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Toll-Like Receptor Agonists Synergistically Increase Proliferation and Activation of B Cells by Epstein-Barr Virus[▽]

Stefanie Iskra,¹ Markus Kalla,¹ Henri-Jacques Delecluse,²
Wolfgang Hammerschmidt,¹ and Andreas Moosmann^{1,3*}

Department of Gene Vectors, Helmholtz-Zentrum München, Munich, Germany¹; Department of Virus-Associated Tumors, German Cancer Research Center, Heidelberg, Germany²; and Clinical Cooperative Group Molecular Oncology, Helmholtz-Zentrum München, and Ludwig-Maximilians-Universität, Munich, Germany³

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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 posttransplant lymphoproliferative disease. Human B-cell proliferation is also driven by ligands of Toll-like receptors (TLRs), 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 of both 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.

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 modes 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, as well as causing 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, 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 such as CD86, a T-cell-costimulatory molecule (24). Additional immune effectors, the cytokines interleukin-6 (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 the expression of TLR7 and its downstream signaling mediators (40). Additional mechanisms have recently been proposed to explain 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 (IM), 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 exog-

* Corresponding author. Mailing address: Helmholtz-Zentrum München, Marchioninistr. 25, 81377 Munich, Germany. Phone: 49-89-7099-202. Fax: 49-89-7099-225. E-mail: andreas.moosmann@helmholtz-muenchen.de.

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enous 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% fetal calf serum (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. Where noted, cyclosporine (1 µg/ml; Novartis) was used in peripheral blood mononuclear cell (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 green fluorescent protein (GFP)-encoding recombinant EBV genome based on EBV strain B95.8 (14). EBV genome-free virus-like particles were produced in a cell line (293/TR⁻) 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 3 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 Biotec, 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 Biotec). 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, 10⁵ purified primary B cells or Raji B cells in 500 µl were infected with various amounts of supernatant. After 1 day, the mean green fluorescence intensity 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 by flow cytometry on day 3 after infection of primary B cells, as a surrogate marker. One lymphoblastoid cell on day 3 after infection was assumed to represent 1 unit of transforming EBV. At least up to a level of 0.1 transforming unit 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 10⁵ 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 unit 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 × 10⁴ cells in 250 µl 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 µg/ml; Pam₃CSK₄, 1 µg/ml; imiquimod, 0.5 µg/ml; lipopolysaccharide (LPS) from *Salmonella enterica* serovar Minnesota, 1 µg/ml; lipoteichoic acid (LTA) from *Staphylococcus aureus*, 1 µg/ml (all from InvivoGen); and inactivated *Staphylococcus aureus* bacteria, 4 µg/ml (Pansorbin, Calbiochem).

Quantification of lymphoblastoid cell outgrowth and flow cytometry. For quantification of lymphoblastoid cell outgrowth, B cells were seeded at 2 ×

10⁵/ml, with or without EBV, EBV TR⁻, or TLR ligands, in 48-well plates in separate 250-µl or 500-µl cultures for each time of analysis and each condition. Immediately before analysis, the complete culture was thoroughly resuspended and directly transferred to a fluorescence-activated cell sorter (FACS) tube containing 20,000 allophycocyanin (APC)-labeled calibration beads (Becton Dickinson), to determine absolute cell numbers, and the membrane-impermeant DNA dye ToPro-3 to a final concentration of 0.1 µM, to discriminate live and dead cells (Molecular Probes). In most cases, 5,000 beads were acquired by 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 that of the resting B-cell population. Cells in the lymphoblastoid gate consistently stained as live cells (negative for ToPro3). For staining of cell surface molecules, 200,000 cells were harvested, washed, and stained with saturating amounts of CD86-APC or CD80-fluorescein isothiocyanate (CD80-FITC) antibodies (BD Pharmingen) in phosphate-buffered saline (PBS)-2% FCS for 15 min on ice. Cells were washed, fixed in 1% formaldehyde (Roth), and stored at 4°C until analyzed 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 ToPro3 staining, and quantified with calibration beads as described above. All flow cytometric analyses were performed on a Becton Dickinson FACSCalibur flow cytometer equipped with CellQuest software.

Quantification of EBV release by LCLs. EBV release by lymphoblastoid cell lines (LCLs) after incubation with CpG or medium alone was quantified by infection of the EBV-free CD40-stimulated B-cell line L5175 (56). This cell line is maintained on fibroblasts expressing CD40L. In the absence of these fibroblasts, L5175 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 min and again at 1,600 × g for 15 min 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 × 10⁵ L5175 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 with a fluorescence microscope.

