β_1 Integrin Expression Increases Susceptibility of Memory B Cells to Epstein-Barr Virus Infection[∇]

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Epstein-Barr virus (EBV) uses nasal mucosa-associated lymphoid tissue (NALT) as a portal of entry to establish life-long persistence in memory B cells. We previously showed that naïve and memory B cells from NALT are equally susceptible to EBV infection. Here we show that memory B cells from NALT are significantly more susceptible to EBV infection than those from remote lymphatic organs. We identify β_1 integrin, which is expressed the most by naïve B cells of distinct lymphoid origin and by memory B cells from NALT, as a mediator of increased susceptibility to infection by EBV. Furthermore, we show that BMRF-2- β_1 integrin interaction and the downstream signal transduction pathway are critical for postbinding events. An increase of β_1 integrin expression in peripheral blood memory B cells provoked by CD40 stimulation plus B-cell receptor cross-linking increased the susceptibility of non-NALT memory B cells to EBV infection. Thus, EBV seems to utilize the increased activation status of memory B cells residing in the NALT to establish and ensure persistence.

Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus that is transmitted via saliva and infects more than 90% of the world's population (21). Much of EBV's medical importance relates to its association with B-cell malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, and post-transplant lymphoproliferative disease (21). The oncogenic potential of EBV is clearly illustrated by its unique capability to transform B cells *in vitro* (21).

In the current paradigm, EBV infects naïve B cells in tonsils *in vivo* (32). EBV is present mainly as a latent virus; upon infection, EBV expresses distinct patterns of its latency genes depending upon distinct B-cell differentiation stages, varying from expression of all 10 known EBV latency genes in naïve B cells to the complete absence of EBV mRNA expression in resting memory B cells. This has led to the model that EBV, by virtue of expression of its latency genes, provides cell survival signals in naïve B cells (32). In particular, recent data suggest that EBV expedites the antigen-driven somatic hypermutation and selection of B cells taking place in germinal centers (GC) (26). Chaganti et al. challenged the current paradigm by showing for patients with primary EBV infection that EBV avoids GC transit and directly infects memory B cells (6). This report

is consistent with *in vitro* experiments showing that EBV is able to infect memory B cells (9, 10), in addition to the well-accepted susceptibility of naïve and GC B cells to EBV.

Irrespective of which B-cell subset is the primary target of EBV, its propagation within the host is linked to proliferation of infected B cells, which deliver latent EBV to daughter cells, or, more rarely, to switching of EBV to lytic infection (21). The latter process can eventually be triggered by the differentiation of infected memory B cells into plasma cells and results in the release of virions that may subsequently infect new B cells (17). Importantly, transmission of EBV to naïve hosts is thought to occur via droplets loaded with virions (21). Thus, lytic replication of EBV takes place best in nasal mucosa-associated lymphoid tissue (NALT), which will release EBV into the saliva, generating infectious droplets. Therefore, the NALT is the point of EBV transmission, i.e., the portal of entry of EBV as well as a shedding organ for further transmission (21).

The attachment of EBV to B cells is mediated by the direct interaction of EBV glycoprotein gp350/220 with cellular CD21, initiating receptor-mediated endocytosis. After binding to CD21, EBV gp42 can interact with host HLA class II molecules, leading to a conformational change in the viral glycoproteins and triggering fusion with the host cell membrane (12, 28). Nevertheless, experimental data suggest that CD21 and HLA class II molecules are dispensable for the infection of B cells (14). Notably, in polarized oropharyngeal epithelial cells, which lack CD21, interactions between β_1 integrin and the EBV glycoprotein BMRF-2 via its Arg-Gly-Asp (RGD) motif are critical for infection (34, 38, 39). The role of β_1 integrin in

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mediating EBV infection of memory B cells from NALT or non-NALT is unknown.

We recently demonstrated that tonsillar memory B cells are much more susceptible to EBV infection than those from the peripheral blood, originating from various lymphoid tissues (9). Thus, tonsillar memory B cells seem to express properties which render them more susceptible to EBV infection than their counterparts of other lymphatic origin.

Here we hypothesized that memory B cells from the NALT exhibit specific properties rendering them highly susceptible to EBV infection. Indeed, in this work, we found that memory B cells from the NALT are distinguishable from memory B cells of other lymphoid tissue by their β_1 integrin expression levels, and thus their activation status, and that this higher expression level is a critical factor in their greater susceptibility to EBV infection.

MATERIALS AND METHODS

Cell culture and viruses. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin (all from Gibco, Basel, Switzerland). The EBV strains used were B95.8; B95.8EBfaV-GFP, carrying a cytomegalovirus enhanced green fluorescent protein (CMV EGFP) cassette (29, 30) (kindly provided by Richard Longnecker, Northwestern University Feinberg School of Medicine, Chicago, IL); and B27, expressing low BMRF-2 levels (38). B95.8EBfaV-GFP and B27 were constantly cultivated in the presence of G-418 (Sigma, Buchs, Switzerland) to maximize the yield of recombinant virus. Flow cytometry analysis after staining for EBER (8) showed that among the recombinant EBV producer cells (B95.8EBfaV-GFP and B27), fewer than 2% were positive for EBER but did not express GFP. Virus production was performed as described previously (9). The same virus stocks were used for all experiments in order to exclude bias from batch-dependent viral titers, except where indicated. Inactivation of EBV was performed by heat inactivation at either 56°C or 95°C for 60 min.

DNase treatment. To remove nonencapsidated EBV DNA before EBV DNA quantification, viral stocks were treated with DNase according to the instructions of the manufacturer (Ambion, Austin, TX).

qPCR assay. Quantification of EBV DNA copies in viral stocks by quantitative PCR (qPCR) was carried out after DNase treatment by using a TaqMan (Applied Biosystems, Rotkreuz, Switzerland) real-time PCR technique with a PCR primer-probe system targeting the conserved EBV BamHI W region, as reported previously (3). Serial dilutions of EBV DNA calibrated to an EBV-specific plasmid were included in every PCR run (3).

