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Distinct Ex Vivo Susceptibility of B-Cell Subsets to Epstein-Barr Virus Infection According to Differentiation Status and Tissue Origin▽

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Epstein-Barr virus (EBV) uses tonsils as the portal of entry to establish persistent infection. EBV is found in various B-cell subsets in tonsils but exclusively in memory B cells in peripheral blood. The in vitro susceptibilities of B-cell subsets to EBV infection have been studied solely qualitatively. In this work, we examined quantitatively the in vitro susceptibilities of various B-cell subsets from different tissue origins to EBV infection. First, we established a centrifugation-based inoculation protocol (spinoculation) that resulted in a significantly increased proportion of infected cells compared to that obtained by conventional inoculation, enabling a detailed susceptibility analysis. Importantly, B-cell infection occurred via the known EBV receptors and infected cells showed EBV mRNA expression patterns similar to those observed after conventional inoculation, validating our approach. Tonsillar naïve and memory B cells were infected ex vivo at similar frequencies. In contrast, memory B cells from blood, which represent B cells from various lymphoid tissues, were infected at lower frequencies than their naïve counterparts. Immunoglobulin A (IgA)-positive or IgG-positive tonsillar memory B cells were significantly more susceptible to EBV infection than IgM-positive counterparts. Memory B cells were transformed with lower efficiency than naïve B cells. This result was paralleled by lower proliferation rates. In summary, these data suggest that EBV exploits the B-cell differentiation status and tissue origin to establish persistent infection.

Epstein-Barr virus (EBV) infects over 90% of the world's population and establishes life-long persistence in the memory B cells of the infected host (32). In vitro, EBV can transform and immortalize B cells to generate lymphoblastoid cell lines (LCLs) (19). That characteristic points at the potential neoplastic role of EBV in several tumors of B-cell origin in humans, including Burkitt's lymphoma, Hodgkin's disease, and posttransplant lymphoproliferative disease (8, 21).

The mucosal lymphoid tissues of the Waldeyer's ring, including tonsils, act as the original site of EBV infection and the reservoir for the virus where B-cell infection initiates during infectious mononucleosis (2–4, 24, 28). EBV is detected in both naïve and memory B cells when infected tonsillar tissue is examined (3). By contrast, EBV is found exclusively in memory B cells when peripheral blood is examined (2, 18). Notably, EBV-infected naïve B cells isolated from tonsils express a lymphoblastoid phenotype corresponding to latent EBV genes, whereas EBV-infected memory B cells from tonsils express a more restricted pattern (3). In contrast, the EBV-infected memory B cells isolated from the peripheral blood display a very restricted pattern of EBV latent gene expression, in which no latent EBV genes, with the possible exception of *LMP2*, are expressed (3). Thus, B cells from distinct compartments show different EBV infection and gene expression patterns. Notably, the cited data from in vivo studies are purely descriptive and no data on the relative EBV susceptibilities of naïve and memory B cells from different compartments are yet available. In particular, we do not know to what extent the pool of EBV-infected memory B cells in the tonsils is derived from naïve EBV-infected B cells that have gone through a germinal-center reaction or how the pools of infected cells are influenced by distinct proliferation rates of naïve and memory B cells following EBV infection. Also, given the different EBV gene expression patterns in naïve and memory tonsillar B cells, EBV-infected cells from different subsets may be subjected to differential recognition by EBV-specific immune responses, which in turn may impact the survival and thus the frequencies of these cells. Information on the relative susceptibilities of the various B cells and the subsequent phenotypic changes would be highly desired for a detailed understanding of EBV pathogenesis.

There are only limited detailed quantitative data available about the susceptibilities to EBV infection of distinct B-cell subsets. It appears that EBV infects various subsets of B cells from tonsils, adenoids, or peripheral blood in vitro (3, 6, 11, 15). In very early studies, the general susceptibility of B cells to EBV infection in vitro was demonstrated by assessing transformation rates of nonseparated (6) or isolated (15) B cells. Transformation, however, provides only indirect information about EBV infectivity and does not take into account the proliferation and apoptosis rates of EBV-infected B-cell subsets. More recent studies isolated tonsillar or adenoidal B cells on the basis of CD10, CD77, and/or surface immunoglobulin isotype expression (3, 11). Nowadays, memory B cells can be more accurately identified thanks to the specific memory B-cell

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marker CD27 (20, 41). Most importantly, published work on the in vitro infection of B-cell subsets has addressed the issue of susceptibility to EBV infection only qualitatively, and the efficiencies of infection of B-cell subsets from distinct tissues have not been studied in detail. However, quantitative differences in susceptibilities of B cells to EBV infection may depend on the tissue origin, and the elucidation of these differences will give further insight into EBV pathogenesis.

The aim of this work was to assess the efficiencies of EBV infection of distinct B-cell subsets from different lymphoid compartments and to compare the transformation behaviors of B-cell subsets infected ex vivo with EBV. To enable these investigations, we established a centrifugation-based inoculation protocol, so-called spinoculation, using a recombinant EBV encoding enhanced green fluorescent protein (EGFP) (39, 40). This procedure resulted in significantly higher proportions of cells infected ex vivo than conventional inoculation and enabled us to identify EBV-infected B-cell subsets as early as 24 h after infection and to characterize EBV-induced proliferation of naïve and memory B cells. Since EBV-specific immune responses may impact the cellular infection and transformation processes, we used primary cells exclusively from EBV-naïve individuals.

MATERIALS AND METHODS

Cell culture. The EBV-infected marmoset cell lines B95.8 (27) and B95.8EBfaV-GFP (39) were maintained in RPMI 1640 medium (Gibco, Basel, Switzerland) with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin, referred to hereafter as complete medium. Primary cells were cultivated in complete medium supplemented with 1 μM sodium pyruvate and 1× nonessential amino acids (Gibco).

Isolation of mononuclear cells. Primary human mononuclear cells were isolated from palatine tonsils obtained from EBV-seronegative patients undergoing routine tonsillectomy or from blood samples taken from healthy EBV-seronegative adult donors. Tonsillar mononuclear cells (TMC) were prepared as described previously (14). Briefly, tonsils were disintegrated with a scalpel in complete medium and passed through a 70-μm-pore-size cell strainer (Falcon, Wohlen, Switzerland). For the isolation of B-cell subsets, TMC were further purified by density gradient centrifugation with Ficoll-Hypaque (GE Healthcare, Stockholm, Sweden). Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque density gradient centrifugation according to the instructions of the Ficoll-Hypaque manufacturer. The viability of primary cells, as determined by trypan blue exclusion, was >99% in all preparations. The EBV serostatuses of the mononuclear cell donors were determined by using the Immunodot Mono G and Mono M kit according to the instructions of the manufacturer (Ruwag Diagnostics, Bettlach, Switzerland). Informed consent was obtained from subjects or parents before the study. The institutional ethics committee approved the collection and use of clinical material.

Preparation of virus stock. B95.8 and B95.8EBfaV-GFP cells were seeded at a density of 10^6 cells/ml and were stimulated to release virus by being cultured for 4 days in complete medium containing 50 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich, Buchs, Switzerland)/ml. Cell suspensions were centrifuged at 400 × *g* for 5 min. Supernatant was passed through a 0.45-μm-pore-size cellulose acetate filter (Millipore, Zug, Switzerland) and stored at −80°C.

Infection of primary cells. EBV inoculation was done as described earlier (23). Briefly, 1 ml of supernatant from EBV-producing cell lines was added to 2×10^6 cells in 1 ml of complete medium for 3 h at 37°C. For so-called conventional inoculation, supernatants from TPA-induced EBV-producing cells lines were used. Cell suspensions were centrifuged at 300 × *g* for 5 min, and cells were resuspended in fresh complete medium.

For spinoculation, EBV-containing supernatants were concentrated using a Vivaspin 20 concentrator with polyethersulfone and a molecular weight cutoff of 1,000,000 according to the instructions of the manufacturer (Sartorius-Stedim, Dietikon, Switzerland). Primary cells were centrifuged at 300 × *g* for 5 min, and the medium was completely replaced by the concentrated virus-containing supernatants to a final concentration of 10^6 cells/ml. The cell suspensions were then centrifuged for 1 h at 800 × *g* at 24°C. After spinoculation, cells were washed in phosphate-buffered saline and resuspended in fresh complete medium.

Flow cytometry. Flow cytometry was carried out on a Cytomics FC500 instrument (Beckman Coulter, Nyon, Switzerland) with FlowJo software, used in accordance with the instructions of the manufacturer (Treestar, Ashland, OR).

