The Epstein-Barr virus induces the expression of the LPAM-1 integrin in B-cells *in vitro* and *in vivo*

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Running title: B-cell homing and EBV infection

Word count abstract: 146

Word count text: 3886

Abstract

The Epstein-Barr virus (EBV) infects the oropharynx but surprisingly frequently induces B-cell proliferations in the gut of immunosuppressed individuals. We found that EBV infection *in vitro* induces the expression of the LPAM-1 integrin on tonsillar B cells and increases it on peripheral blood cells. Similarly, LPAM-1 was induced in the tonsils of patients undergoing primary infectious mononucleosis. EBV-induced LPAM-1 bound to the MAdCAM-1 addressin that allows B-cell homing to the gastrointestinal mucosa-associated lymphoid tissue (GALT). Thus, we hypothesized that EBV-induced LPAM-1 could induce relocation of infected B-cells from the tonsil to the GALT. *In situ* hybridization with an EBER-specific probe revealed the frequent presence of EBV-infected cells in the pericolic lymph nodes of healthy individuals. Relocation of infected B-cells into the GALT would expand the EBV reservoir, possibly protects it from T-cells primed in the oropharynx and explain why EBV induces lymphoid tumors in the gut.

Importance

The Epstein-Barr virus (EBV) causes tumors in multiple organs, in particular the oro- and nasopharyngeal area, but also in the digestive system. This virus enters the body in the oropharynx and establishes a chronic infection in this area. The observation that the virus causes tumors in the digestive system implies that the infected cells can move to this organ. We found that EBV infection induces the expression of integrin beta 7 (ITGB7), an integrin that associates with integrin alpha 4 to form the LPAM-1 dimer. LPAM-1 is key for homing of B cells to the gastrointestinal tract, suggesting that induction of this molecule is the mechanism through which EBV-infected cells to enter

this organ. In favor of this hypothesis, we could also detect EBV-infected cells in the lymph nodes adjacent to the colon and in the appendix.

Introduction

The Epstein-Barr virus (EBV) infects the large majority of the world population (1). The virus is spread through saliva and efficiently infects B lymphocytes that undergo transformation through the expression of nine viral latent genes that belong to the EBNA (Epstein-Barr virus nuclear antigen) and LMP (latent membrane protein) families. This cellular proliferation is usually transitory in immunocompetent individuals, although it can be quite extensive in tonsils of individuals undergoing infectious mononucleosis (IM) (1).

Even if the EBV infection causes no pathology, the virus remains silent in infected subjects (1). Patients with immune deficiencies, e.g. transplant recipients treated with immunosuppressive drugs or HIV-infected individuals, are at increased risk of B-cell lymproliferations driven by EBV (See Van Krieken et al., Raphael et al., Swerdlow et al. in (2) for reviews). These disorders commonly invade the gastrointestinal tract, in particular the colon. A recent series of patients with post-transplant lymphoproliferative disease (PTLD) showed GI tract involvement in 56% of the cases (3). However, the origin of the infected cells remains unclear because the virus enters the body through the oropharynx, is initially found in the tonsil where it persists and from which it recirculates in the peripheral blood (1). Previous investigations showed that EBV-infected cells are 20 times more frequent in the Waldeyer ring than in the spleen or in mesenteric lymph nodes around the liver and spleen (4). Indeed, lesions such as plasmacytic hyperplasia and infectious mononucleosis–like lymphoproliferations that are encountered in children or EBV-naive adult solid organ recipients frequently present in tonsils and adenoids, but this does not explain the high frequency of gut PTLDs (See Swerdlow et al. in (2)). We have readdressed this issue and performed molecular

investigations that revealed that EBV infection induces the expression of integrin beta 7 (ITGB7), an integrin that associates with integrin alpha 4 (ITGA4) to form the LPAM-1 dimer. LPAM-1 binds to the addressin MAdCAM-1 that is expressed on the surface of venules of the mucosa associated lymphoid tissue (MALT) and is key for homing of B cells to the gastrointestinal tract (5). Furthermore, we found EBV-infected cells, sometimes in large numbers, in para-colic lymph nodes, an organ that had not been precisely investigated before and in the appendix.

Material and methods

Human tissues

The samples were provided by the tissue bank of the National Center for Tumor Diseases (DZIF, Heidelberg, Germany) in accordance with the regulations of the tissue bank and the approval of the ethics committee of Heidelberg University. We collected extranodal PTLD samples that developed in the digestive system and tonsils from normal and IM patients. We also investigated paracolic lymph nodes (between 1 and 6 per patient, on average 3), spleens, Peyer's patches, gastric biospsies and appendices from patients without cancer or acute infections.

