Toll-like Receptor Agonists Synergistically Increase Proliferation and Activation of B Cells by Epstein-Barr Virus

Running title: TLR agonists and EBV

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ABSTRACT

Epstein-Barr virus (EBV) efficiently drives proliferation of human primary B cells in vitro, a process relevant for human diseases such as infectious mononucleosis and post-transplant lymphoproliferative disease. Human B cell proliferation is also driven by ligands of Toll-like receptors (TLR), notably viral or bacterial DNA containing unmethylated CpG dinucleotides which triggers TLR9. Here, we quantitatively investigated how TLR stimuli influence EBV-driven B cell proliferation and expression of effector molecules. CpG DNA synergistically increased EBV-driven proliferation and transformation, T-cell costimulatory molecules, and early production of interleukin-6. CpG DNA alone activated only memory B cells, but CpG DNA enhanced EBV-mediated transformation both of memory and naive B cells. Ligands for TLR2 or TLR7/8, or whole bacteria, had a weaker but still superadditive effect on B cell transformation. Additionally, CpG DNA facilitated the release of transforming virus by established EBV-infected lymphoblastoid cell lines. These results suggest that the proliferation of EBV-infected B cells and their capability to interact with immune effector cells may be directly influenced by components of bacteria or other microbes present at the site of infection.
INTRODUCTION

Epstein-Barr virus (EBV), a herpesvirus, is a very successful infectious agent: it establishes and maintains latent infection in >95% of human beings worldwide. This success is related to EBV's varied strategies to maintain itself in its preferred host cell type, the B cell, by establishing different modes of latent infection (46). Some of these modes (latency 0, I, and II) are characterized by a resting B cell phenotype and expression of a very limited set of EBV proteins (from none to four). In contrast, latency III involves the expression of at least 12 EBV latent cycle gene products (10 proteins and 2 RNAs) (30, 31), which in their combined action profoundly alter the B cell's appearance and behavior by inducing B cell activation associated with proliferation, altered receptor expression and cytokine secretion, and enhanced antigen presentation (31).

In these various features, EBV infection of the latency III type resembles physiological activation of B cells in germinal centers even in its molecular details, because EBV closely mimics or constitutively activates some of the B cell's main signaling pathways. Exogenous physiological signals leading to B cell activation have been classified as "signal 1", the stimulation of the B cell receptor (BCR) by antigen binding, "signal 2", the stimulation of CD40 by the CD40 ligand molecule expressed on activated helper T cells, and "signal 3", the stimulation of Toll-like receptors (TLRs) by microbial components such as unmethylated CpG DNA or their mimics. All three signals together are required for maximal proliferation of naive B cells (47). However, the stimulation with TLR ligands alone, for example CpG DNA, is sufficient to cause transient B cell activation, including proliferation and induction of immune effector molecules like CD86, a T-cell costimulatory molecule (24). Additional
immune effectors, the cytokines IL-6, IL-10 and IL-12, are induced when CpG stimulation is combined with strong CD40 stimulation (55).

For primary infection of B cells, it is well established that EBV’s latent membrane proteins LMP2A (10, 39) and LMP1 (22) mimic signaling by the BCR and CD40, respectively. It is less clear whether and how EBV generates a potential signal 3 in the course of primary B cell infection. A role of the TLR7 pathway has been proposed based on the observation that EBV infection of naive B cells elevates their expression of TLR7 and its downstream signaling mediators (40). Additional mechanisms have recently been proposed as to how EBV might trigger TLRs or other pattern recognition receptors in other cellular systems. For example, the Epstein-Barr virus-encoded small RNAs (EBERs) were described to trigger the retinoic acid inducible gene I (RIG-I)—encoded protein, a receptor for various viral RNAs, in Burkitt’s Lymphoma cells (48, 49). TLR2 signaling in monocytes is activated by binding of EBV particles to the cells (21) or by extracellular provision of EBV dUTPase (2).

However, a physiologically relevant signal 3 need not originate in EBV itself. Other microbial agents present at the site of EBV infection might influence EBV infection, B cell transformation, and virus release. For example, infectious mononucleosis, a frequent consequence of primary EBV infection in adolescents and adults, is usually accompanied by tonsillitis with characteristic massive bacterial colonization (50), a likely source of TLR agonists acting on local EBV-infected B cells. Here, we investigate the effects of CpG DNA and other exogenous TLR ligands on EBV-driven B cell proliferation, clonal outgrowth, and induction of activation-associated cellular receptors and cytokines.
MATERIALS AND METHODS

Standard medium for cell culture was RPMI 1640 (high glutamine, Gibco) supplemented with 10% FCS (PAA), 1% Penicillin/Streptomycin (Gibco), and 100 nM sodium selenite (ICN). For continuous culture of EBV producer cell lines, hygromycin (75 μg/mL; PAA) was added. When noted, cyclosporin A (1 μg/mL; Novartis) was used in PBMC or B cell infections.

Production of recombinant EBV and virus-like particles. Transforming EBV was produced in a cell line (2089/293) based on 293 cells stably transduced with a GFP-encoding recombinant EBV genome based on EBV strain B95.8 (14). EBV genome-free virus-like particles were produced in a cell line (TR−/293) carrying a GFP-encoding EBV genome deleted for the terminal repeats (TR), EBV's essential cis-acting packaging signals (15). For virus or virus-like particle production, cells were plated in medium without hygromycin, and EBV's lytic cycle was transiently induced by transfection with an expression plasmid coding for BZLF1 using Metafectene transfection reagent (Biontex). Supernatants containing virus ("EBV") or virus-like particles ("EBV TR−") were harvested three days later and further purified by centrifugation of cell debris (300 × g, 5 min) and filtration (0.8 μm pore size). EBV or EBV TR− preparations were stored at 4°C for up to 3 months.

Preparation of human B cells. Human B cells were prepared from peripheral blood buffy coats from anonymous adult donors (Institute for Transfusion Medicine, Ulm, Germany), from peripheral blood from adult volunteers, or from manually homogenized adenoid tissue obtained after routine tonsillectomies. Mononuclear cells containing B cells were enriched by centrifugation on Ficoll (Biochrom, Berlin, Germany). From this population, B cells were isolated by immunomagnetic labeling and depletion of other cell types (B Cell Isolation Kit II and LS columns, Miltenyi
Biotech, Bergisch Gladbach, Germany) according to the manufacturer's protocols. Purified B cell preparations regularly contained >98% CD20+ cells, as assessed by flow cytometry. Naive (CD27–) and memory (CD27+) B cells were separated by labeling with anti-human CD27 microbeads and two consecutive purifications using paramagnetic MS columns (Miltenyi Biotech). The purity of the subpopulations was assessed by flow cytometry.