Antibodies. Fluorochrome-conjugated monoclonal antibodies directed against human CD19, CD21, CD27, HLA-DR, HLA-DP, HLA-DQ, immunoglobulin D (IgD), IgM, IgG, and IgA were purchased from BD Pharmingen (a division of BD Biosciences, Basel, Switzerland). Unconjugated and fluorescein isothiocyanate (FITC)-conjugated antibodies directed against EBV gp350/220 were purchased from Chemicon. Anti-EBV gp42 was a generous gift from L. Hutt-Fletcher (Shreveport, LA). Rabbit antibodies against poly(ADP-ribose) polymerase (PARP) and actin were from Cell Signaling Technology (Danvers, MA).

Inhibition of EBV infection. Concentrated EBV-containing supernatants were treated with either anti-gp350/220, anti-gp42, or a combination of the two antibodies at dilutions of 1:10, 1:100, and 1:1,000 at 4°C for 1 h. TMC were infected with the concentrated supernatants by spinoculation at 4°C to prevent virus entry and stained with FITC-anti-gp350/220, and the amount of bound virus was determined by flow cytometry. TMC were also analyzed for the expression of EGFP 24 h after inoculation to evaluate the number of EBV-infected cells. Furthermore, TMC were pretreated with anti-CD21 and anti-HLA-DR, anti-HLA-DP, and anti-HLA-DQ at dilutions of 1:10, 1:100, and 1:1,000 for 1 h at 4°C before inoculation with EBV by spinoculation. TMC were analyzed for EBV infection 24 h after spinoculation by flow cytometric quantification of EGFP expression.

Isolation of B-cell subpopulations. B cells were isolated using the B-cell isolation kit II according to the instructions of the manufacturer (Miltenyi Biotech, Bergisch Gladbach, Germany). Further separation into naïve and memory B cells was performed using the naïve-B-cell isolation kit (Miltenyi Biotech) or CD27 microbeads (Miltenyi). IgM-positive B cells were isolated by treating B cells with phycoerythrin-conjugated anti-human IgM and separating the cells with the PE multisort kit (Miltenyi). To obtain IgM-positive memory B cells, cells were further subjected to CD27 microbead isolation. The purity of isolated B-cell subsets was determined by flow cytometry using antibodies to human CD19, CD27, IgD, IgM, IgG, and IgA for discrimination between naïve and memory B cells. Purified cell populations used for experiments were always >95% pure.

RNA extraction and quantitative real-time PCR. The preparation of total RNA was performed using an RNeasy mini kit according to the instructions of the manufacturer (Qiagen, Hombrechtikon, Switzerland). After DNase I digestion (Ambion, Rotkreuz, Switzerland), RNA was reverse transcribed using an Omniscript reverse transcription-PCR kit (Qiagen). Quantitative PCR for EBV gene mRNA was performed using specific primers and probes for *EBNA2*, *EBNA1*, *EBNA3A*, *EBNA3C*, *LMP1*, *LMP2*, or *BZLF1* as described previously (5, 23, 34). All reactions were performed on a real-time PCR machine (ABI Prism 7700; Applied Biosystems) with TaqMan mastermix (Eurogentec, Seraing, Belgium) by using *HMBS* as a housekeeping gene. Cycling conditions were as follows: a 10-min denaturation step at 95°C was followed by 40 cycles of denaturation for 15 s at 95°C and annealing and synthesis for 1 min at 60°C.

Transformation assay. Transformation efficiency was assessed by a modification of the classical protocols (35). Briefly, either unseparated TMC or isolated naïve and memory B cells were infected with B95.8 EBV by either conventional inoculation or spinoculation. The wild-type B95.8 EBV was used to exclude potential interference of the recombinant EBV with the transformation process. To provide similar culture conditions for purified B cells and mononuclear cells, we added B-cell-depleted TMC as a feeder layer for ex vivo EBV-infected naïve and memory B cells immediately after spinoculation (29). One half of the medium was changed every 3 days for 4 weeks in all wells initially used.

Distributions of naïve and memory B cells were evaluated by flow cytometry 1, 2, 3, and 4 weeks after infection. After 4 weeks, wells still showing proliferation were suggested to contain transformed B cells (35). At that time, the proportion of B cells in all measured samples was above 98%. The transformation efficiency was calculated by counting the wells that contained actively proliferating B cells 4 weeks following infection.

Western blotting. For Western blotting, whole-cell extracts were prepared from 10^6 cells with RIPA buffer (50 mM Tris-Cl, pH 6.8, 100 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate) supplemented with complete mini protease inhibitor cocktail (Roche Applied Sciences, Rotkreuz, Switzerland) and 1 mM sodium orthovanadate (Sigma-Aldrich) for 20 min on ice. For the detection of primary antibodies, we used horseradish peroxidase-labeled goat anti-rabbit or anti-mouse antibodies from Pierce Biotechnology (Rockford, IL). Signal detection was performed by using the chemiluminescence substrate from Pierce and scanning directly with the ChemiGenius imaging system (SynGene,

Cambridge, United Kingdom), and signals were quantified with the GeneTools 3.1 image analysis software (SynGene).

ATP uptake assay. For ATP uptake, naïve and memory B cells were seeded at a density of 0.8×10^6 cells per ml in complete medium and were allowed to proliferate for 24 h. ATP uptake was measured using the ViaLight bioassay kit according to the instructions of the manufacturer (Lonzabiosciences, Basel, Switzerland). As a reference, a standard curve was used to determine the absolute cell numbers.

Statistical analyses. All experiments were carried out at least three times, and representative results from the experiments are presented. Statistical analyses of significance (P values) were based on a two-tailed paired t test. P values of <0.05 were regarded as statistically significant.

RESULTS

Spinoculation with concentrated virus substantially increases the proportion of primary B cells infected by EBV ex vivo. We first established an infection protocol that would provide a sufficient number of infected cells for analysis. Standard ex vivo inoculation protocols for primary human mononuclear cells yield proportions of EBV-infected cells of up to 2% of total B cells (40). We thus adapted a protocol originally designed to infect T cells with human immunodeficiency virus type 1 that is based on low-speed centrifugation of cells in human immunodeficiency virus type 1-containing supernatants (spinoculation) (31). The low-speed centrifugation is thought to facilitate virus binding to the cells, in contrast to pelleting of the virus (31). To rapidly identify EBV-infected cells, we used EBfaV-GFP, a recombinant EBV carrying a gene for EGFP that replaces the *LMP2* gene (39). The cytomegalovirus immediate-early promoter is active in naïve and memory B cells (22), drives the constitutive expression of EGFP (40), and thus allows the identification of all EBV-infected B cells as early as 24 h postinfection (40).

Following ex vivo inoculation of TMC with supernatants from the EBV-producer cell line EBfaV-GFP, <1% of B cells were infected at 48 h (Fig. 1A). Conventional inoculation using supernatants from TPA-induced EBfaV-GFP infected up to around 2% of B cells, even if the supernatants were concentrated 50-fold. Spinoculation with supernatants from non-TPA-induced EBfaV-GFP infected around 6% of B cells when the supernatants were concentrated 50-fold. The fraction of infected TMC B cells could be further increased to 50% by adding concentrated EBV-containing supernatants from TPA-induced EBfaV-GFP (Fig. 1A to E). Similarly, ex vivo inoculation of PBMC by spinoculation with TPA-induced and concentrated EBV led to 25-fold-higher infection frequencies than inoculation with nonconcentrated EBV by conventional protocols (data not shown). Thus, spinoculation markedly increased the fraction of infected B cells compared to that obtained by conventional inoculation when either TPA-induced or 50-fold-concentrated EBV was used.

Overall, the percentages of EBV-positive cells increased from <1 to >30% of TMC and from 1 to 5% of PBMC. As expected, B cells constituted a vast majority of the infected cells (Fig. 1E).

EBV binding and the infection of B cells after spinoculation depend on viral glycoproteins and their specific receptors. The attachment of EBV to B cells is mediated by the direct interaction of EBV gp350/220 with cellular CD21, initiating receptor-mediated endocytosis. After binding to CD21, EBV gp42 can interact with host HLA class II, leading to a conformational change in the viral glycoproteins and triggering fusion with the host cell membrane (16, 38). To exclude nonspecific EBV uptake during spinoculation (42, 43), we treated the EBV-containing supernatants with antibodies against EBV gp350/220, gp42, or both. In addition, we treated the target B cells with antibodies against CD21, HLA class II, or both before spinoculation (Fig. 1F and G). Cells were kept at 4°C during spinoculation to inhibit EBV entry (30).