Lymphocyte adhesion and blocking of adhesion

We generated CHO cell lines that stably express pcDNA3.1 expression plasmids that encode MAdCAM-1 (B1621) and the EBV gene BLLF1 whose protein product is gp350 (B702) after selection with G418 (1 mg/ml). Clones that express gp350 or MAdCAM-1 and controls (CHO/gp350, CHO/MAdCAM-1, CHO/pcDNA3.1) were selected for adhesion assays in which they are co-cultivated with EBV-transformed LCLs for 30 minutes at +4°C under constant agitation (6). 2x10E4 transfected CHO cells were mixed with 6x10E4 EBV-infected B-cells in 96 well plates. After three gentle washings with PBS, the percentage of non-adherent B-cells was determined by counting. For blocking experiments, 5x10E4 EBV-infected B-cells were first mixed with either the LPAM-1 blocking antibody Act-1 (provided by NIH, 1:500 dilution) or with an isotype control and co-cultivated with CHO/MAdCAM-1 cells for 30 minutes at 37°C. After three gentle washings with PBS, the percentage of non-adherent B-cells was determined by counting.

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Virus production

HEK293 cells (ATCC: CRL-1573) stably transfected with recombinant B95-8 or M81 EBV-BACs were transfected with expression plasmids encoding BZLF1 (p509) and BALF4 (pRA) to induce lytic replication (7). Three days after transfection, virus supernatants were collected and filtered through a 0.45 µm filter.

B cell infections and *in vitro* transformation experiments

Total CD19-positive B-cells purified from the peripheral blood or tonsils from different donors were exposed to viruses for four hours at a multiplicity of infection (MOI) of 20 virus genomes, as defined by qPCR, per target cell as described previously (7). These assays were approved by the ethics committee of Heidelberg University (392/2005). Infected cells were washed once with PBS and plated in cluster plates in RPMI supplemented with 20% FBS. In some experiments, we performed EBV infections of CD10-positive and CD10-negative cells isolated from tonsillar CD19-positive using MACS beads (Miltenyi Biotec, Germany). We also used the same technology to purify CD27+/IgD- memory and CD27-/IgD+ naïve B-cells from the peripheral blood and infected them in parallel with the virus.

B cell stimulation with CD40-ligand and IL-4

Freshly isolated CD19+ primary B cells were cultured on a 90 Gy-γ-irradiated CD40-ligand (CD40L) feeder cell layer in the presence of 25 ng/ml recombinant human IL-4 (PeproTech, Germany) for 1 week. For long-term expansion, the CD40-ligand feeder cells were replaced at least once per week and the cells were replenished with fresh medium containing 10ng/ml recombinant human IL-4.

Antibodies

We used primary mouse monoclonal antibodies against ITGA4 (Biolegend 1:25 dilution), ITGB1 (Biolegend 1:50 dilution), ITGB7 (Biolegend 1:50 dilution), LPAM-1 (Clone Act-1, 1:500 dilution) (8). The secondary antibodies applied for immunofluorescence staining were goat anti-mouse coupled to Alexa488 (Biolegend 1:300 dilution) or to PE (Biolegend 1:300 dilution). We also used streptavidin-coupled to Alexa 488 (Life technologies 1:200 dilution) to visualize the biotinylated primary antibodies.

Results

1) EBV infection induces LPAM-1 expression in B-cells.

We first performed RNAseq on four B cell lines generated by EBV infection and filtered the list of transcripts for molecules involved in homing. This approached identified CCR7, CD44, CD99, F11R, SELPLG, GLG1, CXCR4, PECAM1, LPAM-1 (ITGA4 and ITGB7), L-Selectin (SELL), LFA-1 (ITGB2 and ITGAL) and VLA-4 (ITGA4 and ITGB1) as potentially up regulated by EBV infection (Table 1) (5). CD44, CCR7, SELL, LFA-1 and VLA-4 were known to be targets of the virus and its LMP-1 protein, but ITGB7 and LPAM-1 have not been reported before as EBV target (9, 10). Flow cytometry analyses were first performed with B-cells from adenoids and from tonsils that were or not exposed to EBV. Non-infected tonsillar B-cells did not express LPAM-1, as previously reported and adenoid cells expressed this molecule only weakly (Fig. 1A) (11). However, EBV infection of adenoid and tonsillar B-cells induced the expression of LPAM-1 strongly, demonstrating that exposure to the virus induced the expression of this integrin pair in hitherto negative cells or in cells weakly expressing it. The induction of LPAM-1 became visible 15 hours after EBV infection (Fig. 1B). We then repeated and extended this assay with resting peripheral blood B-cells, the same B-cells stimulated with CD40L/IL-4 or infected with EBV. The peripheral blood B-cells expressed ITGA4, ITGB7 and LPAM-1 but not ITGB1 as previously described (Fig. 1C) (11, 12). Expression of ITGB7 on these cells is expected as they include cells recirculating cells from the GALT. CD40L/IL-4 stimulated B-cells lost expression of ITGB7 and LPAM-1, but expressed ITGA4 and ITGB1. Peripheral blood B-cells transformed by EBV expressed all these integrins, thereby confirming the results of the RNAseq assay. Importantly, EBV-transformed blasts expressed ITGB7 and LPAM-1 at levels clearly higher than the resting peripheral blood B-cells, an observation in line

