Assessment of the effect of TLR7/8, TLR9 agonists and CD40 ligand on the transformation efficiency of Epstein-Barr virus in human B lymphocytes by limiting dilution assay

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Abstract Infection of human B cells with Epstein-Barr virus (EBV) induces polyclonal activation in almost all infected cells, but a small proportion of infected cells are transformed to immortalized lymphoblastoid cell lines. Since B cells are activated also by CD40 ligand (CD40L) and Toll-like receptor (TLR) agonists via a similar signaling pathway, it is likely that costimulation through these molecules could result in synergistic enhancement of the transformation efficiency of EBV. In this study, the stimulatory effect of TLR7/8 (R848), TLR9 (CpG) agonists and/or CD40L on transformation efficiency of EBV in normal human B cells was assessed using the limiting dilution assay. Costimulation of peripheral blood mononuclear cells (PBMCs) with CpG and R848, but not CD40L, increased significantly the frequency of EBV transformed B cells ($p < 0.001$). Neither synergistic nor additive effects were observed

between TLR agonists and CD40L and also TLR7/8 and TLR9 agonists. Costimulation with R848, CpG and CD40L enhanced the proliferative response of B cells infected with EBV. This effect was more evident when enriched B cells were employed, compared to PBMCs. The promoting effect of TLR agonists stimulation, implies that EBV may take advantage of the genes induced by the TLR stimulation pathway for viral latency and oncogenesis.

Keywords Toll-like receptor · Epstein-Barr virus · CD40 ligand · Transformation · B lymphocyte · Proliferation · Limiting dilution assay

Introduction

Epstein-Barr virus (EBV) is a member of B lymphotropic gammaherpes virus family which infects preferentially human B lymphocytes and epithelial cells (Young and Rickinson 2004). At least 90 % of the human population worldwide is infected with EBV (Middeldorp et al. 2003). EBV infection which is mediated by binding to class II major histocompatibility complex (MHC) and CD21 molecules is responsible for infectious mononucleosis and seems to be implicated in the etiology of a number of human lymphoid and epithelial malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, Sézary disease as well as non-malignant disorders such as systemic lupus erythematosus (SLE)

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(Middeldorp et al. 2003; Young and Rickinson 2004; Quan et al. 2010). In vitro EBV infection results in polyclonal B cell activation which leads to unlimited proliferation and transformation of B cells into immortalized lymphoblastoid cell lines (LCLs), a condition classified as type III latency infection (Young and Rickinson 2004).

Among 100 identified open reading frames (ORF) of EBV genome only nine proteins including six nuclear antigens (EBNA 1,2,3A,3B,3C and –LP) and three latent membrane proteins (LMP-1,2A and 2B) and two non-coding RNA (EBER1, 2) are expressed in LCLs (Middeldorp et al. 2003; Young and Rickinson 2004). Expression of LMP-1 and LMP-2 induces morphological and molecular changes which mimic those induced by B cell receptor (BCR) and CD40 signaling pathways (Kuppers 2003; Mancao and Hammerschmidt 2007; Rastelli et al. 2008).

Similar to LMP-1, Toll-like receptor (TLR) signaling, which has been proposed as the third signal for B cell activation, could influence a wide range of immunological functions of B cells such as up-regulation of activation markers, proliferation, cytokine secretion, class switch recombination, terminal differentiation and antibody production (Hartmann and Krieg 2000; Bekeredjian-Ding and Jegou 2009).

While in vitro exposure of B cells to EBV results in a high infection rate, a small proportion of infected cells becomes finally transformed (about 1–3 %) (Shokrgozar and Shokri 2001; Traggiai et al. 2004). Several protocols have been developed to improve the efficiency of EBV transformation, including the use of γ irradiated fibroblast feeder cells or culture medium supplemented with human plasma to boost the proliferation of transformed B cells (Pelloquin et al. 1986; Manor 2008). Preparation of high titer EBV stock (Oh et al. 2003) and employment of a variety of mitogens such as phytohemagglutinin (Henderson et al. 1977), pokeweed mitogen (Bird et al. 1981), and lipopolysaccharide (Henderson et al. 1977) concomitant to EBV infection have promoting effects on B cell transformation.