ELISA. For cytokine detection, supernatants of infected/stimulated B cells were harvested at different times and stored at -20°C. Enzyme-linked immunosorbent assays (ELISAs) for interleukin-6 (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 antibody against IL-6 (LEAF; Biolegend) or IL-10 (clone 12G8; Mabtech) was directly added to the infection/stimulation mixture. Neutralization was verified by ELISA.

Limiting-dilution analysis and quantification of B-cell clones. B cells (10⁵) were infected as described above in the presence and absence of CpG DNA. Cyclosporine (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 analysis was performed in round-bottom 96-well plates. After an additional 4 weeks, outgrowth of B-cell clones was quantified by the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay. MTT solution (10 µl; 5 mg/ml in PBS) and medium (40 µl) were added to B-cell cultures (50 µl). After 3 h 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 nonoutgrowth, a fixed cutoff value for 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 upon visual inspection.

Quantitative reverse transcription-PCR (RT-PCR). At the time of analysis, total RNA was extracted from primary B-cell cultures or LCL cultures by use of an RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. RNA was treated with DNase I (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 PCRs 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 microliter of 20 µl of cDNA product was used as the template for PCR amplification of selected EBV genes. Real-time PCR was performed with a LightCycler machine (Roche) according to the manufacturer's instructions. Quantification of reverse-transcribed transcripts was carried out by using LightCycler FastStart reaction mix (SYBR green I; Roche). The amplification of PCR products was monitored online and usually stopped after

40 cycles. The following settings were used: initial template denaturation for 10 min at 95°C and cycles of 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 sequences are given in the 5'-to-3' direction): for *EBNA1*, TGA CAA AGC CCG CTC CTA CC and CTC ACC CTC ATC TCC ATC ACC TC; for *EBNA2*, CAC ACG GCA ACC CCT AAC G and GGT CCC TCC ACA TAA TCT TCA TCT G; for *LMP1*, GTG TCT GCC CTC GTT GGA GTT AG and CAT CCT GCT CAT TAT TGC TCT CTA TCT AC; for *BZLF1*, GGG GCA AGC AAA CAC CAC TG and CAA CCG CTC CGA CTG GGT C; for *BMRF1*, TTG AGG TTT TAC AGG TCT GGC ATC and GGT GGC GGA GGT GAA GGA G; for *BLLF1*, ACC GAG CAT TTC TGT TTT TAC GC and GAT GTC TAC TTT CAA GAT GTG TTT GGA AC; for *BALF4*, CTG GGG GGT GAG GAA GTC G and CAA CAC AAC CGT GGG CAT AGA G; and for *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* (for 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 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⁻ cells), 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 which are incapable of transforming B cells (15, 18). These virus-like particles (designated "EBV TR⁻") specifically bind to and are taken up by B cells, in a similar fashion to that for 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 and C). GFP protein transfer to primary B cells was readily detectable on day 1 after infection and was equally mediated by EBV and 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 uptake-ready 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 number and dom-