Quantification of EBV and EBV TR–. Rapid GFP transfer to B cells was used as a surrogate marker for quantifying and adjusting the amounts of virus (EBV) or virus-like (EBV TR–) particles in supernatants of producer cells. For titration, 10^5 purified primary B cells or Raji B cells in 500 µl were infected with various amounts of supernatant. After one day, mean green fluorescence intensities of the entire viable cell population (defined by forward and side scatter) was quantified by flow cytometry. Up to a certain threshold, a linear relationship was observed between the amount of EBV or EBV TR– preparation and fluorescence intensity. This linear relationship was used to adjust EBV and EBV TR– to each other. To standardize the number of transforming infectious EBV particles, we used the outgrowth of strongly GFP-positive, enlarged lymphoblastoid cells, identified in flow cytometry at day 3 after infection of primary B cells, as a surrogate marker. One lymphoblastoid cell on day 3 after infection was assumed to represent one unit of transforming EBV. At least up to 0.1 transforming units per B cell, we observed a linear relationship between the number of lymphoblastoid cells and the amount of EBV used for infection.

Infection and stimulation of human B cells. For EBV infection, EBV TR– pseudoinfection, and TLR agonist stimulation, replicates of 10^5 freshly purified B cells in 500 µL were plated in a well of a 48-well plate in the presence of EBV (0.1 transforming units per B cell) or EBV TR– (in amounts adjusted as described above). TLR ligands were added simultaneously, unless otherwise indicated. When the
medium became acidic, cultures were expanded by adding a defined volume of fresh medium. This was never necessary before day 7 of infection/stimulation. In experiments with unseparated PBMCs, \(5 \times 10^4\) cells in 250 \(\mu\)L per well of a 48-well plate were used. The following TLR agonists were used at the given standard concentrations, unless indicated otherwise: CpG-containing oligonucleotide 2006 (24), \(5'\)-TCG TCG TTT TGT CGT TTT GTC GTT-3', synthesized as full phosphorothioate DNA (Metabion, Martinsried, Germany), 4 \(\mu\)g/ml; Pam\(_3\)CSK\(_4\), 1 \(\mu\)g/ml; Imiquimod, 0.5 \(\mu\)g/ml; lipopolysaccharide (LPS) from Salmonella minnesota, 5 \(\mu\)g/ml; and lipoteichoic acid (LTA) from Staphylococcus aureus, 1 \(\mu\)g/ml (all from InvivoGen); inactivated Staphylococcus aureus bacteria, 4 \(\mu\)g/ml (Pansorbin, Calbiochem).

**Quantification of lymphoblastoid cell outgrowth and flow cytometry.** For quantification of lymphoblastoid cell outgrowth, B cells were seeded at \(2 \times 10^5\)/mL, with or without EBV, EBV TR–, or TLR ligands, in 48-well plates in separate 250 \(\mu\)L or 500 \(\mu\)L cultures for each time of analysis and each condition. Immediately before analysis, the complete culture was thoroughly resuspended and directly transferred to a FACS tube containing 20,000 APC-labeled calibration beads (Becton Dickinson), to determine absolute cell numbers, and the membrane-impermeable DNA dye ToPro-3 to a final concentration of 0.1 \(\mu\)M, to discriminate live and dead cells (Molecular Probes). In most cases, 5000 Beads were acquired in flow cytometry per sample. The cell-to-bead ratio was used to relate the experimental cell number to the total culture volume and, thereby, to the initial cell number at day 0. Lymphoblastoid cells were identified by increased forward and sideward scatter relative to the resting B cell population. Cells in the lymphoblastoid gate consistently stained as live cells (negative for To-Pro3). For staining of cell surface molecules, 200,000 cells were harvested, washed and stained with saturating amounts of CD86-APC or CD80-FITC.
antibodies (BD Pharmingen) in PBS/2% FCS for 15 minutes on ice. Cells were washed, fixed in 1% formaldehyde (Roth), and stored at 4°C until analysed by flow cytometry. For determination of apoptotic cells, the distinct side population of cells with low forward scatter was included in the gate, evaluated for To-Pro3 staining, and quantified with calibration beads as described above. All flow cytometric analyses were performed on a Becton Dickinson FACS caliber equipped with CellQuest Software.

Quantification of EBV release by LCLs. EBV release by LCLs after incubation with CpG or medium alone was quantified by infection of the EBV-free CD40-stimulated B cell line LENL5 (56). This cell line is maintained on fibroblasts expressing CD40L. In the absence of these fibroblasts, LENL5 cells cease to proliferate and gradually die off, but proliferation can be rescued by EBV infection. LCLs were cultivated with or without CpG DNA for 1 day, washed three times, and cultivated for one or four more days. Supernatants were harvested and centrifuged at 300×g for 10 minutes and again at 1,600 g for 15 minutes to remove residual cells. Microcultures were set up in 96-well plates in 48 replicates for each condition by combining 100 µl of LCL supernatant, 1×10^5 LENL5 cells, and 100 µl of medium. A 50 µl portion of medium was exchanged every week for fresh medium. Wells with cell outgrowth were visually identified 7 weeks after infection. All outgrowing cultures expressed GFP, as verified in a fluorescence microscope.