In a recent study CD19 and BCR co-ligation has been shown to promote EBV transformation and establishment of LCLs (Hur et al. 2005). However, little is known about the synergistic effects of CD40-CD40 ligand (CD40L) interaction and TLR engagement on the efficiency of transformation. CD40 ligation of B cells infected with EBV leads to promotion of viral latency by control of EBV

reactivation through downregulation of BZLF-1 which is considered as the major viral activator of lytic EBV replication (Adler et al. 2002). Moreover, it was shown that EBV infection can induce CD40L expression in B cells which is considered critical for transformation and survival of infected cells (Imadome et al. 2003). However, controversial findings have been reported indicating that CD40 ligation can restrain the growth of EBV-transformed LCLs by switching the viral transcription program from the full lymphoblastoid to a more limited latency program (Pokrovskaja et al. 2002). Full activation of B cells infected with EBV also can be triggered endogenously by direct recognition of EBV related pathogen associated molecular pattern (PAMP) via TLR2 (Gaudreault et al. 2007), TLR7 (Martin et al. 2007) and TLR9 (Guggemoos et al. 2008; Gargano et al. 2009), under physiological conditions, or exogenously by other microbial components at the sites of EBV infection (Stenfors and Raisanen 1993; Iskra et al. 2010). Recently, it has been shown that triggering TLR9 and probably other components of the innate immune system inhibits switch of latent EBV infection to lytic replication and promotes EBV-driven B cell transformation and tumor development (Ladell et al. 2007). This supports the observation that TLR9 agonists (unmethylated CpG containing oligonucleotides) can enhance synergistically the efficiency of transformation of peripheral memory B cells by EBV (Traggiai et al. 2004). The promoting effects of the other TLR agonists, especially TLR7/8 and CD40L on B cell transformation have not been studied extensively. In this study, the potential promoting effects of TLR9 and TLR7/8 agonists and CD40 ligation, alone or in combination, on EBV-driven B cell proliferation and transformation was investigated based on limiting dilution assay (LDA).

Materials and methods

Mononuclear cells preparation

Heparinized peripheral blood was obtained from seven healthy adult volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque (Sigma, St. Louis, MO, USA) density-gradient centrifugation. Isolated PBMCs were washed twice with RPMI-1640 culture medium (Gibco, Grand Island, NY, USA) and resuspended in the same medium

supplemented with 10 % fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 IU) and streptomycin (100 µg/ml) (Biosera, Ringmer, East Sussex, UK).

Enrichment of normal B cells by nanomagnetic beads

B cells were isolated from PBMCs of the same volunteers using magnetic-activated cell sorting (MACS) negative selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described (Kazemi et al. 2008). Enrichment was assessed by flow cytometry (before enrichment: 8–15 %, after enrichment: >90 %) and cell viability was more than 98 % based on trypan blue dye exclusion (data not presented).

EBV stock preparation

EBV stock was prepared as described previously (Shokri et al. 1991). Briefly, cell free supernatant from 3 weeks old culture of B95.8 cell line (NCBI C-110, National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran) was filtered by 0.45 µm filter. The collected filtrate was centrifuged in a Beckman ultracentrifuge (L8-80 M; Beckmann, Palo Alto, CA, USA) at 30,000×g for 2 h at 4 °C. The resulting pellets were resuspended in a small volume of complete culture medium to yield 100-fold concentrated viral load as compared to primary B95.8 supernatant. Concentrated EBV stock was aliquoted into 0.1 ml volumes and stored in cryovials at −70 °C. Aliquots stored in −70 °C from the same concentrated stock were employed throughout the study in all experiments to minimize inter-assay variations. At the time of experiment each aliquot was diluted 10 times (1 ml) with complete culture medium prior to incubation with 1×10^6 PBMCs or enriched B cells.

EBV transformation and limiting dilution of B cells infected with EBV

Isolated cells were stimulated with 5 µg/ml of TLR9 (CpG 2006, 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3', Operon Technologies, Cologne, Germany), 1.25 µg/ml of TLR7/8 (R848, InvivoGen, San Diego, CA, USA) agonists or cocultured with CD40L-transfected inactivated mouse fibroblast cells (National Cell Bank of Iran), concomitant with EBV infection.