To quantify the efficiency of EBV binding to cells, we stained cell-associated EBV with FITC-conjugated anti-EBV gp350/220 antibodies and examined the cells by flow cytometry (Fig. 1F). Blocking EBV glycoprotein gp350/220 reduced the mean efficiency of binding of spinoculated EBV to B cells to 65% in a dose-dependent manner, as also observed earlier (26). Blocking the fusion-triggering EBV gp42 also resulted in a loss of binding efficiency to 50% of that in controls, which may be due to sterical hindrance or the loss of the gp350/220-CD21 interaction with the use of blocking antibodies to gp42. Simultaneous blocking of gp350/220 and gp42 reduced the mean binding efficiency of EBV to 22% of that in untreated samples, showing that both attachment via gp350/220 and the subsequent interaction between gp42 and HLA class II are required during the infection of cells by spinoculation (Fig. 1F). Blocking of the EBV receptor and coreceptor with anti-CD21 or anti-HLA class II antibodies reduced EBV attachment to 10 to 15% of that in controls, and the simultaneous use of both antibodies further reduced the mean level of cells showing cell-associated EBV to <10% (Fig. 1F). The blocking of EBV binding by anti-EBV gp350/220 did not influence the detection of bound EBV by FITC–anti-EBV gp350/220 in comparisons of EBV with free gp350/220 to EBV with blocked gp350/220 (Fig. 1F).

Similarly, blocking either gp350/220 or gp42 or both by monoclonal antibodies dose dependently decreased the mean levels of infected B cells from 40 to 50% to less than 5% (Fig. 1G). Blocking CD21, HLA class II, or both dose dependently reduced the mean levels of infected B cells from 40 to 50% to 10 to 20% (Fig. 1G). These results indicate that spinoculation does not bypass and change the requirements for the normal binding and fusion of EBV with B cells.

Infecting lymphoid cells with EBV by spinoculation results in a type III EBV gene mRNA expression pattern. Normally, following the ex vivo infection of B cells by conventional inoculation, EBV undergoes the so-called type III program of latency gene expression (33). To determine if spinoculation has no aberrant effects on EBV gene expression after infection, we compared the EBV mRNA expression profiles after infection by spinoculation with those after infection by conventional inoculation. Indeed, patterns of mRNA expression of EBV latent genes, including *EBNA1*, *EBNA2*, *LMP1*, and *LMP2*, in TMC and PBMC after conventional inoculation or spinoculation with B95.8 EBfaV-GFP were similar (Fig. 2). Nevertheless, mRNA expression levels after spinoculation were 10- to 50-fold higher than those after conventional inoculation (Fig. 2). This increase is likely explained by the 25- to 50-fold enhancement in the proportion of infected cells following spinoculation compared to that following conventional inoculation (Fig. 1). Thus, EBV mRNA expression patterns were similar irrespective of the inoculation protocol used. This finding sug-

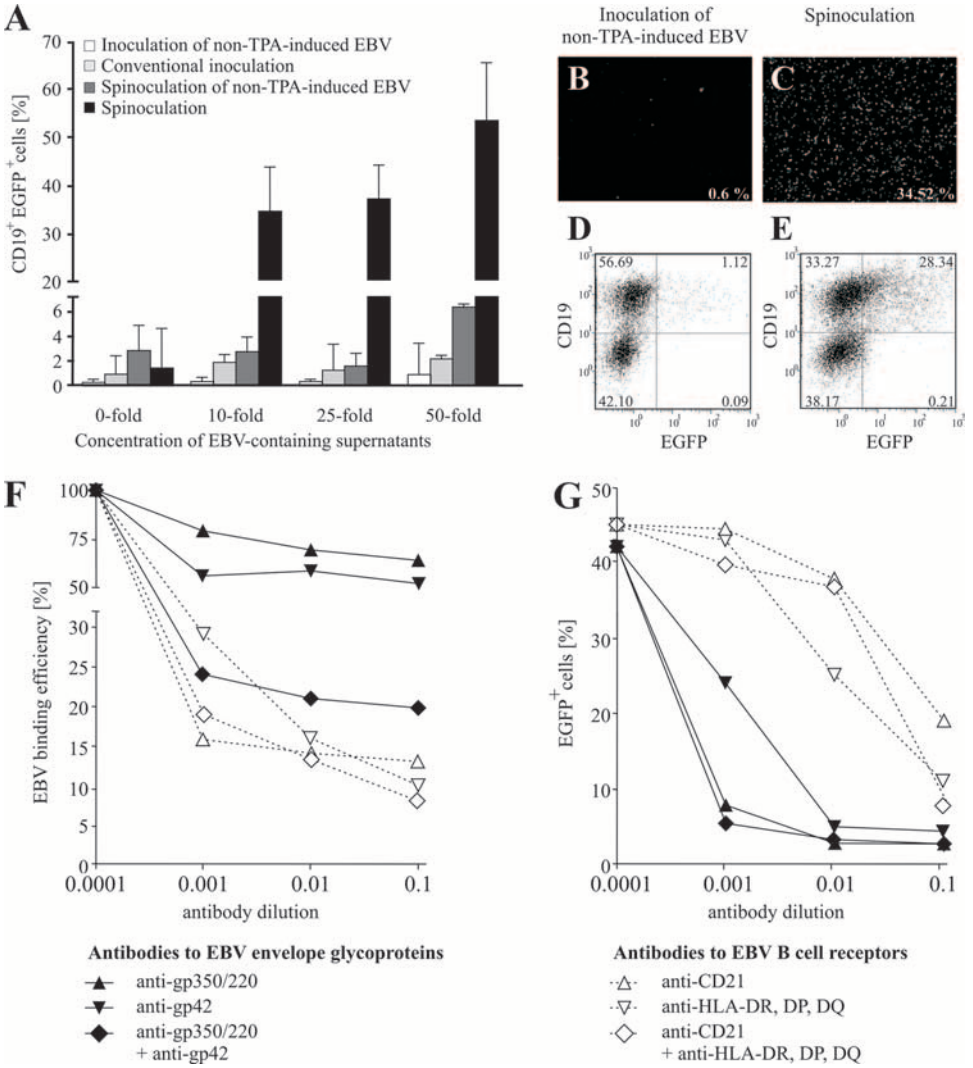

FIG. 1. Spinoculation increases the total numbers of ex vivo EBV-infected B cells without affecting the specificity of infection. (A) Results of inoculation and spinoculation of TMC with different concentrations of TPA-induced or uninduced cultures of the EBV strain B95.8EBfaV-GFP. Cells were counterstained with *R*-phycoerythrin-labeled antibody against the B-cell surface marker CD19 and analyzed by flow cytometry 48 h after inoculation. (B and C) Fluorescence microscopy of TMC 48 h after infection by inoculation with non-TPA-induced supernatants or by spinoculation with 50-fold-concentrated supernatants from TPA-induced cultures of the EBV strain B95.8EBfaV-GFP. The percentages of $EGFP^+$ cells among the total number of cells are presented. (D and E) Flow cytometry quantification of $EGFP^+$ cells 48 h after ex vivo inoculation with non-TPA-induced EBV (D) or spinoculation with 50-fold-concentrated supernatants from TPA-induced cultures of the EBV strain B95.8EBfaV-GFP (E). Cells were counterstained with *R*-phycoerythrin-labeled antibody directed against CD19. Values presented show the mean infection rates of results from four independent experiments. (F) Binding of EBV to B cells. The efficiency of EBV binding to B cells was assessed using 50-fold-concentrated supernatants from TPA-induced B95.8EBfaV-GFP cells. The concentrated supernatants were treated with antibodies against the EBV glycoprotein gp350/220, gp42, or both at serial dilutions or mock treated and used for ex vivo spinoculation of TMC at 4°C. Conversely, TMC were treated with antibodies specifically targeting the two receptors required for EBV entry, CD21 and HLA class II, at serial dilutions (1:10, 1:100, 1:1,000, and 1:10,000) and then subjected to ex vivo spinoculation with 50-fold-concentrated supernatants from TPA-induced B95.8EBfaV-GFP cells. The efficiency of the binding of EBV to B cells was determined by flow cytometry using FITC-labeled anti-EBV gp350/220 immediately after spinoculation. (G) Infection of B cells with EBV. The EBV infection of B cells was quantified by flow cytometry to measure the EGFP fluorescence intensity 24 h after spinoculation. Results from one independent experiment of two are shown.

gested that spinoculation does not elicit an aberrant effect on EBV gene expression.

As in subsequent experiments we planned to use the reporter virus EBfaV-GFP, we wished to determine if it behaved as predicted. Previous studies have reported that the recombinant B95.8EBfaV-GFP infects and transforms B cells as well as the wild-type strain B95.8 (39). With the exception of *LMP2* (which had lower mRNA expression levels after infection with B95.8 EBfaV-GFP), infection with either virus gave the same EBV mRNA expression profile. Although *LMP2* is replaced by *EGFP* in the reporter virus, there is still production of wild-type virus in the producer cells (40), which we believed resulted in the low *LMP2* mRNA expression observed (Fig. 2D).

There is the possibility that the B-cell phenotype has an effect on the EBV mRNA expression pattern, an important feature in the context of the potential transformation ability.

