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Role of Epstein-Barr virus microRNAs in viral immune evasion

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Hiermit versichere ich an Eides statt, dass die vorliegende Arbeit mit dem Titel

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von mir selbständig und ohne unerlaubte Hilfsmittel angefertigt wurde, und ich mich dabei nur der ausdrücklich bezeichneten Quellen und Hilfsmittel bedient habe. Die Arbeit wurde weder in der jetzigen noch in einer abgewandelten Form einer anderen Prüfungskommission vorgelegt.

München 28.2.18,

Manuel Albanese

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Epstein-Barr viral miRNAs inhibit antiviral CD4$^+$ T cell responses targeting IL-12 and peptide processing.

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Abstract

Epstein-Barr virus (EBV) is a tumor virus that establishes lifelong infection in most of humanity, despite eliciting strong and stable virus-specific immune responses. EBV encodes at least 44 miRNAs, most of them with unknown function. Here we show that multiple EBV's miRNAs modulate immune recognition of recently infected primary B cells, EBV's natural target cells. EBV's miRNAs collectively and specifically suppress release of pro-inflammatory cytokines such as IL-12, repress differentiation of naive CD4$^+$ T cells to Th1 cells, interfere with peptide processing and presentation on HLA class II, and thus reduce activation of cytotoxic EBV- specific CD4$^+$ effector T cells and killing of infected B cells. Our findings identify a previously unknown viral strategy of immune evasion. By rapidly expressing multiple miRNAs, which are themselves non-immunogenic, EBV counteracts recognition by CD4$^+$ T cells and establishes a program of reduced immunogenicity in recently infected B cells allowing the virus to express viral proteins required for establishment of lifelong infection.

Publication II

Albanese Manuel*, Tagawa T.*, Bouvet M., Maliqi L., Lutter D., Hoser J., Hastreiter M., Hayes M., Sugden B., Martin L., Moosmann A., Hammerschmidt W. *equal author contributions

Epstein-Barr virus microRNAs reduce immune surveillance by virus-specific $CD8^+$ T cells.

2016, **Proc. Natl. Acad. Sci. USA** 113, E6467–E6475

Abstract

Infection with Epstein-Barr virus (EBV) affects most humans worldwide and persists lifelong in the presence of robust virus-specific T cell responses. In both immunocompromised and some immunocompetent people EBV causes several cancers and lymphoproliferative diseases. EBV transforms B cells in vitro and encodes at least 44 miRNAs, most of which are expressed in EBV-transformed B cells, but their functions are largely unknown. Recently, we showed that EBV's miRNAs inhibit $CD4^+$ T cell responses to infected B cells by targeting IL-12, MHC class II, and lysosomal proteases. Here we investigated whether EBV's miRNAs also counteract surveillance by $CD8^+$ T cells. We have found that EBV's miRNAs strongly inhibit recognition and killing of infected B cells by EBV-specific $CD8^+$ T cells through multiple mechanisms. EBV's miRNAs directly target the peptide transporter subunit TAP2 and reduce levels of TAP1, MHC class I molecules, and EBNA1, a protein expressed in most forms of EBV latency and a target of EBV-specific $CD8^+$ T cells. Moreover, miRNA-mediated downregulation of IL-12 decreases the recognition of infected cells by EBV-specific $CD8^+$ T cells. Thus, EBV's miRNAs employ multiple, distinct pathways allowing the virus to evade surveillance not only by $CD4^+$ but also by antiviral $CD8^+$ T cells.

SUMMARY

Epstein-Barr virus (EBV) is a herpesvirus that infects the majority (>90%) of the human population worldwide. EBV establishes a stable relationship with its host for a lifetime, which is mostly asymptomatic and under efficient control of the host's T cells. As a consequence, in immunocompromised individuals, severe EBV-associated malignancies can develop. In order to prevent these diseases and find possible new therapeutic approaches, it is essential to study the mechanisms of viral immune evasion, which protect the virus-infected cells from eradication.

To date, 25 microRNA (miRNAs) precursors have been identified in EBV's genome and accumulating evidences suggest that they might subvert the host's antiviral immune response. We used deep-sequencing techniques to analyze the contribution of EBV's miRNAs in primary B cells infected with EBV for five days. We found cellular transcripts related to adaptive and innate immunity among the most affected pathways in the presence of EBV's miRNAs.

The combination of RNA-seq and RISC-IP-seq analyses revealed that *IL12B* was the top downregulated gene, but pro-inflammatory cytokines such as IL-6 and TNF-α appeared to be controlled as well. Interestingly, we could validate *IL12B* as the direct target of five different viral miRNAs. We found that reduced level of IL-12 secreted from EBV-infected B cells reduced differentiation of antiviral Th1 cells.

We could also validate the miRNA-mediated reduction of TAP1 and TAP2, two proteins that form the heterodimeric transporter of processed peptides from the cytoplasm to the ER lumen. Furthermore, we observed that MHC class I, all three subclasses of MHC class II molecules (HLA-DR, HLA-DQ and HLA-DP), and several

costimulatory and adhesion molecules were downregulated in the presence of EBV's miRNAs.

The activation of EBV-specific $CD4^+$ T cells was reduced in co-culture experiments with B cells infected with EBV strains expressing viral miRNAs in contrast to B cells infected with a strain incapable of expressing them. Similarly, we found that the recognition and killing of EBV-infected B cells by EBV-specific $CD8^+$ T cells was strongly reduced by EBV's miRNAs.

In addition, other direct targets of viral miRNAs were identified, including viral proteins such as LMP1 and three important lysosomal enzymes, which further contribute to the immune evasive phenotype that we identified.

Overall, our findings provide the first evidence that EBV's miRNAs have an important role in adaptive immune evasion of EBV-infected cells. The viral miRNAs repress several genes involved in different steps of cellular immune recognition.

INTRODUCTION

1. Epstein-Barr Virus

Epstein-Barr Virus (EBV), also known as human herpes virus 4 (HHV-4), belongs to the family of gamma herpes viruses. EBV is an enveloped virus of approximately 180 nm in size with a double-stranded DNA genome of 160 kb that encodes about 85 open reading frames (ORFs). More than 90% of the worldwide population is infected with the virus, in which the virus persists lifelong, often asymptomatically (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2010). However, when the first viral infection does not take place early during childhood but occurs later during adolescence or in adults, EBV can cause acute infectious mononucleosis (IM), which is characterized by high fever, enlarged lymph nodes, and tiredness (Rickinson et al., 2014). These patients have a higher risk of developing malignant diseases (Crawford, 2001) and multiple sclerosis (MS) later in their lives (Achiron, 2004).

EBV predominantly infects B cells, mainly resting B cells, in which the virus establishes a latent infection. During latency, only a limited number of viral genes is expressed, preventing the recognition of the infected B cells by the immune system. Indeed, the EBV infection is never completely cleared by the host immunity (Chen, 2011).

Several malignancies are linked to EBV, such as Hodgkin Lymphoma (HL), Burkitt's lymphoma (BL), and post-transplant lymphoproliferative disorder (PTLD). PTLD occurs in immunocompromised individuals, who have a reduced immune surveillance of EBV-infected B cells (Gottschalk et al., 2005), indicating that the virus causes this disease. EBV-associated diseases other than PTLD, likely emerge from co-morbidities or co-factors, which are mostly unknown.

1.1 EBV's replication cycle

Infection of EBV initiates via the envelope glycoprotein 350 (gp350) that binds to the cellular complement receptor 2 (CR2 or CD21) (Nemerow et al., 1987; Wang and Hutt-Fletcher, 1998)**,** which is present on B cells but also on certain epithelial cells. Infection of B cells *in vitro* induces their activation, proliferation and immortalization leading to the generation of lymphoblastoid cell lines (LCLs). LCLs are a useful model to study and characterize many steps of the viral replication cycle and its impact on infected cells.

The viral glycoprotein 42 (gp42) contributes to the interaction between virions and target cells binding to the major histocompatibility complex II (MHC-II). Differently from B cells, epithelial cells lack MHC-II, therefore *in vitro* infection of these cells is very inefficient (Wang et al., 1998; Shannon-Lowe et al., 2009).

Immediately after B cell infection, several latent and many early-lytic viral genes are expressed. The latter are only transiently expressed early after infection to support survival and cell cycle entry of resting B cells, but not expressed later when latency is established (Kalla and Hammerschmidt, 2012).

Among these lytic genes, two viral homologous of cellular anti-apoptotic BCL-2 proteins, BHRF1 and BALF1, are essential for initial cell transformation (Altmann and Hammerschmidt, 2005).

During the early phase of infection, named EBV's transitional pre-latent phase, the EBV nuclear antigen 2 (EBNA2) is the earliest latent protein detected (Allday et al., 1989), which is essential for the cellular transformation. EBNA2 acts as transactivator of other viral genes, such as the latent membrane protein 1 (LMP1) and 2 (LMP2) and many other cellular genes (Fields et al., 2007; Zimber-Strobl and Strobl, 2001).

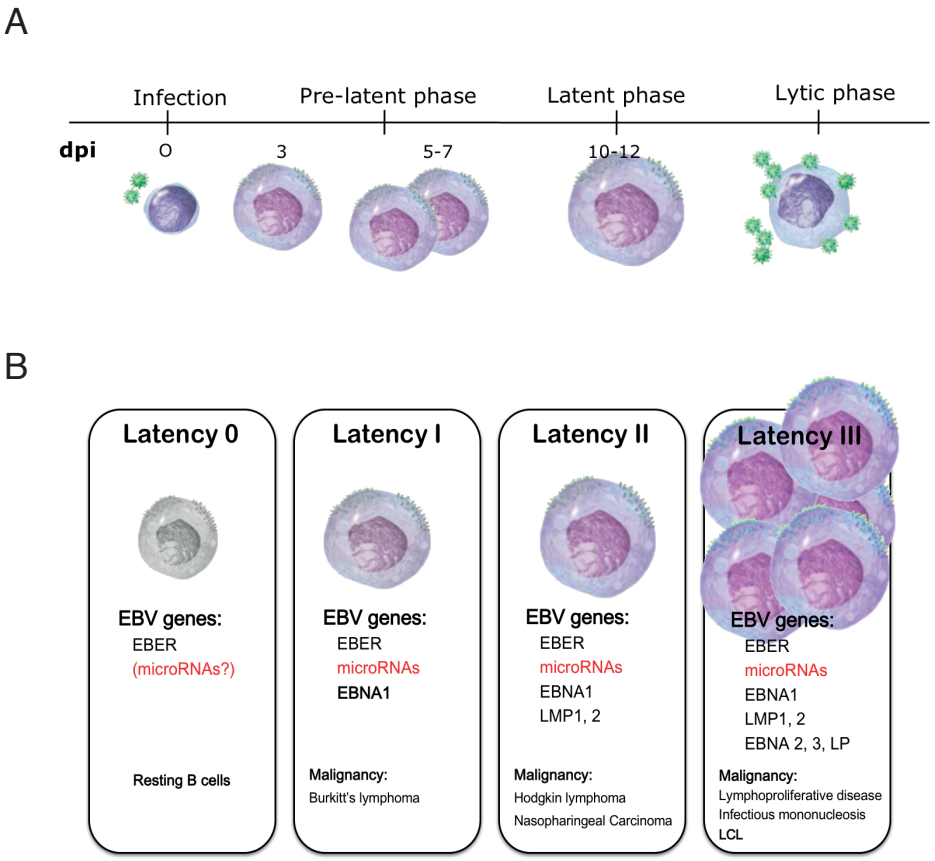
The EBV nuclear antigen 1 (EBNA1) is also expressed early after infection and has many key functions for the virus. During the early phase, EBNA1 is essential for the expression of EBV's transforming latent genes (Altmann et al., 2006), because it acts as a transcription factor on promoters of certain viral latent genes (Kennedy and Sugden, 2003). When the cells start dividing four days post-infection, EBNA1 is necessary for the replication, segregation and maintenance of the viral genome in the infected cells (Yates et al., 1985; Levitskaya et al., 1997).

During the pre-latent phase, the latent membrane proteins LMP1 and LMP2 promote the activation and proliferation of infected B cells. LMP1 mimics a constitutively active CD40 receptor and therefore does not depend on CD40 ligand (CD40L) to be active (Gires et al., 1997; Kutz et al., 2008). Expression of LMP1 is important for B cell-transformation, inducing an activated phenotype (Dirmeier et al., 2003). LMP2 mimics the B-cell receptor (BCR) providing an effective survival signal for infected B cells (Mancao and Hammerschmidt, 2007; Merchant et al., 2001).

Seven to ten days after infection, the virus expresses only eight latent proteins: six EBV nuclear antigens (EBNA1, 2, 3A/B/C and LP) and the membrane proteins (LMP1 and 2). During this phase of latent infection, called "Latency III" or growth program, the cells are fully activated and proliferate, giving rise to lymphoblastoid cell lines (LCLs) (Scheme 1). LCLs established after *in vitro* infection of B cells express the set of genes characteristic of the latency III "growth program". However, because of an efficient control of the immune system *in vivo*, infected B cells further reduce the expression of viral genes, limiting their immunogenicity. The different types of viral latencies are classified depending on the number of proteins expressed: latency II (four proteins), latency I (EBNA1 only) and latency 0 or "true latency" in which no viral proteins are expressed (Babcock and Thorley-Lawson, 2000). Infected B cells in

latency 0 are considered to be peripheral resting B cells that recirculate between the blood and the oropharyngeal lymphoid tissues (Taylor et al., 2015) (Scheme 1). Latently infected memory B cells sporadically reactivate entering the lytic cycle, probably induced by their differentiation to plasma cells or by other signals from the extracellular environment. Activated memory B cells produce progeny virions, which spread to other B cells allowing the virus to survive in its host lifelong. The different types of viral latencies are also observed in many EBV associated diseases: post-transplant lymphoproliferative disease (PTLD; type III), Hodgkin's disease (type II), gastric carcinoma, nasopharyngeal carcinoma (type I/II) and Burkitt's lymphoma (type I) (Rickinson et al., 2014) (Scheme 1).

In addition, EBV expresses different non-coding RNAs, such as Epstein-Barr virus-encoded RNAs (EBER), the BamHI-A rightward transcripts (BARTs), the small nucleolar RNAs (snoRNA1) and EBV's miRNAs (described below). The specific functions of most viral non-coding RNA remain still controversial and unclear, but the EBERs are used as biomarker in clinical practice since they are highly abundant and expressed in all the EBV-infected cells *in vivo* (Skalsky and Cullen, 2015).



Scheme 1. EBV's replication cycle
A. Within the first phase post infection, called pre-latent phase, few viral genes are expressed aiming to immortalize the infected cells and establish its latency. Sporadic lytic reactivation is observed, the production of new virions allows the virus to spread into other target cells and to infect the host lifelong. B. Description of the EBV viral latencies. The latency programs differ according to the gene expression pattern of the infected cells; each type has a different cellular phenotype and is associated with distinct malignancies. dpi = days post infection.

2. Adaptive immune response against EBV

During primary infection, the virus is rapidly detected by components of the innate immune system, which initiate a direct antiviral response and prime the adaptive immunity.

Immune responses are crucial to control the virus both in acute and chronic infections. Adaptive immune responses can be divided into the humoral response, which is antibody mediated, and the cell-mediated response.

2.1 Antibody responses

The humoral response is mediated by plasma B cells, which release antibodies, a soluble form of the BCR with specificity to recognize various types of antigens.

Acute IM patients show a strong production of IgM antibodies specific for the viral capsid antigen (VCA) as an early response against the virus. IgM antibody levels decrease after a few days and are replaced by a class of IgG antibodies directed against VCA, the EBV early antigen (EA-D) and EBNA2 (Taylor et al., 2015). After the acute phase of infection, these antigens rapidly decline and a peak of IgG response against EBNA1 can be detected from three-six months after infection. The IgG response reaches a stable level and persists for life. In seropositive individuals, IgG antibodies against EBNA1 can be always detected, while newly produced IgM antibodies, mainly directed against VCA, are detected only upon viral reactivation (Klutts et al., 2004).

2.2 T cells responses

The cell-mediated response is mediated by the interaction of the Major Histocompatibility complex also known as the human leukocyte antigen (HLA), expressed on target cells and the T cell receptor (TCR), on T cells.

MHC class I molecules are expressed on any nucleated cell and present short antigenic peptides to $CD8^+$ T cells. MHC-II molecules, primarily expressed on antigen presenting cells (APC), present peptides of phagocyted proteins to $CD4^+$ T cells. B cells are considered to be APC because they express both MHC-I and MHC–II molecules, therefore infected B cells are potentially recognized by both $CD4^+$ and $CD8^+$ T cells. The $CD8^+$ T cells are known to be the effector T cells responsible for the clearance of the infected cells and $CD4^+$ T cells are considered to have a role in supporting and shaping the immune response to the pathogen.