EBER *in situ* hybridization. The presence of EBER in single cells was determined by *in situ* hybridization and flow cytometry, as reported previously (8), with the modification that the EBER hybridization probe was linked to Atto620 (Microsynth, Balgach, Switzerland). EBV-negative Ramos Burkitt lymphoma cells and lymphoblastoid cell line (LCL) cells were used as negative and positive controls, respectively.

Antibodies and reagents. Antibodies to CD19, CD20, CD27, CD29, and CD62 ligand (CD62L) were from BD Biosciences (Allschwil, Switzerland). Blocking antibodies against $\alpha_5\beta_1$ integrin and EBV gp350/220 were from Abcam (Cambridge, United Kingdom), the EBV gp42 (clone F2.1) blocking antibody was kindly provided by L. Hutt-Fletcher (Louisiana State University, Shreveport, LA), recombinant β_1 integrin was from Thermo Scientific (Surrey, United Kingdom), and blocking antibodies against CD21 and HLA-DR, DP, DQ were from Biolegend (Lucerne, Switzerland). The inhibitors PP2, wortmannin, cytochalasin D, and AG-82 were from Calbiochem (Dietikon, Switzerland), and Ly-294002 was from Sigma-Aldrich. Antibodies detecting pY397 focal adhesion kinase (FAK) or FAK were from BD Biosciences. Antibodies detecting pY416 c-Src, c-Src, pY458 phosphatidylinositol 3-kinase (PI3K) p85 α , PI3K p85 α , pS3 cofilin, and cofilin and all horseradish peroxidase (HRP)-conjugated secondary antibodies were from Cell Signaling (Allschwil, Switzerland). Anti-human IgM and CD40L were from R&D Systems (Abingdon, United Kingdom). Cells were stimulated with concentrations recommended by the manufacturer.

Isolation of cells. Human mononuclear cells were isolated from tonsils of donors undergoing routine tonsillectomy, from peripheral blood, and from mesenteric lymph nodes of donors undergoing abdominal surgery. Donors were EBV seronegative. Tonsillar and mesenteric lymph node mononuclear cells were pre-

pared as previously described (9). B-cell subset separation was performed by using a B-cell isolation kit II and CD27 microbeads according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of the isolated subsets was determined by flow cytometry and was above 90% in all preparations.

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the institutional review boards of the University Children's Hospital of Zurich (IRB study StV 29/06) and the University Hospital of Zurich (IRB study EK 607). All subjects provided written informed consent for the collection of samples and subsequent analysis.

Infection of cells. EBV infection was performed as described earlier (9), with minor modifications. Briefly, supernatants of B95.8, B95.8EBfaV-GFP, and B27 (BMRF-2^{low}) stocks were centrifuged at 40,000 $\times$ g for 2 h at 4°C. The pellet was resuspended in phosphate-buffered saline (PBS), and 10% high-titer EBV (0.5×10^8 to 2.5×10^8 EBV DNA copies/ml after DNase treatment) was added to the memory B cells. The cells were then subjected to spin inoculation (spinoculation) at 4°C at 800 $\times$ g for 60 min, as conventional inoculation with EBV-containing supernatants normally does not yield sufficient numbers of infected cells to permit detailed susceptibility studies (9). All infection frequencies shown for B95.8EBfaV-GFP were quantified using flow cytometry 24 h after inoculation of cells.

EBV binding assay. Isolated B cells or monocytes from peripheral blood were incubated with either wild-type B95.8 or B27 BMRF-2^{low} EBV at a multiplicity of infection (MOI) of 5 or 25 per cell for 2 h at 4°C. Following extensive washing of the cells with ice-cold PBS, RNA was extracted and the number of bound EBV DNA copies was determined by qPCR as previously described (38).

Inhibition of EBV infection. Inhibition of EBV receptors was performed by incubating memory B cells with either anti-human CD21 (1:100), anti-human HLA-DR, DP, DQ (1:10), dilutions of anti-human CD20, or dilutions of anti- β_1 integrin for 1 h at 4°C, followed by spinoculation of the cells. Inhibition of EBV glycoproteins was performed by incubation of the EBV-containing supernatants with either anti-gp350/220 (1:100) or dilutions of anti-gp42 or soluble recombinant β_1 integrin for 1 h at 4°C prior to spinoculation of memory B cells. Inhibition of integrin signaling was performed by incubating the cells with either PP2 (10 μ M), wortmannin (10 nM), Ly-294002 (10 μ M), AG-82 (10 μ M), or cytochalasin D (10 μ M) for 30 min prior to spinoculation with EBV.

Western blotting. Cells were resuspended in 200 μ l modified RIPA buffer. Total amounts of protein in each sample were quantified and normalized using Nanodrop technology (Wilmington, DE), and samples were separated in a 4 to 12% Bis-Tris NuPAGE gel (Invitrogen, Carlsbad, CA).

Fluorescence microscopy. Cells were washed with ice-cold PBS and were cytospun onto microscope slides. Fixation was performed in 4% paraformaldehyde for 20 min prior to permeabilization for 3 min using 0.1% Triton X-100. Actin was stained using fluorescein isothiocyanate (FITC)-phalloidin (Sigma-Aldrich) for 1 h at room temperature.

Flow cytometry and FACS. Flow cytometry was performed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Nyon, Switzerland), and fluorescence-activated cell sorting (FACS) was done using a MoFlo system (Dako Cytomation, Glostrup, Denmark).

DNA microarrays. RNA from isolated tonsillar or peripheral blood B cells was extracted using the Trizol method (Invitrogen). Quality was verified on a model 2100 bioanalyzer and Nanodrop system. RNA was transcribed to cRNA by using the NuGEN system. The synthesized cRNA was hybridized to U133Plus2.0 GeneChips (Affymetrix) and scanned using an Affymetrix GeneChip scanner (model 3000) per Affymetrix protocols. Results were analyzed using Genespring 9 software (Agilent).