ELISA. For cytokine detection, supernatants of infected/stimulated B cells were harvested at different times and stored at -20°C. ELISAs for IL-6 and IL-10 were performed as proposed by the manufacturer (Mabtech). For neutralization of IL-6 and IL-10, 1 µg/ml of purified monoclonal antibodies against IL-6 (LEAF, Biolegend) or IL-10 (clone 12G8, Mabtech) was directly added to the infection/stimulation. The neutralization was verified by ELISA.
**Limiting dilution and quantification of B cell clones.** B cells ($10^5$) were infected as described above in the presence and absence of CpG DNA. Cyclosporin A (Novartis) was simultaneously added to the culture to prevent long-term inhibitory effects or culture regression mediated by potentially contaminating T cells. Fourteen days after infection, limiting dilution was performed in round-bottom 96-well plates. After additional 4 weeks, outgrowth of B cell clones was quantified by the colorimetric MTT assay. MTT solution (10 µl, 5 mg/ml in PBS) and medium (40 µl) was added to B cell cultures (50 µl). After 3 hours of incubation at 37°C, the reaction product was solubilized by adding 100 µl of 10% SDS. After overnight incubation at room temperature, absorption at 595 nm was determined. To discriminate between outgrowth and non-outgrowth, a fixed cutoff value of absorption was used throughout an experiment. This cutoff value was defined according to the observed absorption of appropriate positive and negative control cultures that unequivocally showed outgrowth or lack of outgrowth in visual inspection.

**Quantitative RT-PCR.** At the time of analysis, total RNA was extracted from primary B cell cultures or LCL cultures using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA was treated with DNaseI (amplification grade, Invitrogen) for 90 min at 37°C to remove contaminating genomic DNA, followed by DNase inactivation (10 min at 65°C). To monitor DNA contamination in the RNA preparation, control PCR reactions were performed with primers specific for an EBV gene. mRNA was reversely transcribed (SuperScript III First-Strand Synthesis Kit, Invitrogen) according to the manufacturer’s protocol. One µl out of 20 µl of the cDNA product was used as template for PCR amplification of selected EBV genes. Real-time PCR was performed with the LightCycler (Roche) according to the manufacturer’s instructions. Quantification of reverse transcribed transcripts was carried out by using the LightCycler FastStart Reaction Mix (SYBR Green I; Roche).
The amplification of PCR products was monitored on-line and usually stopped after 40 cycles. The following settings were used: initial template denaturation for 10 min at 95°C, cycles with 1 s at 95°C, 10 s at 62°C and 10 s at 72°C. The following PCR primer pairs were used for amplification of EBV cDNAs. All primers are given in 5' to 3' direction. 

- **EBNA1**, TGA CAA AGC CCG CTC CTA CC and CTC ACC CTC ATC TCC ATC ACC TC; **EBNA2**, CAC ACG GCA ACC CCT AAC G and GGT CCC TCC ACA TAA TCT TCA TCT G; **LMP1**, GTG TCT GCC CTC GTT GGA GTT AG and CAT CCT GCT CAT TAT TGC TCT CTA TCT AC; **BZLF1**, GGG GCA AGC AAA CAC CAC TG and CAA CCG CTC CGA CTG GGT C; **BMRF1**, TTG AGG TTT TAC AGG TCT GGC ATC and GGT GGC GGA GGT GAA GGA G; **BLLF1**, ACC GAG CAT TTC TGT TTT TAC GC and GAT GTC TAC TTT CAA GAT GTG TTT GGA AC; **BALF4**, CTG GGG GGT GAG GAA GTC G and CAA CAC AAC CGT GGG CAT AGA G; **BFLF2**, GCT CAT CCC CAC ATT CCA GG and CTC CCT TCA CAT CCC AGA GAC C. Levels of EBV gene expression were standardized to levels of the housekeeping gene **GUSB** (β-glucuronidase) (13). **GUSB** cDNA was amplified with the following primers: CAC GAC TAC GGG CAC CTG G and TGC TCC ATA CTC GCT CTG AAT AAT G.
RESULTS

Controlled analysis of infection and transformation by EBV. The aim of our study was to assess the impact of exogenous TLR agonists on EBV-mediated transformation of B cells. However, EBV particles or their components might directly trigger TLR-mediated activation (21) of the target cells. Therefore, it was necessary to control for such effects by using quantified preparations of virus-like particles alongside transformation-competent EBV. We chose to use a recombinant EBV system expressing enhanced green fluorescent protein (GFP) in our studies.

Transforming, GFP-encoding EBV (simply called "EBV" in the experiments below) can be produced by inducing EBV's lytic cycle in human epithelial 293 cells that stably carry genomes of this recombinant EBV as episomes (14). Producer cells carrying a mutant version of this EBV genome that lacks the terminal repeats (293/TR-), which are required for efficient processing and packaging of the viral genome into the capsid, nearly exclusively release virus-like particles with "empty" capsids containing no EBV DNA and incapable of transforming B cells (15, 18). These virus-like particles (designated "EBV TR–" below) specifically bind to and are taken up by B cells in a similar fashion as transforming EBV. Pseudoinfection of primary B cells with EBV TR– results in the efficient uptake of virion proteins, including GFP, and recognition of the B cells by CD4+ T cells specific for EBV structural proteins (1). Incubation of B cells with EBV TR– may therefore serve as a control for various possible effects of EBV infection that are not caused by viral gene transfer, for example effects of virion binding to the cell surface or transfer of virion proteins into the cell (Fig. 1A).

We analyzed the transfer of virion-associated GFP and GFP de novo expression in the B cell after EBV infection or EBV TR– pseudoinfection (Fig. 1B, C). GFP protein
transfer to primary B cells was readily detectable on day 1 after infection and was equally mediated by EBV or EBV TR–, but not by supernatants from parental 293 cells. The entire B cell population exhibited green fluorescence, indicating an excess of GFP-delivering particles over uptaking B cells. On day 3 after infection, a strong increase in green fluorescence was seen in a subpopulation of B cells infected with complete EBV, but not in B cells incubated with EBV TR–, indicating de novo expression of GFP from the viral genome. Thereafter, the GFP-high subpopulation rapidly increased in numbers and dominated the cultures by day 7 after infection, consistent with the anticipated effects of EBV-mediated B-cell transformation (Fig. 1B, C). Taken together, GFP protein transfer, which is rapid, is less intense, and affects all B cells, can be distinguished from GFP de novo expression, which occurs later, is more intense, and affects a distinct subpopulation of B cells. Therefore, direct GFP transfer can be used to comparatively quantify virions in preparations of EBV and EBV TR– (see Materials and Methods). In the following experiments, we used EBV and EBV TR– preparations in quantities adjusted on this basis.