During acute infection, EBV-infected cells are more susceptible to T cells recognition compared to latently infected cells. IM patients show a dramatic expansion of global $CD8^+$ T cells with high frequencies of EBV-specific $CD8^+$ T cells, up to 50% of which recognize lytic antigens (Hislop et al., 2007). Although the numbers of $CD4^+$ T cells do not increase as much as $CD8^+$ T cells, activated EBV-specific $CD4^+$ T cells represent up to 1% of the peripheral $CD4^+$ T cells (Long et al., 2013). It has been reported that EBV-specific $CD4^+$ T cells against lytic antigen show a strong cytotoxic phenotype (Adhikary et al., 2006; Münz et al., 2000; Long et al., 2005).

When the acute infection comes to an end, the levels of EBV-specific T cells decrease with the reduction of circulating virus. In healthy, EBV-positive individuals EBV-specific $CD4^+$ T cells constitute less than 0.1% of the total $CD4^+$ T cell population, but EBV-specific $CD8^+$ T cells may still represent up to 2% of all peripheral $CD8^+$ T cells. Furthermore, T cells specific for peptides derived from lytic antigens are twice as many compare with $CD8^+$ T cells that recognize epitopes of latent antigens (Abbott et al., 2013). In case of lytic reactivation, the adaptive immunity relies on a strong memory response of these EBV-specific T cells.

During latency, EBV-infected B cells are almost invisible to virus-specific T cells because the virus dramatically reduces the expression of viral proteins to limit the amount of antigen**.** This situation accounts for the asymptomatic infection of EBV in the majority of the population. In individuals with an impaired T cell immunity, EBV can promote the development of malignances such as post-transplant lymphoproliferative disease (PTLD) and Hodgkin's disease**,** underlining the important role of T cells, which keep the virus under control.

3. EBV immune evasion

The ability of EBV to establish a latent infection and to infect its host lifelong, despite a strong immunity against the virus, suggests that EBV has developed very efficient strategies to evade the immune system.

During lytic reactivation, EBV expresses more than 80 viral genes. Their products make the virus-infected cells more susceptible to immune surveillance. Among these proteins, more than eight are reported to act as immunoevasins (Ressing et al., 2015), limiting the activation of the immune system that would otherwise eliminate the virus-producing cells, thereby preventing the release of progeny viruses and viral spread to other target cells.

EBV's immunoevasins inhibit several steps of MHC class I and II mediated epitope presentation, some of which are shared with other herpesviruses. BGLF5 is a protein with DNase and RNAse activities that promotes mRNA degradation and reduces the levels of the MHC-I gene transcripts (Zuo et al., 2009). BILF1 is a G-protein coupled receptor that localizes to the plasma membrane. BILF1 can directly interact with MHC class I promoting its lysosomal degradation and reducing its surface levels (Paulsen et al., 2005). Interestingly, HLA-A and B but not HLA-C are affected (Griffin et al., 2013), probably preventing the recognition of the virus producing cells by natural killer (NK) cells. NK cells are particularly sensitive to abnormal HLA-C levels, since the reduction of these molecules leads to NK cells activation (Blais et al., 2011).

Other two important EBV immunoevasins are BNLF2a and the EBV homologue of IL-10, v-IL-10 (also named BCRF1). These two viral proteins inhibit the transporter associated with antigen processing (TAP) through different mechanisms; BNLF2a binds to the cytosolic domains of TAP and blocks both peptide and ATP binding (Horst

et al., 2011; Wycisk et al., 2011), while v-IL-10 decreases the mRNA and protein levels of TAP1 (Zeidler et al., 1997).

The TAP complex is a heteroduplex formed by TAP1 and TAP2 and necessary for the translocation of peptides generated by the proteasome from the cytosol to the endoplasmic reticulum (ER). The peptides transported into the ER are then loaded onto MHC-I molecules and presented on the cell surface to $CD8^+$ T cells. The unique role of the TAP complex in MHC-I presentation makes TAP an appropriate target for viral strategies of immune evasion. In fact, not only EBV but also many other viruses actively reduce TAP activity: US6 encoded by human cytomegalovirus (HCMV) (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997), CPXV012 of several cowpox virus strains (Alzhanova et al., 2009; Byun et al., 2009), ICP47 encoded by herpes simplex virus (HSV)-1 and HSV-2 (Hill et al., 1995; Ahn et al., 1996; Tomazin et al., 1996), UL49.5 encoded by varicella virus, as well as bovine herpes virus (BHV)-1, BHV-5 and equine herpes virus (EHV)-1 and EHV-5 (Koppers-Lalic et al., 2008).

EBNA1 is the only EBV protein that is expressed in almost all types of viral latencies. As such, it is important for EBNA1 to reduce its own presentation via MHC-I limiting the recognition of EBV-infected cells by EBNA1-specific $CD8^+$ T cells. EBNA1 contains a repetitive amino acid sequence of glycin-alanine (GAr) that inhibit translation of EBNA1 itself and interferes with its proteasome degradation and peptide presentation via MHC-I (Levitskaya et al., 1995, 1997; Yin et al., 2003). Despite these EBNA1-specific immune evasive mechanisms, EBNA1-specific $CD8^+$ as well as $CD4^+$ T cells are prevalent in the majority of EBV-positive individuals and are probably important to keep the virus under control.

In contrast to the well described strategies of EBV immune evasion during lytic reactivation, it is not clear how the infected B cells can evade T cell recognition

immediately after infection, for example during the pre-latent phase, or during latency III. In these phases the infected B cells show an activated phenotype that support an efficient antigen presentation. During latency III, more than eight EBV proteins are expressed. Similarly, in the pre-latent phase, dozens of viral proteins are expressed and functionally presented. Thus, these fully activated B cells need to reduce their recognition by EBV-specific T cells. It was shown that BNLF2a and v-IL-10, known to act during the lytic phase, are also expressed and active during the pre-latent phase (Jochum et al., 2012a). The BNLF2a protein inhibits $CD8^+$ T cells recognition by downregulating the TAP complex and the viral IL-10 reduces cytokines important for anti-viral $CD4^+$ T cell activity.

During latency III, EBNA1, LMP1, and LMP2 are proteins with described immune evasive functions. LMP1, similar to EBNA1 reduces the presentation of its own peptides in cis (Smith et al., 2009). LMP2 was reported to downregulate the activation of $CD8^+$ T cells by reducing ligands of the Natural Killer Group 2D (NKG2D) receptor (Rancan et al., 2015). Despite these known immunoevasive functions, B cells infected with EBV mutants that do not express these immunoevasins are still recognized by T cells in pre-latent phase and in latency III (Rancan et al., 2015; Jochum et al., 2012a). It is likely that other viral immunoevasins, which have not been identified so far, are responsible to protect the infected cells from immune surveillance to establish and maintain a persistent infection *in vivo*. EBV's miRNAs seem to be the perfect immunoevasins candidates: they are expressed in all known phases of EBV (Cai et al., 2006; Seto et al., 2010), they are not immunogenic, since they are non-coding RNA and they are indistinguishable from miRNAs of the host cells. In addition, miRNA are present in EBV virions and passively transferred to newly infected B cells (Jochum et

al., 2012b), indicating that they can be functional even prior to *de novo* transcription from the incoming viral genome.

In fact, there are few reports describing viral miRNAs as immunoevasins. Two reports come from analyzing human cytomegalovirus (HCMV): Stern-Ginossar et. al. showed that miR-UL112 targets and reduces the expression of MICA, a stress induced immune ligand (Stern-Ginossar et al., 2008); Kim et al. showed that the viral miRNA miR-US4-1 reduces the recognition of $CD8^+$ T cells by directly targeting the endoplasmic reticulum aminopeptidase 1 (ERAP1) (Kim et al., 2011). ERAP1 is an important protein that trims the peptides in the ER optimizing the stable binding between peptides and MHC-I molecules (Saric et al., 2002). Another report of viral miRNAs acting as immunoevasins comes from simian virus (SV40): it was shown that SV40 encoded miRNAs downregulate the viral T antigen, thus reducing antigen levels and the activation of T cells specifically directed against this viral protein (Sullivan et al., 2005).

4. EBV's microRNAs

microRNAs (miRNAs, miRs) are small, single-stranded RNAs of about 21 to 24 nt in length that cause translational inhibition or deadenylation followed by the degradation of the targeted mRNA transcripts. The miRNA's region that binds the mRNA target is called the "seed sequence" and consists of six nucleotides located at the 5' end (from nucletotide 2 to 8) of the miRNA molecule(Bartel, 2004). The miRNA binds to the targets mainly within their 3'-untranslated regions (3'-UTRs) and by Watson-Crick partially bulged complementary base pairing. Therefore, each miRNA species can have multiple targets and, in the same way, one mRNA can be targeted by

many miRNAs due to the only partial complementarity between miRNA and the mRNA target (Ambros, 2004; Bartel, 2004).

EBV encodes 44 different mature miRNAs (Cai et al., 2006; Pfeffer et al., 2004), divided into three clusters: BHRF1 cluster, BART cluster 1 and 2. The EBV genome of the laboratory strain B95.8 (NC-007605.1) has a deletion of about 11.8 kb (Skare et al., 1982), which contains the majority of BART miRNA cluster. Interestingly, this strain can still infect and immortalized B cells both *in vitro* and *in vivo* (Miller et al., 1972), indicating that the missing miRNAs in this EBV strain are not essential to establish latent infections in B cells.

Our comprehension of EBV's miRNAs and their functions is still limited. Certain viral miRNAs clearly play a role in B cell "transformation" by EBV. Experiments performed by our group and others using viruses devoid of all or certain miRNAs underline the role of miRNAs of the BHRF1 cluster in cell cycle progression and B cells immortalization (Seto et al., 2010; Vereide et al., 2013; Feederle et al., 2011b). EBV mutants with a knockout (KO) of this cluster can still immortalize B cells and establish a latent infection *in vivo,* but with a delayed kinetics (Wahl et al., 2013).

Several groups used high-throughput approaches based on a combination of immunoprecipitation of the RNA-induced silencing complex (RISC-IP), the complex that mediates the interaction between miRNA and target, and RNA-sequencing to discover new targets. These approaches generated long lists of putative targets, which mostly have not been functionally validated (Riley et al., 2012; Skalsky et al., 2012; Vereide et al., 2013). Surprisingly, the published lists of predicted targets show only very small overlaps (Klinke et al., 2014), which might be due to different long-term cultivated cell lines used by the different groups.

To date, few targets of EBV's miRNAs have been confirmed. Many of the identified targets encode pro-apoptotic proteins such as *BBC3*, *BCL2L11* or *CASP3* (Choy et al., 2008; Vereide et al., 2013; Marquitz et al., 2011). Only a small number of targets are involved in innate immunity and inflammation. The EBV-miR-BART13-5p was shown to target the mRNA of NLRP3 and repress the inflammasome's activation and the subsequent release of the pro-inflammatory cytokine interleukine 1β (IL-1 β) (Haneklaus et al., 2012).

The IFN-inducible T-cell attracting chemokine *CXCL11* has been reported to be a target of EBV miR-BHRF1-3 (Xia et al., 2008). These authors suggested that this suppression might serve as an immunomodulatory mechanism in EBV-associated lymphomas.

MICB is targeted by EBV miR-BART2-5p and its downregulation allows the infected B cells to escape recognition and elimination by NK cells (Nachmani et al., 2009). Interestingly, the same group found that the 3'UTR of *MICB* is also a target of miRNAs of other herpesviruses (KHSV and HCMV), suggesting that the reduction of this NK ligand is important for viral immune escape.

Interestingly, EBV's miRNAs are also reported to target several viral mRNAs including LMP1 (Lo et al., 2007), BZLF1 (Jung et al., 2014) and LMP2 (Lung et al., 2009) most likely to reduce the antigenicity caused by the expression of these viral proteins.

4.1 EBV's miRNAs support cell proliferation and prevent apoptosis

EBV encodes the largest number known among human herpesviruses (Kincaid et al., 2012). To date, the role of the majority of EBV's miRNAs is not well characterized.

We and others reported that one of the important functions of EBV's miRNAs early after infection is to support survival of infected B cells by promoting cell cycle, proliferation and suppressing apoptosis (Feederle et al., 2011b; a; Vereide et al., 2013; Seto et al., 2010). This feature of viral miRNAs is now better characterized and many viral miRNAs' targets involved in these pathways have been found, such as the pro-apoptotic genes *BBC3* (*PUMA*) (Choy et al., 2008), *BAX* (Strasser, 2005), *CASP3* (Vereide et al., 2013), and *BCL2L11* (*BIM*) (Marquitz et al., 2011). The virus has therefore a better chance to reach its latent phase and to establish its lifelong infection. In these studies, isolated B cells have been used and the effects of miRNAs appear to be beneficial but not essential for the infected B cells to survive. Since EBV is orally transmitted via saliva, primary infection targets submucosal B cells are surrounded by a variety of other cell types, including immune cells. Furthermore, when the virus enters lytic reactivation and progeny virions are produced, the infected B cells are probably in a highly immune competent micro-environment (Laichalk and Thorley-Lawson, 2005; Tugizov et al., 2003).

For these reasons, we established a model of infection that closely resembles the *in vivo* situation. Isolated peripheral blood mononuclear cell (PBMC) were infected with two EBV strains that differ in their capacity to encode viral miRNAs. After 6 weeks, MTT assays were performed to assess the viability of the cells (Fig 1A). Interestingly, B cells infected with the wt/B95.8 virus that encodes viral miRNAs grew out in up to 30 out of 30 wells, while surviving B cells infected with the ΔmiR strain of EBV were

only found in at most five wells of 30 wells indicating a very strong impact of viral miRNAs on the survival of B cells in this complex model with PBMCs (Fig. 1A). Since we miss the non-infected control, we cannot exclude that the B cells that outgrew in the PBMCs infected with ΔmiR are a consequence of the reactivation of the field strain virus, since the donors used for the experiment were all EBV positive.

The contribution of the viral miRNAs to B-cell survival is much stronger than the one observed before with isolated B cells (Vereide et al., 2013; Seto et al., 2010; Feederle et al., 2011b; a), suggesting that miRNAs are also involved in mechanisms other than pure regulation of pro-apoptotic cellular functions. Our experiment suggested that miRNAs could reduce cell-cell communication with immune cells thereby increasing the chance of EBV to establish a latent infection.

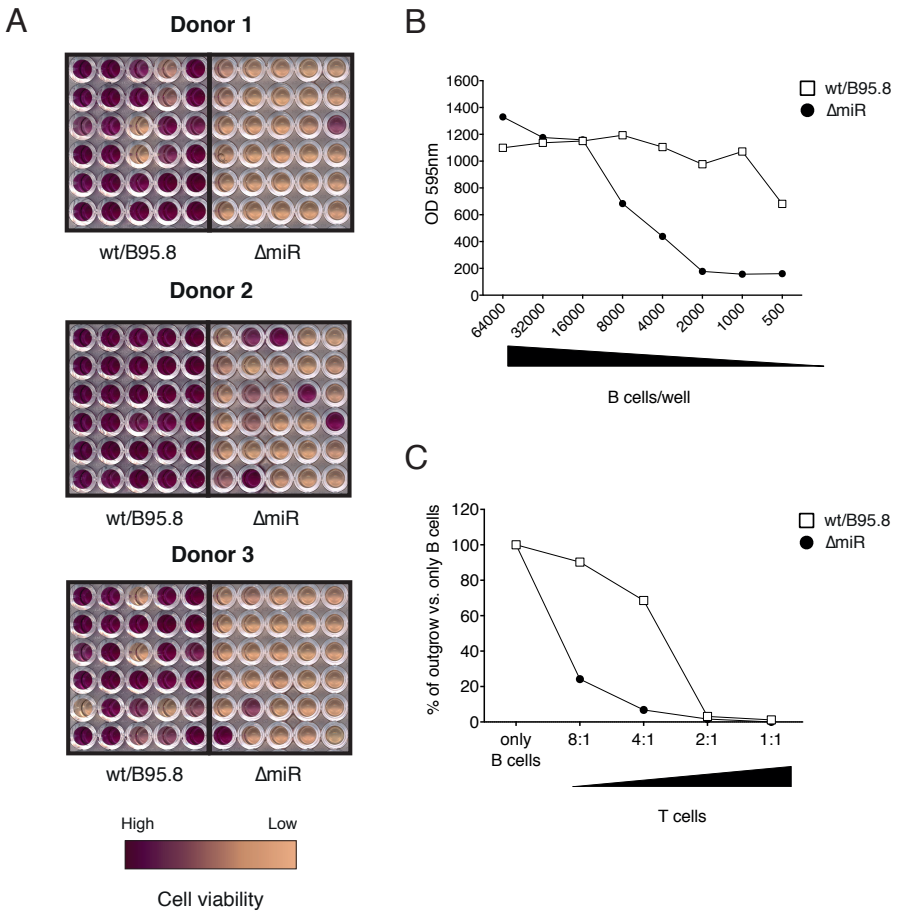


Fig. 1. EBV's miRNAs are important to establish a viral latent infection
A. PBMCs were isolated by Ficoll gradient centrifugation from three EBV-positive donors and infected with wt/B95.8 or ΔmiR EBV strains at an MOI 0.1. The medium of the infected cells was refreshed weekly and a MTT assay was performed. To assess the viability of the cells after 6 weeks. The experiment was performed in 30 replicates. A strong purple color indicates cellular proliferation of EBV-infected cells. B. $CD19^+$ B cells were isolated from PBMCs by positive selection using $CD19^+$ beads. Isolated B cells were infected with wt/B95.8 or ΔmiR EBVs at an MOI 0.1 and seeded in a 96 wells plate in different conditions (from 500 to 64,000 cells/well). The medium was refreshed weekly. Four weeks after infection the viability of the cells was addressed by MTT assay (OD 595nm). The experiment was performed in 6 replicates. C. $CD19^+$B cells and $CD8^+$ T cells were isolated from PBMCs of EBV-positive donors with magnetic beads coupled with appropriate antibodies. B cells were infected and seeded using 32,000 cells/well. At this condition, no difference in proliferation was observed between the two viral strains (B). Isolated $CD8^+$ T cells were added to the infected B cells at different B: T cells ratios. The medium was refreshed weekly. Four weeks after infection the viability of the cells was analyzed by MTT assay. Values (%) are normalized with respect to control B cells, only, set to 100%. The experiment was performed in 6 replicates. One donor out of 3 is shown as a representative experiment.