Statistics. Statistical analyses were performed using GraphPad Prism software (La Jolla, CA). Dual comparisons were analyzed using a two-tailed Mann-Whitney *t* test. *P* values below 0.05 were considered statistically significant.

RESULTS

Susceptibility of memory B cells from various lymphoid tissues to EBV infection. Even though peripheral blood resting memory B cells are the main reservoir for EBV persistence *in vivo* (26), memory B cells from peripheral blood appear to be less susceptible to EBV infection *in vitro* than their tonsillar counterparts (1, 9). This may indicate that EBV preferentially infects memory B cells from the NALT rather than those from other lymphoid tissues.

FIG. 1. EBV preferentially infects memory B cells expressing CD62L. (A) EBV infection of memory B cells from tonsils, peripheral blood, and mesenteric lymph nodes. EBV infection was performed by spinoculation of B95.8EBfaV-GFP supernatants. Results shown are percentages of EGFP⁺ cells gated for CD19⁺ CD27⁺ memory B cells. (B) Percentages of memory B cells (CD19⁺ CD27⁺) from tonsils, peripheral blood, and mesenteric lymph nodes expressing CD62L. (C) EBV infection of CD62L⁺ and CD62L⁻ memory B cells from tonsils, peripheral blood (PB), and mesenteric lymph nodes (LN). (D) EBV infection frequency of tonsillar memory B cells following blocking of CD62L by blocking antibodies. Data are percentages of EGFP⁺ cells gated for CD19⁺ CD27⁺ cells. Results shown are means $\pm$ standard deviations (SD) for 3 to 8 replicates. *P* values were calculated by using the Mann-Whitney *t* test. *, *P* = 0.05; **, *P* = 0.03; ***, *P* = 0.02; ****, *P* = 0.01; *****, *P* = 0.002.

To investigate tissue-specific EBV infection susceptibility in detail, we performed infection assays of lymphoid cells from tonsils, peripheral blood, and mesenteric lymph nodes by spinoculation of the EBV strain B95.8EBfaV-GFP to quantify the EBV-infected memory B cells by flow cytometry as previously described (9). Notably, following spinoculation, EBV enters B cells by the well-known EBV receptors, i.e., CD21 and HLA-DR (9). Memory B cells were identified by expression of CD19 and CD27 (31).

Memory B cells from tonsils, which are part of the Waldeyer's ring forming the NALT, showed the most susceptibility to EBV infection, with 83.1% $\pm$ 9.1% of cells being infected at 24 h postinoculation (Fig. 1A). In contrast, memory B cells

from peripheral blood, which represent a mixture of memory B cells from all lymphatic compartments, exhibited a lower infection frequency (30.3% $\pm$ 13.6%; *P* = 0.002), and memory B cells from mesenteric lymph nodes exhibited an even lower infection frequency (6.7% $\pm$ 3.1%; *P* = 0.02). This suggested that EBV preferentially infects memory B cells originating from NALT (Fig. 1A).

Since the expression of L-selectin (CD62L) on memory B cells is strongly associated with a NALT origin (5), we used this cell surface marker to further characterize the origin of EBV-infected memory B cells from the various lymphoid tissues. Tonsils, where NALT-originating memory B cells are generated, contained 81.6% $\pm$ 6.7% CD19⁺ CD27⁺ memory B cells expressing CD62L, indicating that <20% of the memory B cells homed to the tonsil from remote lymphatic organs (Fig. 1B). In contrast, only 21.5% $\pm$ 6.9% (*P* = 0.002) and 16.0% $\pm$ 5.3% (*P* = 0.01) of the CD19⁺ CD27⁺ peripheral blood and mesenteric lymph node memory B cells, respectively, expressed CD62L (Fig. 1B), documenting that only a minority of the memory B cells in these lymphoid tissues express the NALT-associated cell surface marker.

CD19⁺ CD27⁺ CD62L⁺ memory B cells isolated from tonsils, peripheral blood, and lymph nodes showed higher EBV infection susceptibility frequencies (75.8% $\pm$ 5.0%, 67.8% $\pm$ 7.9%, and 68.0% $\pm$ 9.2%, respectively) than their CD19⁺ CD27⁺ CD62L⁻ counterparts (40.0% $\pm$ 7.0%, 16.0% $\pm$ 4.7%, and 14.2% $\pm$ 3.1%, respectively) (Fig. 1C). Importantly, neither exposure to nor infection with EBV *in vitro* altered the levels of CD62L expression on memory B cells (data not shown). Thus, the presence of CD62L on the cell surface was associated with a significantly higher susceptibility to EBV infection than that in the absence of CD62L.

To investigate whether CD62L might be a cellular receptor for EBV, in addition to the identified EBV receptors on B cells, i.e., CD21 and HLA class II molecules, we performed EBV infection experiments using tonsillar memory B cells and CD62L-blocking antibodies. We could not observe any inhibition of EBV infection by CD62L-blocking antibodies, even at very high antibody concentrations (Fig. 1D). Thus, CD62L defines a subset of NALT-originating memory B cells that is phenotypically characterized by increased susceptibility to EBV infection but appears not to be involved in the cell infection process.

Expression of $\alpha_5\beta_1$ integrin on memory B cells from various lymphoid tissues. Next, we were interested in identifying, among cell surface structures, potential candidates contributing to preferential infection of memory B cells from NALT compared to those from other secondary lymphoid tissues. EBV is able to infect epithelial cells despite their lack of CD21, the main receptor for EBV on B cells. Thus, we hypothesized that epithelial cell surface structures facilitating or mediating EBV entry are present and active on memory B cells. Recently, it was described that the integrin receptor family, including the $\alpha_5\beta_1$ integrin, plays an important role in EBV attachment to epithelial cells (38, 39). This process is mediated by the EBV glycoprotein BMRF-2 (39).