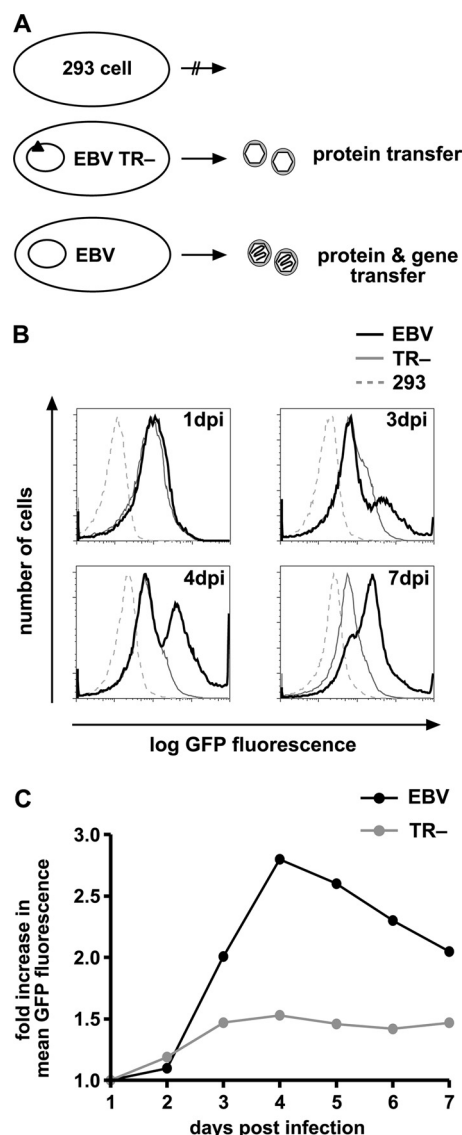


FIG. 1. System to compare the biological effects of EBV and EBV-derived virus-like particles. (A) Virus and virus-like particles were produced by cell lines based on 293 human epithelial kidney cells stably transfected with recombinant EBV genomes coding for GFP. Producer cells that carry EBV genomes 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 a 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 analyzed 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⁻.

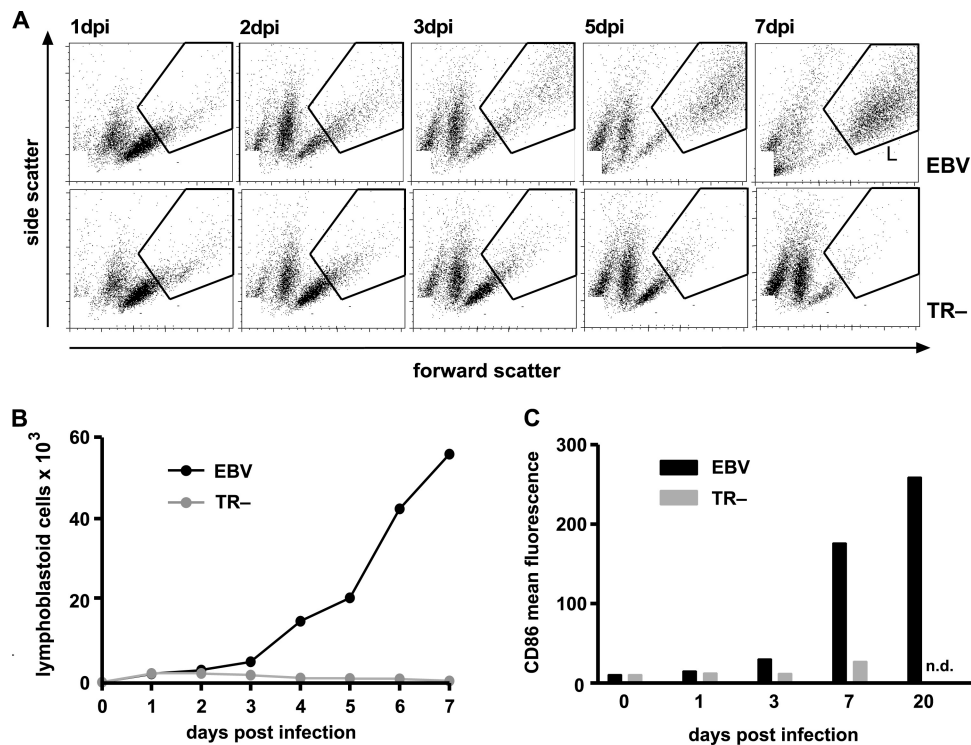


FIG. 2. Rapid activation and proliferation of B cells in response to EBV. (A) Rapid emergence of lymphoblastoid B cells after infection with EBV but not after pseudoinfection with EBV TR⁻. Primary peripheral B cells were treated with virus or virus-like particles and analyzed by flow cytometry after various times. Seven days after EBV infection, viable cells were 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 were no longer viable).

inated the cultures by day 7 after infection, consistent with the anticipated effects of EBV-mediated B-cell transformation (Fig. 1B and C). Taken together, GFP protein transfer, which is rapid and 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 in response to EBV. Upon infection with EBV, resting B lymphocytes assume a lymphoblastoid phenotype, with a larger cell size, enlarged cytoplasm, expression of activation markers, and proliferation, and they ultimately grow out as transformed stable cell lines called lymphoblastoid cell lines (LCLs) (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 levels emerged and continuously expanded thereafter (Fig. 2A and 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 by flow cytometry. Such cells were invariably GFP positive (not shown); however, because scatter analysis allowed an even clearer discrimination of B-cell subpopulations than that by GFP fluorescence (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 lymphoblas-