4.2 EBV's miRNAs prevent the recognition by antiviral T cells

EBV-infected B cells are constantly under immune surveillance *in vivo*. Among the immune cells, cytotoxic $CD8^+$ T cells (CTL) are mainly responsible for the killing of latently infected cells. To analyze if EBV's miRNAs affect the CTL immune surveillance, we developed a simple but effective model (Publication 2 Fig. 1). Isolated B cells from EBV-positive donors were infected with wt/B95.8 or ΔmiR viruses and seeded under different conditions (Fig. 1B). As expected, B cells infected with the wt/B95.8 strain proliferated under all conditions, while B cell infected with ΔmiR EBV showed a strongly reduced outgrowth.

To address the effects of EBV's miRNAs on CTL immune surveillance, we used purified B cells infected with the two EBV viruses (wt/B95.8 or ΔmiR) and seeded at 32,000 cells/well. At this initial cell density, all wells contained proliferating B cells four weeks after infection, irrespective of the EBV strain used (Fig. 1B). The initially seeded cells were co-cultured with isolated $CD8^+$ T cells from the same donor using different B to T cells ratios (Fig. 1C). In the presence of T cells and already at low T cells ratios the outgrowth of B cells infected with the virus devoid of miRNAs (ΔmiR) was dramatically decreased. Similarly, about four times more T cells were required to affect the outgrowth of wt/B95.8-infected B cells. These data indicate that EBV's miRNAs can prevent, directly or indirectly, the eradication of EBV-infected B cells by CTL. This finding can also explain why in PBMCs from EBV-positive donors only B cells infected with the EBV strain wt/B95.8 but not B cells infected with ΔmiR EBV grew out efficiently (Fig. 1A).

4.3 Why miRNAs?

Many EBV immunoevasins have been described, but the ones identified so far are all viral proteins. As such, they are processed and presented in the infected B cells to both $CD8^+$ and $CD4^+$ T cells and their expression could be counterproductive for the virus by increasing the immunogenicity of the infected cells. The virus establishes latency by switching off the expression of as many proteins as possible to become almost "invisible" to the immune system rather than using additional viral proteins for immune evasion.

EBV's miRNAs are known to be expressed in all phases of EBV infection (Qiu et al., 2011), including all the known forms of viral latency. EBV's miRNAs are potentially active even immediately after viral infection, since they have been identified to be contained in EBV virions (Jochum et al., 2012b).

It is appealing to consider that EBV uses its miRNAs as immunoevasins to escape from host immune surveillance, since they cannot be "seen" by T cells and they do not trigger any pattern recognition receptors (PRR) that would activate innate immune responses against the virus. As such, miRNAs can be a perfect immune evasive tool for viruses such as EBV.

With this working hypothesis at hand and our preliminary experiments shown in Fig. 1, we decided to study the role of EBV's miRNAs in immune evasion to understand what their molecular functions are (Tagawa et al., 2016; Albanese et al., 2016).

AIM OF THE THESIS

The aim of this study was to investigate the role of EBV's miRNAs in immune evasion. We sought to achieve a better understanding of how they contribute to hiding the presence of the virus from the host's immunity and to establish and maintain a lifelong infection. Our approach followed two main lines:

I) Identification and validation of new EBV's miRNAs targets

Isolated B cells can be easily infected with EBV *in vitro,* leading to the generation of immortalized LCLs. We used two viruses based on the B95.8 EBV genome previously established in our lab (Delecluse et al., 1998) called wt/B95.8 EBV here. This virus expresses only 13 out of 44 miRNAs, because it has a deletion of approximately 11.8 kb which includes the majority of the BART cluster encoding many viral miRNAs.

In this study, we compared B cells infected with wt/B95.8 EBV with B cells infected with a mutant virus devoid of all miRNAs (ΔmiR), which was also established in our lab (Seto et al., 2010). Because we are interested in the first initial phase of infection, the so-called pre-latent phase, we characterized newly infected B cells to avoid a possible bias introduced by long-term culture of lymphoblastoid cell lines.

We used a combination of different next generation sequencing techniques to identify putative direct targets of EBV's miRNAs. Ideally, a perfect target should be differentially expressed in RNA-seq analysis, indicating that the expression of the transcript is reduced in the presence of EBV's miRNAs. The ideal target should also be enriched in RISC-IP-Seq, indicating that one or more EBV's miRNAs directly interact with the target mRNA, usually within its 3'UTR. We then used bioinformatics tools (e.g. RNAhybrid or Targetscan) to determine which viral miRNA is predicted to

regulate a certain target. If a target fulfilled all these criteria, we introduced its 3´UTR into a dual-luciferase reporter plasmid and tested it together with the predicted single or multiple miRNAs. Finally, the reduced expression of the target had to be confirmed using quantitative PCR (qPCR) of its mRNA and western blot (WB) detection of its protein level.

II) Interference of EBV's miRNAs with immune cells functions

To address whether EBV's miRNAs interfere with adaptive immune responses, we established different polyclonal or monoclonal EBV-specific T cells.

Monoclonal EBV-specific T cell can be rapidly established by stimulating T cells isolated from PBMCs of EBV-positive donors with autologous or HLA matched antigen presenting cells (APCs) loaded with specific peptides (Bollard et al., 2008; Moosmann et al., 2010). EBV-specific T cell clones can be used as tools to evaluate the presentation of a specific peptide using infected B cells as APC. The activation of the T cells depends on the ability of the APCs to present the specific peptide. Once activated, EBV-specific T cells release a proportional amount of IFN-γ that can be quantified by ELISA.

Polyclonal EBV-specific T cells can be established by stimulating T cells ($CD8^+$ or $CD4^+$) isolated from PBMCs of EBV-positive donors with autologous LCLs, every second week. The peculiarity of these EBV-specific T cell lines is that they recognize a broad range of antigens from different viral proteins, providing a more diverse view of antigens presentated by autologous or HLA-matched target cells.

B cells infected with wt/B95.8 or ΔmiR EBV strains were co-cultured together with EBV-specific T cells. This model can be used to evaluate the activities of these T cells as a function of the immunomodulatory impact of EBV's miRNAs. This approach is

extremely sensitive and allows the identification of differences with respect to cytokines release and T cells mediated killing of antigen presenting cells.

PUBLICATIONS

Publication I

Tagawa T.*, **Albanese Manuel***, Bouvet M., Moosmann A., Mautner J., Heissmeyer V., Zielinski C., Lutter D., Hoser J., Hastreiter M., Hayes M., Sugden B., Hammerschmidt W., 2016. **Epstein-Barr viral miRNAs inhibit antiviral CD4$^+$ T cell responses targeting IL-12 and peptide processing.** J. Exp. Med. 213, 2065–2080.

*equal author contributions

I designed all the experiments together with Tagawa T. and Hammerschmidt W. and I personally performed the experiments of Fig. 4, 5 and 6. I wrote the section Material and Methods, Introduction and the following paragraphs of the Result section: "Viral miRNAs directly and indirectly control antigen presentation" and "EBV miRNAs inhibit recognition of infected B cells by EBV-specific CD4$^+$ T cells". I revised the entire manuscript together with Tagawa T., Hammerschmidt W., Bouvet M. and Moosmann A..

Individual contributions:

Fig.1: Co-authors and my contribution as in panel E
Fig.2: Co-authors and my contribution as in panels B and D
Fig.4: my contribution (panel D by co-authors)
Fig.5: my contribution (panel B by co-authors)
Fig.6: my contribution
Fig.S2: my contribution

All the experiments and data in the figures as indicated above were performed, analyzed, and illustrated by me.

Takanobu Tagawa

Manuel Albanese

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Epstein-Barr viral miRNAs inhibit antiviral CD4$^+$ T cell responses targeting IL-12 and peptide processing

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Epstein-Barr virus (EBV) is a tumor virus that establishes lifelong infection in most of humanity, despite eliciting strong and stable virus-specific immune responses. EBV encodes at least 44 miRNAs, most of them with unknown function. Here, we show that multiple EBV miRNAs modulate immune recognition of recently infected primary B cells, EBV's natural target cells. EBV miRNAs collectively and specifically suppress release of proinflammatory cytokines such as IL-12, repress differentiation of naive CD4$^+$ T cells to Th1 cells, interfere with peptide processing and presentation on HLA class II, and thus reduce activation of cytotoxic EBV-specific CD4$^+$ effector T cells and killing of infected B cells. Our findings identify a previously unknown viral strategy of immune evasion. By rapidly expressing multiple miRNAs, which are themselves nonimmunogenic, EBV counteracts recognition by CD4$^+$ T cells and establishes a program of reduced immunogenicity in recently infected B cells, allowing the virus to express viral proteins required for establishment of life-long infection.

INTRODUCTION

EBV is both ubiquitous and immunogenic. This oncogenic herpesvirus (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2010) has evolved multiple genes to fend off immune responses when its infection is established (Hislop et al., 2002; Rowe et al., 2007; Ressing et al., 2008; Zuo et al., 2009; Qiu et al., 2011; Rancan et al., 2015). Despite these measures, EBV-specific T cells constitute a considerable fraction of the memory T cell repertoire of the latently infected human host (Hislop et al., 2002) and are essential in controlling latent EBV infection (Moosmann et al., 2010). In fact, immunocompromised patients have an increased incidence of EBV-associated malignancies (Gottschalk et al., 2005).

EBV infects nondividing B lymphocytes, activates them, and drives them to proliferate, thus amplifying the load of viral genomes. Once activated, infected B cells acquire properties of antigen-presenting cells. After infection, they rapidly present epitopes of structural proteins from incoming virus particles and transiently express lytic genes that are otherwise characteristic of EBV's productive cycle (Kalla and Hammerschmidt, 2012). This prelatent phase of infection includes expression of two genes coding for viral immunoevasins, BNLF2a and BCRF1 (Jochum et al., 2012), which inhibit the recognition of the infected cells by EBV-specific effector T cells and natural killer cells, respectively. These two viral proteins are insufficient, however, to overcome T cell recognition (Jochum et al., 2012). Within 7–10 d, EBV establishes a latent infection in the infected B cells and expresses only few or no viral genes, which reduces their risk of becoming eliminated by the immune-competent host.

Thus, early infection could be EBV's Achilles heel, a window when the infected cell expresses and presents many viral antigens to immune cells but is inadequately protected from the host's immune response. We have now established that EBV's miRNAs overcome this vulnerability; they protect newly infected B lymphocytes from immune eradication by CD4$^+$ T cells, supporting EBV's lifelong success.

EBV encodes at least 44 microRNAs (miRNAs; Barth et al., 2011), which are small RNA regulatory molecules of ~22 nt in length (Bartel, 2004). miRNAs encoded by herpesviruses

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Abbreviations used: CTSB, cathepsin B; LCL, lymphoblastoid cell line; LMP, latent membrane protein; miRNA, microRNA; RISC, RNA-induced silencing complex.

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are reported to play important roles in cell proliferation, development, immune regulation, and apoptosis in infected cells (Skalsky and Cullen, 2010). The EBV-encoded miRNAs have been found to control expression of several cellular genes with antiapoptotic functions, but they also reportedly down-regulate *MICB* (Nachmani et al., 2009), *CXCL11* (Xia et al., 2008), and *NLRP3* (Haneklaus et al., 2012) and thus interfere with innate immune responses and inflammation. Interestingly, *MICB*, a gene encoding a ligand for the activating receptor NKG2D expressed on T and NK cells, is also targeted by miRNAs of Kaposi sarcoma–associated herpesvirus and human cytomegalovirus (Nachmani et al., 2009; Grundhoff and Sullivan, 2011). These studies imply that certain miRNAs encoded by herpesviruses target pathways involved in innate immune recognition.

EBV's miRNAs have been studied by several groups with established, EBV-infected cell lines obtained from biopsies of nasopharyngeal carcinoma and Burkitt's lymphoma, or lymphoblastoid cell lines (LCLs) derived from infecting primary B lymphocytes with EBV in vitro (Dölken et al., 2010; Gottwein et al., 2011; Kuzembayeva et al., 2012; Riley et al., 2012; Erhard et al., 2013). High-throughput target screens using immunoprecipitation of the RNA-induced silencing complex (RISC) and deep sequencing have identified many potential targets of EBV miRNAs, but the catalogs of predicted targets assembled by different groups have a surprisingly small overlap (Klinke et al., 2014). This lack of consensus may be due to the accumulation of profound differences in gene expression between different long-term, cultivated EBV-infected cell lines that do not reflect the impact of EBV's miRNAs in vivo.

To circumvent these problems, we developed an experimental approach using primary human B lymphocytes, and analyzed them during their initial days of EBV infection (Seto et al., 2010; Vereide et al., 2014). We infected the B lymphocytes with two EBV strains with and without miRNA genes, compared the gene expression in the infected cells, and examined them for their immune recognition.

We found that EBV-encoded miRNAs regulated several immune pathways, which affected $CD4^+$ T cell differentiation and activation. In addition, key molecules important for interactions with $CD4^+$ T cells were down-regulated. EBV miRNAs repressed the secretion of IL-12, which resulted in suppression of type 1 helper T cell (Th1) differentiation. Viral miRNAs controlled gene expression of HLA class II and three lysosomal enzymes important for proteolysis and epitope presentation to $CD4^+$ T cells. Such a wholesale inhibition of adaptive immune responses by multiple miRNAs of a single pathogen is unprecedented. Our findings explain the abundance of miRNAs in complex persisting viruses, and clarify how EBV can escape elimination for the lifetime of its host in spite of intense adaptive immune responses.

RESULTS

EBV miRNAs control immune regulatory pathways

We searched for cellular targets of EBV's miRNAs, using an experimental system that closely mimics human infection in vivo. Two strains of EBV, a laboratory strain (wt/B95-8) that expresses 13 miRNAs, and its derivative (ΔmiR) that expresses none (Seto et al., 2010) were used to infect freshly isolated B lymphocytes from six donors. We used carefully titrated virus stocks and infected the cells with optimal doses of both viruses (Steinbrück et al., 2015). No differences in the percentage of infected cells were seen when comparing cells infected with wt/B95-8 versus ΔmiR EBV. RNAs were isolated on day 5 after infection and sequenced (available from GEO under accession no. GSE75776; see Materials and methods). Genes that were differentially expressed in cells infected with wt/B95-8 versus ΔmiR EBV were identified with those having an absolute z-score >1.6 (Fig. 1 A and Table S1). These genes included the published miRNA targets *LY75/DEC205* (Skalsky et al., 2012) and *IPO7* (Dölken et al., 2010). Genes that were consistently down-regulated in wt/B95-8 EBV-infected cells were grouped according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway categories (Fig. 1 B). Down-regulated genes were predominant in pathways linked to apoptosis, cell cycle regulation, and p53 signaling, which were previously proposed to be regulated by EBV miRNAs (Seto et al., 2010; Feederle et al., 2011a,b; Vereide et al., 2014). Unexpectedly, EBV's miRNAs also regulated a wide array of genes with functions in immunity, such as cytokine–cytokine receptor interactions, antigen processing, and HLAs and co-stimulatory molecules (Fig. 1, B and C; and Table S1). We immunoprecipitated RISC (RISC-IP) and found that 14.5% (±2.4% SD) of all miRNAs were of viral origin in wt/B95-8 EBV-infected cells, dominated by miRNAs of the BHRF1 gene cluster (Fig. 1 D). No appreciable viral miRNA reads were found in cells infected with ΔmiR EBV (Fig. 1 D), suggesting that the B lymphocytes of six donors were free of EBV field strains. In wt/B95-8 EBV-infected cells, we detected viral miRNAs as early as day 1 after infection, which reached high levels 5 days post infection (dpi; Fig. 1 E). In RISC-IP, detection of mRNAs was variable among infected B cells of the different donors, a phenomenon that was reported earlier using a related model of established infection and PAR-CLIP experiments (Skalsky et al., 2012; GEO accession no. GSE41437). Therefore, we focused our analyses on candidate mRNAs that were uniformly regulated in all samples (Fig. 1 C), and used RISC-IP results to confirm them (Table S1).