To identify whether the differential susceptibility of memory B cells from NALT and non-NALT might be due to dissimilar $\alpha_5\beta_1$ integrin expression levels, we measured the expression profiles of integrins in B cells from tonsils and peripheral

FIG. 2. $\alpha_5\beta_1$ integrin is expressed preferentially on memory B cells from NALT. (A) Gene expression profiling of integrin-related genes, comparing tonsillar and peripheral blood B cells. The scale indicates fold regulation. (B and C) Immunofluorescence of $\alpha_5\beta_1$ integrin on memory B cells from tonsils (B) and peripheral blood (PB) (C). Bar, 50 μ m. (D to U) Quantification of $\alpha_5\beta_1$ integrin expression on memory B cells (D to F, J to L, and P to R) and naive B cells (G to I, M to O, and S to U) by flow cytometry. Data shown are percentages of $\alpha_5\beta_1$ integrin-positive memory and naive B cells from tonsils (D, E, G, and H), peripheral blood (J, K, M, and N), and lymph nodes (P, Q, S, and T) and relative fluorescence intensities of β_1 integrin on memory and naive B cells from tonsils (F and I), peripheral blood (L and O), and mesenteric lymph nodes (R and U). Data shown are means $\pm$ SD for 3 or 4 biological replicates. *P* values were calculated by using the Mann-Whitney *t* test. *, *P* = 0.001; **, *P* = 0.03; ***, *P* = 0.0001; n.d., not detected.

blood, respectively. The integrin subunits α_5 and β_1 and the integrin-related gene products TIF6 and PTK2 were found to be upregulated significantly in tonsillar B cells (Fig. 2A), indicating that signaling via $\alpha_5\beta_1$ integrin is particularly important in B cells originating from the tonsils. To confirm these data at the protein level, we isolated memory B cells from tonsils, peripheral blood, and mesenteric lymph nodes and subjected them to immunofluorescence staining for $\alpha_5\beta_1$ integrin and to flow cytometry. Immunofluorescence showed weaker expres-

sion of $\alpha_5\beta_1$ integrin on memory B cells from peripheral blood than on those from tonsils, even though all cells expressed at least low levels of $\alpha_5\beta_1$ integrin (Fig. 2B and C). This observation was further validated by quantification of the $\alpha_5\beta_1$ integrin expression levels by flow cytometry, showing that $95.1\% \pm 4.4\%$, $83.6\% \pm 3.1\%$, and $54.01\% \pm 11.5\%$ of memory B cells from tonsils, peripheral blood, and mesenteric lymph nodes, respectively, were $\alpha_5\beta_1$ integrin positive. Notably, two distinct populations could be distinguished based on

their $\alpha_5\beta_1$ integrin expression levels (Fig. 2D, G, and J). Cells with high expression levels of $\alpha_5\beta_1$ integrin were predominant in tonsils, with only a minority of cells expressing small amounts of $\alpha_5\beta_1$ (Fig. 2D and E). In contrast, in peripheral blood, the majority of $\alpha_5\beta_1$ integrin-positive memory B cells expressed low integrin levels (Fig. 2G and H), and in mesenteric lymph nodes, virtually all $\alpha_5\beta_1$ integrin-positive memory B cells showed low expression levels (Fig. 2J and K). Comparing the frequencies of $\alpha_5\beta_1$ integrin-positive memory B cells in the two complementary lymphoid tissues, i.e., tonsils and mesenteric lymph nodes, with the percentage of CD62L⁺ NALT-originating memory B cells, it is obvious that the $\alpha_5\beta_1$ integrin-positive phenotype is restricted to memory B cells selected in the NALT. Analysis of the mean fluorescence intensity (MFI) of $\alpha_5\beta_1$ integrin on these cells revealed that $\alpha_5\beta_{1\text{high}}$ memory B cells from tonsils and peripheral blood expressed 8- to 10-fold higher levels of $\alpha_5\beta_1$ integrin than did $\alpha_5\beta_{1\text{low}}$ memory B cells (Fig. 2F and I), whereas no $\alpha_5\beta_{1\text{high}}$ cells were found in mesenteric lymph nodes (Fig. 2L). In contrast, virtually all naïve B cells from tonsils, peripheral blood, and mesenteric lymph nodes invariably expressed amounts of $\alpha_5\beta_1$ integrin as high as those in the $\alpha_5\beta_{1\text{high}}$ tonsillar memory B cells (Fig. 2G, H, I, M, N, O, S, T, and U). Thus, tonsillar memory B cells differ from their counterparts from the other lymphatic tissues investigated by virtue of their $\alpha_5\beta_{1\text{high}}$ expression, a characteristic common to the naïve B cells from these tissues.

Binding of EBV to $\alpha_5\beta_1$ integrin-expressing memory B cells.

Next, we addressed the question of whether EBV uses $\alpha_5\beta_1$ integrin to attach to NALT-originating memory B cells, like it does with epithelial cells. Two cellular receptors are important for EBV binding and entry into B cells, namely, CD21 and HLA class II molecules (12, 28). Notably, both receptors are similarly expressed by memory B cells from NALT and non-NALT (9). CD21 (CR2, C3R), which is a member of the complement receptor family, acts as the main attachment factor for EBV on B cells. Attachment of EBV to CD21 via the viral glycoprotein gp350/220 has been shown to induce receptor-mediated endocytosis. Within the endosomal compartment, a trimer of EBV glycoproteins consisting of gH, gL, and gp42 binds to HLA class II molecules. This interaction, in turn, triggers a pH-dependent membrane fusion of EBV with the endosomal membrane and allows nuclear transport of the capsid (12, 28).