with the infection experiments performed with adenoid cells. We then compared the activation status of CD40L/IL-4 stimulated B cells and EBV-infected cells using CD80 expression as a readout. This assay showed that both B-cell populations were activated at comparable levels, suggesting that the differences in integrin expression between these populations could not be ascribed to a general activation level (Fig. 1D). We then infected naïve and memory B-cells isolated from peripheral blood and transformed by EBV that were found to express LPAM-1 at high level after transformation (Fig. 1E). Therefore, EBV infection induces ITGB7 expression in B-cells independently of their initial differentiation status. Infection of CD10-positive and CD10-negative cells from an adenoid sample also enhanced ITGB7 and LPAM-1, although the effect was stronger in the CD10-negative population (Fig. 1F). We extended our investigations to the EBV-negative Burkitt's lymphoma cell lines DG75, BJAB and Akata (EBV-negative clone) that were found to be LPAM-1 negative (Fig. 1G). Similar results were obtained with the EBV-positive cell line Akata. However, we found a weak expression of LPAM-1 on the Burkitt's lymphoma cell line Raji, that was one order of magnitude lower than in LCLs. We infected BJAB cells with a recombinant B95-8 virus and selected the transfected cells with hygromycin. This generated a cell line that expressed the GFP gene that is cloned onto the recombinant viral genome in more than 98% of the cells. FACS staining of this cell line showed that it weakly expressed LPAM-1 at the levels seen in Raji cells. Thus, expression of ITGB7 after EBV infection is observed with primary B-cells and irregularly and weakly in Burkitt's lymphoma cell lines (Compare Fig. 1C and 1G).

We then attempted to identify the EBV proteins involved in the induction or amplification of ITGB7 expression. To this end, tonsillar B-cells were infected with a virus mutant lacking the latent EBV protein LMP1 but we found that the infected cells

expressed ITGB7 at same levels as B-cells transformed by wild type viruses (Fig. 1H). Similar results were obtained with B-cells infected with other EBV mutants lacking other viral latent proteins or non-coding RNAs such as the EBERs or the BHRF1 and BART microRNAs (Fig. 1H and data not shown). To determine the role of EBNA2 in LPAM-1 induction, we studied EREB cells that are peripheral blood B cells transformed with a conditional EBNA2 that is responsive to estrogen (13). Inactivation of EBNA2 after a three-day estrogen withdrawal did not affect its expression (Fig. 1I). We expanded our analyses to a pair of Burkitt's lymphoma cell lines that either express the full (MUTU III, latency III) or a restricted set of latent proteins (MUTU I, latency I) but could not detect LPAM-1 in any of the samples (Fig. 1J). This suggests that the EBNA and LMP proteins with the exception of EBNA1 are not involved in the induction of LPAM-1. Therefore, we stained an LCL generated with an EBNA1 null mutant with an antibody specific to LPAM-1 (14). This cell line expressed LPAM-1, suggesting that EBNA1 is not involved in its regulation in LCLs (data not shown). To determine whether LPAM-1 expression requires infection with a functional EBV genome, we infected adenoid B-cells with EBV virus-like particles (VLPs) that are devoid of viral DNA and are therefore unable to transform infected cells. Whilst LPAM-1 expression was found in cells infected with wild type virus, we could not detect LPAM-1 expression after exposure to EBV VLPs (Fig. 1K). Thus, it remains unclear how the viral infection induces LPAM-1 but this process depends on the presence of the EBV genome.

In an attempt to demonstrate that ITGB7 induction after EBV infection also takes place *in vivo,* we stained tissue sections from tonsils of patients with or without acute EBV infection. While EBV-negative tonsils showed no or only faint ITGB7 expression in both follicular and interfollicular B-cells, confirming the data of the literature and our

FACS stains, the IM tonsils strongly expressed this integrin in areas of the section where tonsillar cells were infected with EBV in the seven investigated cases (Fig. 2A), suggesting that EBV infection also induces ITGB7 *in vivo*. Immunostaining of tissues from tumors generated in humanized mice infected by the virus (n=4) and of gastrointestinal PTLD cases (n=8) revealed that these lesions all expressed ITGB7 (Fig. 2B). Thus, EBV infection induces or potentiates the expression of a molecule on activated B lymphocytes that allows entry into the mucosal immune system both *in vitro* and *in vivo*.

2) EBV-infected cells can be captured by cells expressing MAdCAM-1

LPAM-1 interacts with MAdCAM-1, an addressin selectively expressed by the endothelial cells of high endothelial venules in the small and the large intestine and the GALT, as well as in the intestinal lamina propria (15). We tested whether LPAM-1 expressed at the surface of EBV-infected B cells can interact with MAdCAM-1 by transfecting CHO cells with the human MAdCAM-1 gene and with the EBV-specific BLLF1 gene whose protein product gp350 avidly binds to CD21 expressed by B cells (1). Cells transfected with an empty pcDNA3.1 plasmid served as a negative control. We assessed the ability of these cell lines to retain EBV-infected cells. Fig. 3A shows that the expression of MAdCAM-1 doubles the number of EBV-infected cells retained by CHO cells after 30 minutes of co-culture at 4°C, with an efficacy close to the one observed after transfection of gp350. To further validate that the binding to MAdCAM-1 was attributable to LPAM-1 expression, we treated EBV-infected B cells with an antibody against LPAM-1 (Act-1) that blocks the binding to MAdCAM-1. Compared to cells that were treated with an isotype control antibody, treatment with Act-1 led to a 3.1-fold decrease in the number of bound B-cells (Fig. 3B). We conclude that the

LPAM-1 molecule expressed by EBV-infected cells interacts with MAdCAM-1, its ligand (15).