**Rapid activation and proliferation of B cells by EBV.** Upon infection with EBV, resting B lymphocytes assume a lymphoblastoid phenotype with larger cell size, enlarged cytoplasm, expression of activation markers, and proliferation, and ultimately grow out as transformed stable cell lines called lymphoblastoid cell lines (LCL) (31). To reassess the timing of this process in its early stages, we infected primary peripheral B cells with EBV or EBV TR– at a multiplicity of infection of 0.1 (see Materials and Methods), and analyzed the cells’ scatter characteristics by flow cytometry. On day 3 after infection with EBV, but not EBV TR–, a distinct population of lymphoblastoid cells with high forward and side scatter emerged and continuously expanded thereafter (Fig. 2A, B). As a marker of B cell activation, we analyzed surface expression of the costimulatory molecule CD86 early after infection of
primary B cells with EBV or EBV TR– (Fig. 2C). On EBV-infected B cells, CD86 was detectable on day 3, highly expressed on day 7, and remained stably expressed thereafter. There was no induction of CD86 on B cells treated with EBV TR– (Fig. 2C). Therefore, emerging EBV-transformed B cells can be detected from day 3 after infection and quantitated by a simple forward and side scatter analysis in flow cytometry. Such cells were invariably GFP-positive (not shown); however, because scatter analysis allowed an even clearer discrimination of B cell subpopulations than GFP fluorescence (compare Fig. 1B), we used scatter to quantitate lymphoblastoid B cells in the experiments below.

**CpG DNA synergistically enhances B cell transformation by EBV.** We hypothesized that agents of microbial origin might influence EBV-mediated B-cell transformation. DNA containing unmethylated CpG dinucleotides, such as bacterial or viral DNA, is a ligand of TLR9, activates human B cells and induces their proliferation for several days (24). It was also shown that CpG DNA increases clonal outgrowth of EBV LCLs in limiting dilution reactions in the presence of feeder cells (54). Therefore, CpG DNA was a likely candidate to directly modulate EBV transformation. We infected purified primary B cells with EBV or EBV TR– in the presence or absence of CpG DNA. On day 7 after infection (Fig. 3A), infection with EBV alone resulted in outgrowth of two lymphoblastoid cells from one resting B cell in the starting population. The effect of CpG DNA together with EBV TR– was much weaker, and EBV TR– alone produced no lymphoblastoid cells. When combined, EBV and CpG DNA induced the outgrowth of seven lymphoblastoid cells per B cell in the starting population, a clearly synergistic effect. The immediate presence of CpG DNA at the time of EBV infection was critical: more than half of the CpG-mediated increase in the number of lymphoblastoid cells was lost when CpG DNA was added 12 hours after infection (Fig. 3B). Conversely, when B cells were EBV-infected with
some delay after CpG stimulation, outgrowth of B cells was reduced in absolute terms compared to simultaneous infection (Fig. 3C). However, the relative potential of initial CpG treatment to rescue B cell outgrowth was even greater for delayed EBV infection than for simultaneous infection. For example, when EBV infection was delayed for one day, CpG-pretreated B cells yielded 10 times more lymphoblastoid cells than medium-pretreated cells (Fig. 3C).

To test whether other immune effector cells could influence this EBV-CpG synergism, we repeated the experiment with total PBMC, instead of purified B cells, in the presence or absence of cyclosporin A, an inhibitor of T cell activation. PBMCs from EBV-positive donors were used, in order to detect a possible effect of EBV-specific memory T cells. However, the effect of CpG DNA on EBV-mediated lymphoblastoid cell outgrowth from PBMCs (Fig. 3D) and from purified B cells (Fig. 3A) was very similar, and inhibition of T cell activation by cyclosporin A had no effect (Fig. 3D). This experiment confirmed that the EBV-CpG synergism was mediated by direct effects of EBV and CpG DNA on B cells, and showed that there was no rapid control of outgrowth by T cells.

These experiments, however, addressed only the first seven days after EBV infection. Because CpG DNA alone activates B cells only transiently, the possibility remained that some of the lymphoblastoid B cells detected after EBV infection in the presence of CpG DNA might not be permanently transformed by EBV. To investigate this possibility, we infected B cells with EBV in the presence or absence of CpG DNA, washed out remaining CpG DNA after 14 days, submitted the B cells to limiting dilution, and counted outgrowing EBV-transformed B cell clones after four more weeks (6 weeks after infection). EBV-transformed B cells grew out in limiting dilution at a similar efficiency from 14-day cultures previously infected in the presence or absence of CpG DNA (not shown). When we calculated the clonal outgrowth
efficiency with respect to starting numbers of B cells at the time of EBV infection (Figure 3E), we found that CpG DNA increased the yield of EBV-transformed clones by a factor of 4.4, similar to the increase in lymphoblastoid cells detected after shorter periods (Figure 3A, D). Therefore, the presence of CpG DNA at the time of infection led to a long-term increase in the number of EBV-transformed B cell clones.

We investigated whether the effect of CpG DNA might be connected to reduced death of EBV-infected B cells. By flow cytometry, we determined live/dead cell ratios in EBV/CpG versus EBV-infected B cell cultures. Interestingly, the addition of CpG DNA together with EBV in early infection led to a 6-fold better relative survival on day 3, the day when lymphoblastoid cell outgrowth was initiated (Figure 3F). This result showed that although CpG DNA had to be present within a day after infection to have an effect (Figure 3B), it mediated better survival of EBV-infected B cells for several days after infection. Taken together, these results show that the presence of a TLR ligand at the site of EBV infection increases the efficiency of infection and transformation of B cells.

