoptosis rate of B cells, compared with the group treated HCMV first + anti-BAFF-R second and medium-only groups (*, $P < 0.05$). Similar effects were observed when HCMV were replaced by ODN 2006 (*, $P < 0.05$).

Neutralizing Abs treated alone did not induce the apoptosis of B cells, and the apoptosis rate significantly increased when B cells were infected with HCMV (*, $P < 0.05$). The apoptosis rate of B cells significantly decreased with pre-treatment with ODN2006 for two hours was followed with neutralizing Abs, compared with neutralizing Abs treatment alone with or without ODN2006 treatment (*, $P < 0.05$).

Figure6. The effect of sequence of treatment with neutralizing Abs to BAFF-R and HCMV infection on IgG production of B cells

Pretreatment with neutralizing Abs to BAFF-R for two hours, then infected with HCMV, did not impact the IgG production ability of B cells, compared with the group treated by the contrary order ($P > 0.05$). However, IgG levels significantly increased in HCMV-treated group no matter whether neutralizing Abs to BAFF-R was present or not, compared with medium-only group (*, $P < 0.05$; **, $P < 0.01$).

Pretreatment with neutralizing Abs to BAFF-R for two hours then treatment with ODN2006, significantly downregulates the IgG secretion ability of B cells, compared with the group treated by the contrary order, or ODN2006-treated alone group (**, $P < 0.01$; ***, $P < 0.001$).

IgG secretion ability of B cells significantly increased in ODN2006-treated group with or without neutralizing Abs to BAFF-R, compared with B cells in neutralizing Abs-treated alone group (***, $P < 0.001$).

Table1. Activation state of B cells when HCMV infection

	Primary B cells (CD19 ^{hi} CD38 ^{hi} IgD ⁺)	Activated B cells (CD19 ^{hi} CD38 ^{lo} IgD ⁻)	Plasma cells (CD19 ^{lo/-} CD38 ^{hi} IgD ⁻)
0 day	87.21±12.30	9.49±8.59	1.94±3.81
1st day	23.58±18.32*	58.76±21.6*	12.19±14.23*
3rd day	16.89±9.26*	52.59±10.48*	46.32±6.96*
5th day	7.95±13.47*	42.36±11.63*	59.43±12.43*
7th day	—	32.15±15.32*	52.15±15.3*

Notes: Data were presented with means±SD according to mean fluorescence intensity (MFI)(%).
 $P < 0.05$ was considered to be significant. * represented there was significant difference compared with data at 0 day.