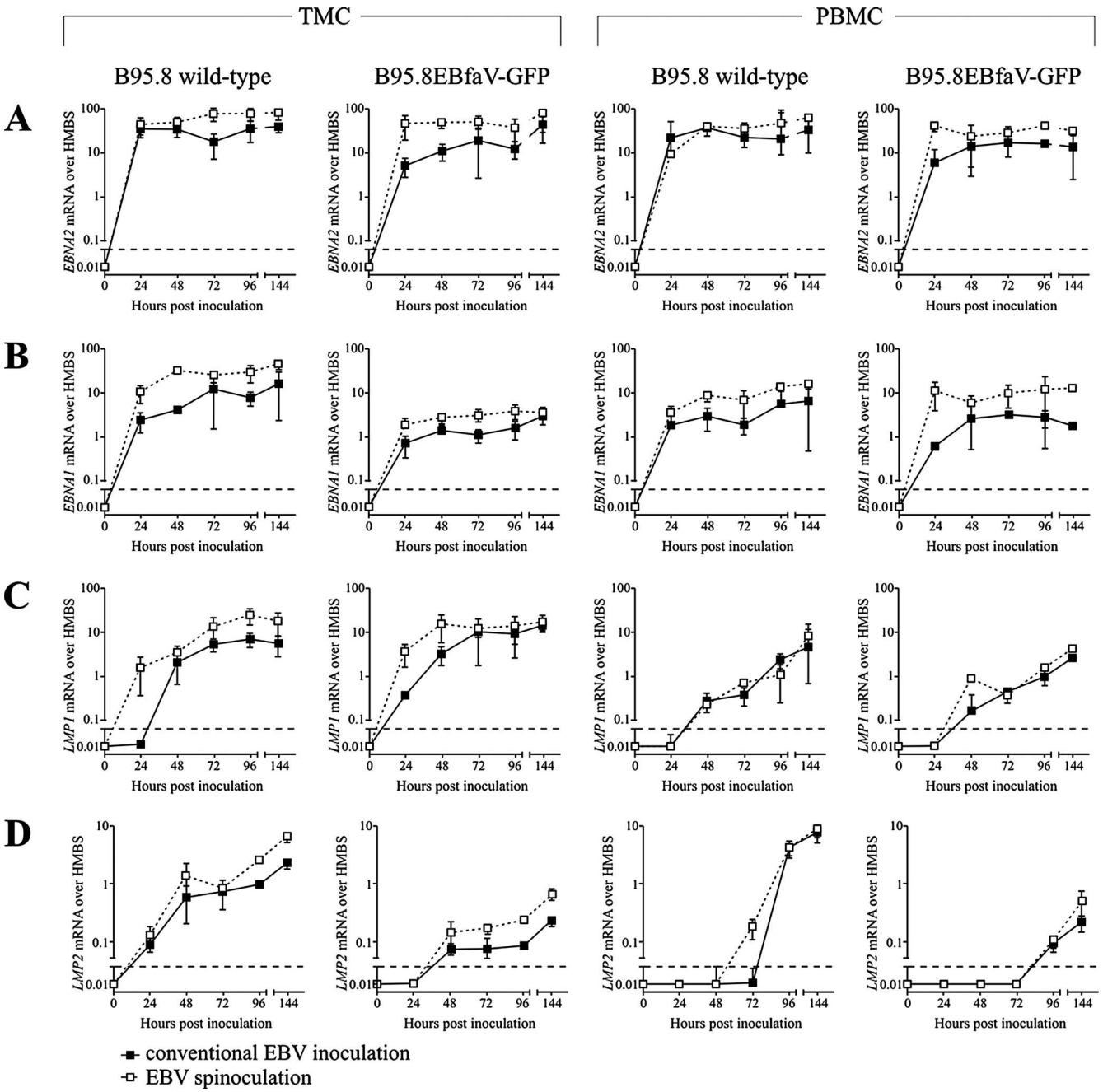

FIG. 2. Spinoculation and conventional inoculation of TMC and PBMC with EBV yielded similar mRNA expression patterns. TMC and PBMC infection by spinoculation results in EBV gene mRNA expression patterns similar to those resulting from infection by conventional inoculation using either the wild-type B95.8 EBV or B95.8EBfaV-GFP. Longitudinal EBV gene mRNA expression was assessed by specific quantitative real-time PCR analysis. The mRNA expression of the genes encoding EBV nuclear antigen, *EBNA2* (A) and *EBNA1* (B), and those encoding the EBV latent membrane proteins, *LMP1* (C) and *LMP2* (D), showed a sequential activation of EBNA2 and EBNA1, followed by LMP1 and LMP2. The increased rate of infection of the cells with EBV after spinoculation was mirrored in the mRNA expression levels of EBV genes. The use of the recombinant B95.8EBfaV-GFP EBV did not influence EBV gene mRNA expression compared to that in wild-type EBV. All EBV gene mRNA expression data were normalized to the mRNA expression of the housekeeping gene for hydroxymethylbilane synthase (*HMBS*). Results shown are from TMC from three donors and are expressed as means ± standard errors of the means (SEM).

We therefore isolated naïve and memory B cells from TMC at 24-h intervals during the first 7 days after spinoculation with EBV and quantified EBV mRNA expression by quantitative real-time PCR. The master regulator of all other EBV latent genes, *EBNA2*, and the latent EBV genes *EBNA1*, *EBNA3A*, *EBNA3C*, *LMP1*, and *LMP2* showed almost identical patterns of expression in naïve and memory B cells (Fig. 3A to F). No expression of the initiator and master regulator gene of lytic infection, *BZLF1*, was detected in either B-cell subset (Fig. 3G). Also, the expression of lytic genes downstream of *BZLF1*

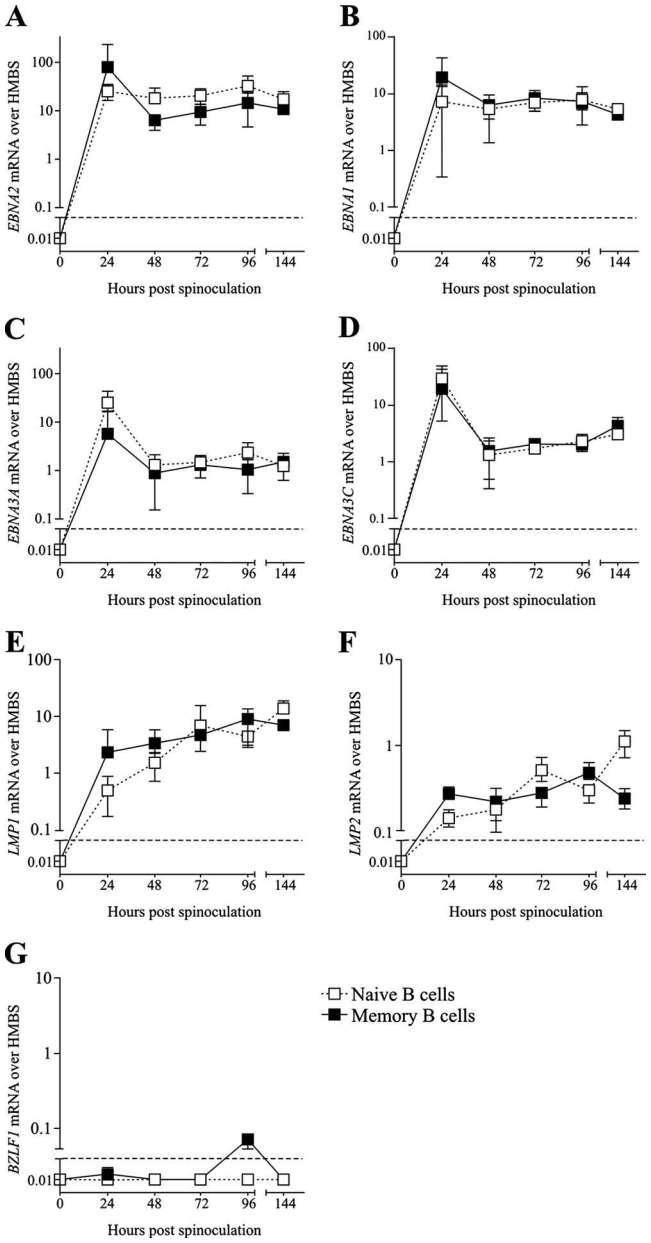

FIG. 3. EBV gene expression patterns are similar in tonsillar memory and naïve B cells after infection. (A to F) Comparison of the longitudinal EBV gene mRNA expression patterns in tonsillar naïve and memory B cells after ex vivo infection by spinoculation. Both memory and naïve B cells expressed the EBV latency genes *EBNA2* (A), *EBNA1* (B), *EBNA3A* (C), *EBNA3C* (D), *LMP1* (E), and *LMP2* (F). (G) Neither B-cell subset initiated EBV lytic infection, as documented by the absence of mRNA expression of the EBV immediate-lytic gene *BZLF1*. After spinoculation of TMC with EBV ex vivo, memory and naïve B cells were isolated with magnetic beads at different times. The purity of the B-cell subsets was >98%. Results shown are from TMC from three donors and are expressed as means ± SEM. HMBS, hydroxymethylbilane synthase mRNA.

was absent in naïve and memory B cells (data not shown). This finding is consistent with those in previously published reports suggesting that EBV manifests primarily as a latent type III infection in TMC after ex vivo infection and verifies that EBV establishes latency in memory B cells in a fashion similar to that in naïve B cells (1). The isolation of naïve B cells and that of memory B cells before EBV infection showed comparable results, thereby excluding possible effects of the cell isolation on the EBV mRNA expression (data not shown).