**CpG DNA increases activation of B cells by EBV.** Control by antigen-specific T cells is decisive for the course of EBV infection. The efficient interaction of antigen-presenting cells and antigen-specific T cells depends on costimulatory molecules like CD80 and CD86 of the B7 family. CD80 and CD86 are not expressed on resting human B cells, but are induced by EBV infection (Fig. 2C) or stimulation with CpG DNA (24). We were interested whether CpG might modify the expression of such effector molecules by B cells after EBV infection. We infected peripheral B cells with EBV or EBV TR– in the presence or absence of CpG DNA, and followed the induction of CD80 over 7 days by flow cytometry. Indeed, CD80 upregulation was fastest and highest after EBV infection in the presence of CpG DNA (Figure 4A).
Activated B cells secrete cytokines such as interleukins 6 and 10 (IL-6 and IL-10). Both these cytokines influence B cell growth and differentiation. IL-6 was described as an autocrine growth factor of established LCLs (53, 58). IL-10 favours EBV transformation of B cells (9) and proliferation of LCLs (4). IL-6 and IL-10 are induced by CpG or CD40 stimulation of primary B cells, and both stimuli together synergistically increase IL-6 or IL-10 secretion (55, 56). We investigated how the combined action of CpG DNA and EBV would influence the secretion of these cytokines. We found that IL-6 was rapidly induced by CpG DNA and EBV together (Figure 4B), detectable already on day 1 after infection. In contrast, the secretion of IL-10 was more delayed and appeared to reflect the number of lymphoblastoid B cells throughout the different reaction conditions (Figure 4C, compare Figure 3A). Depletion of available IL-6 or IL-10 by blocking antibodies had no effect on lymphoblastoid cell outgrowth in the early days after infection, neither after EBV infection alone nor after infection in the presence of CpG DNA (Figure 4D, E). These results are in line with previous observations that IL-10 depletion does not strongly reduce outgrowth of EBV-infected B cells during the first 7 days after infection, but only later (9), when cell density is higher or cytokine accumulates over time in vitro. We conclude that the enhanced IL-6 or IL-10 release is not a major factor in the rapid CpG DNA-mediated increase of outgrowth in the first days after EBV infection.

Susceptibility of naive and memory B cell subsets to EBV transformation in the presence of CpG DNA. Naive and memory B cells differ in TLR expression and in their responsiveness to TLR agonists. It was described that naive B cells express less TLR9 than activated or memory B cells, and react with much weaker proliferation to CpG DNA than memory B cells (5). However, there is controversy on this issue (27), possibly related to the use of different criteria to distinguish naive and memory B cells (57). Many questions remain open regarding the susceptibility of
different B cell subsets to EBV infection, but there appears to be a consensus that tonsillar naive and memory B cells are equally susceptible to short term EBV infection in vitro (16, 17).

We were interested whether the EBV–CpG synergism acted on memory B cells, naive B cells, or both. We isolated B cells from peripheral blood and separated them into naive (CD27−) and memory (CD27+) B cells. Cells were infected with EBV or EBV TR− in the presence or absence of CpG DNA. CpG DNA synergistically increased EBV-mediated cellular outgrowth both from naive and from memory B cells, and this effect was quantitatively very similar for both B cell subpopulations 7 and 14 days after infection (Figure 5). As might have been expected, at an early time point (day 4) we observed reactivity against CpG DNA in the absence of EBV, and this effect was only seen in memory but not naive B cells. In these experiments, the purity of the B cell subpopulations was limited (CD27+, 98%; CD27−, 81%), but sufficient to support the conclusion that the EBV–CpG synergism targets both the naive and the memory B cell compartment, although only memory B cells respond to CpG DNA alone.

**Effect of CpG DNA on early activation of EBV latency genes.** EBV transformation is mediated by a set of at least nine EBV latency-associated proteins. Essential roles are played by EBNA1, EBNA2, and LMP1, whose functions include EBV genome maintenance and replication, transcriptional activation of various cellular and viral genes, and activation of intracellular signaling pathways such as the NF-kB pathway. We speculated that CpG might enhance EBV-mediated cellular transformation by upregulating these EBV factors. We infected primary B cells with EBV in the presence or absence of CpG DNA and analyzed expression levels of the EBNA1, EBNA2, and LMP1 genes at several time points between 6 hours and 3 days after infection, normalized to expression of the β-glucuronidase gene. We could clearly
visualize the subsequent induction of EBNA2, EBNA1 and LMP1 genes during EBV infection (Figure 6). However, the presence or absence of CpG DNA made little difference. It appears that EBV’s transforming gene expression programme is not significantly altered by CpG DNA. TLR stimulation might rather support EBV transformation by directly accessing cellular pathways leading to B cell activation (44).

**Effect of CpG DNA on the induction of EBV’s lytic cycle.** Our previous results suggest that TLR agonists might contribute to the expansion of EBV’s physiological reservoir, latently infected B cells. One step further, TLR agonists might contribute to the mobilization of EBV from this reservoir by favouring lytic EBV replication. Previous studies on MHV-68, EBV’s homolog in mice, suggest this (19, 20), although other studies appear to contradict (23, 35). Because the productive lytic EBV program is suppressed in the early days of B cell infection (29), we investigated the effect of CpG DNA on the lytic cycle in established lymphoblastoid cell lines. LCLs were cultivated in the presence or absence of CpG DNA, and expression levels of five genes from EBV’s immediate-early, early and late lytic program were determined by quantitative RT-PCR. Most of these lytic-cycle genes were modestly downregulated by CpG DNA (Fig. 7); only BALF4, a glycoprotein that is produced at low amounts, limiting infectivity of some EBV strains (42), was occasionally upregulated by CpG DNA. However, alterations by CpG treatment were limited and did not exceed a 2-fold upregulation or 3.5-fold downregulation. In contrast, when we tested whether supernatants of these LCLs contained EBV that could infect and transform a sensitive B cell line, we obtained transformants exclusively with supernatants from CpG-treated, not from untreated LCLs (Table 1). Although EBV release from LCLs was not particularly efficient, we take this experiment as a first hint
that CpG DNA might indeed favour EBV release by latently infected B cells without necessarily requiring global upregulation of lytic cycle genes.

**Effects of various TLR ligands or bacteria on EBV transformation of tonsillar or peripheral B cells.** We extended our studies to the effects of other TLR agonists on EBV-driven B cell activation. These were Pam₃CSK₄, a TLR2 ligand, imiquimod (TLR7), LPS (TLR4), lipoteichoic acid (TLR2), and whole fixed *Staphylococcus aureus* bacteria. We infected tonsillar B cells with EBV in the presence of these TLR ligands or CpG DNA at various concentrations. Lymphoblastoid cell outgrowth was analysed seven days after infection (Figure 8A). Several TLR ligands favoured the outgrowth of EBV-infected B cells. CpG DNA had the strongest effect, followed by *S.aureus* and the TLR2 ligands Pam₃CSK₄ and lipoteichoic acid. Imiquimod was active only at high concentration, and lipopolysaccharide had no effect. CpG DNA appeared to have the fastest effect on EBV-mediated B cell outgrowth (Figure 8B), but also some of the other TLR agonists discernably supported B cell outgrowth on day 5 after infection. In the presence of EBV TR– particles, effects of all TLR ligands were small, and no activated B cells remained on day 7 (Figure 8B, lower panel).