First, we aimed at identifying whether the $\alpha_5\beta_{1\text{high}}$ memory B-cell subpopulation in tonsils is indeed more susceptible to EBV infection. Infection of tonsillar memory cells, unsorted or sorted by flow cytometry, showed a 20% to 30% higher infection frequency of $\alpha_5\beta_{1\text{high}}$ than $\alpha_5\beta_{1\text{low}}$ memory B cells ($P = 0.05$) (Fig. 3A). FACS sorting was performed by gating on CD19⁺ CD27⁺ cells, followed by selection of $\alpha_5\beta_{1\text{high}}$ and $\alpha_5\beta_{1\text{low}}$ populations. Thereby, we could guarantee that the immunoglobulin isotype composition of the sorted cell populations reflected that of unsorted memory B cells. The preferential infection susceptibility of $\alpha_5\beta_{1\text{high}}$ memory B cells was also evident for memory B cells from peripheral blood, where $\alpha_5\beta_{1\text{high}}$ memory B cells exhibited a 30% higher infection frequency than that of their $\alpha_5\beta_{1\text{low}}$ counterparts ($P = 0.025$) (Fig. 3B). Although the observed infection frequencies in the $\alpha_5\beta_{1\text{high}}$ memory B-cell compartment were increased only 20% to 30% compared to those in the $\alpha_5\beta_{1\text{low}}$ memory B-cell com-

partment, these differences might be sufficient *in vivo* to allow preferential infection of $\alpha_5\beta_{1\text{high}}$ memory B cells.

Next, to test whether EBV uses $\alpha_5\beta_1$ integrin on NALT memory B cells for attachment, we performed experiments using antibodies blocking EBV binding to $\alpha_5\beta_1$ integrin or soluble recombinant β_1 integrin to compete with EBV binding to cellular β_1 integrin. As expected, blocking of CD21 or blocking of HLA class II molecules resulted in a significant reduction of the EBV infection frequency, legitimizing the use of spinoculation as a means to test EBV entry, as already shown in previous work (9) (Fig. 3C). Using antibodies to CD20, which is expressed by B cells but is irrelevant for EBV infection, we found no changes in EBV infection frequencies compared to those with mock treatment. However, with $\alpha_5\beta_1$ integrin-blocking antibodies, used either alone or in combination with anti-CD21 blocking antibodies, a dose-dependent reduction of the EBV infection frequency could be observed in tonsillar memory B cells (Fig. 3C). Since the combination of CD21- and $\alpha_5\beta_1$ integrin-blocking antibodies showed a nearly complete inhibition of EBV entry, it is unlikely that receptors other than CD21, HLA-DR, and $\alpha_5\beta_1$ integrin play a pivotal role in EBV entry into memory B cells. Finally, recombinant soluble β_1 integrin showed a similar dose-dependent inhibition of EBV infection of memory B cells from tonsils in the presence of additional inhibition of the EBV glycoprotein gp350/220 (Fig. 3D). As positive controls, we also included blocking of gp350/220 alone or in combination with dilutions of anti-gp42 to block EBV penetration of the endosomal membrane. Thus, we could show by either blocking cellular $\alpha_5\beta_1$ integrin or competing with recombinant β_1 integrin that EBV indeed uses β_1 integrin as a factor to attach to memory B cells.

Lack of BMRF-2 impairs infection of but not binding to B cells. Since $\alpha_5\beta_1$ integrin was recently shown to be critical for infection of epithelial cells by EBV (34, 37–39), we chose to employ the previously described BMRF-2^{low} EBV strain B27 to test whether the enhanced infection susceptibility of $\alpha_5\beta_1$ integrin-expressing memory B cells is due to BMRF-2– $\alpha_5\beta_1$ integrin interactions. The BMRF-2^{low} B27 and B95.8EBfaV-GFP strains did not contain significant amounts of wild-type EBV (<2%) (see Materials and Methods), and therefore we could exclude any significant interference of wild-type EBV. EBVs produced from both B95.8EBfaV-GFP- and B27-infected cells as well as wild-type B95.8-infected cells were used to infect B cells isolated from tonsils. EBV infection was determined by flow cytometry for GFP fluorescence 24 h following inoculation of the cells. As expected, inoculation with wild-type EBV showed no GFP fluorescence (Fig. 4A) and inoculation with B95.8EBfaV-GFP showed an increase of GFP fluorescence in around 50% of the inoculated cells (Fig. 4B). In contrast, <5% GFP fluorescence was observed 24 h after inoculation of the cells with BMRF-2^{low} B27 EBV, despite use of the same number of EBV DNA copies, as measured by qPCR after DNase treatment of the viral stock preparation (Fig. 4C).

Next, we compared the abilities of BMRF-2^{low} B27 EBV and wild-type EBV to bind to B cells by performing binding experiments followed by quantification of bound EBV DNA copies by quantitative PCR to determine the number of EBV particles bound per cell. Both wild-type and BMRF-2^{low} EBVs were able to bind to B cells, with comparable, dose-dependent

efficiencies (Fig. 4D). These results indicate that binding of EBV to B cells is not influenced by BMRF-2- $\alpha_5\beta_1$ integrin interaction and are in line with CD21 being the main receptor for EBV binding to B cells. To verify this observation, we performed binding experiments on monocytes, which lack the expression of CD21. Binding to monocytes was impaired for BMRF-2^{low} EBV compared to that for wild-type EBV (Fig. 4E). Surprisingly, wild-type EBV binding to B cells and monocytes was similar. This might indicate that additional binding sites for EBV exist that partially compensate for the lack of CD21.

Taken together, these data indicate that BMRF-2 is nonessential for the formation of enveloped EBV particles which are still able to bind to B cells via CD21 and/or HLA-DR but exhibit reduced binding capacity for monocytes, which lack expression of CD21.