3) **EBV-infected cells are present in paracolic mesenteric lymph nodes and in the appendix.**

The detection of LPAM-1 at the surface of infected B-cells suggested that these cells should be able to enter the GALT. Therefore, we screened a panel of lymphoid tissues from immunocompetent individuals without cancers or infections with a probe specific for the EBV-specific viral non-coding RNA EBER. This analysis detected, as expected, rare EBV-positive cells in 70% of investigated tonsils (Fig. 4, Table 2). The frequency of infection in pericolic lymph nodes and appendices reached 40% and 44% of the investigated samples, respectively. We could not find evidence for EBER-positive cells in the investigated splenic or ileal samples (Fig. 4, Table 2).

We then determined the density of EBER-positive cells and found that it was more than 2 times higher in the pericolic lymph nodes, relative to tonsils or appendices (Table 2). The investigated tissues were subjected to EBV-specific qPCR that confirmed the absence of viral DNA in the spleen and its presence in the tissues that contain EBER-positive cells. Only one sample of pericolic lymph nodes showed discrepancies between both methods, being positive by qPCR and not for EBER (Table 2). As we could investigate only a limited proportion of lymph nodes from individual healthy virus carriers, and, considering the abundance of the GALT system, it is thus likely that, at least some individuals host far more resting EBV-infected cells in the GALT than in the tonsils.

4) EBV can colonize acquired MALT structures in the stomach with follicular gastritis.

The identification of EBV-positive gastric carcinomas raises the question of how the virus enters the stomach. However, the normal stomach does not contain any organized lymphoid structures (16). This changes after infection with *H. pylori* that can lead to the development of an acquired mucosa-associated lymphoid tissue (MALT) tissue with development of B-cell follicles, generating a follicular gastritis (16). We EBER-stained three cases of these lesions and could detect the presence of the virus in all three cases that were also PCR-positive (Fig. 4, Table 2). We also tested seven additional samples from gastritis without B-cell follicles but could not detect the virus in these tissues.

Discussion

In an attempt to explain the frequent localization of EBV-associated tumors to the GI tract, we screened multiple RNA seq libraries of EBV-infected B cells for molecules involved in B-cell homing and found that this virus induced the expression of LPAM-1. Furthermore, LPAM-1 expression in B-cells allowed functional interaction with cells that express MAdCAM-1, its ligand, that can be blocked by an antibody against LPAM-1 (15). This strongly suggests that EBV-infected B-cells can access the GALT. Indeed, a strong expression of LPAM-1 on lymphocytes, and in particular on B immunoblasts, is sufficient for their homing to the GALT through interaction with the MAdCAM-1 addressin (11). This molecular interaction is essential as knockout mice that lack MAdCAM-1 or ITGB7 possess a severely atrophic GALT system (17, 18).

LPAM-1 is expressed by a subset of B-cells located in the Peyer's patches and in the lamina propria of the small intestine (19). This molecule is also present on peripheral blood B-cells that comprise cells recirculating from the GALT (11, 12). However, LPAM-1 is expressed neither by resting nor by proliferating B-cells located in the tonsil or in the spleen (11, 12, 20). We could confirm that tonsillar B-cells do not express ITGB7 and that low levels of ITGB7 and LPAM-1 expression can be detected on peripheral blood B-cells and cells from adenoids. However, EBV infection clearly upregulated ITGB7 and LPAM-1 expression in all of these cell types, the amplification being maximum in tonsillar B-cells. LPAM-1 induction upon EBV infection took place after infection of different B cell subsets including CD10+ and CD10- cells, as well as naïve and memory B cells. INTB7 upregulation also took place in tonsils of patients with IM. Unfortunately, the ITGB7-specific antibody is not suitable for double stains with EBERs that would have been ideal to directly demonstrate that the EBV-infected cells express ITGB7. However, this protein was clearly expressed only in areas where

the infected cells could be found. In contrast, B-cells stimulated by CD40L and IL-4 and Burkitt's lymphoma cells, some of which were EBV-positive, did not express ITGB7/LPAM-1, although B-cells stimulated by CD40L and IL-4 and LCLs expressed CD80 at similar levels. On the other hand, Raji and BJAB cells infected with a recombinant B95-8 virus expressed LPAM-1 but at much lower levels than LCLs. Thus, strong and constant LPAM-1 upregulation after EBV infection takes place only in primary B lymphocytes and is probably the result of a complex process that does not involve that key latent proteins EBNA2 and LMP-1. Furthermore, LPAM-1 induction following EBV infection bypasses the B-cell priming for gut homing conferred by intestinal dendritic cells (20). This is, to our knowledge the first example of a virus infection that can induce a change in the homing of B-cells. HIV selectively infects LPAM-1 positive T-lymphocytes to access the gut in which the virus can replicate (21). However, HIV does not induce expression of the LPAM-1 integrin as EBV does.