EBV miRNAs inhibit secretion of proinflammatory cytokines and antigen presentation

We confirmed that EBV's miRNAs regulate cytokines central to immune function. Supernatants from B cells infected with the two strains of EBV were assayed for the levels of IL-6, IL-10, TNF, IL12B (IL-12p40), IL-12 (p35/p40), and IL-23 (p19/p40). We added CpG DNA, which stimulates TLR9, for the detection of IL-6 secreted from EBV-infected cells (Iskra et al., 2010). B cells infected with wt/B95-8 EBV secreted less IL-6, TNF, and IL-12p40 than B cells infected with ΔmiR EBV. In contrast, release of the anti-inflamma-

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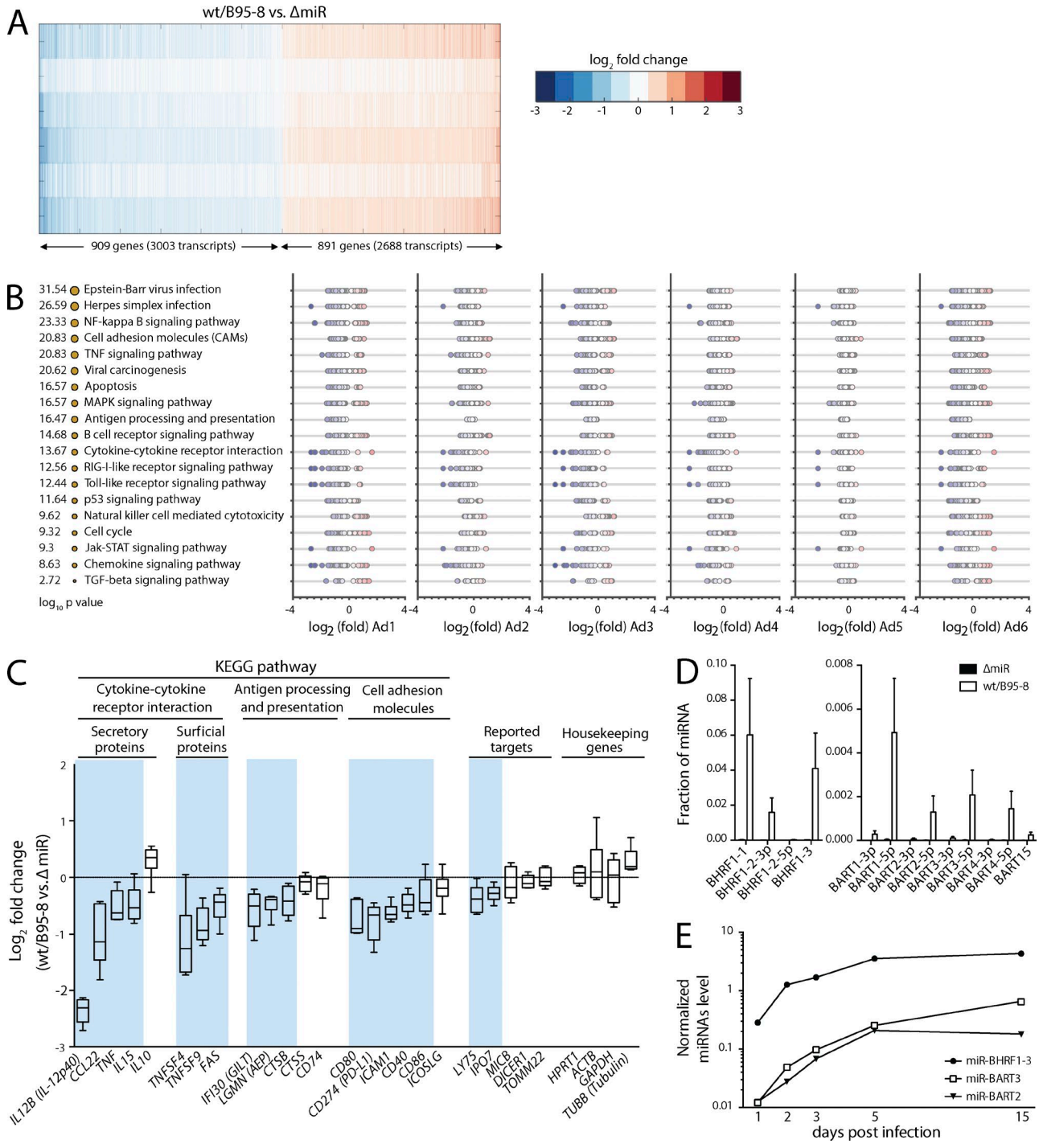

Figure 1. **EBV miRNAs affect major pathways of immunity.** (A) A heat map of the most strongly regulated genes in wt/B95-8 or ΔmiR EBV–infected B cells of six donors (donor Ad1-Ad6) 5 dpi shows differentially expressed gene transcripts with absolute z-scores >1.6. Blue and red indicate down- and up-regulated transcripts, respectively, in wt/B95-8 compared with ΔmiR EBV–infected cells. (B) Shown are gene functions according to KEGG pathway categories with the identified pathways sorted by statistical significance. The sizes of the orange dots indicate $-\log_{10}$ P value scores. For each of the six donors, fold change values of differentially expressed transcripts are plotted. As in A, blue and red indicate down- or up-regulation by EBV miRNAs, respectively. Enrichment of specific pathways was estimated by a hypergeometric distribution test via the KEGG API Web service. (C) Inhibition of selected transcripts associated with adaptive immune responses is shown together with previously reported targets of EBV miRNAs and common housekeeping genes. Blue background shadings indicate genes down-regulated by viral miRNAs (Table S1). (D) Levels of indicated EBV miRNAs in B cells infected with wt/B95-8 or ΔmiR EBV–infected B cells of six donors (donor Ad1-Ad6) 5 dpi were quantified by RISC-IP-seq. Mean ± SD are shown. (E) Three miRNAs, which represent different primary miRNA transcripts in EBV-infected B cells, were quantified with stem-loop qPCR over time. One of two independent experiments is shown.

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tory cytokine IL-10 appeared to be unaffected by viral miRNAs (Fig. 2 A) consistent with our transcriptome analysis (Fig. 1 C). Secretion of IL-12 (p35/p40 or IL-12p70) and IL-23 (p19/p40), both of which contain the IL-12p40 subunit (Szabo et al., 2003), encoded by the *IL12B* gene, was significantly reduced in wt/B95-8 EBV-infected cells compared with ΔmiR EBV-infected cells (Fig. 2 A). Viral miRNAs also inhibited the secretion of IL-12p40 from PBMCs infected with wt/B95-8 EBV (Fig. 2 B). IL-12p40 secretion from PBMCs infected with ΔmiR EBV was reduced when B cells were removed from the PBMCs, indicating that B cells are the main contributors to release of IL-12p40 in PBMCs. Remarkably, our transcriptome analysis revealed the consistent reduction of *IL12B* mRNA with EBV's miRNAs, reducing it by 80% in all six donors' B lymphocytes (Fig. 2 C). Quantitative RT-PCR confirmed this finding (Fig. 2 D).

Multiple EBV miRNAs target *IL12B* and prevent Th1 differentiation of naive CD4$^+$ T cells

We investigated whether *IL12B* was a direct target of EBV miRNAs. EBV's miR-BART1, miR-BART2, and miR-BHRF1-2 repressed the luciferase activity of the *IL12B* reporter (Fig. 3 A). The mutation of predicted binding sites of miR-BART1, miR-BART2, or miR-BHRF1-2 abrogated their ability to inhibit the *IL12B* reporter (Fig. 3 A and Fig. S1), confirming the direct control of *IL12B* by these miRNAs. We similarly analyzed miR-BART10 and miR-BART22, which are present in field strains of EBV but not in wt/B95-8 EBV. For these miRNAs, mutations of their predicted target sites only partially relieved inhibition (Fig. 3 A and Fig. S1), suggesting the presence of additional binding sites in the *IL12B* transcript. In summary, these experiments validated *IL12B* as a direct target of multiple viral miRNAs.

IL-12 is critical for differentiation of Th1 cells (Szabo et al., 2003). Therefore, we co-cultured naive CD4$^+$ T cells with autologous EBV-infected B cells (Fig. 3 B). Relative to ΔmiR EBV, wt/B95-8 EBV-infected B cells repressed Th1 differentiation (Fig. 3, C and D). An antibody that neutralizes the functions of IL12B, but not an isotype control antibody, suppressed Th1 differentiation when T cells were co-cultured with ΔmiR EBV-infected cells (Fig. 3 E), indicating that IL-12, secreted from EBV-infected and activated B cells, was responsible for generation of Th1 cells. Thus, EBV miRNAs suppress the release of IL-12 from infected cells and thereby interfere with formation of Th1 cells, which are important antiviral effectors.

Viral miRNAs directly and indirectly control antigen presentation

Having shown that EBV miRNAs interfere with CD4$^+$ T cell differentiation, we turned our attention to molecules that are involved in recognition of infected cells by specific CD4$^+$ T cells. We quantified levels of surface proteins with a role in HLA class II antigen presentation (Fig. 4). All three subclasses of HLA class II (HLA-DR, HLA-DQ, and HLA-DP) tested were reduced in wt/B95-8 relative to ΔmiR EBV-infected B cells (Fig. 4, A and B), as were many co-stimulatory and adhesion molecules 5 and 15 dpi (Fig. 4 C). MHC class I molecules were also affected, but to a lesser extent.

Among the many co-receptors and adhesion molecules down-regulated in cells infected with wt/B95-8 EBV (Fig. 4 C), we searched for direct targets of EBV miRNAs but found only *CD40* and *FAS* in RISC-IPs (Table S1), which we could not confirm in subsequent luciferase assays using miRNAs encoded by wt/B95-8 EBV. Interestingly, the viral latent membrane protein 1 (LMP1) activates the CD40 pathway, inducing important immune co-receptors (Kieser and Sterz, 2015), but several viral BART miRNAs were reported to control LMP1 expression (Lo et al., 2007; Riley et al., 2012; Verhoeven et al., 2016). We tested these findings in our model of newly infected B cells, and found reduced but highly variable levels of LMP1 transcripts (Fig. 4 D) and protein (Fig. 4 E) in B cells infected with wt/B95-8 EBV compared with ΔmiR EBV 5 dpi. We identified miR-BART3 and miR-BART16 as inhibiting LMP1 in reporter assays (Fig. 4 F). miR-BART3 is encoded in wt/B95-8 EBV, whereas miR-BART16 (Fig. 4 F) is only present in field strains of EBV. We also tested miR-BART1 and miR-BART17, which were reported together with miR-BART16 to target LMP1 3′-UTR (Lo et al., 2007), but failed to confirm that miR-BART1 and miR-BART17 target LMP1 (Fig. 4 F). Collectively, our results showed that viral miRNAs limit LMP1 gene expression and thereby indirectly inhibit surface expression of some immune co-receptors and adhesion molecules.

Viral miRNAs target lysosomal enzymes and inhibit antigen processing

According to our transcriptome analysis, genes encoding lysosomal enzymes actively involved in MHC class II peptide processing (Blum et al., 2013) were inhibited by EBV miRNAs (Fig. 1 C). These included *IFI30* (coding for IFN-γ–regulated thiol reductase GILT), *LGMN* (coding for asparagine endopeptidase AEP alias legumain), and *CTSB* (coding for the peptidase cathepsin B). Expression of all three genes was reduced by EBV miRNAs (Fig. 1 C), which we verified by quantitative RT-PCR (Fig. 5 A). We found that EBV's miR-BART1, miR-BART2, and miR-BHRF1-2 could directly regulate *IFI30*, *LGMN*, and *CTSB* gene expression via their 3′-UTRs in luciferase reporter assays (Fig. 5 B and Fig. S1). Importantly, the knock-down of these three genes (Fig. 5 C) resulted in reduced antigen presentation of exogenously loaded protein (Fig. 5 D). Collectively, our results show that EBV miRNAs interfere with processes involved in MHC class II antigen presentation at multiple levels, including lysosomal protein degradation, HLA class II expression, and co-stimulatory molecule expression.

EBV miRNAs inhibit recognition of infected B cells by EBV-specific CD4$^+$ T cells

Next, we asked whether these multiple levels of regulation ultimately resulted in reduced MHC class II–mediated recognition

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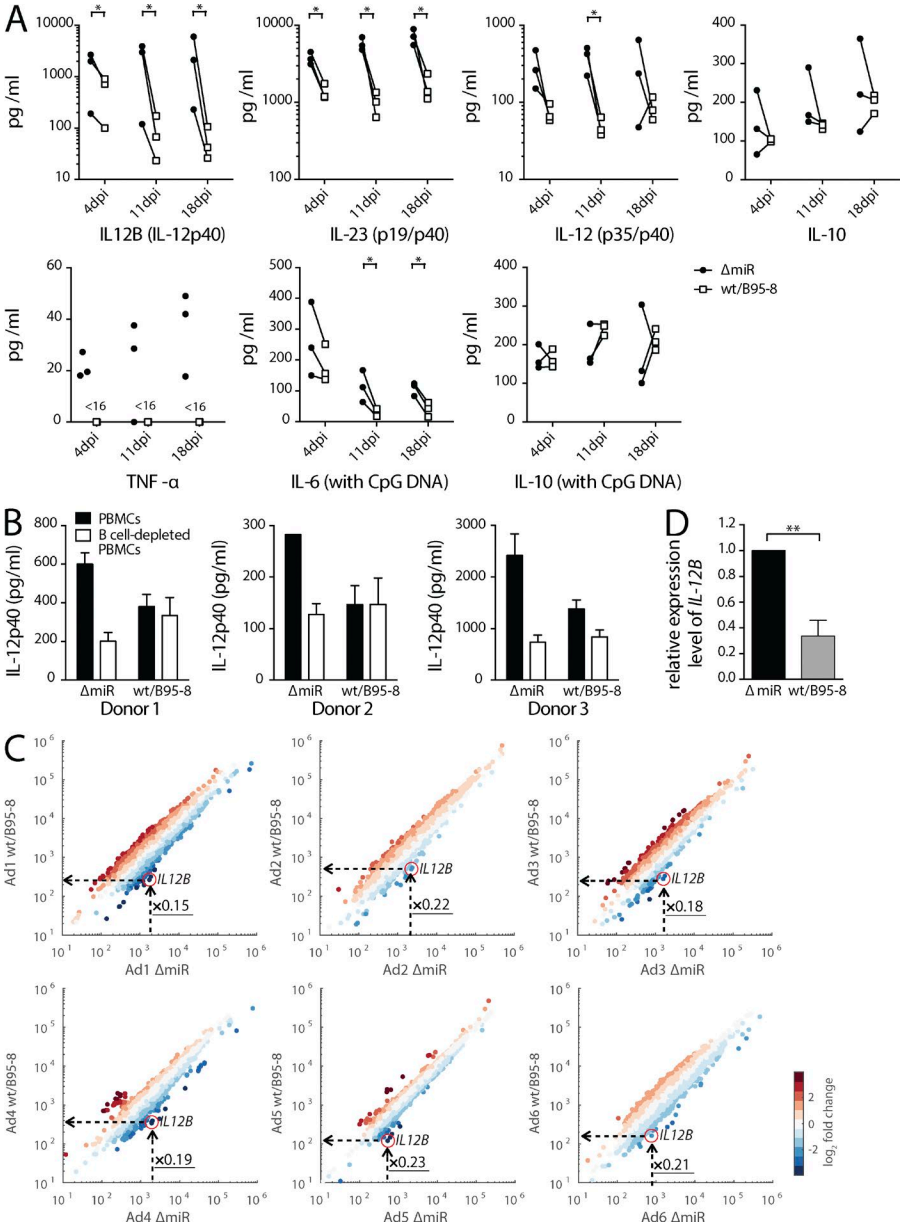

Figure 2. **EBV miRNAs inhibit secretion of proinflammatory cytokines.** (A) B cells infected with wt/B95-8 or ΔmiR EBV for 4, 11, or 18 d were cultivated for an additional 4 d to determine levels of selected cytokines by ELISA (n = 3). CpG DNA was added where indicated. Paired samples from individual donors are connected by solid lines. P values were calculated by a paired two-tailed t test. <16, under the detection limit (16 pg/ml); *, $P < 0.05$. (B) PBMCs or PBMCs depleted of B cells (n = 3) were infected with either wt/B95-8 or ΔmiR EBV, and concentrations of IL-12p40 in the supernatants of the infected B cells after 5 d were determined by ELISA. (C) Shown are scatter plots of transcriptomes of B cells from six donors infected with wt/B95-8 or ΔmiR EBV for 5 d. Fold changes of transcript levels are indicated as blue or red dots, indicating down- and up-regulated transcripts, respectively. The individual *IL12B* transcripts are highlighted by red circles and the calculated fold changes (x-values) are provided. (D) Transcript levels of *IL12B* were measured with quantitative RT-PCR in RNA preparations of B cells infected with wt/B95-8 or ΔmiR EBV for 5 d (n = 4). **, $P < 0.01$.

of EBV-infected cells by antiviral $CD4^+$ T cells. $CD4^+$ T cells from EBV-positive individuals were enriched for EBV-specific T cells by repeated stimulation with irradiated wt/B95-8 EBV-infected autologous LCLs. The EBV-specific $CD4^+$ T cells were then co-cultured with autologous B cells that had been infected with the two EBV strains 5 d earlier (Fig. 6 A, top). Release of IFN-γ by EBV-specific $CD4^+$ T cells was substantial when co-cultured with ΔmiR EBV-infected cells as targets, but was consistently reduced when co-cultured with wt/B95-8 EBV-infected B cells at all cell ratios tested (Fig. 6 B). Activation of EBV-specific $CD4^+$ T cells, measured as IFN-γ release, was observed in autologous and partially matched but not in HLA-mismatched conditions (Fig. 6 C and Table S2), indicating that the activation was HLA class II-restricted.