Role of integrin signaling in EBV entry into NALT memory B cells. Even though the requirement of $\alpha_5\beta_1$ integrin for EBV binding to and infection of epithelial cells has been shown previously (38, 39), the participation of signaling events downstream of $\alpha_5\beta_1$ integrin initiated through EBV binding and their contribution to EBV entry have not been elucidated. The first evidence that $\alpha_5\beta_1$ integrin may be involved in the EBV infection process in B cells originating from the tonsil arose from our microarray analysis of tonsillar B cells showing significant upregulation of the TIF6 and PTK2 genes (Fig. 3A), two genes downstream of the $\alpha_5\beta_1$ integrin gene. We therefore dissected the integrin signaling pathway (11, 25) following EBV binding to memory B cells. One of the initial steps in integrin signaling involves activation of focal adhesion kinase by phosphorylation of ³⁹⁷Tyr, followed by downstream signaling through PI3K p85 α , c-Src kinases, and cofilin to finally activate the depolymerization of the actin cytoskeleton (Fig. 5A) (11, 13, 22, 24, 40). To investigate whether EBV itself activates integrin signaling, thereby enhancing infection efficiency, we (i) followed the phosphorylation status of FAK, PI3K, and cofilin over time upon binding of EBV to tonsillar memory B cells treated or not treated with inhibitors of FAK, c-Src, and PI3K p85 α activation prior to infection with EBV and (ii) investigated the specificity of the BMRF-2-integrin interac-

FIG. 3. Integrin $\alpha_5\beta_1$ is required for efficient attachment and entry of EBV on memory B cells. (A) Infection frequencies of $\alpha_5\beta_1^{\text{low}}$ and $\alpha_5\beta_1^{\text{high}}$ CD19⁺ CD27⁺ memory B cells after inoculation of either pooled tonsillar memory B cells or fluorescence-activated cell-sorted memory B-cell populations with B95.8EBfV-GFP by spinoculation. (B) Infection frequencies of $\alpha_5\beta_1^{\text{low}}$ and $\alpha_5\beta_1^{\text{high}}$ CD19⁺ CD27⁺ memory B cells after inoculation of pooled peripheral blood memory B cells with B95.8EBfV-GFP by spinoculation. (C) Inhibition of cellular receptors required for EBV infection (CD21 and HLA class II molecules), $\alpha_5\beta_1$ integrin, and nonrelevant surface proteins (CD20) on memory B cells from tonsils, using different concentrations and combinations of blocking antibodies. (D) Inhibition of the viral glycoproteins gp350/220 and gp42 in combination with competition inhibition of recombinant soluble β_1 integrin, using tonsillar memory B cells. Data shown in panels A and B are means $\pm$ SD for 3 biological replicates. *P* values were calculated using the Mann-Whitney *t* test. Data shown in panels C and D are from one representative experiment of three. *, *P* = 0.05; **, *P* = 0.025.

FIG. 4. Lack of BMRF-2 impairs infection but not binding to B cells. (A to C) Flow cytometry to detect GFP fluorescence 24 h after spinoculation of isolated tonsillar B cells with either wild-type B95.8 (A), B95.8EBfaV-GFP (B), or B27 BMRF-2^{low} EBV (C). The viral stocks used for spinoculation were normalized to 2×10^8 EBV DNA copies/ml after DNase treatment. Data shown are from one of three representative experiments. (D and E) Binding efficiencies of wild-type EBV and B27 BMRF-2^{low} EBV for either B cells (D) or CD14⁺ monocytes (E), as determined by quantitative PCR detecting the number of EBV DNA copies bound per cell. Data shown are means $\pm$ SD for three independent experiments.

tion by employing a recombinant EBV strain containing only trace amounts of BMRF-2 protein (38, 39).

Binding of EBV to tonsillar memory B cells led to upregulation of pFAK within 20 min, followed by phosphorylation of c-Src, PI3K p85 α , and cofilin (Fig. 5B). The kinetics of c-Src phosphorylation, which is much faster than that of PI3K p85 α , indicates that c-Src actually phosphorylates PI3K p85 α in the process of EBV entry. Furthermore, protrusions in the actin cytoskeleton similar to those observed upon activation of integrin signaling via fibronectin were readily detectable in memory B cells when EBV binding took place (Fig. 5D). This was abolished using heat-denatured EBV or heat-inactivated EBV, indicating that a conformationally active BMRF-2 protein might be required for interaction with $\alpha_5\beta_1$ integrin (Fig. 5D).

We also conducted experiments using a recombinant EBV containing only trace amounts of BMRF-2 (38). Following challenge of memory B cells with B27 BMRF-2^{low} EBV, activation of FAK and all downstream effectors was abolished (Fig. 5C), and rearrangements of the actin cytoskeleton did not occur (Fig. 5D). Given that the B27 BMRF-2^{low} EBV strain still contains residual BMRF-2, efficient activation of integrin signaling obviously requires a certain threshold amount of incorporated BMRF-2 that is present in wild-type EBV but not BMRF-2^{low} EBV. Memory B cells could not be infected with B27 BMRF-2^{low}, as determined by flow cytometry after stain-

ing for EBER (8) 48 h after spinoculation, even if B27 BMRF-2^{low} was used at an up to 4-fold higher DNA concentration than that of wild-type EBV B95.8 (data not shown). These results indicate that interaction between EBV BMRF-2 and integrins on memory B cells is crucial for the activation of the integrin signaling pathway.

To further investigate the dependence of EBV entry into memory B cells on integrin signaling, we used small-molecule phosphorylation inhibitors of FAK (AG-82), PI3K (wortmannin and Ly-294002), and c-Src (PP2) prior to infection of memory B cells by EBV-EGFP (Fig. 6A and B). These experiments showed that the whole integrin signaling pathway is indeed crucial for EBV entry, as all inhibitors used significantly reduced the susceptibility of the cells to infection with EBV. Dependence on the actin cytoskeleton rearrangement was tested by pretreating the memory B cells with cytochalasin D, which disrupts the actin microfilaments (23), and the results showed increased EBV infection of memory B cells (Fig. 6A and B).