LPAM-1 is considered to be the most important protein for lymphoid cells to access the GALT and its induction could thus enable EBV-infected B cells to access the GALT (5). We tested this hypothesis by staining tissues from the GALT and could identify EBV-infected B cells in the appendix and in pericolic lymph nodes. Thus, we are confident that EBV-infected B-cells can be found outside the blood and the oropharyngeal area. The analysis of a larger number of samples from the GALT will give more precise information on the size of the EBV reservoir in these organs.

However, we could not detect any EBV-infected cells in the Peyer's patches of adults. It is possible that EBV-infected cells are more frequent in the larger Peyer's patches of younger individuals (22). CCR9 and CCR10, two chemokine receptors that increase B-cell retention in the small intestine or colon, are not expressed in EBV-infected B-cells (Table 1) (5, 23). However, EBV-infected cells expressed L-selectin

that binds to the peripheral node addressin, and CCR7, a chemokine receptor that interacts with the CCL19 and CCL21 chemokines (10). This unusual combination of integrins allowing entry in different lymphoid structures at the surface of EBV-infected cells might lead to preferential persistence in the paracolic mesenteric lymph nodes within the GALT (23).

Primary EBV infection takes place in the oropharynx and B-cells become infected in this area (1). Indeed, individuals with infectious mononucleosis have enlarged tonsils and adenoids containing a large number of EBV-infected cells (1). The detection in the present study of EBV-infected B-cells in mesenteric lymph nodes implies that these infected cells originate from these oropharyngeal lymphoid organs. The homing of B-cells in general from the Waldeyer ring to the GALT in humans has not been investigated in details, although one study suggested that its efficacy is poor (24). This cell migration could be greatly facilitated by an EBV infection.

The identification of EBV-infected B-cells in the GI tract has implications for our understanding of the interactions between the virus and its host. Previous models have considered that the Waldeyer ring and not the mesenteric lymph nodes are the main reservoir of virus, although it was recognized that a few EBV-infected cells can be found in these latter organs (4). Importantly, this study focused on mesenteric lymph nodes adjacent to the liver and the spleen and not on the paracolic lymph nodes (4). This model was mainly based on the observation that viral replication takes place in the oropharynx and virus-infected B-cells outside the Waldeyer ring were considered to be dead ends in terms of virus spread (4). EBV-infected cells express not only LPAM-1 but also CD44, L-selectin, LFA-1 and VLA-4, a set of molecules that allow entry in the Waldeyer ring and inflammatory sites (15). However, EBV-infected cells are found at

variable levels in the peripheral blood and could include recirculating infected B-cells from the oropharynx and B cells from the GALT that could return to the oropharynx.

Our findings have consequences for the pathogenesis of EBV-associated diseases. Many PTLD develop in the GI tract of immunodeficient individuals who present in 20% of the cases with enlarged mesenteric lymph nodes (25). The frequent detection of latently infected B-cells in the paracolic lymph nodes or in the intestinal wall suggests that their uncontrolled proliferation gives rise to these adenopathies and are the source of the transformed cells that invade the GI tract. Interestingly, an early form of polyclonal PTLD frequently develops in the oropharyngeal areas in children (2). This fits with the concept that the virus initially enters the body though the oropharynx, but establishes long-term persistence in larger areas of the body, including the GI tract. Inflammation enhances MAdCAM-1 expression (26, 27). Thus LPAM-1 expression in inflammatory bowel disease colonic tissues could explain the reports of an increased presence of EBV-infected cells in these samples (28-30). In the same vein, EBV has been detected in patients with gastritis cases, with or without adjacent carcinoma (30). Inflammation could also explain the development of EBV-associated diffuse large B-cell lymphomas in body cavities of patients with tuberculosis treated by artificial pneumothorax and in patients with osteomyelitis, metallic implant or chronic skin ulcer (See Chan et al. in (2)).

What would be the advantages of virus to persist in the GALT? On the one hand, it would expand the reservoir of virus in infected individuals, thereby increasing the chances of the virus to persist in the infected host. On the other hand, it would also reduce the likelihood of being detected by EBV-specific T-lymphocytes. Indeed, upon encounter with their cognate antigen in a lymph node, T-lymphocytes become imprinted to the territory drained by this lymph node (31). Thus, if the primary encounter with the

virus takes place in the tonsil, the likelihood for an EBV-infected cell to be recognized by T-cells in the GALT is reduced. In favor of this hypothesis, Andrew Hislop and colleagues found that EBV specific CD8-positive T-cells home to oropharyngeal sites during IM (32). The reduced ability of the T-cells to target EBV-infected cells in the gut would lead to a preferential proliferation of infected cells located in this organ in case of immunosuppression.