We also tested an antigen-specific $CD4^+$ T cell clone (Fig. 6, A [bottom] and D) directed against the FGQ peptide, an epitope derived from the viral glycoprotein gp350 (Adhikary et al., 2006). We observed dramatically reduced T cell activities with target B cells infected with wt/B95-8 EBV compared with ΔmiR EBVs 5 dpi (Fig. 6 D). T cell activities were much reduced at 15 dpi, but a difference between wt/B95-8 and ΔmiR EBV-infected target cells remained detectable. Weak recognition on day 15 is in accordance with gp350 protein being delivered as a component of the virion (Adhikary et al., 2006) but not synthesized during prelatency or latency (Kalla et al., 2010). Interestingly, expression of cell surface HLA class II levels peaked between 4 to 10 dpi (Fig. 6 E) suggesting the importance of

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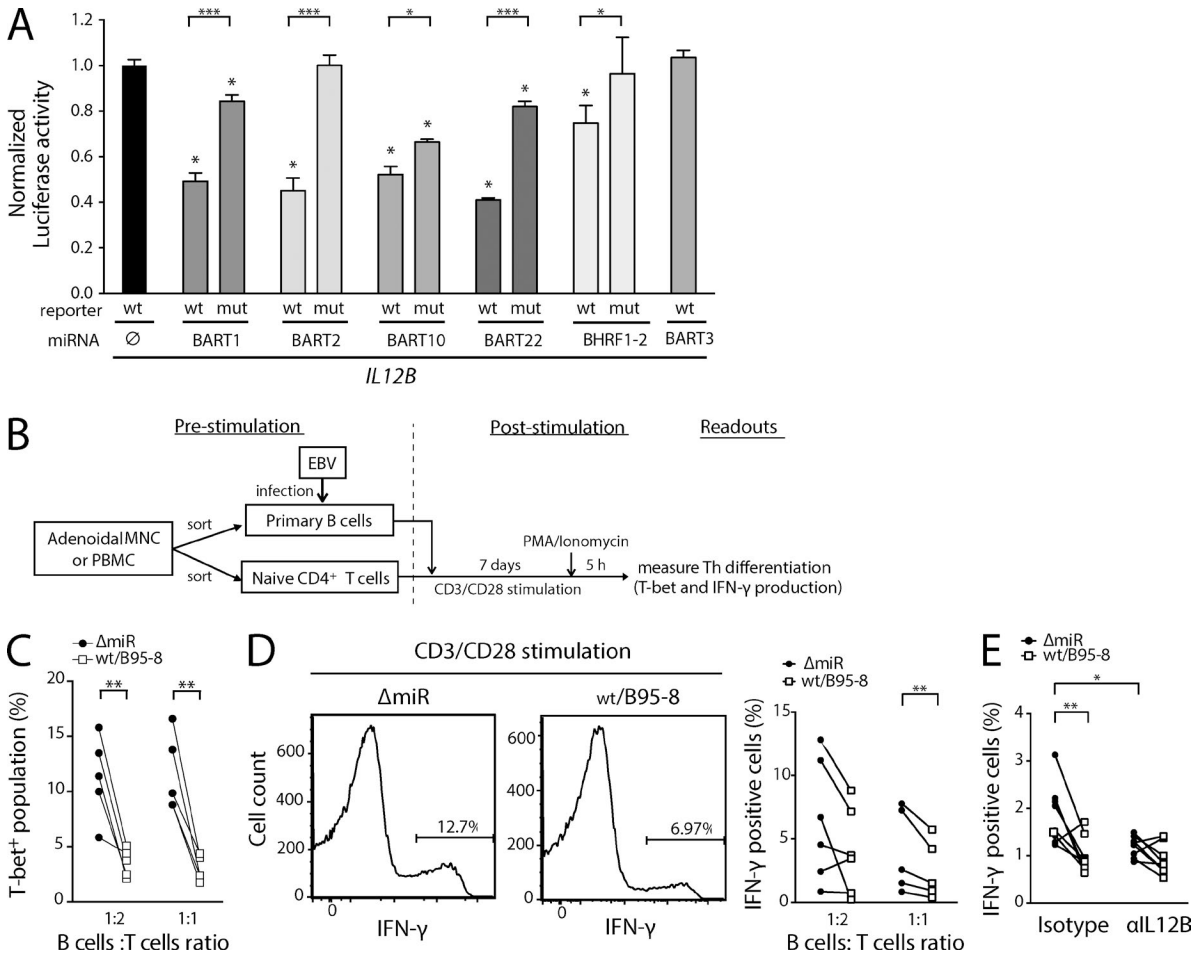

Figure 3. **EBV miRNAs inhibit *IL12B* directly and prevent Th1 differentiation.** (A) HEK293T cells were cotransfected with miRNA expression vectors and luciferase reporter plasmids carrying a wild-type or mutated 3′-UTR (Fig. S2) as indicated ($n = 3$). The luciferase activities were normalized to lysates from cells cotransfected with the wild-type 3′-UTR reporter and an empty plasmid. wt, wild-type 3′-UTR; mut, mutated 3′-UTR; ∅, empty plasmid. P-values were calculated by an unpaired two-tailed Student's *t* test. *, $P < 0.05$; ***, $P < 0.001$, with respect to the luciferase activity of the wild-type reporter cotransfected with empty plasmid. (B) Schematic representation of the steps for experiments shown in C and D. Primary B cells sorted from adenoids or PBMCs were infected with either wt/B95-8 or EBV ΔmiR EBV and co-cultured with autologous naive $CD4^+$ T cells, which were stimulated with αCD3/αCD28 antibody-conjugated beads for 7 d. Th1 differentiation was assessed by intracellular staining of T-bet and IFN-γ after stimulation with PMA/ionomycin for 5 h. (C and D) Naive $CD4^+$ T cells were cultivated for 7 d with autologous, newly infected B cells and αCD3/αCD28 antibody-conjugated beads at indicated ratios ($n = 5$–6). Proliferating PMA- and ionomycin-restimulated Th1 cells were quantified by intracellular T-bet (C) and IFN-γ (D) staining. (D, left) Representative flow cytometry analyses; (right) summary of all experiments. Solid lines indicate paired samples from five to six individual donors. (E) Naive $CD4^+$ T cells were cocultivated with wt/B95-8 or ΔmiR-infected B cells at a B/T cell ratio of 1:1 ($n = 8$) as shown in (B). An anti-IL12B antibody was administered at a concentration of 5 µg/ml, and an irrelevant antibody of the same isotype was used as a control. Solid lines indicate paired samples from individual donors. *, $P < 0.05$; **, $P < 0.01$.

viral miRNAs that counteract $CD4^+$ T cell recognition in the early days of infection.

EBV-specific $CD4^+$ T cells have cytolytic activity (Adhikary et al., 2006). In allogeneic, partially HLA-matched conditions, EBV-specific $CD4^+$ T cells consistently showed stronger cytolysis of target B cells infected with ΔmiR EBV than cells infected wt/B95-8 EBV (Fig. 6 F).

Collectively, we have discovered that EBV miRNAs inhibit the recognition and elimination of infected B cells by HLA class II–restricted $CD4^+$ T cells. Apparently, EBV utilizes multiple miRNAs to interfere with proinflammatory cytokines, antigen processing, and epitope presentation of the infected B lymphocyte to evade EBV-specific and antiviral $CD4^+$ T cells responses early after infection.

DISCUSSION

EBV infects its human hosts for their lifetime, residing in nonproliferating B cells largely invisible to the host's immune response (Thorley-Lawson, 2005). EBV, to be the successful pathogen that it is, however, must both establish a latent in-

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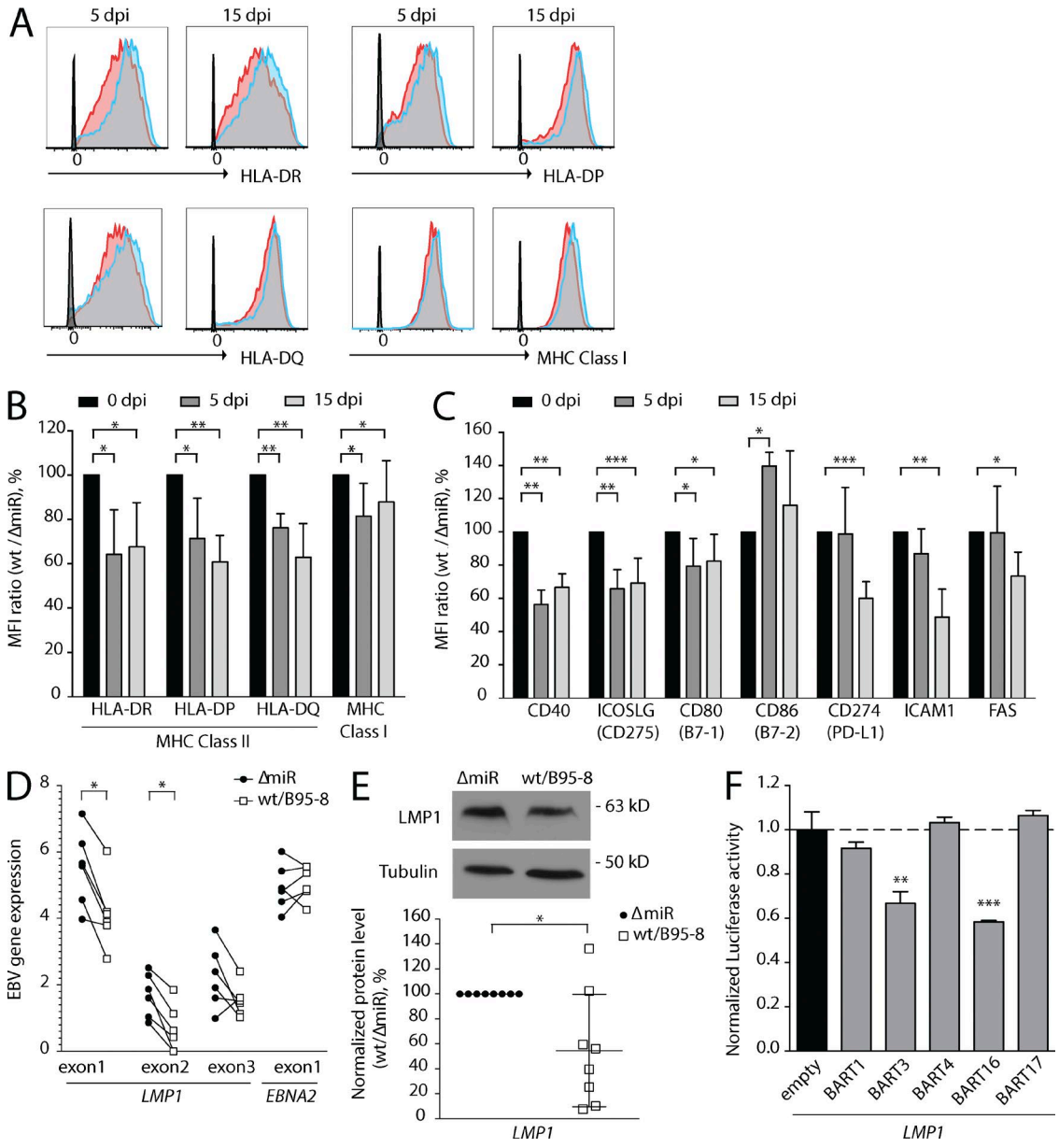

Figure 4. **EBV miRNAs control cell surface levels of HLAs and co-receptors.** (A) FACS panels show the expression profiles of three HLA class II gene families and HLA class I protein on B cells on 5 dpi. One representative example is shown as a histogram for each condition. (B and C) Cell surface expression of HLA molecules (B) and of co-stimulatory and adhesion molecules (C) was measured after immunostaining for proteins inhibited by EBV miRNAs. Ratios (wt/B95-8 divided by ΔmiR EBV–infected B cells) are shown as median fluorescence intensity (MFI). Means ± SD of experiments with infected B cells from 5–10 donors are shown. P-values were calculated by a paired two-tailed Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (D) Viral gene expression obtained from the transcriptome data shown in Fig. 1 C was quantified for exons to analyze splicing variants precisely. The y-axis shows natural-log values ($n = 6$) with paired samples from individual donors connected by solid lines. Statistical significance was assessed with the repeated-measurements ANOVA. *, adjusted $P < 0.01$. (E) Cell lysates were prepared from B cells infected with wt/B95-8 or ΔmiR EBV for 5 d and analyzed by Western blotting for expression of LMP1 and tubulin. An example (top) and the quantification of all results (bottom) are shown. Protein levels were measured relative to tubulin and LMP1 levels in ΔmiR EBV-infected cells were set to 100%. Mean ± SD are shown ($n = 8$). (F) Dual luciferase reporter assays with LMP1 3′-UTR are shown ($n = 3$). P-values were calculated by an unpaired two-tailed Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

fection and produce and disseminate progeny virus, all in the face of robust innate and adaptive immune responses.

Such responses include EBV-specific $CD4^+$ T cells, which have an important role in controlling EBV infection and disease. For example, patients with EBV-associated tumors treated with virus-specific T cell preparations showed better clinical responses if the preparations contained larger fractions of $CD4^+$ T cells (Haque et al., 2007; Icheva et al.,

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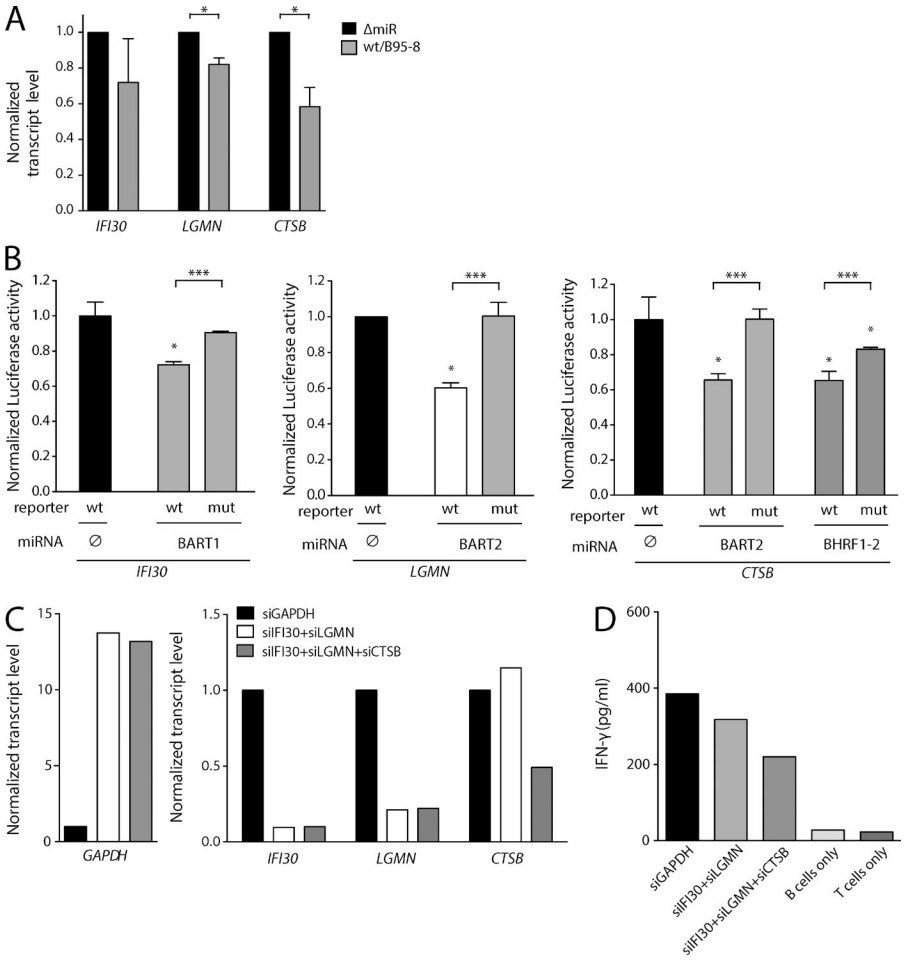

Figure 5. **Viral miRNAs inhibit two lysosomal endopeptidases and a thiol reductase needed for antigen presentation.** (A) Transcript levels of *IFI30*, *LGMN*, and *CTSB* encoding GILT, AEP, and CTSB, respectively, were measured with quantitative RT-PCR (n = 3). P-values were calculated by a paired two-tailed Student's t test. *, $P < 0.05$. (B) HEK293T cells were cotransfected with different miRNA expression vectors and luciferase reporter plasmids carrying 3′-UTRs as indicated (n = 3). The luciferase activities were normalized to lysates from cells cotransfected with the wild-type 3′-UTR reporter and an empty plasmid. wt, wild-type 3′-UTR; mut, mutated 3′-UTR; ∅, empty plasmid. P-values were calculated by an unpaired two-tailed Student's t test. *, $P < 0.05$; ***, $P < 0.001$, with respect to the luciferase activity of the wild-type reporter cotransfected with an empty expression plasmid. (C) Transcript levels after RNAi knock-down of lysosomal enzyme-encoding genes were investigated. DG-75 cells were transduced with commercial siRNAs directed against *GAPDH*, *IFI30*, *LGMN*, or *CTSB* as indicated and transcript levels were quantified with RT-PCR. Means of three technical replicates are shown. (D) DG-75 cell transduced with siRNAs directed against three lysosomal enzymes as in (C) were loaded with purified influenza M1 protein and co-cultured with M1-specific $CD4^+$ T cells (epitope LENL; HLA-DRB1*1301-restricted) for 1 d. After another 16 h, IFN-γ secretion was assessed by ELISA. One of two independent experiments is shown as means of three technical replicates.