The CD40L-transfected cell line employed in this study displayed a wide range of cell populations with different expression levels of CD40L as assessed by flow cytometry. We cloned this cell line and tested a number of clones for CD40L expression and subsequently a clone with the highest expression level (90 %) was selected for our experiments. After 3 h of costimulation at 37 °C with periodic agitation, the cells were collected by gently pipetting up and down, without dissociating CD40L-transfected inactivated cells, washed with RPMI-1640 and resuspended in complete culture medium. Treated cells were seeded subsequently into 60 wells of a 96 -well microtiter plate at three different densities on human fetal foreskin fibroblasts (HFFF-PI6) (NCBI C-170; National Cell Bank of Iran). The fibroblast cells were inactivated with γ-irradiation (6,000 rad) and plated at 15,000 cells per well in 96-well microtiter plate overnight before distribution of cells infected with EBV. Culture medium contained 1 µg/ml of cyclosporin A to prevent the regression of immortalized EBV-infected cells by EBV-specific memory T lymphocytes in seropositive individuals. Following 3 weeks, the frequency of transformation was evaluated based on the number of wells containing growing transformed B cells. Using the standard EBV transformation protocol in normal peripheral PBMCs, we and others (Chang et al. 2006) have already shown that transformed B cells are established within 3–5 weeks after EBV infection. In the present study we employed an improved transformation protocol using irradiated fetal fibroblast feeder cells and tenfold concentrated EBV stock which accelerates the transformation process (data not presented). Nevertheless, we checked a number of the plates 5 weeks after EBV infection to evaluate the rate of transformation, but no difference was observed as compared to the results obtained after 3 weeks infection.

Determination of the efficiency of EBV transformation in PBMCs and enriched B cells

This determination had been performed previously in our laboratory for studies carried out to estimate the frequency of B cells specific for hepatitis B surface antigen (HBsAg) or RhD antigen in immunized subjects (Shokrgozar and Shokri 2001; Pasha et al. 2004). PBMCs and enriched B cells were infected with EBV (as described above) and seeded separately at

250, 500 and 1,000 (for PBMCs) or 30, 60 and 120 (for enriched B cells) cells/well into 96-well microtiter plates. These cell densities were selected based on a pilot study on a number of normal individuals to enable analysis of EBV transformation frequency. Each cell density was distributed into 20 wells. The frequency of transformed B cells in PBMCs was evaluated based on the Poisson statistical analysis taking into consideration the percentage of B cells in PBMCs which was determined by flow cytometry using FITC-conjugated anti-CD19 monoclonal antibody (eBioscience, San Diego, CA, USA). The emergence of LCLs was evaluated following the first week and the actual number of wells that were positive and negative for the presence of growing LCLs in each plate was determined microscopically starting from the third week of infection. The data were transferred to a semilogarithmic graph on which the *x*- and *y*-axes represent dilution of cells and the negative fraction, respectively. A best-fit line was constructed based on the experimental data for each donor. This analysis shows that at a cell dilution where 37 % of wells are negative for growing LCLs (negative fraction = 37 %) there is, on average, one precursor B cell in each well (Yarchoan et al. 1983). Afterward, the efficiency of EBV transformation in PBMCs was estimated based on the Poisson statistical analysis formula by determination of negative fraction and percentage of B cells in the PBMCs population as described previously (Yarchoan et al. 1983; Pasha et al. 2004).

Cell proliferation assay

A total of 1.5×10^5 PBMCs or 5×10^4 enriched B cells were resuspended in 200 μ l of complete culture medium and incubated in 96-well microtiter plates at 37 °C in a humidified 5 % CO₂ incubator. PBMCs and B cells were stimulated with CpG-2006 at 5 μ g/ml or R848 at 1.25 μ g/ml in presence or absence of concentrated EBV stock. For CD40L stimulation the same number of cells was cocultured with γ -irradiated CD40L-transfected in 200 μ l of complete culture medium as described above. After 72 h of incubation, cells were pulsed with 1 μ Ci of [³H]-thymidine (Amersham, Aylesbury, UK) and harvested 18 h later onto glass-fiber filters. [³H]-thymidine incorporation was measured by β -scintillation counter as previously described (Shokri et al. 1991). Stimulation index (SI) is defined as counts per minute (c.p.m) of radiation in

presence of stimuli divided by c.p.m of radiation in absence of stimuli.