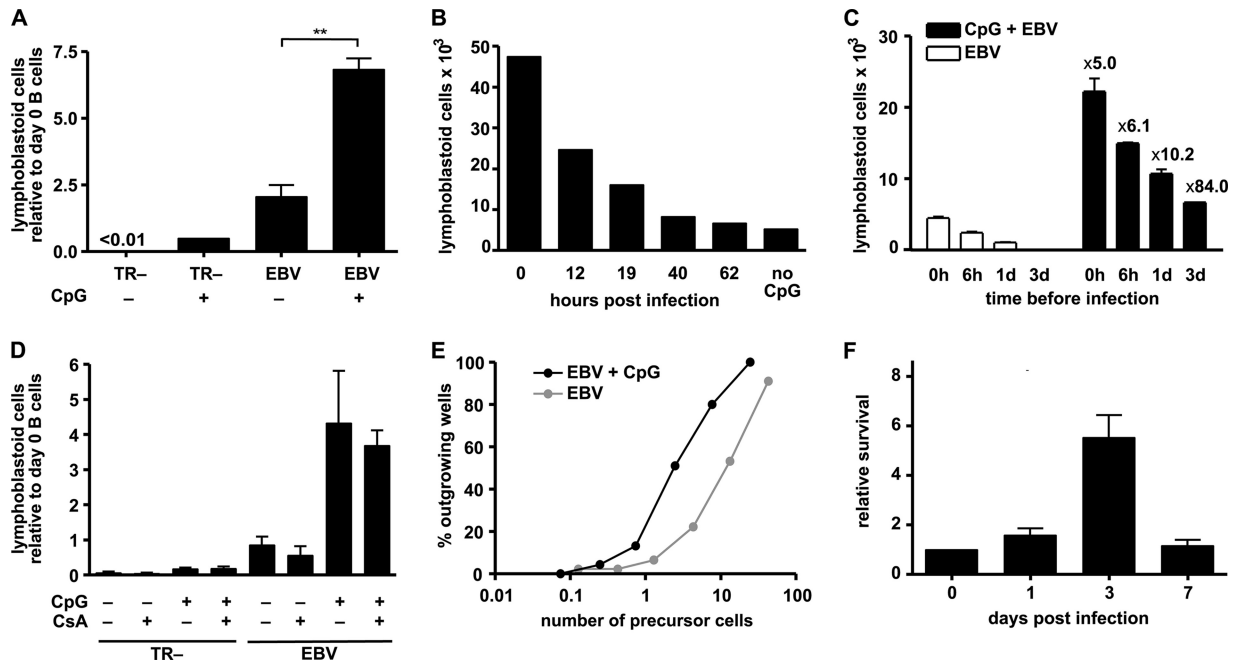


FIG. 3. CpG DNA synergistically enhances B-cell transformation by EBV. (A) Freshly purified peripheral blood B cells were treated with EBV or EBV TR⁻ in the presence or absence of CpG DNA. Numbers of lymphoblastoid cells were determined on day 7 by flow cytometry. The graph shows these numbers divided 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 h) or 6 h, 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/or cyclosporine (CsA). Lymphoblastoid cell outgrowth was analyzed at 7 days postinfection. (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 analysis 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.

toid cells. When combined, EBV and CpG DNA induced the outgrowth of seven lymphoblastoid cells per B cell in the starting population, which is clearly a 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 h after infection (Fig. 3B). Conversely, when B cells were infected with EBV with some delay after CpG stimulation, outgrowth of B cells was reduced in absolute terms compared to that for 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 1 day, CpG-pretreated B cells yielded 10 times more lymphoblastoid cells than did medium-pretreated cells (Fig. 3C).

To test whether other immune effector cells could influence this EBV-CpG synergism, we repeated the experiment with total PBMCs instead of purified B cells, in the presence or absence of cyclosporine, 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 effects of CpG DNA on EBV-mediated lymphoblastoid cell outgrowth from PBMCs (Fig. 3D) and from purified B

cells (Fig. 3A) were very similar, and inhibition of T-cell activation by cyclosporine 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 7 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 transformed permanently by EBV. To investigate this possibility, we infected B cells with EBV in the presence or absence of CpG DNA, washed out the remaining CpG DNA after 14 days, subjected 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 assays 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 (Fig. 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 (Fig. 3A and D). Therefore, the presence of CpG DNA at the time of