Acknowledgments

The mouse monoclonal antibody Act-1 (cat#11718) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. SF was supported by the German Center for Infection Research (DZIF). We thank the microarray unit of the Genomics and Proteomics Core Facility, German Cancer Research Center (DKFZ), for providing excellent services. We thank Dr. B.Kempkes for the EREB cells and Dr. A. Schepers for the LCL transformed by the EBNA1-null mutant.

Conflict of Interests

The authors declare no conflict of interests.

Figure legends

Fig. 1 Expression of integrins in various B-cell populations. A) Resting (CD19+) and EBV-infected adenoid and tonsillar B-cells (LCL) were stained with antibodies against LPAM-1 (red) or isotype control (blue) and analyzed by flow cytometry (n=3 for each type of sample). B) Resting (CD19+) adenoid B cells were exposed to EBV. Cell populations were stained with antibodies against LPAM-1 and analyzed by flow cytometry 15 hours and 40 days post-infection (dpi) (n=2). C) The expression of ITGA4, ITGB1, ITGB7 and LPAM-1 (red) or isotype control (blue) was assessed by flow cytometry in resting blood B-cells (CD19+), EBV-infected blood B-cells (LCL) or blood B-cells stimulated by CD40L and IL-4 (CD40L) (n=5). D) The expression of CD80 (red) or isotype control (blue) was assessed by flow cytometry in resting blood B-cells (CD19+), EBV-infected blood B-cells (LCL) or blood B-cells stimulated by CD40L and IL-4 (CD40L) (n=2). E) Three EBV-negative Burkitt's lymphoma cell lines (Akata-, DG75, BJAB) and two EBV-positive Burkitt's lymphoma cell line (Akata+ and Raji) were stained for LPAM-1 (red) or isotype control (blue) (n=1). F) Expression of LPAM-1 (red) or isotype control (blue) in cell lines generated by infection of memory and naïve blood B-cells (n=2). G) LPAM-1 (red) or isotype control (blue) expression in EBV-infected adenoid CD10+ and CD10- B-cells (n=3). H) Expression of LPAM-1 in B cell populations infected with various viral mutants. Resting (CD19+) adenoid B cells were infected with either M81 Wt virus (Wt) or a M81 mutant lacking the LMP1 or LMP2 gene (ΔLMP1 or ΔLMP2). Cell populations were stained with antibodies against LPAM-1 and analyzed by flow cytometry at day 7 post-infection. I) EREB cells were grown in the presence (Estrogen+) or absence (Estrogen -) of estrogen. LPAM-1 (red) or isotype control (blue) expression is shown in the FACS dot

plots. J) Two EBV-positive Burkitt's lymphoma cell lines that express the full (MUTU III, latency 3) or restricted set of viral latent proteins (MUTU I, latency 1) were stained for LPAM-1 (red) or isotype control (blue). K) Expression of LPAM-1 (red) or isotype control (blue) in resting (CD19+) B cells and in B cells exposed to DNA-free virus-like particles (VLP) for 24 hours (n=2).

Fig. 2 Expression of integrins and localization of EBV-infected B-cells. A) Consecutive histological sections of a tonsil removed from a patient with IM was stained for EBER (left top panel) or ITGB7 (right top panel) (n=7). The top and middle panel show pictures taken at low (x20) and high (x40) power. Similar investigations performed in the tonsil of a patient without EBV acute infection (bottom panel) (n=4). B) The expression of ITGB7 in various EBV-infected tumors from humans or immunosuppressed mice was assessed by immunohistochemistry (n=4 and n=8, respectively).

Fig. 3 EBV-transformed B cells are retained by CHO cells that express MAdCAM-1. A) CHO cells were stably transfected with pcDNA3.1 vectors expressing MAdCAM-1, EBV gp350, or with the pcDNA3.1 parental vector. These cell lines were incubated with 4 independent EBV-transformed B cell samples. The dot plot shows the percentage of B cells adherent to the stably transfected CHO clones. B) Lymphocyte adhesion to CHO cells that stably express MAdCAM-1 was blocked by an antibody directed against LPAM-1 (Act-1). An antibody of the same class and type but without target specificity served as isotype control (IC). Three replicates of two independent LCLs were analyzed and the results are shown in the dot plot (n=6).

Fig. 4 EBV-infected cells can be detected in various lymphoid organs. Tissues from tonsils, paracolic lymph nodes, appendices, Peyer's patches, follicular gastritis, and spleen collected from immunocompetent individuals were subjected to *in situ* hybridization with an EBER specific probe (For the number of investigated samples, also see Table 2).