2013). $CD4^+$ T cells target a wide repertoire of EBV antigens from all phases of latent and lytic infection (Adhikary et al., 2007; Long et al., 2011). $CD4^+$ T cells with specificity for structural EBV proteins play a prominent role: they are a universal component of the T cell repertoire, rapidly detecting EBV-infected B cells and killing them directly (Adhikary et al., 2006, 2007). Several EBV proteins expressed during its lytic phase can inhibit recognition of EBV-infected cells by $CD4^+$ T cells (Ressing et al., 2015). The broad-ranging functions of the host shut-off gene product BGLF5 include reduction of HLA class II molecules on the cell surface during EBV's lytic, productive phase (Rowe et al., 2007). The late glycoprotein gp42, encoded by BZLF2, was shown to associate with HLA class II and to hinder recognition by $CD4^+$ T cells sterically (Ressing et al., 2003). Both mechanisms are unlikely to be operational in newly infected B lymphocytes, because the two viral proteins appear not to be expressed in the prelatent phase (Kalla et al., 2010). Viral IL-10, encoded by BCLF1, is an immunomodulatory protein expressed early in infection, but its effects on B cell elimination by $CD4^+$ T cells were limited (Jochum et al., 2012). Two additional viral gene products reported to affect $CD4^+$ T cell recognition, BDLF3 (Quinn et al., 2015) and BZLF1 (Zuo et al., 2011), may act during EBV primary infection, because BDLF3 is in the virus particle (Johannsen et al., 2004) and BZLF1 has been found to be expressed early during infection (Wen et al., 2007; Kalla et al., 2010). BDLF3 transcripts were present at very low levels, only, whereas BZLF1 transcripts were not mapped in our RNA-Seq analysis. Thus, how EBV infection escapes detection and elimination by EBV-specific T cells during the early phase of infection has remained uncertain.

Here, we present an answer to this question and show that EBV uses its large repertoire of miRNAs to target $CD4^+$ T cell differentiation and recognition of infected cells. It appears that EBV's immunoevasive strategy uses miRNAs, which are themselves nonimmunogenic (Boss and Renne, 2011), rather than viral proteins which themselves would be antigenic. EBV induces a state of reduced immunogenicity in infected and recently activated B cells with viral miRNAs, which allows the virus to express its latency-associated antigens avoiding the recognition and elimination by $CD4^+$ T cells. Because activated B cells are professional antigen-pre-

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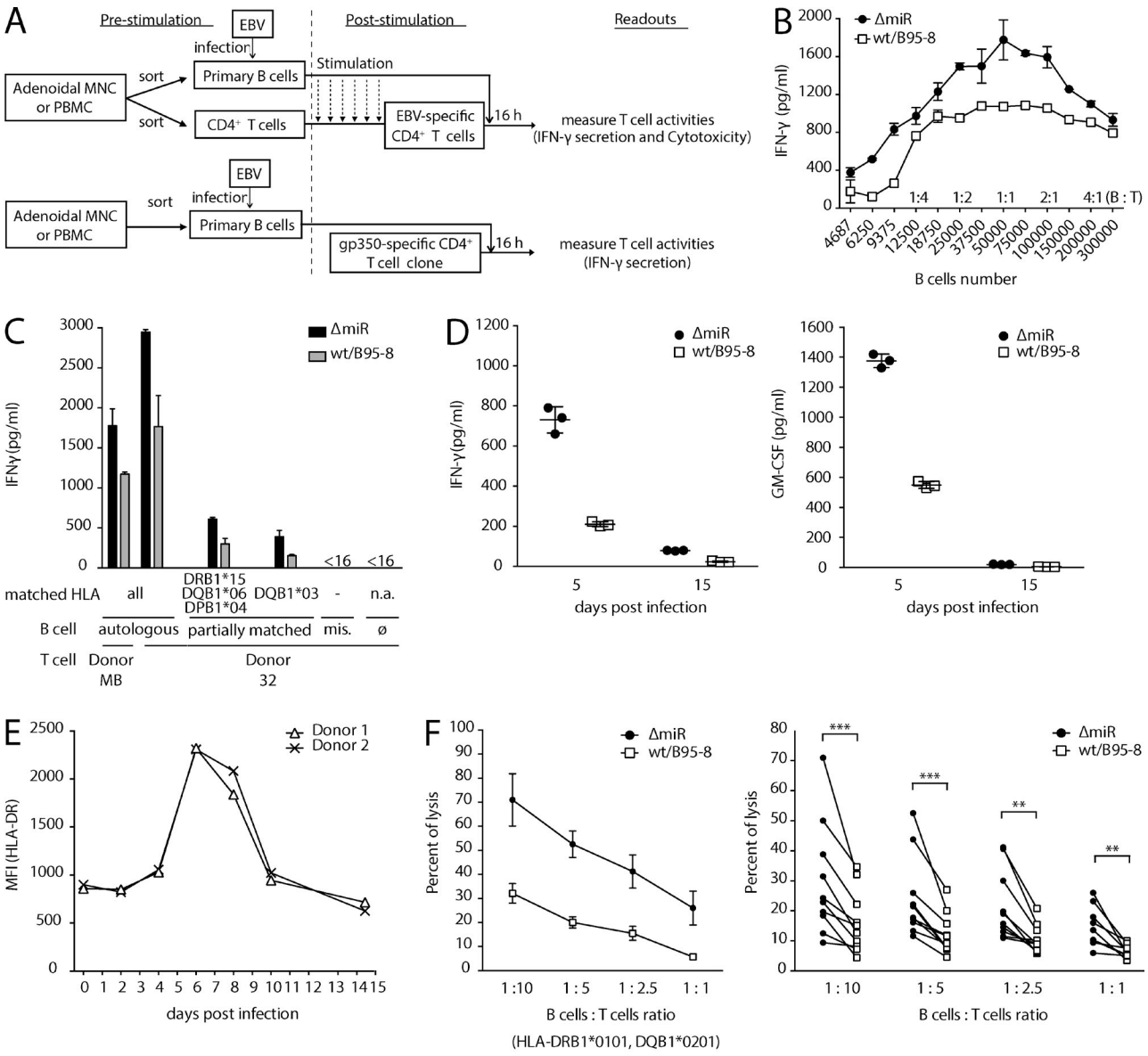

Figure 6. **EBV miRNAs inhibit recognition and killing of infected B cells by EBV-specific CD4⁺ T cells.** (A) Overview of the co-culture experiments used in B–F. Primary B cells sorted from adenoids or PBMCs were infected with either wt/B95-8 or ΔmiR EBV and co-cultured with polyclonal (top) or monoclonal (bottom) EBV-specific CD4⁺ T cells. Polyclonal antiviral CD4⁺ T cells were selected through stimulation (once in every two weeks) with irradiated LCLs infected with wt/B95-8 EBV. (B) Polyclonal EBV-specific CD4⁺ T cells were co-cultured for 16 h with autologous B cells that had been infected 5 d earlier. Levels of secreted IFN-γ were quantified by ELISA ($n = 3$). Several B/T cell ratios were used as indicated. Means ± SD are shown. (C) Autologous, partially HLA-matched, or mismatched (mis.) B cells infected with wt/B95-8 or ΔmiR EBV ($n = 3$; Table S2) were cocultivated with polyclonal EBV-specific CD4⁺ T cells and secreted IFN-γ was quantified by ELISA after 16 h. The B/T cell ratio was 1:1. Matched HLA class II alleles are indicated. Means ± SD are shown. <16 is under the detection limit (16 pg/ml); ∅, only T cells; n.a., not applicable. (D) The gp350-specific CD4⁺ T cell clone, epitope FGQ (HLA-DRB1*1301), was used as effector cells together with autologous B cells from donor JM (Table S2) as targets. B cells had been infected for 15 d with the two EBV strains as indicated and were used at an B/T cell ratio of 1:1. After 16 h of co-culture, levels of secreted IFN-γ and GM-CSF were quantified by ELISA. Means ± SD are shown. (E) MFIs measured in flow cytometry analysis of cell surface HLA-DR in primary B cells infected with wt/B95-8 EBV from two different donors were calculated. (F) Killing of EBV-infected B cells by EBV-specific CD4⁺ T cells was analyzed at various B/T cell ratios by Calcein release assays. A representative experiment with partially matched EBV-infected target B cells (left; $n = 3$) and a summary of all independent experiments with partially matched B cells (right) are shown. Paired samples from individual donors are connected by solid lines. Means ± SD are shown. P-values were calculated by a paired two-tailed Student's t test. **, $P < 0.01$; ***, $P < 0.001$.

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senting cells and express multiple immune-activating molecules (Wiesner et al., 2008), the need for EBV to control its host cell is more urgent than for other complex viruses that do not rely on professional antigen-presenting immune cells for their life-cycle.

In our experiments, recognition of early-stage infected B cells by $CD4^+$ T cells was strongly inhibited by multiple mechanisms, pointing to the biological importance of the immunoevasive functions of viral miRNAs. First, *IL12B* is dramatically repressed in wt/B95-8 EBV-infected B cells compared with ΔmiR EBV-infected B cells, leading to the down-regulation of three IL-12 family cytokines, IL-12B (IL-12p40), IL-12 (p35/p40), and IL-23 (p19/p40). At least five different viral miRNAs control the fate of the *IL12B* transcript, targeting multiple sites within its 3′-UTR, indicating its critical role in immune regulation by EBV-infected B cells and a redundancy or even cooperativity of viral miRNAs. Interestingly, the miRNAs controlling *IL12B* originate from different viral transcripts (Barth et al., 2011), suggesting a robust control of *IL12B* in all phases of EBV's life cycle and in the many cell types EBV infects, which show different patterns of miRNA expression (Cai et al., 2006; Qiu et al., 2011). Repression of *IL12B* may not only reduce $CD4^+$ T cell differentiation, as shown here, but also regulate T cell effector functions (Curtsinger and Mescher, 2010).

Second, lysosomal proteolysis is regulated. Transcripts of lysosomal endopeptidases, AEP and CTSB, and a thiol reductase, GILT, which are involved in proteolytic degradation and HLA class II epitope generation (Blum et al., 2013), are direct targets of the miRNAs miR-BART1, miR-BART2, and miR-BHRF1-2. An siRNA-mediated knock-down of these three genes in human B cells reduced their recognition by clonal epitope-specific $CD4^+$ T cells (Fig. 5, C and D; Milosevic et al., 2005) suggesting an important role of the three lysosomal enzymes in antigen processing and presentation via MHC class II molecules in human B cells.

Third, HLA class II surface levels are down-regulated in B cells infected with wt/B95-8 EBV (Fig. 4 B). We also tested if EBV miRNA targeted MHC class II molecules directly. miRNAs encoded in wt/B95-8 EBV failed to inhibit consistently four HLA-DRB1 alleles tested in dual luciferase reporter assays and also lacked functional miRNA-binding sites. Thus, the reduction of HLA class II molecules is likely indirect and may be a consequence of altered lysosomal processing of epitopes. Such phenomena might be particularly important early during infection when the EBV-activated B cells present antigenic peptides of virion components (Fig. 6 D) and the expression of HLA class II molecules peaks at the cell surface (Fig. 6 E). Together, these findings demonstrate that EBV miRNAs redundantly and robustly inhibit specific immune functions in newly infected B cells that may otherwise metabolize viral proteins into HLA class II-presented peptides for recognition by antiviral $CD4^+$ T cells.

A limited number of observations have been made before on immunomodulatory functions of EBV miRNAs. The RNAs encoding the innate immune effector molecules *MICB* (Nachmani et al., 2009) and *NLRP3* (Haneklaus et al., 2012) have been found in cell lines to be inhibited by EBV's miRNAs. They were not down-regulated in our experiments (Fig. 1 C, Table S1, and GSE75776), which examined their levels during early infection of primary B cells. Our studies with primary B cells likely avoided the adaptive changes that arise during long-term culturing in vitro.

It is not immediately apparent why B lymphocytes release proinflammatory cytokines upon infection with EBV. One explanation is based on two viral, noncoding RNAs, termed EBERs, which are contained in virions and are transcribed in all EBV-infected cells. EBERs are known to trigger the endosomal TLR3 receptor or the cytosolic RIG-I sensor signaling pathway and induce type I interferon and IL-6 synthesis in infected B cells (Samanta et al., 2006; Wu et al., 2007; Iwakiri et al., 2009), which might lead to the expression of proinflammatory cytokines found in higher concentrations in the supernatants of B cells infected with ΔmiR than with wt/B95-8 EBV.

Interestingly, LMP1, a viral membrane protein that is predominantly expressed in the latent phase but also early upon B cell infection, activates the CD40 pathway, and induces IL-6, adhesion molecules, and important immune co-receptors (Kieser and Sterz, 2015). Several viral BART miRNAs have been reported to control LMP1 expression (Lo et al., 2007; Verhoeven et al., 2016). We confirmed that miR-BART16, which is not in the wt/B95-8 EBV strain, and miR-BART3, which was not previously known to target LMP1 directly, do target it (Fig. 4 F). In addition, miR-BART3 was recently reported to reduce LMP1 protein levels in HEK 293T cells (Verhoeven et al., 2016). We failed to identify direct targets among the many co-receptors and adhesion molecules down-regulated in cells infected with wt/B95-8 EBV (Fig. 4 C). It is possible that, on average, BART miRNAs repress LMP1 levels in cells infected with wt/B95-8 EBV, and thereby indirectly inhibit surface expression of some immune co-receptors and adhesion molecules, further reducing immune recognition of EBV-infected B lymphocytes.

EBV induces proliferation of the B cells it initially infects, and fosters their survival. We have found that EBV encodes miRNAs that regulate multiple facets of a host's adaptive immune response in newly infected B cells. EBV-infected B cells lacking viral miRNAs are deficient both in regulating these responses and in other miRNA-dependent functions, including an inhibition of apoptosis (Seto et al., 2010). These latter defects have precluded comparisons of B cells newly infected with wt/B95-8 or ΔmiR in humanized mouse models. In infection experiments with these mice, we observed defects in persistence after infection with ΔmiR EBV compared with wt/B95-8 EBV, possibly resulting from a combination of decreased survival and enhanced immune control (unpublished data; C. Münz, personal communication).

Collectively, our studies of a model that closely mimics physiological infection in the early phase show that EBV's miRNAs interfere with $CD4^+$ T cell control through multiple mechanisms. They inhibit the secretion of cytokines,

inhibit antigen processing and presentation, inhibit the differentiation of $CD4^+$ T cells, and counteract recognition and elimination of infected B cells by EBV-specific $CD4^+$ effector T cells. The breadth of EBV's use of its miRNAs to inhibit adaptive immune responses is unprecedented and contributes to its efficient establishment of a lifelong infection.

MATERIALS AND METHODS

Patient samples

Surgically removed adenoids and PBMCs were obtained from anonymous patients and anonymous volunteer blood donors, respectively, from Munich, Germany. The use of this human material was approved by the local ethics committee (Ethikkommission bei der LMU München) in writing.

Separation of human primary cells

Human primary B and T cells were prepared from adenoidal mononuclear cells (MNCs) or PBMCs by Ficoll-Hypaque gradient centrifugation with Pancoll (PAN-Biotech). B cells, $CD4^+$ T cells, $CD8^+$ T cells, and naive $CD4^+$ T cells were separated from adenoidal MNCs or PBMCs using MACS separator (Miltenyi Biotec) with CD19 MicroBeads, CD4 MicroBeads, CD8 MicroBeads, and Naive $CD4^+$ T cell Isolation kit II, respectively.