Effects of the various TLR agonists on EBV-mediated activation of tonsillar (Figure 8B) and of peripheral blood B cells (Figure 8C) were closely similar. We conclude that not only agonists of TLR9, but also those of other TLRs like TLR2 support EBV-driven B cell activation and early transformation.
DISCUSSION

In this study, we analyzed the effect of Toll-like receptor agonists on the activation and growth transformation of human B cells by EBV, focusing on the early days after infection. We found that agonists for several TLRs enhanced the outgrowth of activated primary B cells by EBV. Among TLR ligands, CpG DNA, which activates TLR9, had an especially strong effect. CpG DNA, applied alone (24) or in combination with ligation of CD40, of the B-cell receptor, or both (5, 55), had been found to mediate activation and proliferation of B cells for a period of several days. We now found that CpG DNA has an even stronger and synergistic effect on EBV-mediated proliferative transformation. This effect is already prominent a few days after infection, but has long-term consequences, because the number of EBV-transformed B cell clones capable of long-term outgrowth is correspondingly increased. Our results are in accordance with the observation of Traggiai et al., who qualitatively reported that CpG DNA can improve the yield of EBV-transformed B cell clones in limiting dilution cultures containing feeder cells (36, 54). In a more limited set of experiments, we also showed that CpG DNA mobilized the release of EBV from latently infected B cells. We hypothesize that TLR ligand-bearing pathogens such as bacteria co-localized at sites of EBV infection or replication – for example in tonsillitis during infectious mononucleosis – might favour EBV establishment and spread by increased proliferation of infected B cells and, possibly, by favouring lytic EBV replication.

The transformation of primary human B cells by EBV in vitro is remarkably efficient (45) and has therefore served as a standard experimental model of EBV infection and EBV-mediated oncogenesis (34). EBV-mediated transformation, although previously termed “immortalization”, does not confer immortality on the transformed
B cells (51, 56), but nonetheless produces long-lasting cellular proliferation in association with major changes in cellular phenotype and in patterns of gene expression and cytokine secretion. Although transformation is the default result of the infection of primary B cells with EBV infection in vitro, EBV-transformed B cells are undetectable in most asymptomatic EBV carriers. Still, they likely emerge continuously on a very small scale, considering that healthy EBV carriers frequently have a considerably expanded repertoire of memory T cells specific for the antigens EBNA3A, B, and C that are expressed only in EBV’s transforming mode, latency III (25, 41). These T cell specificities, among others, may be required to prevent the outgrowth and tumor formation by transformed B cells, an event that regularly occurs in patients with severe suppression or depletion of their T cell compartment, especially after bone marrow or solid organ transplantation; as a consequence, these patients can develop EBV-associated post-transplant lymphoproliferative disease (PTLD). PTLD is often associated with bacterial infection, which is usually interpreted as an independent indicator of an impaired immune response (43). However, our results suggest the hypothesis that bacterial or fungal co-infection could influence and possibly aggravate PTLD by triggering TLRs on EBV-infected B cells and thereby supporting their proliferation or modifying lytic EBV reactivation.

Another EBV-associated disease, less severe than PTLD but much more frequent, is infectious mononucleosis (IM). Primary EBV infection beyond early childhood is the predominant cause of IM, a benign lymphoproliferative disease associated with general malaise, fever, lymphadenopathy, pharyngitis, and tonsillitis (12). In acute IM, up to a few percent of peripheral B cells may be EBV-infected. In IM-associated tonsillitis (33) as well as in IM-unrelated tonsillitis in EBV carriers (3), there is EBV-driven clonal expansion of infected B cells, many of which show the proliferative latency III pattern of EBV gene expression. Tonsillitis in IM is accompanied by a
characteristic and massive bacterial colonization on the tonsillar surface (50). An increase in intratonsillar colonization by anaerobic gram-negative bacteria is also observed, and the use of antibiotics specifically targeting anaerobic bacteria alleviates tonsillitis and shortens the duration of fever in IM (7, 8). Therefore, bacterial components capable of triggering B–cell-expressed TLRs are present in high amounts at this important site of EBV infection and replication, and it seems possible that they will to some degree influence EBV infection and transformation of co-localized B cells. It is unknown what factors predispose some adolescents and adults to IM, while in others primary EBV infection remains asymptomatic. Individual variations in the nature and magnitude of microbial co-infection might subtly influence the course and intensity of IM. The history of previous infections (shaping the available immune effector repertoire), effects of a coexistent microbial infection on immune effector cells, and direct effects of microbial components on EBV-infected B cells might act together here. Thus, it appears promising to investigate these connections in more detail.

In contrast to PTLD and IM, microbial infection was proposed long ago to be a cofactor for EBV-associated Burkitt’s lymphoma, which mainly occurs in regions where malaria is endemic (28). It was recently observed that a *Plasmodium falciparum* protein present on infected erythrocytes is mitogenic for B cells and increases replication of the viral genome in EBV-infected cells (11), although the relationship of viral reactivation and lymphomagenesis is not clear yet. An alternative hypothesis states that EBV and the malaria parasite may combine to promote B cell transformation and genomic mutation involving activation-induced cytidine deaminase (52). The possible impact of heterologous infectious agents on γ-herpesvirus infection is further demonstrated by a recent study showing that exogenous ligands of the TLRs 3, 4, 5, and 9 directly stimulate in vivo reactivation of
murine herpesvirus (MHV) 68, leading to increased numbers of latently infected B cells (19). However, TLR9 also has a role in the control of viral infection in vivo, because mice lacking TLR9 had higher viral loads than wild-type mice (23). In contrast, in mice lacking the TLR signaling mediator MyD88 levels of latent virus were reduced (20), indicating that the net result of TLR triggering by MHV-68 is to facilitate the establishment of latent infection.