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568

transcript	sample 1 (in RPKM)	sample 2 (in RPKM)	sample 3 (in RPKM)	sample 4 (in RPKM)	mean	SD
ITGA4	27,50	25,50	18,58	19,00	22,64	4,53
ITGAL	8,36	16,16	13,01	19,07	14,15	4,59
ITGB1	11,34	9,05	8,07	8,91	9,34	1,40
ITGB2	14,91	7,67	17,23	9,74	12,39	4,44
ITGB3	3,02	2,27	5,12	4,59	3,75	1,33
ITGB7	12,74	13,25	13,57	11,76	12,83	0,79
SELL	38,46	105,06	46,65	82,10	68,07	31,09
SELPLG	3,15	7,09	18,16	16,48	11,22	7,26
GLG1	7,88	8,20	6,46	8,00	7,64	0,79
CD44	12,87	18,50	18,39	27,25	19,25	5,95
CCR7	8,17	37,54	30,38	49,69	31,44	17,45
CCR9	0,02	0,01	0,00	0,01	0,01	0,01
CCR10	1,71	1,02	0,93	0,67	1,08	0,44
CXCR4	2,96	8,78	11,06	8,85	7,91	3,47
CXCR5	0,09	0,18	0,01	0,07	0,09	0,07
PECAM1	1,30	2,00	1,53	2,51	1,83	0,54
CD99	17,89	17,24	20,91	20,00	19,01	1,73
F11R	3,68	2,54	6,92	5,59	4,69	1,95
JAM3	0,66	1,11	0,83	1,05	0,91	0,21

Table 1: Expression of B-cell transcripts involved in lymphocyte B homing in four EBV-infected cell lines. RPKM: reads per kilobase per million mapped reads

	number of positive samples (EBER)	EBER positive cells per mm^2 (mean)	number of positive samples (qPCR)	qPCR titer (mean in cop/cell)
gut lymph nodes (patients/samples)	4/10 / 16/38	0,139	5/10	5,77E-04
Tonsil	7/10	0,049	7/10	3,49E-03
Appendix	4/9	0,066	4/9	4,06E-04
Peyer's Patches	0/5	0,000	0/5	0
Spleen	0/5	0,000	0/5	0
Gastritis	3/10	0,143	8/10	4,92E-03

Table 2: Frequency of EBV infection in various human tissues.