Memory and naïve B cells from tonsils, but not those from peripheral blood, are equally susceptible to EBV infection. CD21 and HLA-DR are absolutely required for the successful infection of B cells with EBV. Among TMC and PBMC, naïve ($CD19^+$ $CD27^-$) and memory ($CD19^+$ $CD27^+$) B cells showed similar levels of CD21 (Fig. 4A and B) and HLA-DR (Fig. 4C and D) expression (summarized in Fig. 4E and F). Conventional inoculation of TMC (Fig. 4G) or PBMC (Fig. 4H) resulted in very low levels of infected $CD19^+$ B cells (naïve B cells, 0.9% ± 0.1% and 0.5% ± 0.3%, respectively; memory B cells, 0.02% ± 0.01% and 0.01% ± 0.01%, respectively). Spinoculation increased the proportions of infected naïve and memory B cells dramatically compared to those obtained by conventional inoculation. Strikingly, the proportions of infected cells among the B-cell subsets of TMC were rather similar (39.1% ± 2.2% EBV-positive memory B cells versus 49.6% ± 16.7% EBV-positive naïve B cells) (Fig. 4I). However, among PBMC, the proportion of infected naïve B cells (69.2% ± 5.1%) was one-third higher than the proportion of infected memory B cells (25.6% ± 4.8%; $P = 0.016$) (Fig. 4J).

Tonsillar memory B cells are more susceptible to EBV infection after class switching than before. Ehlin-Henriksson et al. reported that tonsillar B-cell subpopulations are equally susceptible to EBV infection in vitro, irrespective of immunoglobulin isotype expression (11). That study did not discriminate between naïve ($CD27^-$) and IgM-expressing memory ($CD27^+$) B cells (44). We therefore characterized in detail the isotype expression in tonsillar memory ($CD27^+$) B cells susceptible to EBV infection. Thus, we isolated memory ($CD27^+$) B cells from TMC and further segregated them into IgM-negative and IgM-positive populations. Forty-eight hours after inoculation with B95.8EBfaV-GFP, we analyzed the infection frequencies among memory B cells expressing IgM, IgA, and IgG (Fig. 5A). We found that IgM-expressing memory B cells showed a significantly lower frequency of EBV infection than memory B cells expressing IgA or IgG, suggesting that class-switched memory B cells are more susceptible to EBV infection than their non-class-switched counterparts. We had to exclude that EBV infection ex vivo alters the relative frequencies of the B-cell subpopulations expressing IgM, IgA, or IgG. To this end, we used isolated tonsillar memory ($CD27^+$) B cells and infected them ex vivo with B95.8EBfaV-GFP. Forty-eight hours after infection, mock-treated and EBV-exposed memory B cells showed similar relative amounts of cells expressing IgM, IgA, or IgG (Fig. 5B).

We next asked whether, within the populations of EBV-exposed cells, the relative distributions of isotype expression among EBV-infected and non-EBV-infected cells were similar. Populations of EBV-infected cells showed a reduced frequency of IgM-positive memory B cells compared to that in populations of non-EBV-infected cells ($P = 0.003$). This finding again suggested that, among memory B cells, EBV preferentially infects cells expressing IgA or IgG, i.e., memory B cells after class switching. These observations are consistent with

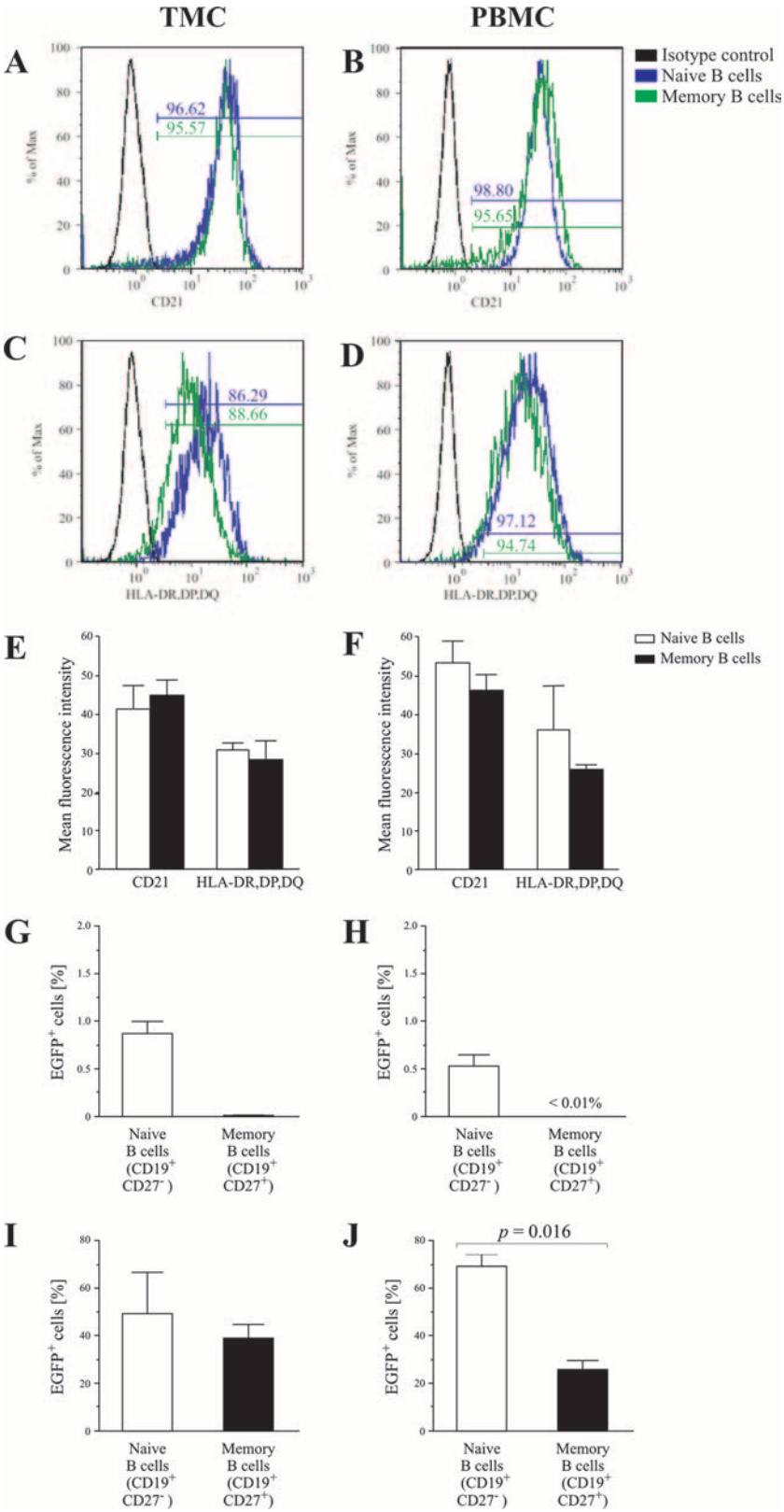

FIG. 4. B-cell subsets from tonsils, but not those from peripheral blood, are equally susceptible to EBV infection. (A to D) Numbers of naïve and memory B cells expressing the EBV receptors CD21 and HLA-DR. Cells were stained with antibodies against CD19, CD27, CD21, and HLA-DR, HLA-DP, and HLA-DQ. Flow cytometry plots are gated for CD19-expressing B cells and CD27-negative naïve or CD27-positive memory B cells. (E and F) Mean fluorescence intensities for CD21 and HLA-DR, HLA-DP, and HLA-DQ on naïve and memory B cells from tonsils and peripheral blood. (G and H) Hardly any infected memory B cells were detected among TMC (G) or PBMC (H) subjected to conven-