Taken together, these results indicate that EBV binds to $\alpha_5\beta_1$ integrin on NALT memory B cells via its glycoprotein BMRF-2 and initiates activation of the integrin signal transduction pathway. Starting with the phosphorylation of FAK, c-Src, PI3K p85 α , and cofilin, this leads to depolymerization of

FIG. 5. EBV binding to integrin via BMRF-2 initiates signaling which is mandatory for virus entry into memory B cells. (A) Proposed signal transduction cascade initiated by EBV BMRF-2 binding to $\alpha_5\beta_1$ integrin. Following binding of BMRF-2 to cellular $\alpha_5\beta_1$ integrin, rapid phosphorylation of FAK initiates signal transduction via phosphorylation of the downstream mediators c-Src, p85 α , and cofilin and activation of the regulatory small GTPase RhoA, analogous to that initiated by binding of fibronectin to $\alpha_5\beta_1$ integrin (20). The resulting actin reorganization then facilitates EBV entry. (B and C) Phosphorylation (p-) of FAK, c-Src, p85 α , and cofilin following binding of B95.8 wild-type EBV (B) or B27 BMRF-2^{low} EBV (C) to isolated tonsillar memory B cells. (D) Initiation of actin filament reorganization (top) triggered by binding of EBV, fibronectin, heat-denatured EBV, heat-inactivated (h.i.) EBV, or B27 BMRF-2^{low} EBV to isolated tonsillar memory B cells. Actin was stained using FITC-phalloidin. Cells were additionally stained with DAPI (4',6-diamidino-2-phenylindole) to visualize nuclei (bottom [merge]). Bar, 20 μ m.

the actin cytoskeleton and thus to a more efficient nuclear translocation of the EBV capsid.

Induction of β_1 integrin expression in memory B cells isolated from non-NALT increases their susceptibility to EBV infection and transformation. Since a high level of expression of β_1 integrin is linked to increased susceptibility to EBV infection in memory B cells from NALT, we aimed at inducing β_1 integrin expression in memory B cells from non-NALT to investigate whether this would increase their susceptibility to EBV infection. Given that the expression and activity of integrins in B cells are regulated mainly via B-cell-activating signals received by CD40 (18) or the B-cell receptor (27), we triggered memory B cells from either tonsils or peripheral blood with CD40L plus anti-IgM antibody prior to EBV infection. Even though IgM-positive memory B cells make up only a minority of tonsillar memory B cells, stimulation with anti-IgM and CD40L has been shown to be sufficient for activation (36). The expression of β_1 integrin was monitored by flow cytometry 18 h after stimulation of cells. Whereas the percentage of memory B cells expressing high levels of β_1 integrin could not be increased by triggering tonsillar memory B cells (Fig. 7A), a 3-fold increase was observed upon triggering of peripheral blood memory B cells with CD40L plus anti-IgM (Fig. 7B and

C). The susceptibility to EBV infection of memory B cells from tonsils was not increased following *in vitro* triggering of these cells, as demonstrated by spinoculation with B95.8EBfaV-GFP (Fig. 7D), but EBV infection of memory B cells from peripheral blood triggered with CD40L plus anti-IgM could be increased to a percentage (60%) matching that for their tonsillar counterparts (Fig. 7E). Thus, the proportion of memory B cells from peripheral blood expressing high levels of β_1 integrin, as well as their susceptibility to EBV infection, can be increased substantially *in vitro*. In contrast, tonsillar memory B cells already show maximal percentages of cells expressing high levels of β_1 integrin and susceptibility to EBV infection prior to *in vitro* triggering.

DISCUSSION

In this work, we studied the mechanisms underlying the increased susceptibility to EBV infection of memory B cells from tonsils as opposed to memory B cells from other lymphoid sites. Our experimental data indicate that (i) high-level expression of $\alpha_5\beta_1$ integrin is unique to memory B cells from the NALT, and EBV indeed uses β_1 integrin as a cofactor to attach to memory B cells; (ii) triggering the $\alpha_5\beta_1$ integrin

FIG. 6. Activation of the integrin signal transduction pathway is crucial for EBV entry into memory B cells. (A and B) Inhibition of signaling molecules c-Src, PI3K, and FAK, activated downstream of integrin, impacts the susceptibility of tonsillar memory B cells to EBV infection by B95.8EBfaV-GFP viral supernatants. Inhibitors used were AG-82 (FAK), PP2 (c-Src), wortmannin (PI3K), and Ly-294002 (PI3K). Cytochalasin D was included to show the dependence of infection susceptibility of memory B cells on actin cytoskeleton depolymerization. Cells were counterstained for CD19 and CD27 to identify memory B cells and determine their infection frequency by flow cytometry. Results shown are from one representative experiment of two experiments.

signaling pathway is a key event for EBV entry and thus for tonsillar B cells' susceptibility to *in vitro* infection; and (iii) inducing expression of β_1 integrin by activating memory B cells with CD40L plus anti-IgM antibody increases the susceptibility of memory B cells to EBV infection. We concluded that memory B cells from tonsils exhibit increased susceptibility to EBV infection, by virtue of their high basal activation status reflecting their high level of β_1 integrin expression, which mediates

FIG. 7. Augmenting β_1 integrin expression on memory B cells increases their susceptibility to EBV infection and transformation. (A to C) Expression of β_1 integrin on memory B cells isolated from tonsils (A) or peripheral blood (B and C), without or following 18 h of treatment with CD40L plus anti-IgM. (D and E) Infection susceptibility to EBV of memory B cells from tonsils (D) or peripheral blood (E) treated for 18 h as described above, as determined by flow cytometry 24 h following inoculation of memory B cells with B95.8EBfaV-GFP by spinoculation.

increased attachment and entry of the virus, compared to memory B cells from other lymphatic tissues where B-cell-activating factors or antigen is less abundant.