Thus, it may be predicted that, in humans, TLR activation has multiple effects in during primary EBV infection, some of which may favour viral latency or reactivation, and some of which may facilitate immune control. TLR activation of B cells triggers the increased proliferation of EBV-infected B cells, but the increased expression of molecules like the costimulatory ligands CD80 or CD86 on the B cells’ surface might favour elimination of infected B cells by cytotoxic CD8 or CD4 T cells. Recruitment and actions of T cells and other immune effectors might be further modulated due to increased secretion of IL-6, IL-10, or other mediators. Recognition of EBV-infected B cells might not be exclusively beneficial, because the debilitating symptoms of IM are largely ascribed to an "overshooting" response by large expansions of EBV-specific (and, possibly, additional non-specific) T cell populations recognizing EBV-carrying B cells (12). A further level of complexity is added by the observation that primary CD4 T cells reactive to EBV-infected B cells may exert "helper" function, by favouring rather than restricting outgrowth of EBV-transformed B cells (38). It remains an open question how the combined effects of TLR triggering would alter the spread and proliferation of EBV-infected B cells, the expansion of reactive T cells, and the associated immunopathological symptoms during primary EBV infection.

In this study, we addressed how exogenous agonists of TLRs can influence the fate of cells after EBV infection. In addition, EBV itself is a source of endogenous ligands of TLRs and other pattern-recognition receptors (PRR). Like other herpesviruses,
EBV activates TLR2 by binding of virions to the surface of monocytes, eliciting the secretion of proinflammatory cytokines (21). In our experiments, we did not observe that B cell activation or proliferation were triggered by viral particles without a transforming genome (EBV TR−, Figure 8). Because the expression of TLR2 is relatively low on resting human B cells (26), it may be that virion binding alone does not suffice to induce strong TLR2-mediated signaling in these cells. Recently and unexpectedly, purified viral dUTPase, when added to human monocytes, was found to trigger TLR2 (2), and the authors speculated that dUTPase could be released by lytically infected cells and contribute to inflammation. The influence of EBV infection on expression levels of TLRs was studied by Martin et al. (40). They observed that EBV, even when inactivated by UV irradiation, induced TLR7 expression in human naive B cells but rapidly suppressed TLR9 expression. This effect could readily explain our observation that CpG DNA has a strong effect on EBV-mediated B cell proliferation only when it is supplied within the first hours after infection (Figure 3B). It appears paradoxical that TLR9 expression in human B cells is increased by BCR or CD40 stimulation (5, 6), but decreased by EBV infection (supposed to mimic BCR and CD40 signaling) (40). The mechanisms underlying this difference deserve further investigation. Using a TLR7 antagonist, Martin et al. additionally showed that EBV-mediated TLR7 activation contributed to cell viability after EBV infection, and viability of EBV-infected B cells was even further increased by adding a synthetic TLR7 agonist (40). This result is in line with our observation that exogenous TLR ligands favour EBV-mediated B cell proliferation. In analogy to other herpesviruses (32, 37), EBV itself might be capable of triggering TLR9, mediated by hypomethylated CpG motifs in the viral DNA genome. Under physiological conditions, both EBV’s own display of pathogen-associated molecular patterns and exogenous TLR agonists might contribute to TLR signaling in infected B cells.
Human B cells strongly respond to TLR stimulation (24). It seems plausible that EBV, a virus that probably depends on B cell activation for its maintenance and spread, exploits this characteristic TLR reactivity of B cells. Here we present in vitro evidence that this is indeed the case. It remains to be investigated whether and how endogenous and exogenous TLR agonists might cooperate in shifting the balance between infected B cells and protective antiviral immunity, or between innocuous viral latency and disease.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

FIG. 1. A system to compare the biological effects of EBV and of EBV-derived virus-like particles. (A) Virus and virus-like particles were produced by cell lines based on 293 human epithelial kidney cells stably transfected with recombinant EBV genomes coding for GFP. Producer cells that carry EBV genomes deleted for the terminal repeats turn out virus-like particles ("EBV TR–"), which carry EBV structural proteins and GFP, but no viral genomes (15, 18). EBV TR– particles may activate target B cells by binding and may mediate protein transfer, but not gene transfer, to B cells. Producer cells that carry complete EBV genomic constructs turn out EBV particles with similar protein content, including GFP, but additionally contain a transforming EBV genome (14). EBV TR– serves as a control for effects that are unrelated to EBV gene transfer and viral transformation. Supernatants from unmodified 293 cells serve as another negative control. (B) GFP fluorescence intensity discriminates between virion-mediated protein transfer and de novo gene expression in infected cells. Peripheral blood B cells were incubated with EBV, EBV TR– or 293 cell supernatant and analysed by flow cytometry at various times. (C) Time course of GFP transfer and GFP synthesis after infection of peripheral blood B cells with EBV or EBV TR–.

FIG. 2. Rapid activation and proliferation of B cells by EBV. (A) Rapid emergence of lymphoblastoid B cells after infection with EBV, but not after pseudoinfection with EBV TR–. Primary peripheral B cells were treated with virus or virus-like particles and analyzed by flow cytometry after various times. Seven days after EBV infection, viable cells are concentrated within the lymphoblastoid cell gate ("L"). (B) Increase in B cells with a lymphoblastoid phenotype over time. (C) Induction of the costimulatory molecule CD86 on peripheral blood B cells after EBV infection. Total viable cells
were stained with a monoclonal anti-CD86 antibody and analyzed by flow cytometry; n.d., not determined (20 days after treatment with EBV TR–, cells are no longer viable).