Statistical analysis

The frequency of transformed B cells (efficiency of EBV transformation) was calculated based on LDA and Poisson analysis (Pasha et al. 2004). The differences between frequencies of EBV transformation in presence or absence of TLR agonists or CD40L were determined using descriptive Chi Square test. Differences of stimulation index were determined using two-independent-samples test. P values of less than 0.05 were considered significant. Statistical analysis was performed using the SPSS package for Windows (SPSS Inc, Chicago, IL, USA).

Results

Influence of TLR7/8, TLR9 agonists and CD40L on efficiency of EBV transformation in PBMCs

Peripheral blood mononuclear cells (PBMCs) of seven adult donors were stimulated with CpG, R848 and CD40L-transfected cells, concomitant to EBV infection and seeded at 250, 500 and 1,000 cells/well densities into 60 wells of a 96-well microtiter plate on γ -irradiated feeder cells. Following 3 weeks of stimulation, the frequency of transformation was evaluated by enumeration of wells containing proliferating transformed LCLs. Culture of PBMCs alone did not result in appearance of growing LCLs following 3 weeks of culture. In absence of EBV, the proliferative response of PBMCs to CpG or R848 stopped within 1–2 weeks and there were no growing cells at day 21 of culture (data not shown). Costimulation with CpG or R848 resulted in the highest frequency of transformation compared to EBV alone ($p < 0.001$, Fig. 1). Costimulation of PBMCs infected with EBV by a combination of CpG and R848, neither induced additive effects nor significantly enhances the transformation efficiency of EBV. No significant differences were observed following CD40L costimulation. However, costimulation of PBMCs infected with EBV by a combination of CD40L and TLR agonists, either R848 or CpG, enhanced the transformation efficiency of EBV, but to a lesser extent than TLR agonists alone ($p < 0.05$, Fig. 1).

Fig. 1 Promoting effects of costimulation with TLR agonists and CD40L stimulation on efficiency of EBV transformation in PBMCs. PBMCs were isolated from healthy adults, infected with EBV and costimulated with CpG, R848 and/or CD40L. Treated cells were then seeded at 250, 500 and 1,000 cells/well into 96-well culture plates over inactivated feeder cells in the presence of cyclosporine A. Following 3 weeks after infection, the percentage of wells containing growing LCLs was determined. Vertical and horizontal bars represent SEM and mean of percentage of positive wells, respectively; * $p < 0.05$ and ** $p < 0.001$

Influence of TLR7/8, TLR9 agonists and CD40L on efficiency of EBV transformation in enriched B cells

To clarify the direct or indirect stimulatory effect of TLR agonists and CD40L on EBV transformation, our study was extended using enriched B cells from PBMCs from five individual subjects. B cells infected with EBV were costimulated with CpG, R848 or CD40L. R848 and CpG costimulation induced a higher transformation efficiency in enriched B cells compared to PBMCs ($p < 0.001$, Fig. 2). In absence of EBV, the proliferative response of B cells to TLR agonists stopped within 1–2 weeks and there were no growing cells at day 21 of culture (data not shown). Costimulation of B cells infected with EBV by CD40L also significantly enhanced the efficiency of B cell transformation by EBV ($p < 0.05$, Fig. 2).

Frequency analysis of EBV transformed cells following costimulation with TLR agonists and/or CD40L

The frequency of transformed lymphoblastoid cells was determined by limiting dilution assay based on

Fig. 2 Promoting effects of TLR agonists and CD40L stimulation on the efficiency of EBV transformation in B cells. Enriched B cells isolated from PBMCs of five healthy adults were infected with EBV and costimulated with CpG, R848 or CD40L. The cells were seeded subsequently at 30, 60 and 120 cells/well into 96-well culture plates over irradiated feeder cells. Following 3 weeks incubation, the percentage of wells containing growing transformed LCLs was determined. Vertical and horizontal bars represent SEM and mean of percentage of positive wells, respectively; * $p < 0.05$ and ** $p < 0.001$

Poisson statistical analysis as explained in the materials and methods. Infection of PBMCs with EBV alone gave a transformation frequency of 0.93 % (0.5–1.8 %) (Fig. 3). Costimulation with R848 and CpG enhanced the transformation efficiency of EBV by a mean of 2.5-fold (2.4 %) and 1.7-fold (1.6 %), respectively (Fig. 3). While the transformation efficiency of EBV in enriched B cells was comparable to PBMCs (0.9 %) costimulation with TLR agonists or CD40L enhanced the frequency of transformation leading to a mean of threefold increase for CpG (2.7 %), 6.2-fold increase for R848 (5.5 %) and 1.8-fold increase for CD40L (1.6 %) (Fig. 4).