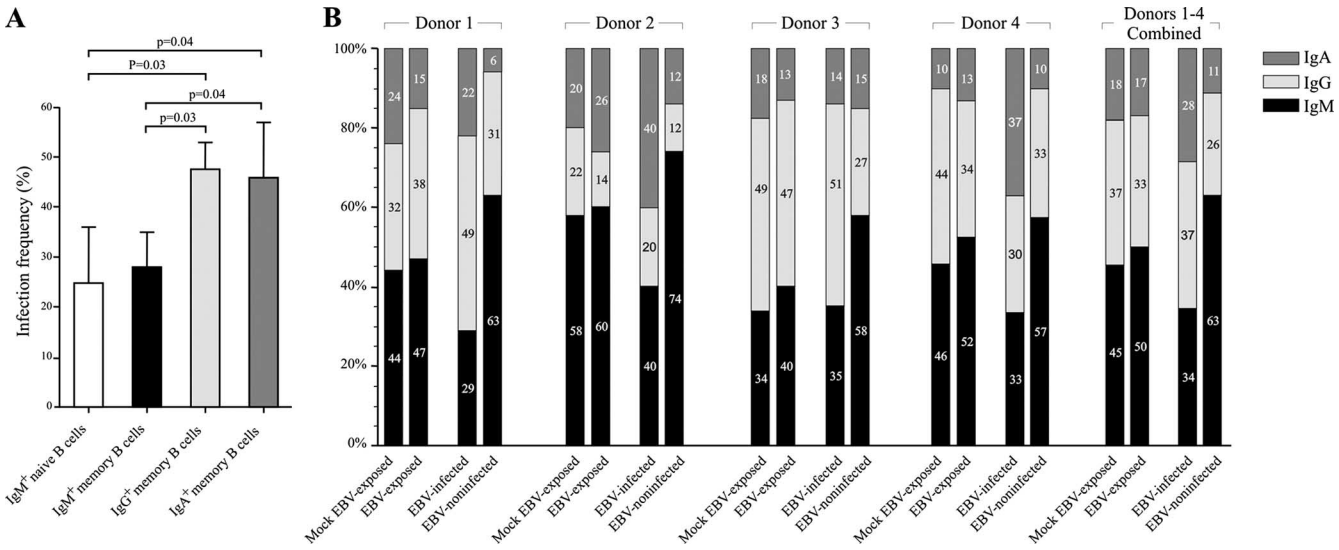

FIG. 5. Class switch recombination renders tonsillar memory B cells more susceptible than naïve B cells to EBV infection. (A) Infection frequencies for isolated IgM-positive and IgM-negative tonsillar memory B cells. Memory B-cell populations that did not undergo class switch recombination (IgM^+) showed significantly fewer infected cells 48 h postinoculation than their class-switched counterparts (IgG^+ and IgA^+). (B) Immunoglobulin isotype distribution among ex vivo EBV-infected tonsillar memory B cells 48 h postinoculation. The $EGFP^+$ (EBV-infected) population of the infected cells showed fewer IgM-positive cells than the corresponding $EGFP^-$ (uninfected) population. The whole population of infected cells showed no significant change in immunoglobulin composition compared to that of mock-infected controls. Results shown are from TMC from four donors and are expressed as means ± SEM. *P* values were calculated by paired Student's *t* test.

the findings in a recent report showing that EBV is preferentially excluded from the IgM-expressing memory B cells in vivo (36).

Tonsillar memory B cells are transformed by EBV at lower frequencies than tonsillar naïve B cells. Tonsillar memory B cells and naïve B cells showed similar EBV mRNA expression patterns after ex vivo infection; nevertheless, to test if their EBV-associated growth transformation potentials were identical, we infected whole populations of TMC, in addition to isolated tonsillar naïve and memory B cells, with B95.8 by spinoculation. We used wild-type B95.8 EBV in all transformation assays to exclude bias from the absence of LMP2 in the recombinant virus (B95.8EBfaV-GFP). The cells were cultured on an autologous feeder layer for 4 weeks to assess the transformation of EBV-infected B cells. Whole TMC populations and isolated tonsillar naïve B cells and tonsillar memory B cells infected with EBV showed the typical formation of cellular aggregates 48 h after spinoculation (data not shown), and the number of aggregates and their sizes steadily increased over the following 4 weeks (Fig. 6A to C) (29, 35). Conventional inoculation of TMC resulted in a transformation efficiency of around 60% when starting with 200 cells per well. In contrast, the spinoculation of a whole TMC population and isolated naïve B cells and memory B cells increased the transformation efficiency to more than 90% (Fig. 6B to D). Nevertheless, comparing the initial frequencies of infection of TMC that we observed using conventional inoculation or spinoculation with the transformation efficiencies suggested that using the conventional transformation assay (35) led to a state of initial saturation with EBV-infected B cells, which may lead to underestimation of the transformation efficiency. Thus, we performed a modified transformation assay, adding a limiting dilution of EBV-infected cells to a fixed number of B-cell-depleted autologous feeder cells. The transformation efficiency for naïve B cells was significantly higher than the one for memory B cells when cells were seeded at densities of 10 to 100 cells per well (Fig. 6E). Thus, memory B cells are transformed by EBV with a lower efficiency than naïve B cells. The micrographs in Fig. 6 show naïve and memory B cells at a cell density optimized for growth transformation. As seen in Fig. 6E, with the use of more than 100 naïve or memory B cells per well for transformation, no difference in transformation efficiency compared to that obtained with 100 cells per well is detectable.

LCLs from memory B cells exhibit less apoptosis and slower growth than LCLs from naïve B cells. In vitro, naïve B cells exhibit more apoptosis than memory B cells (7). We hypothesized that a change in apoptotic behavior after EBV infection may explain the differences in growth transformation efficiencies between naïve and memory B cells. As a marker for apoptosis, we analyzed the cleavage of PARP by Western blotting

tional EBV inoculation, which led to a very low rate of infection of the $CD19^+$ B cells. (I and J) Spinoculation led to a significant rise in the overall infection rate of the B-cell population. Among TMC, the proportions of naïve ($CD19^+$ $CD27^-$) and memory ($CD19^+$ $CD27^+$) B cells infected with EBV were similar (I), but among PBMC, the proportion of memory cells infected with EBV was lower than that of naïve B cells infected with EBV (J). Data are expressed as means ± SD of results from 10 independent experiments (A to F) and 3 independent experiments (G to J). *P* values were calculated by paired Student's *t* test.