FIG. 3. CpG DNA synergistically enhances B cell transformation by EBV. (A) Freshly purified peripheral blood B cells were treated with EBV or EBV TR– in the presence or absence of CpG DNA. Numbers of lymphoblastoid cells were determined on day 7 by flow cytometry. The graph shows these numbers divided by the number of total B cells on day 0. (B) Effect of delayed stimulation with CpG. Peripheral B cells were infected with EBV, and CpG was added after the indicated period. On day 7, the numbers of lymphoblastoid cells were determined by flow cytometry. (C) Effect of prior CpG stimulation. Freshly purified peripheral B cells received medium with or without CpG DNA. EBV was added at the same time (0 hours) or 6 hours, 1 day or 3 days later. Six days after EBV infection, lymphoblastoid cells were counted by flow cytometry. The ratio of cell yields (cell number obtained with CpG divided by cell number obtained without CpG) is shown numerically for each time condition. (D) Unseparated PBMCs were infected with EBV in the presence or absence of CpG DNA and of cyclosporin A. Lymphoblastoid cell outgrowth was analyzed 7 days post infection. (E) Effect of CpG on clonal outgrowth of EBV-infected B cells. Purified B cells were infected with EBV in the presence or absence of CpG DNA. After 14 days, limiting dilution was performed. The number of outgrowing EBV-transformed B cell clones was determined 14 days later. (F) Relative survival of peripheral blood B cells infected with EBV in the presence versus absence of CpG DNA. For several time points after infection, the graph shows the live/dead cell ratio in the presence of CpG DNA, divided by the live/dead cell ratio in the absence of CpG DNA.
FIG. 4. CpG DNA increases activation of B cells by EBV. (A) Expression of CD80 after infection of peripheral blood B cells with EBV or EBV TR– with or without CpG DNA was analyzed by flow cytometry. Secretion of IL-6 (B) and IL-10 (C) under the same treatment was analyzed by ELISA. A symbol (#) indicates that the amount of cytokine was below the detection limit. The effect of adding neutralizing IL-6 or IL-10-specific antibodies on the outgrowth of lymphoblastoid cells early after infection is shown in (D) and (E).

FIG. 5. Susceptibility of naive and memory B cells to EBV transformation and its enhancement by CpG DNA. Peripheral B cells were separated into CD27– (naive) and CD27+ (memory) subpopulations and infected with EBV or EBV TR– in the presence or absence of CpG DNA. At the indicated times, the number of lymphoblastoid cells was assessed by flow cytometry.

FIG. 6. Effect of CpG DNA on the expression of EBV latency genes at early times after infection. Primary B cells were infected with EBV immediately after preparation in the presence or absence of CpG DNA. After the indicated periods, cells were harvested for total RNA extraction. Quantities of EBNA1, EBNA2, and LMP1 mRNA relative to mRNA of β-glucuronidase (GUS) were determined by quantitative RT-PCR.

FIG. 7. Effect of CpG DNA on the expression of EBV lytic-cycle genes by established lymphoblastoid cell lines. Four different LCLs carrying EBV strain M-ABA, strain B95.8, or GFP-expressing recombinant EBV were cultivated in the presence or absence of CpG DNA for 2 or 5 days. Total RNA was extracted and levels of mRNAs coding for BZLF1 (immediate-early), BMRF1 (early), BLLF1 (late), BALF4 (late), and
BFLF2 (early) were determined by quantitative RT-PCR, relative to mRNA of β-glucuronidase (GUSB).

FIG. 8. Effect of various TLR ligands or whole bacteria on EBV transformation of tonsillar or peripheral B cells. (A) Outgrowth of lymphoblastoid cells after EBV infection of tonsillar B cells in the presence of various TLR ligands or fixed S. aureus bacteria at different concentrations. Lymphoblastoid cells were enumerated on day 7 by flow cytometry. In control experiments with EBV TR– instead of EBV, less than 0.1×10^3 lymphoblastoid cells were detected after 7 days of treatment with each TLR ligand (not shown). (B, C) Lymphoblastoid cell outgrowth was assessed by flow cytometry at several points of time after EBV infection of tonsillar B cells (B) or peripheral blood B cells (C) in the presence of various TLR ligands or fixed S. aureus. Note the smaller range of the y axis in the control experiment with EBV TR–.
TABLE 1. Release of transforming EBV by LCLs after treatment with CpG DNA.

<table>
<thead>
<tr>
<th>source of EBV</th>
<th>day 2 supernatants</th>
<th>day 5 supernatants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-CpG</td>
<td>+CpG</td>
</tr>
<tr>
<td>LCL LT-M-ABA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LCL LT-B95.8</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>LCL JN-B95.8</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>LCL ST-EBV/GFP</td>
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<td>0</td>
</tr>
<tr>
<td>medium</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EBV preparation</td>
<td>48</td>
<td>48</td>
</tr>
</tbody>
</table>

*Four different LCLs were cultivated in the presence or absence of CpG DNA for one day, washed, and cultivated in medium. On day 2 or day 5, culture supernatants were harvested, centrifuged to remove residual cells, and added to CD40-activated B lymphoblasts (LENL5) in 48 replicate cultures for each condition. Seven weeks later, cultures were visually inspected for outgrowth of EBV-transformed B cells. As controls, medium alone or a standard EBV preparation were added to LENL5 cells instead of LCL supernatants. All outgrowing B cells displayed green fluorescence.*
Figure 1

A

293 cell

EBV TR– protein transfer

EBV

protein & gene transfer

B

log GFP fluorescence

days post infection

1.0
1.5
2.0
2.5
3.0

TR– EBV

1dpi 3dpi

4dpi 7dpi

1 2 3 4 5 6 7

C

fold increase in mean GFP fluorescence

1.0
1.5
2.0
2.5
3.0

days post infection

EBV

TR–
Figure 2

(A) Side scatter and forward scatter plots of lymphoblastoid cells at various time points post infection. EBV-positive (EBV) and EBV-negative (TR-) populations are indicated.

(B) Graph showing the increase in lymphoblastoid cells x 10^3 over days post infection for EBV and TR- populations.

(C) Bar graph depicting the mean fluorescence of CD86 for EBV and TR- populations over days post infection, with n.d. indicating not determined.
Figure 4

A

B

C

D

E
Figure 5

![Graph showing lymphoblastoid cells x 10^3 over days post infection with various TR-EBV and CpG conditions.](http://jvi.asm.org/)

- **memory (CD27+)**
- **naive (CD27-)**

Days post infection:
- 1
- 4
- 7
- 14
Figure 6
Figure 7

LCL LT-M-ABA
LCL LT-B95.8
LCL JN-B95.8
LCL ST-EBV/GFP

day 2  day 5  day 2  day 5  day 2  day 5  day 2  day 5

relative expression
(target gene/β-actin)

CpG  –       +                        –       +           –        +                        –       +           –        +

0.01  0.1  1  10  0.01  0.1  1  10  0.01  0.1  1  10
Figure 8

A

B

C

EBV

TLR ligand (µg/ml)

CpG

P3CSK4

Imiquimod

LPS

LTA

S. aureus

1dpi

5dpi

7dpi

lymphoblastoid cells x 10^3

lymphoblastoid cells x 10^6

lymphoblastoid cells x 10^9

EBV

TR–