Cell lines and cell culture

Burkitt's lymphoma cell lines Raji (EBV-positive), DG-75 (EBV-negative), HEK293-based EBV producer cell lines (Seto et al., 2010), infected human primary B cells, and T cells were maintained in RPMI-1640 medium (Thermo Fischer Scientific). HEK293T cells were maintained in DMEM medium. All media were supplemented with 10% FBS (Thermo Fischer Scientific), penicillin (100 U/ml; Thermo Fischer Scientific), and streptomycin (100 mg/ml; Thermo Fischer Scientific). Cells were cultivated at 37°C in a 5% CO_2 incubator.

Preparation of infectious EBV stocks and infection of human primary B cells

Infectious EBV stocks were prepared as previously described (Seto et al., 2010). In brief, EBV producer cell lines for ΔmiR (p4027) and wt/B95-8 (p2089) EBV strains were transiently transfected with expression plasmids encoding BZLF1 and BALF4 to induce EBV's lytic cycle. We collected supernatants 3 d after transfection, and debris was cleared by centrifugation at 3,000 rpm for 15 min. Virus stocks were titrated on Raji cells as previously reported and used at a multiplicity of infection (MOI) of 0.1 Green Raji units (Seto et al., 2010) for infecting primary B lymphocytes with an optimal virus dose (Steinbrück et al., 2015). For virus infection, primary B cells were cultivated with each virus stock for 18 h. After replacement with fresh medium, the infected cells were seeded at an initial density of 5×10^5 cells per ml.

RNA-Seq and RISC-IP

At 5 dpi of human primary B cells, we extracted total RNAs with TRIzol (Thermo Fischer Scientific) and Direct-Zol RNA MiniPrep kit (Zymo Research) from six different donors (Ad1–Ad6; Fig. 1) for RNA-Seq, according to the manufacturers' protocols. In parallel, we performed RISC immunoprecipitation (RISC-IP) as described previously (Kuzembayeva et al., 2012). In brief, lysed cells were incubated with anti-Ago2 antibody (11A9)-conjugated Dynabeads (Thermo Fischer Scientific), washed, and coprecipitated RNA was extracted. The cDNA libraries were prepared (Vertis Biotechnologie AG). For RNA-Seq, total RNAs were depleted of rRNAs by Ribo-Zero rRNA Removal kit (Illumina), fragmented by ultrasonication, and subjected to first strand synthesis with a randomized primer. For RISC-IP, RNAs were poly (A)-tailed, ligated with an RNA adapter at 5′-phosphates to facilitate Illumina TruSeq sequencing, and subjected to first strand synthesis with an oligo-(dT) primer. The cDNAs were PCR-amplified and sequenced with an Illumina HiSeq2000 instrument at the University of Wisconsin Biotechnology Center DNA Sequencing Facility.

Analysis of deep sequencing

For RNA-Seq, processing of paired-end reads (poly-A tail filtering, N-filtering, and adapter removal) was done using FastQC and R2M (RawReadManipulator). Reads were mapped to the human genome (hg19 'core' chromosome-set) by STAR (Dobin et al., 2013) and feature counts per transcript were determined using featureCounts and GENCODE version 19 annotations, together with EBV's annotation (available from GenBank under accession no. AJ507799). To screen differentially regulated genes by viral miRNAs, we used a simple but efficient scoring algorithm based on donor/replicate-wise fold changes ranks. For each gene g and replicate k, we calculate the gene-specific rank score as

$$r_g = \frac{1}{m}\sum_{k=1}^{n} r_{gk},$$

where n is the number of all replicates, m the number of all genes/transcripts, and r_{gk} is the rank of gene g in sample k. To select highly differentially expressed genes, we transformed the rank score into a z-score and selected all transcripts with an absolute z-score >1.6.

For RISC-IP the mapped reads were normalized using size factors estimated with the R package DEseq2 and filtered for reads mapped to annotated 3′-UTR regions using GENCODE version19. To identify local quantitative differences in the read enrichments on 3′-UTRs between wt EBV compared with ΔmiR EBV-infected B cells, we calculated a donor-wise relative enrichment score. For each genomic position p, the relative expression es_p was calculated as

$$es_p = \frac{e_{tp}}{e_{tp} + e_{cp}} \times n_{pu},$$

where e_{tp} is the enrichment value of sequenced reads at position p in wt/B95-8 EBV-infected cells and e_{cp} the local enrichment value in ΔmiR EBV-infected B cells, respectively.

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The normalization factor $n_{pu} = e_{tp}/max(e_u)$ was introduced to correct for local maxima in the UTR sequence of interest, where $max(e_u)$ is the maximum enrichment value in the UTR sequence u. Finally, we used a Gaussian filter to minimize local noise. To select 3′-UTRs bound by viral miRNAs, we set the threshold as follows: enrichment score >0.6 for a stretch of >20 nt in the 3′-UTRs in two or more donors. To quantify viral miRNAs incorporated into the RISC in infected cells, we mapped reads from the RISC-IP Seq to miRNA entries registered in miRBase 21 and calculated fractions of each viral miRNAs out of total miRNA read counts.

KEGG enrichment pathway

Enrichment of specific pathways was estimated by performing a hypergeometric distribution test via the KEGG API Web Service. All calculations were done using Matlab (Mathworks).

ELISA

To detect cytokine secretion from infected B cells, 10^6 cells were seeded in 6-well plates at 4 or 11 dpi, cultivated for 4 d with cyclosporine (1 µg/ml; Novartis). Supernatants were harvested and stored at −20°C. ELISAs for IL-6, IL-10, IL12B (IL-12p40), IL-12, IL-23, and TNF were performed following the manufacturer's protocols (Mabtech). For IL-6 and IL-10, CpG DNA were added as previously described (Iskra et al., 2010) to stimulate infected B cells. ELISA for IFN-γ levels was performed following the manufacturer's protocol (Mabtech).

To detect IL-12p40 secretion from PBMCs or PBMCs depleted of B cells using the MACS separator and CD19 MicroBeads (Miltenyi Biotec), the cells were infected with either wt/B95-8 or ΔmiR EBV at MOIs of 0.1 Green Raji units (Steinbrück et al., 2015). After 5 d of incubation, supernatants were collected and ELISA for IL-12p40 levels was assessed following the manufacturer's protocol (Mabtech).

Luciferase reporter assays

The 3′-UTRs of *IL12B* (Ensembl ENST00000231228), *IFI30* (Ensembl ENST00000407280), *LGMN* (Ensembl ENST00000334869), *CTSB* (Ensembl ENST00000353047), and LMP1 (available from GenBank under accession no. AJ507799) were cloned downstream of Renilla luciferase (*Rluc*) in the expression plasmid psiCHECK-2 (Promega). To construct the viral miRNA expression vectors, we cloned TagBFP (Evrogen) under the control of the EF1α promoter into pCDH-EF1-MCS (System Biosciences). Single miRNAs of interest were cloned downstream of the TagBFP-encoding gene. Viral miRNAs were obtained by PCR from the p4080 plasmid (Seto et al., 2010). 50 ng of the psiCHECK-2 reporter and 150 ng of the pCDH-EF1 miRNA expressor plasmid DNAs were cotransfected into 1×10^5 HEK293T cells by Metafectene Pro (Biontex). After 24 h of transfection, we measured luciferase activities with the Dual-Luciferase Assay kit (Promega) and the Orion II Microplate Luminometer (Titertek-Berthold). The activity of Rluc was normalized to the activity of Fluc (Firefly luciferase) encoded in the psiCHECK-2 reporter plasmid. We performed in silico prediction of EBV miRNA-binding sites on 3′-UTRs primarily with TargetScan (Garcia et al., 2011) and used RNAhybrid (Rehmsmeier et al., 2004) to screen for 6mer binding sites (Bartel, 2009). We performed site-directed mutagenesis with overlapping oligo DNAs and Phusion polymerase (NEB).

Quantitative RT-PCR

To quantify mRNA levels RNAs were reverse-transcribed with SuperScript III Reverse transcription (Thermo Fischer Scientific) and quantitative PCR was performed with LightCycler 480 SYBR Green I Mix (Roche) and LightCycler 480 Instrument II (Roche) according to the manufacturers' instructions. The following primers were used for the detection: *HPRT1* 5′-TGACCTTGATTTATTTTGCATACC-3′ and 5′-CGAGCAAGACGTTCAGTCCT-3′; *HMBS* 5′-CTGAAAGGGCCTTCCTGAG-3′ and 5′-CAGACTCCTCCAGTCAGGTACA-3′; *IL12B* 5′-CCCTGACATTCTGCGTTCA-3′ and 5′-AGGTCTTGTCCGTGAAGACTCTA-3′; *IFI30* 5′-CTGGGTCACCGTCAATGG-3′ and 5′-GCTTCTTGCCCTGGTACAAC-3′; *LGMN* 5′-GGAAACTGATGAACACCAATGA-3′ and 5′-GGAGACGATCTTACGCACTGA-3′; *CTSB* 5′-CTGTGGCAGCATGTGTGG-3′ and 5′-TCTTGTCCAGAAGTTCCAAGC-3′.

To quantify miRNA levels, stem-loop qPCRs were performed with TaqMan MicroRNA Reverse Transcription kit (Thermo Fischer Scientific) and TaqMan Universal Master Mix II (Thermo Fischer Scientific) according to the manufacturer's protocols. *RNU6B* was used for normalization. Following TaqMan MicroRNA assays, specific primers (Thermo Fischer Scientific) were used for detection: ebv-miR-BART2: 197238_mat; ebv-miR-BART3: 004578_mat; ebv-miR-BHRF1-3 197221_mat; *RNU6B*: 197238_mat.

Establishment of EBV-specific effector T cells and T cell clones

EBV-specific $CD4^+$ T cell clones were established from polyclonal T cell lines that were generated by LCLs or mini-LCL stimulation of PBMCs, as previously described (Adhikary et al., 2007).

Flow cytometry and antibodies

After immunostainings with fluorophore-conjugated antibodies, single-cell suspensions were measured with LSRFortessa or FACSCanto (BD) flow cytometers and the FACSDiva software (BD). Acquired data were analyzed with FlowJo software Ver. 9.8 (FlowJo). The following fluorophore-conjugated antibodies reactive to human antigens were used: anti–human IFN-γ APC (4S.B3, IgG1; BioLegend), anti-CD40 PE (5c3, IgG2b; BioLegend), anti–ICOS-L (B7-H2) PE (2D3, IgG2b; BioLegend), anti–PD-L1 (B7-H1) APC (29E.2A3, IgG2b; BioLegend), anti-CD86 (B7-2) PE (37301, IgG1; R&D Systems), anti-CD54 (ICAM-1) APC (HCD54, IgG1; BioLegend), anti–HLA-ABC APC (W6/32, IgG2a;

BioLegend), anti-CD80 PE-Cy5 (L307.4; BD), anti-FAS (CD45) PE (Dx2, IgG1; BioLegend), anti–HLA-DR unlabeled (L234, IgG2a; BioLegend), anti–HLA-DQ unlabeled (SPV-L3, IgG2a ; AbD Serotec), anti–HLA-DP unlabeled (B7/21, IgG3; Abcam), anti–mouse F(ab')2 APC (polyclonal, IgG; eBioscience), isotype IgG1 PE (MOPC-21; BioLegend), isotype IgG2b PE (MPC-11; BioLegend), isotype IgG1 APC (MOPC-21; BD), isotype IgG2a APC (MOPC-173; BioLegend), and isotype IgG2b APC (MG2b-57; BioLegend).

Western blotting

We lysed cells with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, and 0.5% DOC) and boiled the extracts with Laemmli buffer. Proteins were separated on SDS-PAGE gels (Carl Roth) and transferred to nitrocellulose membranes (GE Healthcare) using Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories). Membranes were blocked for 30 min with Roti-Block (Carl Roth), followed by antibody incubation. Secondary antibodies conjugated with horseradish peroxidase were used (Cell Signaling Technology) and exposed to CEA films (Agfa HealthCare). Protein levels were quantified with the software ImageJ. The following primary antibodies reactive to human proteins were used: anti–human Tubulin (B-5-1-2; Santa Cruz Biotechnology, Inc.). The monoclonal antibody (1G6-3) reactive to the EBV protein LMP1 was provided by E. Kremmer (Institute of Molecular Immunology, Helmholtz Zentrum München, München, Germany).

RNAi knock-down and recognition by M1-specific CD4$^+$ T cells

4×10^5 DG-75 cells were incubated in 1 ml Accell Delivery Media (GE Healthcare) and 1 nmol siRNAs directed against *GAPDH*, *IFI30*, *LGMN*, *CTSB,* or combinations thereof for 48 h. Influenza M1 protein purified as previously described (Nimmerjahn et al., 2003) was added to the medium, and the cells were further incubated for 24 h and co-cultured with M1-specific CD4$^+$ T cell clone E5 for 16 h (Milosevic et al., 2005). IFN-γ levels were detected with ELISA.

T cell differentiation and recognition

Th1 differentiation was assessed by co-culture of sorted naive CD4$^+$ T cells and infected B cells 5 dpi. 1×10^5 naive CD4$^+$ T cells stained with CellTrace Violet (Thermo Fischer Scientific) and 0.5 or 1×10^5 infected B cells were cultured in 96-well plates with Dynabeads Human T-Activator CD3/CD28 (Thermo Fischer Scientific) and cultivated for 7 d. The neutralizing antibody against IL12B (C8.6; BioLegend) or the corresponding isotype control antibody (MOPC-21; BioLegend) were added for certain experiments at 5 µg/ml. Cells were restimulated with PMA and ionomycin (Cell Stimulation Cocktail; eBioscience) for 5 h and treated with Brefeldin A and Monensin (BioLegend) for 2.5 h before fixation. Th1 population was measured by intracellular IFN-γ staining with FIX and PERM Cell Fixation and Cell Permeabilization kit (Thermo Fischer Scientific) and subsequent flow cytometry analysis. The Th1 population was defined as IFN-γ$^+$ T cells in the fraction of proliferating T cells identified via CellTrace Violet staining. EBV-specific effector T cells' activities were measured with ELISA and Calcein release assays. For IFN-γ detection from T cells, effector and target cells were seeded at 5×10^4 cell per ml (1:1 ratio) each and co-cultured for 16 h in a 96-well plate (V bottom). IFN-γ levels were detected with ELISA. IFN-γ concentrations <16 pg/ml were considered as not detected.

T cell cytotoxicity assays

Primary infected B cells were purified by Ficoll-Hypaque gradient centrifugation, and 5×10^5 target cells were labeled with calcein at 0.5 µg/ml. After three washing steps with PBS, target and effector cells were co-cultured in a 96-well plate (V bottom) with different ratios in RPMI red phenol-free medium to reduce background signals. After 4 h of co-culture, fluorescence intensity of the released calcein was measured by the Infinite F200 PRO fluorometer (Tecan). As controls, spontaneous calcein release of target cells cultivated without effector cells and cells lysed with 0.5% Triton-X100 were used to define the levels of no and fully lysed target cells, respectively.

Statistical analysis

We used Prism 6.0 software (GraphPad) for the statistical analysis. A two-tailed ratio Student's *t* test was applied unless otherwise mentioned.

Online supplemental material

Fig. S1 shows the predicted miRNA-binding sites and mutations tested in 3′-UTR reporter assays. Table S1, available as an Excel file, lists the gene transcripts controlled by viral miRNAs. Table S2 lists the HLA allele information of donors used in co-culture experiments. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20160248/DC1.