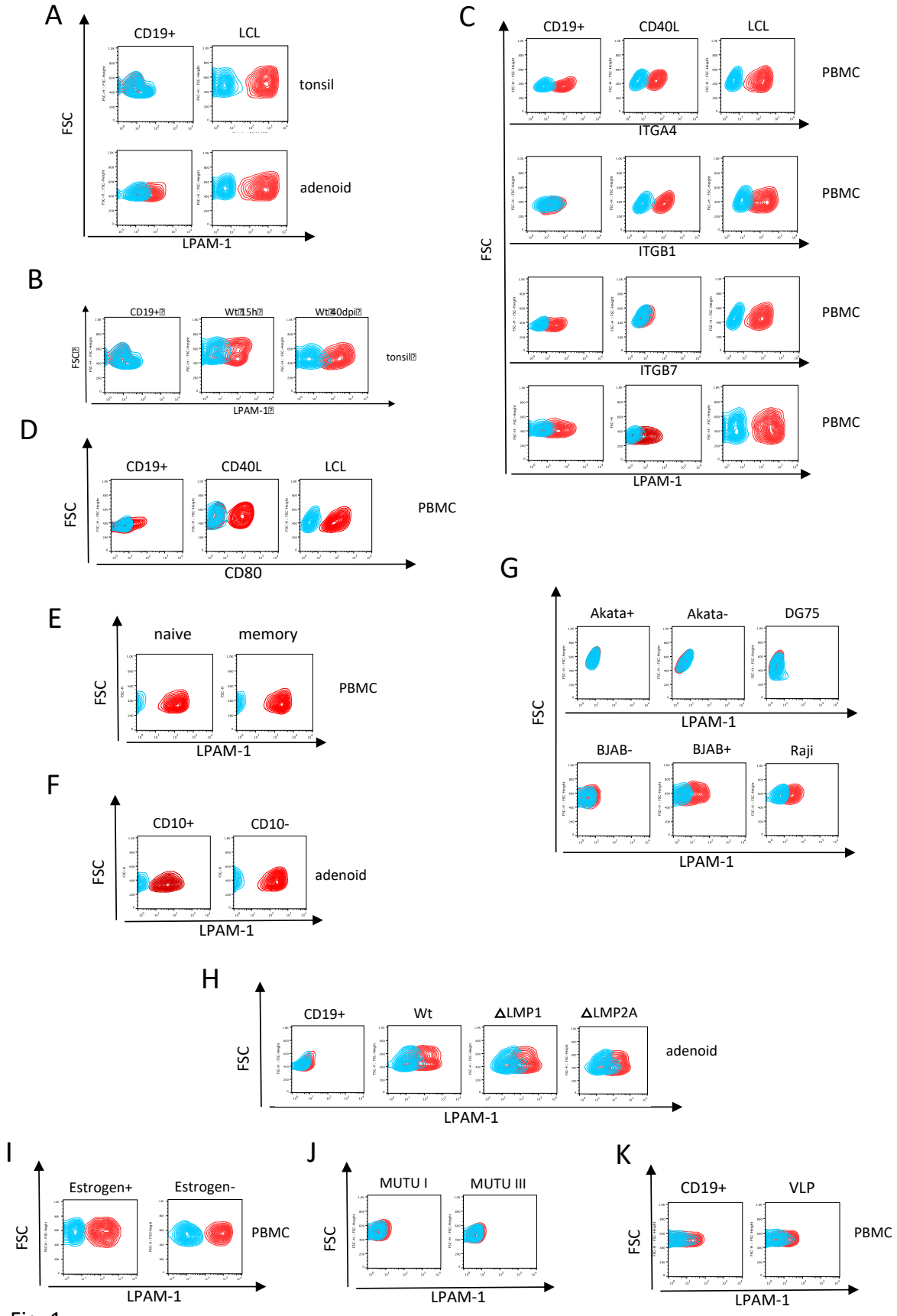


Fig. 1

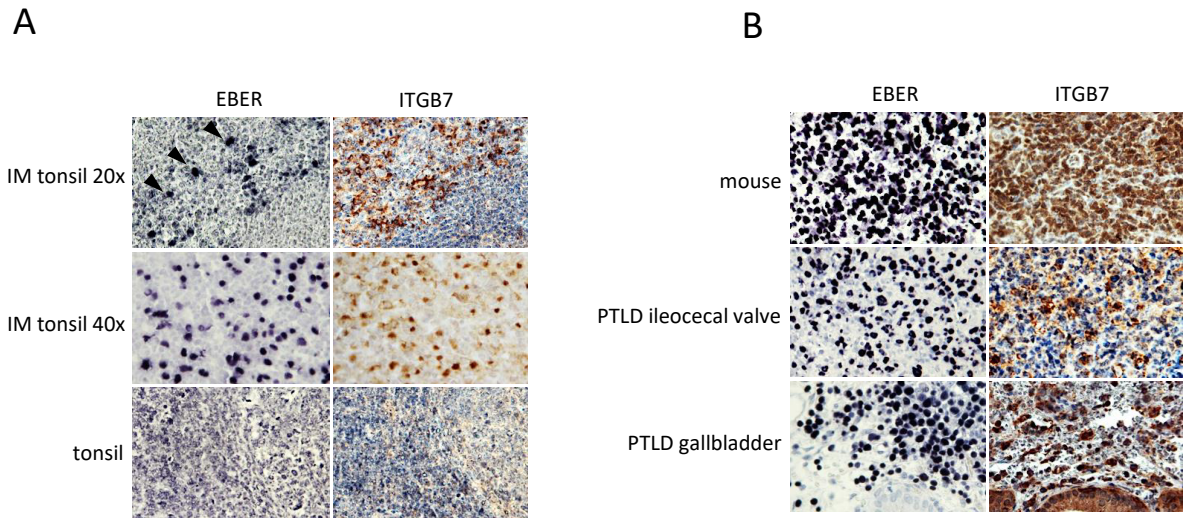
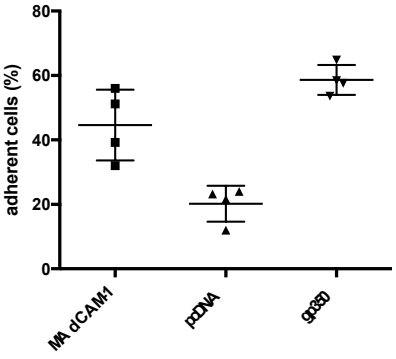


Fig. 2

A



B

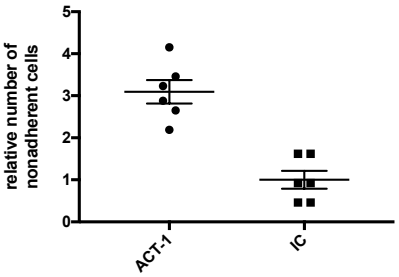


Fig. 3

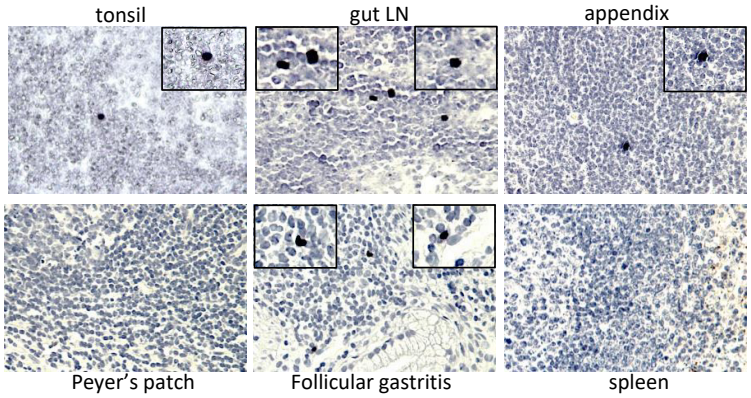


Fig. 4