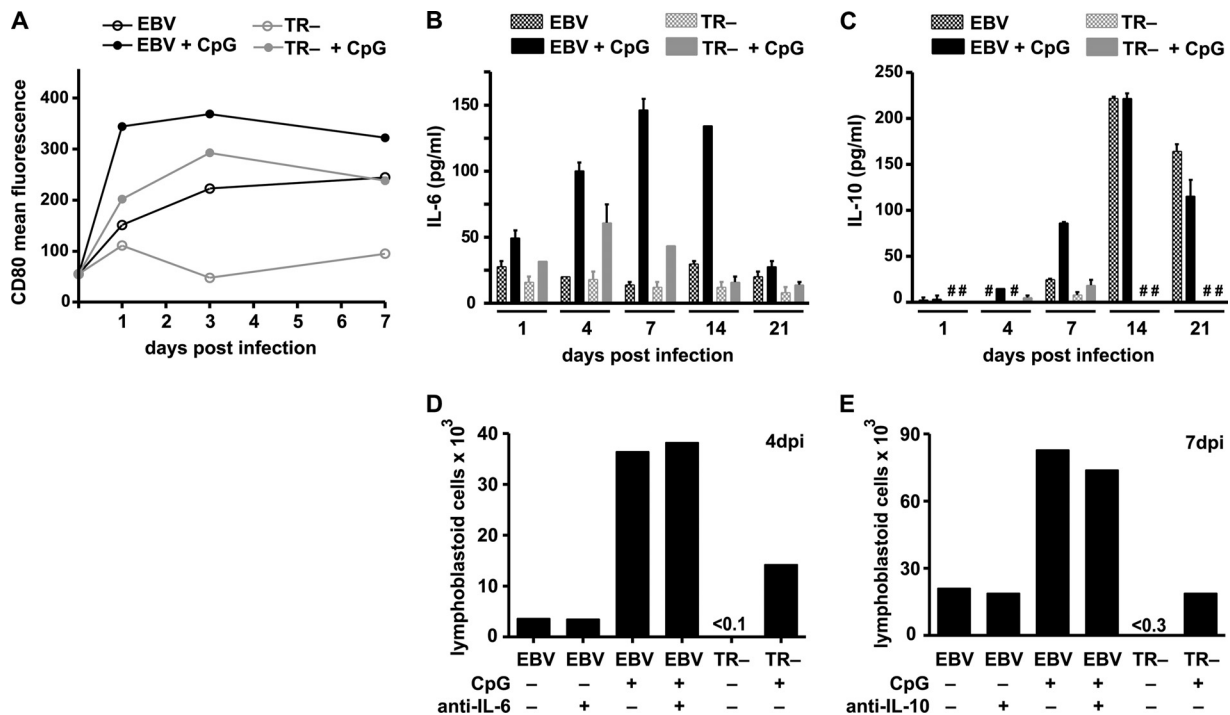


FIG. 4. CpG DNA increases activation of B cells by EBV. (A) The expression of CD80 after infection of peripheral blood B cells with EBV or EBV TR⁻, with or without CpG DNA, was analyzed by flow cytometry. Secretion of IL-6 (B) and IL-10 (C) under the same treatment conditions was analyzed by ELISA. #, the amount of cytokine was below the detection limit. (D and E) Effects of adding neutralizing IL-6 (D)- or IL-10 (E)-specific antibodies on the outgrowth of lymphoblastoid cells early after infection.

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. Using flow cytometry, we determined live/dead cell ratios in EBV-infected/CpG-treated versus EBV-infected B-cell cultures. Interestingly, the addition of CpG DNA together with EBV in early infection led to a 6-fold higher relative survival rate on day 3, the day when lymphoblastoid cell outgrowth was initiated (Fig. 3F). This result showed that although CpG DNA had to be present within a day after infection to have an effect (Fig. 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 such as 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 in 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 (Fig. 4A).