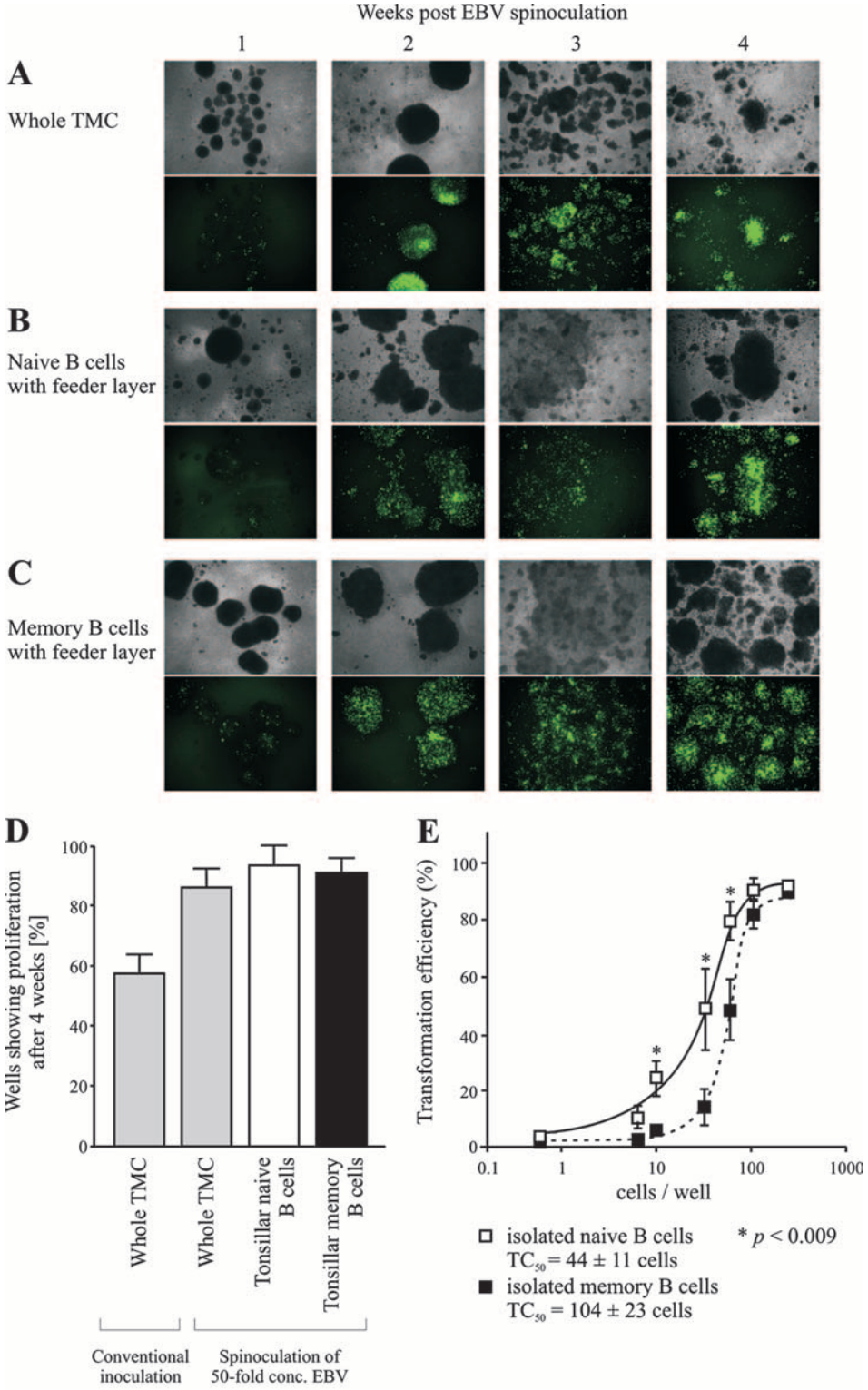

FIG. 6. Tonsillar memory B cells are less efficiently transformed by EBV than naïve B cells. (A to C) Whole TMC populations, naïve B cells, and memory B cells isolated from EBV-seronegative donors showed the classical picture of cell aggregate formation and the outgrowth of transformed cells 24 weeks after ex vivo infection using B95.8EBfaV-GFP. (D) Transformation efficiencies after conventional EBV inoculation and EBV spinoculation using the wild-type B95.8. TMC (2×10^5/well) infected with B95.8 were seeded into 96-well plates, and cells in wells showing proliferation after 4 weeks and B-cell levels above 98% were considered to be transformed. For the transformation of different B-cell subsets, samples of 250 naïve or memory B cells were spinoculated using B95.8, mixed with 2×10^5 B-cell-depleted TMC, and seeded into each well in 96-well plates. After 4 weeks, cells in wells showing proliferation and B-cell levels above 98% were considered to be transformed. conc., concentrated. (E) Transformation efficiencies of tonsillar naïve and memory B cells under conditions of limiting dilution. Naïve and memory tonsillar B cells were inoculated with B95.8 supernatants by spinoculation and were plated in 96-well plates together with an autologous feeder layer (2×10^5 cells/well). Cells in wells still showing growth and B-cell levels above 98% after 4 weeks were regarded to be transformed. Results are means ± SD from three independent experiments. *P* values were calculated by paired Student's *t* test. TC_{50}, 50% transformation concentration.

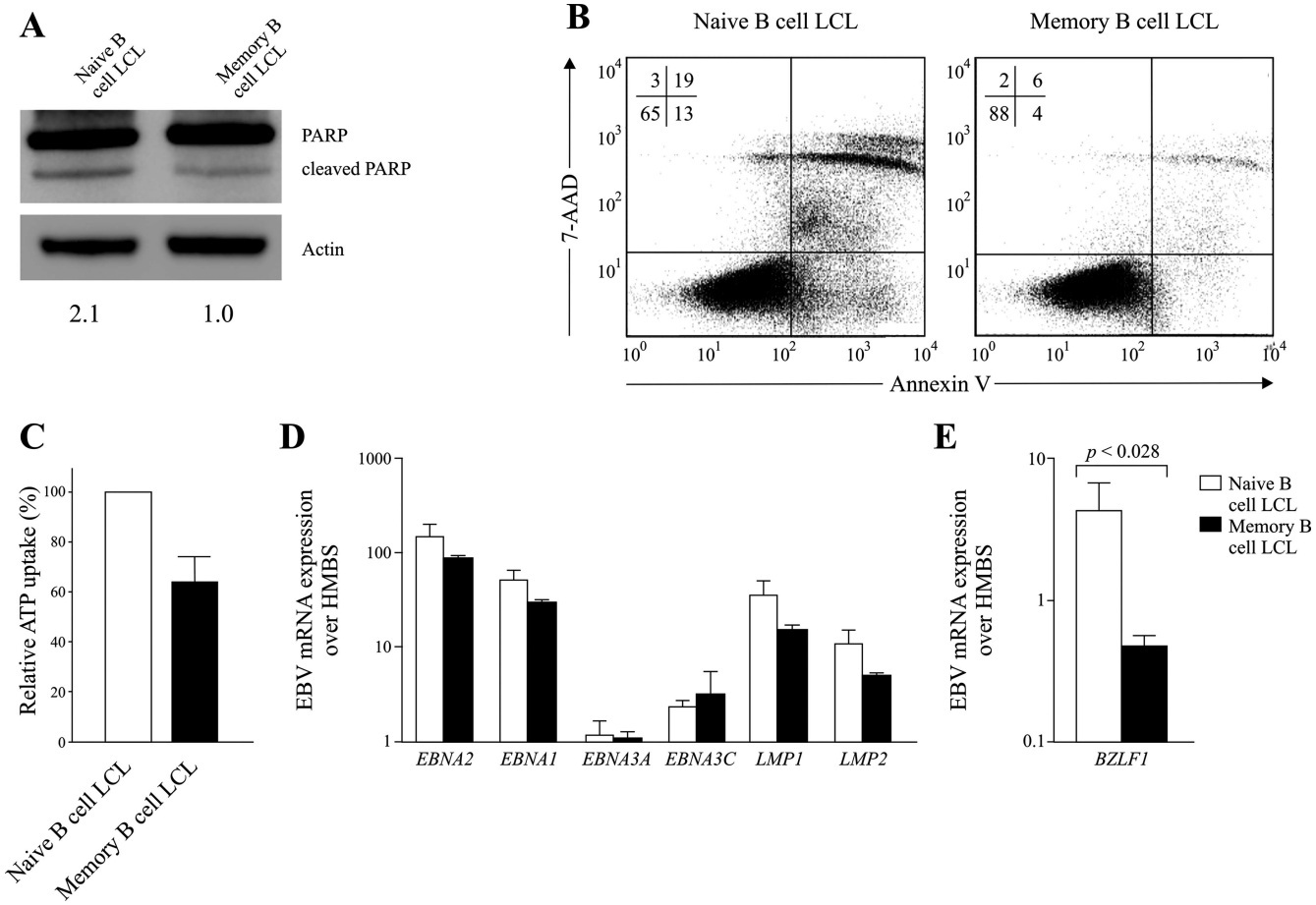

FIG. 7. Naïve and memory B cells retain their distinct apoptosis and proliferation characteristics after EBV-induced transformation. (A) Naïve and memory B cells show different apoptotic behaviors after EBV growth transformation. LCLs from naïve B cells are more susceptible to apoptosis than the autologous memory B cells, as assessed by Western blotting to detect the cleavage of PARP. Numbers below the blot indicate the level of PARP cleavage (*n*-fold) as normalized to actin and to PARP cleavage in memory B-cell LCLs. (B) LCLs from memory B cells show a lower proportion of cells undergoing apoptosis than LCLs from naïve B cells as measured by staining with Annexin V and 7-AAD. (C) LCLs from memory B cells as assessed by ATP uptake. (D) LCLs from naïve B cells and those from memory B cells express mRNA of the same set of EBV latency genes at equal levels, as determined by real-time PCR analysis. (E) EBV-transformed naïve B cells show a higher frequency of cells undergoing lytic EBV reactivation than EBV-transformed memory B cells, as measured by the mRNA expression of *BZLF1*. HMBS, hydroxymethylbilane synthase mRNA.

(10). We also determined the numbers of naïve and memory B cells undergoing spontaneous apoptosis after EBV-associated growth transformation into LCLs by staining with Annexin V and 7-amino-actinomycin D (7-AAD). LCLs from naïve B cells showed twofold-higher levels of PARP cleavage than LCLs from memory B cells (Fig. 7A). The frequency of apoptosis in LCLs from naïve B cells was around threefold higher than that in LCLs from memory B cells, i.e., 13.1% ± 6.5% compared to 4.2% ± 2.8% ($P = 0.038$) (Fig. 7B), when determined by staining with Annexin V and 7-AAD. Thus, after EBV transformation, naïve B cells showed significantly more apoptosis than did memory B cells. Therefore, these results alone could not explain the somewhat lower transformation efficiency of memory B cells than of naïve B cells and led us to hypothesize that the difference in transformation efficiency was linked to unequal proliferation rates.

We assessed the proliferation characteristics of naïve and memory B cells before and after EBV-induced growth transformation into LCLs by measuring the ATP uptake by the cells over 24 h. ATP uptake in newly isolated memory B cells was 47% ± 15% that in their naïve counterparts, implying that memory B cells grow more slowly (data not shown). EBV-transformed memory B cells showed 64% ± 9% of the ATP uptake shown by EBV-transformed naïve B cells (Fig. 7C). These results indicate that naïve and memory B cells retain their distinct apoptotic and proliferation behaviors following EBV transformation. That is, EBV-transformed memory B cells exhibit a lower frequency of apoptosis and proliferate more slowly than EBV-transformed naïve B cells.