Assessment of the effects of TLR agonists and CD40L on proliferation of B cells infected with EBV

In a complementary set of experiments the effect of costimulation with TLR agonists or CD40L on proliferation of PBMCs infected with EBV was assessed. As shown in Fig. 5, while CpG and R848 induced significant proliferation in both PBMCs

Fig. 3 Limiting dilution assay for determination of the frequency of transformed lymphoblastoid cells following infection of PBMCs with EBV. PBMCs infected with EBV were costimulated with TLR agonists and/or CD40L then seeded at 250, 500 and 1,000 cells/well over irradiated feeder cells. After 21 days of culture the number of wells that were positive or negative for the presence of growing LCLs was

enumerated and the efficiency of transformation was determined based on the Poisson statistical analysis, taking into consideration the percent of B cells (CD19⁺ cells) in PBMCs of all samples tested. Each line is constructed using the mean data points obtained from the three cell densities for all individuals tested

Fig. 4 Limiting dilution assay for determination of the frequency of transformed lymphoblastoid cells following infection of enriched B cells with EBV. Enriched B cells collected from five healthy subjects were infected with EBV and costimulated with TLR agonists or CD40L and seeded at three different cell densities (120, 60 and 30 B cells/well). The mean data points obtained for the three cell densities of B cells infected with EBV in presence or absence of TLR agonists or CD40L are depicted on each line

($p = 0.002$, $p = 0.001$, respectively) and enriched B cells ($p = 0.008$, $p = 0.01$, respectively), EBV alone did not induce a substantial proliferative response. Stimulation through CD40L induced a significant proliferation in enriched B cells ($p = 0.008$), but not in PBMCs ($p = 0.30$). Costimulation with CpG or

R848 boosted the proliferative response of PBMCs infected with EBV by a mean of twofold ($p = 0.2$) and B cells infected with EBV by a mean of 2.4- and 2.7-fold, respectively ($p = 0.07$), but this increase did not reach statistical significance most likely due to variation within samples and the small sample size.

Fig. 5 Influence of costimulation with TLR agonists and CD40L on proliferative response of PBMCs or enriched B cells infected with EBV. A total of 1.5×10^5 PBMCs or 5×10^4 enriched B cells were stimulated with R848, CpG or CD40L in the presence or absence of EBV. Following 72 h of incubation, cells were pulsed with [3 H]-thymidine and harvested after 18 h. The results represent mean and standard deviation of stimulation index values obtained from five healthy subjects for each stimulant

Costimulation with CD40L enhanced the stimulation index of the B cells and PBMCs infected with EBV, but the difference was statistically significant only for B cells ($p = 0.01$).

Discussion

Interplay between microbes and innate immune system, in particular through TLR, play a decisive role in the fate of infection. Primary EBV infection occurs predominantly in infants and toddlers and is usually asymptomatic (Young and Rickinson 2004). In adults, however, it may cause infectious mononucleosis (IM), which is characterized by massive lymphocyte proliferation, fever and organomegaly in lymph nodes, tonsils, liver and spleen (Middeldorp et al. 2003). In vitro infection with EBV leads to activation and transformation of human B cells into LCLs (Young and Rickinson 2004). Like other herpesviruses, the outcome of EBV infection is predisposed by the innate immune responses (Mossman and Ashkar 2005).

Lytic replication of EBV is blocked if nuclear factor kappa B (NF- κ B), the master transcription factor related to innate immune response genes, over-expresses in cells infected with EBV (Brown et al. 2003). Recently, it has been shown that lytic

replication of EBV is inhibited following triggering of TLR9 signaling in infected cells (Ladell et al. 2007). Similar to TLR, stimulation of B cells via CD40L could affect the infection and transformation potential of EBV (Adler et al. 2002; Pokrovskaja et al. 2002; Imadome et al. 2003).