Activated B cells secrete cytokines such as IL-6 and IL-10. Both of these cytokines influence B-cell growth and differentiation. IL-6 was described as an autocrine growth factor of established LCLs (53, 58). IL-10 favors 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 (Fig. 4B), as it was 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 under the different reaction conditions (compare Fig. 4C to Fig. 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, either after EBV infection alone or after infection in the presence of CpG DNA (Fig. 4D and 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 the cell density is higher or as the cytokine accumulates over time *in vitro*. We concluded that 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

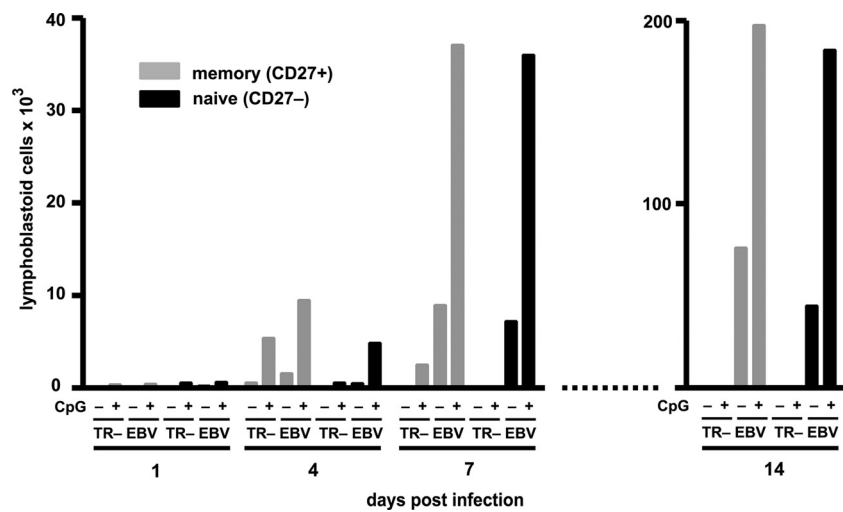


FIG. 5. Susceptibility of naive and memory B cells to EBV transformation and its enhancement by CpG DNA. Peripheral B cells were separated into CD27⁻ (naive) and CD27⁺ (memory) subpopulations and infected with EBV or EBV TR⁻ in the presence or absence of CpG DNA. At the indicated times, the number of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

TLR9 than do activated or memory B cells and react with much weaker proliferation to CpG DNA than do 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 in 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 from both naive and memory B cells, and the effects were quantitatively very similar for both B-cell subpopulations 7 and 14 days after infection (Fig. 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 B cells, not naive B cells. In these experiments, the purity of the B-cell subpopulations was limited (CD27⁺ cells, 98%; CD27⁻ cells, 81%) but sufficient to support the conclusion that the EBV-CpG synergism targets both the naive and the memory B-cell compartments, 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- κ B 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 h

and 3 days after infection, normalized to expression of the β -glucuronidase gene. We could clearly visualize the subsequent induction of the EBNA2, EBNA1, and LMP1 genes during EBV infection (Fig. 6). However, the presence or absence of CpG DNA made little difference. It appears that EBV's transforming gene expression program 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 suggested that TLR agonists might contribute to the expansion of EBV's physiological reservoir, latently infected B cells. Taking this one step further, TLR agonists might contribute to the mobilization of EBV from this reservoir by favoring lytic EBV replication. Previous studies on murine herpesvirus 68 (MHV-68), EBV's homolog in mice, suggest that this is the case (19, 20), although other studies appear to contradict it (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

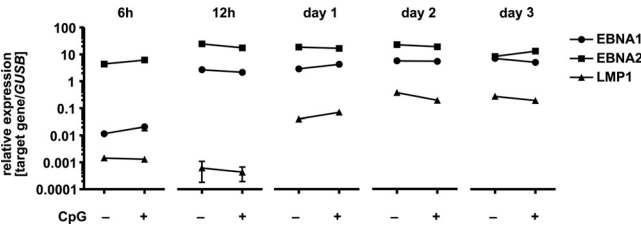


FIG. 6. Effect of CpG DNA on expression of EBV latency genes at early times after infection. Primary B cells were infected with EBV immediately after preparation in the presence or absence of CpG DNA. After the indicated periods, cells were harvested for total RNA extraction. Quantities of *EBNA1*, *EBNA2*,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