The starting points of our hypothesis were the observations that tonsillar memory B cells are particularly susceptible to *ex vivo* EBV infection (9) and that Janz et al. recently reported the possible existence of additional receptors for EBV on B cells, besides CD21 and HLA class II molecules (14). Thus, we hypothesized that memory B cells from the NALT exhibit specific properties rendering them highly susceptible to EBV infection, and we assumed that memory B cells primed in the tonsils would keep their susceptibility to EBV infection when circulating in the blood. To address this hypothesis, we employed the unique CD19⁺ CD27⁺ CD62L⁺ phenotype of NALT-originating memory B cells (4, 5) to compare the relative infection susceptibilities of NALT-originating memory B cells in distinct lymphoid compartments and memory B cells selected at other lymphoid sites. Although NALT-originating (CD62L⁺) memory B cells exhibit a higher level of susceptibility to EBV infection irrespective of whether they are isolated directly from tonsils, peripheral blood, or mesenteric lymph nodes, we excluded the possibility that CD62L itself functions as a coreceptor for EBV by using neutralizing antibodies against CD62. We concluded that CD62L phenotypically marks a population of memory B cells that have been selected in the NALT and have retained or obtained receptors required for an efficient infection with EBV.

Recently, it was found that the interaction of an EBV envelope protein, BMRF-2, with $\alpha_5\beta_1$ integrin plays a role in the infection of polarized epithelial cells (38; R. Speck, unpublished data), which do not express the known B-cell receptors CD21 and HLA class II molecules, both of which are crucial for EBV entry. Here we found that memory B cells from the NALT express $\alpha_5\beta_1$ integrin at least 10-fold more than their counterparts from peripheral blood or mesenteric lymph nodes. Notably, we identified two distinct populations, defined as $\alpha_5\beta_1^{\text{high}}$ and $\alpha_5\beta_1^{\text{low}}$ cells, and found most tonsillar memory B cells expressing large amounts of $\alpha_5\beta_1$ integrin and most peripheral blood or mesenteric lymph node memory B cells expressing small amounts of $\alpha_5\beta_1$ integrin. Given that blocking and saturating $\alpha_5\beta_1$ integrin by use of antibodies and the recombinant extracellular domain of β_1 integrin, respectively, resulted in a clear decrease of EBV entry into memory B cells, β_1 integrin has a key role as a cofactor in the viral entry process. Notably, memory B cells from distinct lymphatic tissues express equal amounts of the cell surface molecules CD21 and HLA class II (9), which constitute the EBV receptor complex: this consequently does not explain the distinct susceptibilities to EBV infection. In contrast, the level of $\alpha_5\beta_1$ integrin expression discriminates between degrees of susceptibility of memory B cells to EBV infection.

As we show here, the correlation of susceptibility to EBV infection with the $\alpha_5\beta_1$ integrin expression level is mediated by binding of EBV's structural protein BMRF-2 to $\alpha_5\beta_1$ integrin. Furthermore, we show that each step of β_1 integrin-mediated signaling leading to the activation of the downstream targets (20) is crucial for EBV entry into B cells. Very importantly, activation of memory B cells from peripheral blood with CD40L plus anti-IgM resulted in a vigorous upregulation of β_1 integrin expression. Thereby, the susceptibility of peripheral

blood memory B cells to infection by EBV, i.e., of memory B cells which were not from the NALT, was increased. Stimulation of the B-cell receptor results in increases of the expression levels of integrin, whereas CD40L, which binds to CD40 on B cells and, in addition, to $\alpha_5\beta_1$ integrin (18), may activate the downstream integrin signaling pathway and in turn may lead to an increased uptake of EBV via actin cytoskeleton reorganizations. Thus, these data demonstrate clearly that the susceptibility of memory B cells to EBV infection is greatly dependent on their activation status and the resulting high expression levels of β_1 integrin. In fact, in a primary immune reaction, the CD40L expressed on activated T cells has a key role in subsequent activation of B cells by binding to CD40 (35). Thus, the events described above may be operative during primary or secondary immune responses in the NALT, explaining its marked susceptibility to EBV infection and its central role in EBV pathogenesis. Whether the interaction of EBV's essential envelope protein gH with $\alpha_v\beta_6$ or $\alpha_v\beta_8$ integrin, which very recently was shown to trigger epithelial cell fusion by EBV (7), plays a role in B-cell infection remains to be investigated.

We propose a model in which EBV takes advantage of targeting activated CD62L⁺ $\alpha_5\beta_1^{\text{high}}$ memory B cells which preferentially reside in or home to NALT and, following circulation, are likely to home back to NALT. We have previously shown that naïve B cells from distinct tissues are equally highly susceptible to EBV infection *ex vivo* (9). Here we report that naïve B cells from distinct tissues essentially express the $\alpha_5\beta_1^{\text{high}}$ phenotype, in contrast to memory B cells expressing the $\alpha_5\beta_1^{\text{high}}$ phenotype preferentially when originating from NALT. Our data speak in favor of a direct infection of tonsillar memory B cells with EBV which, in addition to primary infection of naïve (33) and GC B cells (2, 19), contributes to a more efficient establishment of EBV persistence. In NALT, the memory B cells may encounter recall antigen and subsequently either propagate EBV as a latent infection in progeny cells or undergo differentiation into plasma cells, resulting in lytic replication of EBV, which then can be transmitted via saliva to new susceptible hosts. Triggering of CD40 and the B-cell receptor mediated by exposure to microorganisms may be involved in this process, as such triggering increased the proportion of cells expressing high levels of β_1 integrin. NALT is readily accessible to a wide variety of antigens, explaining why memory B cells generated within and homing back to the NALT are much more readily found in an activated, i.e., high-level β_1 integrin-expressing, state than remote lymphoid organs, which are not accessible by antigen or pathogens directly but only following initial immune recognition by sentinel immune cells such as dendritic cells. The idea that EBV establishes persistence by direct infection of memory B cells was also proposed by Kurth et al., who examined EBV-infected B cells in infectious mononucleosis (15, 16). Our findings give a detailed insight of EBV B-cell infection and biology.

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