In this study, the effect of TLR agonists and CD40L costimulations on the transformation efficiency of EBV was investigated. Addition of TLR7/8 (R848) or TLR9 (CpG) agonists at the time of EBV infection improves significantly the efficiency of transformation in PBMCs and enriched B cells infected with EBV ($p < 0.001$) (Figs. 1 and 2) leading to threefold increase for CpG and 6.2-fold increase for R848 of clonal outgrowth of B cells infected with EBV based on limiting dilution assay (Fig. 4). This promoting effect was more evident, particularly after CD40L costimulation, when enriched B cells were used instead of PBMCs which implies induction of other immune effector cells within PBMCs by TLR agonists and CD40L. Potential activation of these cells, including monocytes, NK cells and dendritic cells through TLR7/8 and 9 and monocytes and dendritic cells through CD40L may result in secretion of inflammatory mediators and type I anti-viral interferons (IFN-I), which confer antiviral state to B cells, leading to a lower transformation rate of B cells infected with EBV (Megyeri et al. 1995; Tomai et al. 1995; Roda et al. 2005). In addition, stimulatory effect of CpG, may arm natural killer (NK) cells to eradicate B cells infected with EBV (Sivori et al. 2004; Roda et al. 2005).

CD40L costimulation displayed a promoting effect on EBV transformation of enriched B cells, although this effect was less than that obtained for TLR agonists (Figs. 2 and 4). Interestingly, the promoting effects of TLR agonists and to a lesser extent CD40L costimulation on transformation efficiency of EBV seem to be correlated with proliferative capacity of the stimulated cells (Fig. 5). TLR agonists and CD40L induced more potent proliferative response in B cells as compared to PBMCs (Fig. 5). Costimulation with CpG, R848 and CD40L resulted in 2.4-, 2.7- and 2.75-fold increase in stimulation index of EBV-driven B cells proliferation, respectively.

The proliferative response of B cells stimulated with TLR agonists and CD40L alone did not last longer than 10 days. Lack of growing LCL cells after 3 weeks of culture of TLRs or CD40L stimulated or

non-stimulated cells in absence of EBV, indicates that outgrowth of EBV stimulated B cells was not due to reactivation of endogenous EBV that was already existing in a small fraction of B cells from seropositive normal individuals or due to TLR- or CD40L-derived B cell proliferation. Taking into consideration the low number of PBMC or enriched B cells seeded in each well in our limiting dilution experiments and the very low frequency of EBV infected B cells in seropositive normal subjects, which has been reported to be less than 0.001 of PBMC or less than 2.8 per 10^6 of B cells (Tosato et al. 1984), the chance of spontaneous transformation occurrence seems to be extremely low.

Using RT-PCR we have recently demonstrated comparable levels of TLR7, 8 and 9 mRNA expression in EBV infected and uninfected B cells. TLR agonist stimulation and EBV infection have no substantial effect on TLR7 and 8 transcription levels (Younesi et al. 2010). Thus, the differential effects of TLR agonists on proliferation of EBV-infected B cells as compared to EBV-untreated B cells do not seem to be associated with the expression status of these TLR in these cells.

The present finding regarding the supportive effect of TLR agonists on proliferation of B cells infected with EBV has been reported previously using the TLR7 agonist R837 (Martin et al. 2007). Most recently, the promoting potential of the TLR9 agonist CpG on the efficiency of EBV transformation was reported (Iskra et al. 2010). In the latter study, however, outgrowth of EBV-transformed LCLs was not determined clonally, based on a quantitative limiting dilution assay. A flow cytometric analysis was performed to determine the increase in the forward and side scatter of transformed cells relative to resting B cells after 7 days of infection. This methodology, as opposed to the LDA assay employed in the present study, could not determine quantitatively the transformation efficiency of EBV and should be considered as a qualitative tool.