Next, we asked whether the observed differences in proliferation were due to unequal EBV mRNA expression levels. LCLs obtained after the infection of naïve or memory B cells showed similar patterns of mRNA expression of EBV latent genes involved in B-cell transformation, with expression levels in naïve cells being slightly higher (Fig. 7D). By contrast, levels of *BZLF1* mRNA in LCLs from memory B

cells were significantly lower than those in LCLs from naïve B cells (Fig. 7E).

Compared to EBV-transformed naïve B cells, EBV-transformed memory B cells exhibit reduced apoptosis and lytic EBV gene expression. However, we believe that the slower proliferation of EBV-transformed memory B cells likely explains the lower EBV transformation efficiency of these cells in the dilution series experiments.

DISCUSSION

In this work, we aimed to assess the efficiencies of infection of distinct B-cell subsets from different lymphoid compartments and to compare the transformation behaviors of B-cell subsets infected ex vivo with EBV. Indeed, we found that tonsillar naïve and memory B cells were equally susceptible to ex vivo EBV infection but that peripheral blood memory B cells were less susceptible than naïve B cells. These data indicate that the tissue origin of memory B cells impacts the susceptibility to EBV infection. Moreover, among tonsillar memory B cells, the subset expressing IgM was less susceptible to EBV infection than the subsets expressing IgA or IgG, suggesting that class switching also impacts the susceptibility to EBV infection. Finally, even though tonsillar naïve and memory B cells displayed very similar patterns of EBV gene expression that is crucial to establish B-cell transformation, memory B cells showed significantly lower EBV-associated transformation efficiency than naïve B cells. This finding may be due to the inherently higher proliferation rates of naïve B cells than of memory B cells. Our findings, thus, reveal that the susceptibilities of B-cell subsets to EBV infection and their transformation capacities are not uniform and suggest that EBV exploits the status of differentiation and the compartment of origin of B cells to optimize the establishment of latency in the host.

Conventional protocols for EBV infection inoculate target cells with supernatant from EBV-positive cell lines that have been driven into lytic infection by mitogens or by the cross-linking of the B-cell receptor (9, 27). Such inoculation results in EBV infection of less than 2% of primary B cells (40). This low proportion hampers the clear dissection of what subsets of B cells are susceptible to EBV (39). To investigate the susceptibilities of distinct B-cell subsets, we needed to reach a higher infectivity rate. By using spinoculation with concentrated EBV, we increased the proportion of $CD19^+$ B cells infected with EBV to more than 50% at 48 h after inoculation. The entry process of EBV was almost completely inhibited when antibodies against the EBV envelope gp350/220 and gp42 or the B-cell receptors CD21 and HLA class II were added before spinoculation. This result clearly indicates that the increased infection frequency resulted from specific virus entry through the known EBV receptors and not from an unspecific uptake. Thus, the ex vivo EBV infection of B cells by spinoculation likely mimics the natural B-cell infection pathway and thus enabled us to study EBV infection in B-cell subsets from primary cells. Importantly, by using primary cells exclusively from EBV-negative individuals, we excluded bias from preexisting EBV-specific immunity or the necessity to apply immunosuppressive drugs in vitro in transformation experiments.

Indeed, using this optimized infection protocol, we found that memory B cells isolated from tonsils are susceptible to ex vivo EBV infection at frequencies similar to those of their naïve counterparts. This observation is in agreement with our finding of similar densities of the receptors for EBV (i.e., CD21 and HLA-DR) on tonsillar naïve and memory B cells, as defined by the expression of CD27. In contrast, among PBMC, the proportion of infected memory B cells corresponded to only one-third of that of infected naïve B cells (Fig. 2). Strikingly, the densities of receptors CD21 and HLA-DR on naïve and memory B cells among PBMC were similar. This observation of a tissue origin-dependent susceptibility rate of memory B cells was unexpected. We speculate that the priming of naïve B cells at different anatomic sites results in the distinct susceptibility of memory B cells to EBV infection and that EBV succeeds in optimally exploiting unknown cellular factors present exclusively in tonsillar memory B cells. Notably, the tonsils are the portal of entry for EBV. Our finding that naïve B cells from peripheral blood were susceptible to EBV ex vivo contrasts with the absence of EBV-infected naïve B cells in peripheral blood in vivo (17). Likely explanations for this discrepancy are that naïve B cells infected at the portal of entry for EBV are too short-lived to gain access to the circulation as long as they do not differentiate into long-lived memory B cells and that EBV virions or productively infected memory B cells are hardly found in the peripheral blood (2).

B cells expressing CD27 correspond to functional memory B cells, i.e., B cells having completed antigen-driven selection and differentiation and thus exiting or having left germinal centers (41). Our data, which show that EBV infects $CD27^+$ tonsillar B cells and $CD27^-$ tonsillar B cells with equal efficiencies, suggest that the degree of susceptibility to EBV infection is retained in B cells exiting the germinal center. We propose that functional memory B cells represent one direct target of EBV at its natural portal of entry in vivo. The infection of memory B cells in addition to naïve B cells at the portal of entry would provide EBV with an additional important biological advantage to increase the pool of infected B cells and thus secure persistence in the host. The targeting of functional memory B cells by EBV, either directly or indirectly via the infection of naïve B cells subsequently driven through a germinal-center reaction by the virus (37), holds the advantage that the EBV-infected B cell is subjected to proliferation and differentiation signals from cognate antigens when homing back to the tonsils. Tonsillar memory B cells are certainly more frequently exposed to antigens than, e.g., memory B cells from lymph nodes draining normally sterile body tissues. This scenario in turn would ensure that latent EBV is handed over to progeny cells. In addition, following the differentiation of the EBV-infected memory B cells into plasma cells, which represent the site of lytic replication (25), EBV could gain access to other susceptible B cells or epithelial cells and, via saliva, to susceptible new hosts.

Our data clearly demonstrate that among $CD27^+$ memory B cells, those expressing IgA or IgG are significantly more susceptible to EBV infection than those expressing IgM. This fact suggests that if EBV uses tonsillar memory B cells as a direct target, the virus preferentially accesses isotype-switched cells. In agreement with our observations, the vast majority of EBV DNA in peripheral blood cells from EBV carriers has been detected in surface-IgA-positive B cells (12). Moreover, recent work on peripheral blood B cells isolated from patients with

infectious mononucleosis found that EBV-infected cells were preferentially excluded from the IgM isotype in the $CD27^+$ memory B-cell pool (36). Based on this finding and on the presence of more frequent somatic hypermutations in EBV-infected than in non-EBV-infected $CD27^+$ B cells, the authors of the study concluded that EBV impacts the infected cells themselves by promoting their germinal-center reaction with isotype switching (36). Thus, it appears that EBV infects B cells at different differentiation stages.

A remarkable finding of this work is that tonsillar memory B cells manifest significantly lower EBV-induced transformation efficiency than their naïve counterparts. The infection of B cells with EBV in vitro is well-known to result in the expression of the so-called latency III viral gene expression program. This program is characterized by the expression of six nuclear antigens (EBNA1 to EBNA6), LMP1, and LMP2 (33), which are required for establishing EBV latency and EBV-induced growth transformation and immortalization (13). Both tonsillar B-cell subsets expressed mRNA of *EBNA2*, the master regulator of latency, and mRNAs of *EBNA1*, *LMP1* and *LMP2*, and *EBNA3A* and *EBNA3C*, which are also important for EBV growth transformation (1) following infection with EBV in vitro. Importantly, the mRNA expression levels of the latency genes involved in cellular transformation were similar in naïve and memory B cells, suggesting what would let us expect similar EBV-induced transformation efficiencies for the two B-cell subsets. The higher apoptosis frequency and higher mRNA expression levels of *BZLF1* in transformed naïve than in memory B cells, indicating greater initiation of spontaneous EBV lytic infection in LCLs from naïve B cells, would rather suggest a lower transformation rate of naïve B cells, which is, however, not the case. Therefore, qualitative and quantitative EBV gene mRNA expression patterns alone do not allow firm conclusions on the host-pathogen interactions.

Another explanation for the distinct transformation capacities of B-cell subsets may be a difference in cell proliferation rates. In fact, we found that the lower transformation efficiency of memory B cells was paralleled by their reduced rate of proliferation compared to that of naïve B cells. Importantly, the proliferation rates of these two B-cell subsets differed before and after EBV-induced transformation. This result suggests that infection with EBV does not significantly alter the proliferation characteristics associated with B-cell differentiation.

In conclusion, our data demonstrate that spinoculation enhances EBV infection without bypassing the requirements for the normal binding of EBV to or the entry of EBV into B cells and, therefore, offers a highly valuable in vitro tool to study EBV infection, host-pathogen interactions, and pathogenesis. This tool allowed the first-time quantification of striking differences in susceptibility to EBV infection and EBV-induced transformation efficiency between B-cell subsets. These differences were dependent on the tissues of origin and the differentiation statuses of the cells, suggesting that EBV exploits the characteristics of B-cell subsets to optimize the establishment of latent infection.

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