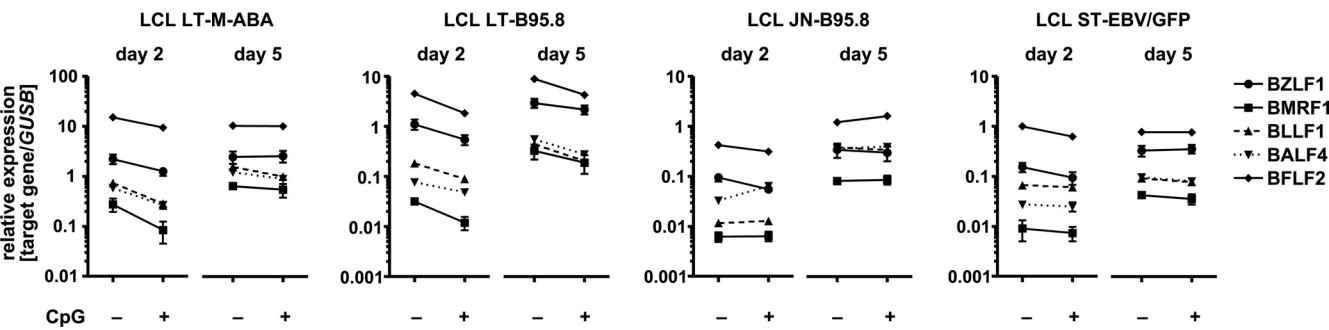


FIG. 7. Effect of CpG DNA on expression of EBV lytic-cycle genes by established lymphoblastoid cell lines. Four different LCLs, carrying EBV strain M-ABA, EBV strain B95.8, or GFP-expressing recombinant EBV, were cultivated in the presence or absence of CpG DNA for 2 or 5 days. Total RNA was extracted,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

of five genes from EBV's immediate-early, early, and late lytic programs 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 in small 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 particles that could infect and transform a sensitive B-cell line, we obtained transformants exclusively with supernatants from CpG-treated LCLs, not 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 favor 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 (TLR2 ligand), imiquimod (TLR7 ligand), LPS (TLR4 ligand), lipoteichoic acid (TLR2 ligand), and whole fixed *Staphylococcus aureus* bacteria.

Source of EBV	No. of outgrowing cultures ^a			
	Day 2 supernatants		Day 5 supernatants	
	–CpG	+CpG	–CpG	+CpG
LCLs				
LT-M-ABA	0	0	0	0
LT-B95.8	0	2	0	1
JN-B95.8	0	3	0	0
ST-EBV/GFP	0	0	0	0
Medium	0		0	
EBV preparation	48		48	

^a Four different LCLs were cultivated in the presence or absence of CpG DNA for 1 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 cells) 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 was added to LENL5 cells instead of LCL supernatants. All outgrowing B cells displayed green fluorescence.

We infected tonsillar B cells with EBV in the presence of these TLR ligands or CpG DNA at various concentrations. Lymphoblastoid cell outgrowth was analyzed 7 days after infection (Fig. 8A). Several TLR ligands favored 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 a high concentration, and lipopolysaccharide had no effect. CpG DNA appeared to have the fastest effect on EBV-mediated B-cell outgrowth (Fig. 8B), but some of the other TLR agonists also discernibly 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 (Fig. 8B, lower panel). Effects of the various TLR agonists on EBV-mediated activation of tonsillar (Fig. 8B) and peripheral blood (Fig. 8C) B cells were closely similar. We concluded that not only agonists of TLR9 but also those of other TLRs, such as TLR2, support EBV-driven B-cell activation and early transformation.

DISCUSSION

In this study, we analyzed the effects 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 found in the present study 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