Costimulation with CD40L plus TLR agonists did not improve the transformation efficiency of EBV (Figs. 1 and 3). This part of results indicates that synergism between CD40L/TLR or TLR/TLR signaling, which has been reported in several studies (Wagner et al. 2004; Napolitani et al. 2005; Roelofs et al. 2005; Ma et al. 2007; Ahonen et al. 2008), is not a

predetermined property and may fluctuate in different biological conditions. Indeed, the effect of TLR agonists was weakened by the addition of CD40L, though the difference was not statistically significant. The lower B cell transformation efficiency induced by the combination of TLR agonists and CD40L as compared to TLR agonists alone could be attributed to some differences observed in their signaling pathways. Activation of IRF3 and IRF7 via MyD88 or TRIF requires TRAF3 (Oeckinghaus et al. 2011). Thus, TRAF3-deficient cells can not display appropriate immune responses following TLR signaling. Interestingly, CD40 signaling induces TRAF3 degradation (Brown et al. 2001). Therefore, CD40 signaling negatively regulates signals induced by receptors that rely on TRAF3 for signaling such as TLRs. Furthermore, EBV infection results in expression of LMP1 protein which induces upregulation of the A20 gene, a negative regulator of NF- κ B (Laherty et al. 1992). LMP1 induces a signaling cascade which mimics the CD40 signaling pathway in many aspects (Young and Rickinson 2004; Rastelli et al. 2008). Altogether, degradation of TRAF3 and upregulation of A20 gene upon CD40L stimulation could weaken TLR promoting effects on EBV transformation.

Surprisingly, while both TLR7/8 and TLR9 agonists enhanced the transformation efficiency of EBV, combination of these two agonists neither induced synergism nor additive effects on the transformation efficiency of EBV. Although paired TLR agonists were found to display a stronger vaccine adjuvant over a single TLR agonist, costimulation of B cells or other immune cells with a combination of TLR agonists may induce additive, synergistic or antagonistic effects depending on the signaling pathways induced by each agonist (Zheng et al. 2008). It has been demonstrated that combination of TLR7/8 and TLR9 agonists may counter-regulate each other through intracellular mechanisms in different immune cells, such as PBMCs, B cells and plasmacytoid dendritic cells (Gorden et al. 2006; Berghofer et al. 2007; Marshall et al. 2007; Booth et al. 2010). Using paired TLR agonists, it has recently been shown that activation of both MyD88-dependent and MyD88-independent pathways results in a synergistic effect, while antagonistic or inert effect is delivered by paired TLR agonists that both act through the same signaling pathway (Bagchi et al. 2007), a finding similar to our

results using TLR7, 8 and 9 agonists which are all acting through the MyD88 pathway.

Since TLR signaling through NF- κ B pathway is critical for early step of immune response, modulation of NF- κ B pathway is an important immunoevasion mechanism (Bowie and Unterholzner 2008). On the other hand, given that NF- κ B signaling has antiapoptotic and proliferative effects, some viruses such as EBV have developed mechanisms to actively induce NF- κ B activation for their life cycle (Unterholzner and Bowie 2008).

Recently, we reported that EBV infection causes significant inhibition of stimulatory effect of TLR7/8 and TLR9 agonists, but not CD40 ligand in human B lymphocytes at early stage, within the first week of EBV infection (Younesi et al. 2010). Thus, our previous data together with the present findings suggest that EBV might employ NF- κ B activity in a biphasic manner, as a physiological advantage (Hiscott et al. 2001). In early stage of EBV infection, when the virus is vulnerable and could easily be eradicated by the innate immune system, NF- κ B activity is hampered, but subsequent to establishment of infection, EBV will take advantage of proliferative effect of the NF- κ B pathway.

In summary, the present findings indicate that TLR7/8 and TLR9 agonists enhance the transformation efficiency of EBV, perhaps through activation of the NF- κ B signaling pathway which downregulates the lytic replication of EBV in infected B cells (Ladell et al. 2007). The lower promoting effect of CD40L stimulation on efficiency of EBV transformation could be attributed to activation of different downstream signaling molecules following CD40-CD40L interaction with no beneficial effect to the replication cycle of EBV. Although CD40 ligation of B cells infected with EBV was shown to promote viral latency (Adler et al. 2002), however, controversial results have been reported showing diminished growth of EBV-transformed LCLs following CD40 ligation (Pokrovskaja et al. 2002). More investigations are required to get further insights into the biological role of CD40 ligation in the course of EBV infection and transformation.

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