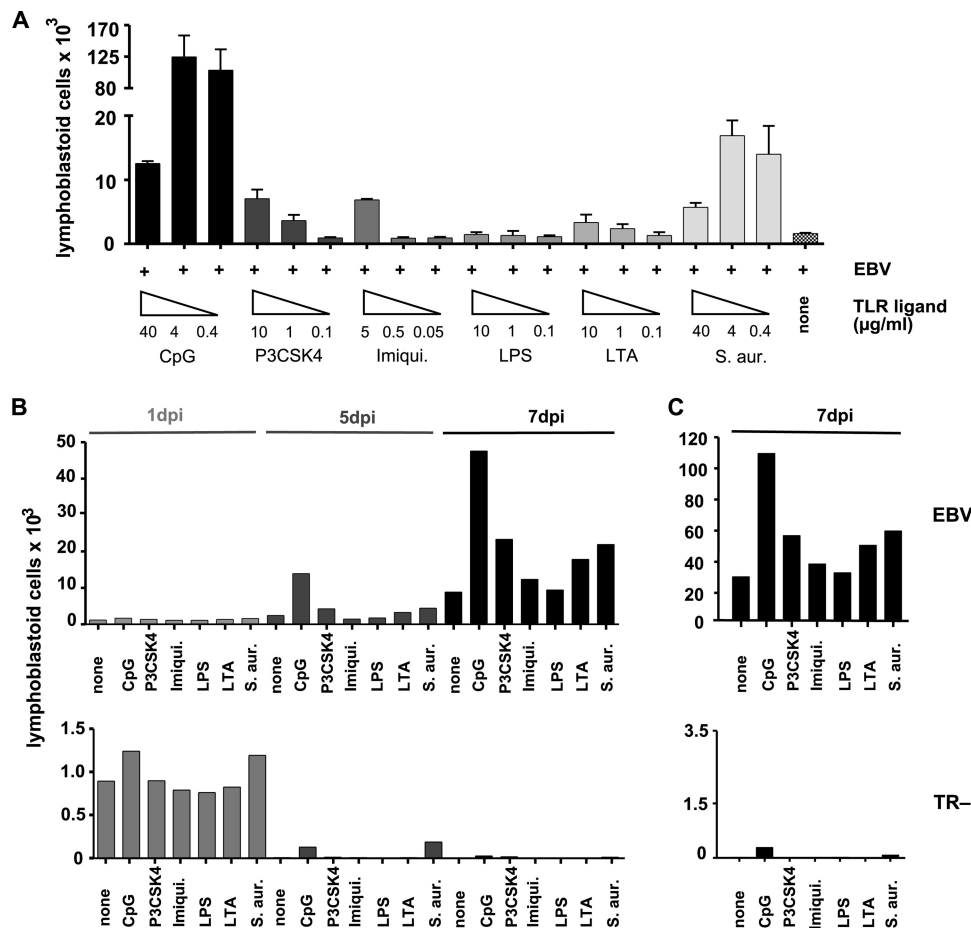


FIG. 8. Effects of various TLR ligands or whole bacteria on EBV transformation of tonsillar or peripheral B cells. (A) Outgrowth of lymphoblastoid cells after EBV infection of tonsillar B cells in the presence of various TLR ligands or fixed *S. aureus* bacteria at different concentrations. Lymphoblastoid cells were enumerated on day 7 by flow cytometry. In control experiments with EBV TR⁻ instead of EBV, fewer than 0.1×10^3 lymphoblastoid cells were detected after 7 days of treatment with each TLR ligand (not shown). (B and C) Lymphoblastoid cell outgrowth was assessed by flow cytometry at several time points 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⁻. Imiqui., imiquimod.

bacteria colocalized at sites of EBV infection or replication—for example, in tonsillitis during infectious mononucleosis—might favor EBV establishment and spread by increased proliferation of infected B cells and, possibly, by favoring 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 (33). 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 *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,

which are expressed only in EBV’s transforming mode, latency III (25, 41). These T-cell specificities, among others, may be required to prevent 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 posttransplantation 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 coinfection 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, which is less severe than PTLD but much more frequent, is 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 percentage points of peripheral B cells may be EBV infected. In IM-associated tonsillitis (34) 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 large 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 colocalized B cells. The factors that predispose some adolescents and adults to IM, while in others primary EBV infection remains asymptomatic, remain unknown. Individual variations in the nature and magnitude of microbial coinfection 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. 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 yet clear. 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 gammaherpesvirus infection is further demonstrated by a recent study showing that exogenous ligands of TLRs 3, 4, 5, and 9 directly stimulate *in vivo* reactivation of 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 did 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 during primary EBV infection, some of which may favor 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 such as the costimulatory ligands CD80 and CD86 on the B-cell surface might favor elimination of infected B cells by cytotoxic CD8 or CD4 T cells. Recruitment and actions of T cells and other immune effectors might be modulated further 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 nonspecific) 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 a "helper" function, by favoring rather than restricting outgrowth of EBV-transformed B cells (38). 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 remains an open question.

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 (PRRs). Like other herpesviruses, EBV activates TLR2 by binding of virions to the surfaces of monocytes, eliciting the secretion of proinflammatory cytokines (21). In our experiments, we did not observe that B-cell activation or proliferation was triggered by viral particles without a transforming genome (EBV TR⁻) (Fig. 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 added to human monocytes was found to trigger TLR2 (2), and it was 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 (Fig. 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 the 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 favor 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 respond strongly 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.

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