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SUPPLEMENTAL MATERIAL

Tagawa et al., http://www.jem.org/cgi/content/full/jem.20160248/DC1

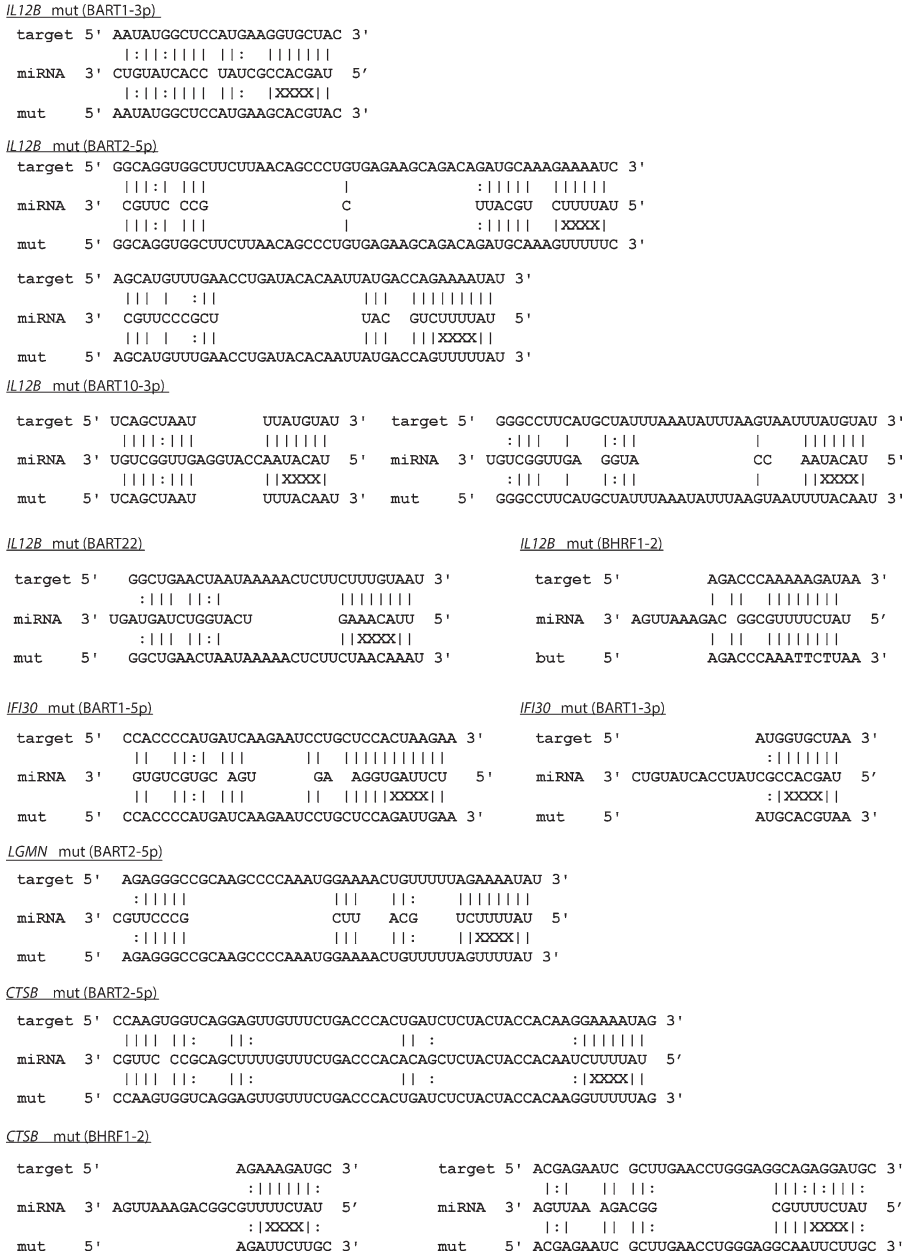

Figure S1. **miRNA binding sites and mutations in 3′–UTR reporters.** Partial sequences of 3′-UTRs of selected transcripts, which were analyzed in Fig. 3 A and Fig. 5 (A and B) are shown together with corresponding miRNAs and mutations within the 3′-UTRs in reporter vectors. Complementarities are based on in silico predictions according to the RNAhybrid algorithm and depicted as Watson-Crick ('|') or G:U (':'). Nonmatching nucleotide residues are indicated (X). They result from mutated mRNA target sequences in the reporter plasmids.

Table S1, available as an Excel file, is a list of gene transcripts down-regulated by EBV miRNAs in infected B cells. Transcript IDs (Ensembl Annotation Release 75), corresponding gene symbols, mean of log_2 fold changes, and z-scores are shown. In addition, transcripts are indicated (yes/no) according to the criteria of the RISC-IP enrichment score as defined in the Materials and methods.

Table S2. **Donor HLA alleles**

Donor	HLA class I	HLA class II
MB	n.a.	DRB1*0301,*0701
JM	n.a.	DRB1*0801,*1301; DQB1*0402,*0603; DPB1*0401,*1301
32	A*0201,*0301; B*3501,*1501; C*0102, *0401	DRB1*1501,*0701; DQB1*0602,*0303; DPB1*0401

List of the donors' HLA alleles (determined by MVZ Martinsried, Germany) whose B and T cells have been used in co-culture experiments in this study. The HLA alleles were identified by deep-sequencing. n.a., not available.

Publication II

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*equal author contributions

I designed all the experiments together with Tagawa T. and Hammerschmidt W.. I wrote the the manuscript entirely together with Tagawa T., Hammerschmidt W., and Moosmann A.. I performed the majority of the experiments with the exception of Fig. 3D and Fig. S2. I revised the entire manuscript together with Tagawa T., Hammerschmidt W., Bouvet M. and Moosmann A..

Individual contributions:

Fig.1: my contribution
Fig.2: my contribution, experiments of panel E were performed with Maliqi L.
Fig.3: my contribution (panel D by co-authors)
Fig.4: my contribution, experiments of panel D were performed with Maliqi L.
Fig.S1: my contribution
Fig.S3: my contribution
TableS1: my contribution

All the experiments and data in the figures as indicated above were performed, analyzed, and illustrated by me.

Takanobu Tagawa

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PNAS PLUS

Epstein–Barr virus microRNAs reduce immune surveillance by virus-specific CD8$^+$ T cells

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Infection with Epstein–Barr virus (EBV) affects most humans worldwide and persists life-long in the presence of robust virus-specific T-cell responses. In both immunocompromised and some immunocompetent people, EBV causes several cancers and lymphoproliferative diseases. EBV transforms B cells in vitro and encodes at least 44 microRNAs (miRNAs), most of which are expressed in EBV-transformed B cells, but their functions are largely unknown. Recently, we showed that EBV miRNAs inhibit CD4$^+$ T-cell responses to infected B cells by targeting IL-12, MHC class II, and lysosomal proteases. Here we investigated whether EBV miRNAs also counteract surveillance by CD8$^+$ T cells. We have found that EBV miRNAs strongly inhibit recognition and killing of infected B cells by EBV-specific CD8$^+$ T cells through multiple mechanisms. EBV miRNAs directly target the peptide transporter subunit TAP2 and reduce levels of the TAP1 subunit, MHC class I molecules, and EBNA1, a protein expressed in most forms of EBV latency and a target of EBV-specific CD8$^+$ T cells. Moreover, miRNA-mediated down-regulation of the cytokine IL-12 decreases the recognition of infected cells by EBV-specific CD8$^+$ T cells. Thus, EBV miRNAs use multiple, distinct pathways, allowing the virus to evade surveillance not only by CD4$^+$ but also by antiviral CD8$^+$ T cells.

adaptive immunity | immune evasion | herpesvirus | CD8 T cells | microRNA

Epstein–Barr virus (EBV) is a ubiquitous herpesvirus that infects the majority of the human population worldwide. Although EBV infection persists for life, most carriers remain asymptomatic due to a stringent control by virus-specific immunity. An important component of this immunity is EBV-specific CD8$^+$ T cells, which often expand to high numbers in healthy carriers or after primary infection. Conversely, the absence of EBV-specific CD8$^+$ T cells predicts the emergence of EBV-associated disease in patients after stem cell transplantation or when afflicted with AIDS (1–3). Dangerous EBV-mediated complications can be reversed or prevented by transfer of EBV-specific T cells (4, 5), which further confirms the important role of continuous T-cell control of EBV infection. Among EBV-specific T cells, CD8$^+$ T cells predominate; about 0.05–1% of all CD8$^+$ T cells in healthy donors are typically specific for EBV latent antigens and about twice as many for lytic antigens (6, 7).

EBV predominantly infects B cells and establishes a latent infection before production of progeny virus becomes possible (8). Four distinct programs of EBV latent infection have been defined according to their expression profiles of latent viral genes (9–11). One of these programs, known as latency III or the "growth program," is characterized by the expression of a restricted set of approximately eight viral proteins, which activate B cells and drive their proliferation, thus increasing the viral reservoir. Latency III is found in EBV-associated malignancies in immunosuppressed patients (9) and likely reemerges continuously in healthy carriers (9, 12), indicating that its control is critical for the health of an EBV carrier. Only at a later stage of infection (13) can the virus enter its lytic phase in infected cells to produce progeny virions, a phase requiring expression of the majority of viral proteins. Some of these lytic-cycle viral proteins are immunoevasins that interfere with CD8$^+$ T-cell recognition: the TAP inhibitor BNLF2a (14, 15); the G protein-coupled receptor BILF1 that associates with MHC class I/peptide complexes, diverts them from the exocytic pathway and the cell surface, and induces their lysomal degradation (16, 17); and the protein BGLF5 that reduces MHC I expression and CD8$^+$ T-cell recognition as a consequence of its generalized host-shutoff function (18, 19). Recently, BDLF3 was identified as an additional lytic-cycle protein that targets MHC molecules for degradation (20). BNLF2a is also expressed early after infection in the prelatent phase (13 for a recent review) and reduces CD8$^+$ T-cell recognition in the first days of infection but does not impair T-cell recognition in established latency (21, 22). It is unclear, though, how latently EBV-infected B cells escape elimination by T cells, in particular during the latency III program that is characterized by a considerable antigenic load and an activated state expected to increase the immunogenicity of B cells (23). In latency III, MHC I, MHC II, and T-cell–coactivating molecules are highly expressed (24, 25), but nonetheless many epitopes are suboptimally recognized by CD8$^+$ T cells (26, 27). Therefore, it is likely that unknown immunoevasive mechanisms operate in these latently infected B cells.

Significance

Most humans are infected for their lifetime with Epstein–Barr virus (EBV), which can cause cancer and other EBV-associated diseases. Infected individuals develop strong immune responses to this virus, in particular cytotoxic CD8$^+$ T cells, but viral infection is never cleared nor is EBV eliminated from the body. This suggests that certain viral molecules might prevent effective elimination of EBV-infected cells by CD8$^+$ T cells. EBV is rich in genes coding for microRNAs, many with unknown function. We show that viral microRNAs interfere with recognition and killing of EBV-infected cells by CD8$^+$ T cells. Multiple mechanisms and molecules are targeted by microRNAs to achieve this immune evasion. Therefore, targeting of viral microRNAs may improve antiviral immunity and therapy.

Author contributions: M.A., T.T., A.M., and W.H. designed research; M.A., T.T., M.B., and L. Maliqi performed research; L. Martin contributed new reagents/analytic tools; M.A., T.T., D.L., J.H., M. Hastreiter, and M. Hayes analyzed data; and M.A., T.T., B.S., A.M., and W.H. wrote the paper.

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A hallmark of EBV is its array of 44 microRNAs (miRNAs) (28–31), which is the largest number of miRNAs identified in a human pathogen to date. Many EBV miRNAs have no known function. A function of viral miRNAs in innate immunity was suggested earlier by findings showing that some regulate the inflammasome component NLRP3 (32), the natural killer group 2D (NKG2D) ligand MICB (33), and the chemokine CXCL11 (34).

Recently, we found that multiple viral miRNAs limit the control of infected B cells by $CD4^+$ T cells early in EBV infection (35). Several viral miRNAs reduce secretion of IL-12, expression of HLA class II molecules, and expression of lysosomal enzymes important for antigen presentation to $CD4^+$ T cells. EBV miRNAs also regulate many molecules of potential importance in HLA class I presentation and $CD8^+$ T-cell recognition (35).

These findings have led us to determine if viral miRNAs inhibit surveillance of EBV by $CD8^+$ T cells. We have found that viral miRNAs do prevent virus-specific activation of $CD8^+$ T cells and killing of infected B cells; we have also delineated the mechanisms underlying this viral evasion of the immune response.

Results

EBV MiRNAs Support Survival of Infected B Cells in the Presence of $CD8^+$ T Cells from EBV-Positive Donors. We used in vitro infection of primary human B cells as a simple but representative model of EBV infection (Fig. 1*A*) to evaluate if EBV miRNAs counteract immune surveillance by EBV-specific $CD8^+$ T cells. B cells ($CD19^+$) isolated from peripheral blood mononuclear cells (PBMCs) from EBV-positive donors were infected with two EBV strains: the laboratory strain B95-8 (WT/B95-8), expressing 13 viral miRNAs, and its derivative ΔmiR, expressing no viral miRNAs (36). Twelve hours later, autologous $CD8^+$ T cells were added, and the cells were cocultured for 4 wk (Fig. 1*B*), when cell viability was tested in MTT assays. Surviving cells were further analyzed by flow cytometry for their identification (Fig. S1).

In the absence of T cells, we observed robust proliferation of B cells infected with ΔmiR or WT/B95-8 EBV (Fig. 1*B*). In the presence of T cells, the survival and outgrowth of infected B cells was decreased. A strong reduction of viable cells was achieved by fewer $CD8^+$ T cells for cells infected with ΔmiR EBV than cells infected with WT/B95-8 EBV (Fig. 1*B*). Flow cytometry analyses showed that B cells represented the viable cells in most of the cultures (Fig. S1). These results indicated that EBV miRNAs protect EBV-infected B lymphocytes from eradication by antiviral $CD8^+$ T cells under these conditions.

EBV MiRNAs Inhibit Recognition, Killing, and Expansion of EBV-Specific $CD8^+$ T Cells. Earlier studies had shown that B-cell survival could be compromised in cells infected with the ΔmiR EBV devoid of miRNAs, because some viral miRNAs contribute to EBV-associated cellular transformation in the early phase of infection (36–38). To evaluate a possible role of viral miRNAs in controlling immune functions of EBV-specific $CD8^+$ T cells, we established polyclonal EBV-specific $CD8^+$ T cells from different donors. Sorted primary $CD8^+$ T cells were stimulated every 2 wk with irradiated lymphoblastoid cell lines (LCLs), which had been established by infecting primary autologous B cells with WT/B95-8 EBV (Fig. 2*A*). For T-cell effector assays, EBV-specific $CD8^+$ T cells established in this way were cocultured with B cells that had been infected with WT/B95-8 or ΔmiR EBV 15 d earlier. T-cell activation was quantified by measuring IFN-γ concentration in the cell culture supernatants after 16 h or by determining cytolysis of infected cells after 4 h. We observed significantly reduced IFN-γ secretion in response to cells infected with WT/B95-8 relative to cells infected with ΔmiR EBV, both in autologous (Fig. 2*B* and Table S1) and HLA-matched conditions (Fig. 2*C*). Importantly, T cells were not activated by HLA-mismatched infected B cells or in B-cell–free cultures, indicating that the observed activation was HLA-restricted and

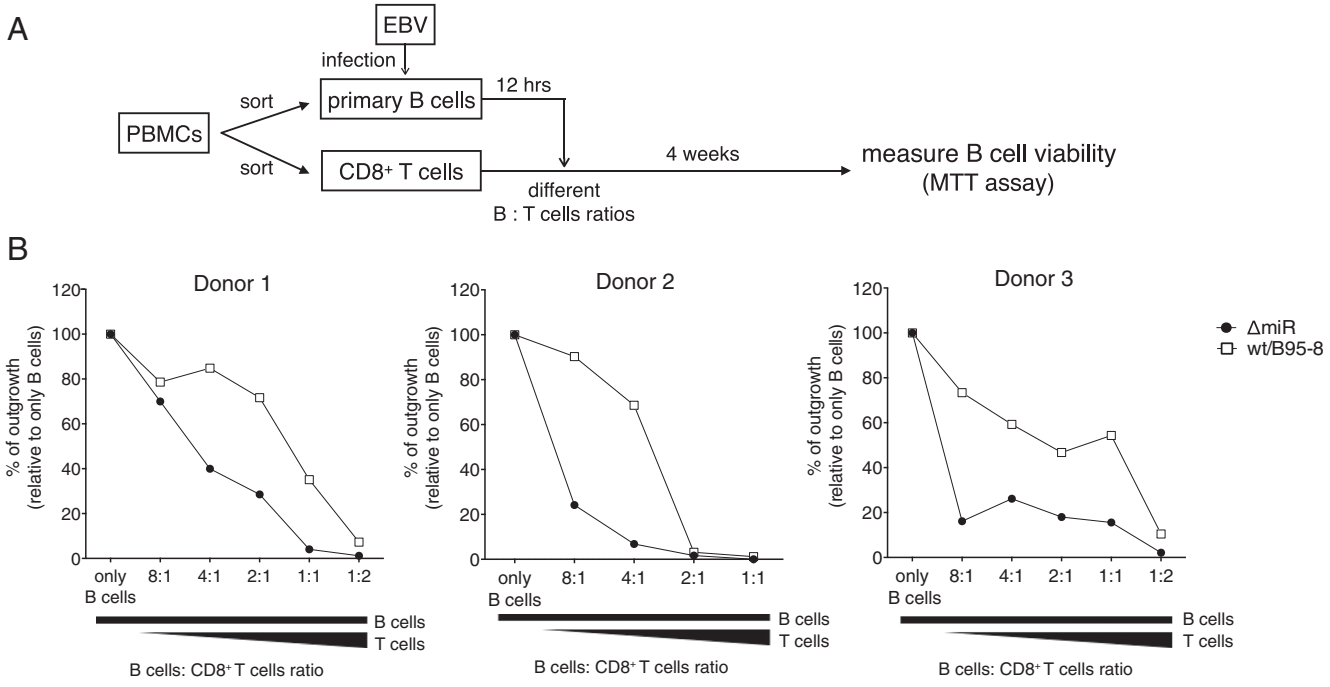

Fig. 1. EBV miRNAs support infected B cells to abrogate $CD8^+$ T-cell responses. (*A*) Schematic overview of the experimental system. (*B*) $CD19^+$ B cells were isolated from PBMCs of three different EBV-positive donors and infected with WT/B95-8 EBV or ΔmiR EBV stocks. Twelve hours later, infected B cells were extensively washed to remove free virions. In 96-well microtiter plates 32,000 EBV-infected B cells were seeded per well and $CD8^+$ T cells isolated from the autologous donors were added at different ratios as indicated. After 4 wk, total cell viability was assessed by MTT assay. Outgrowth of B-cell–only conditions (without T cells) was set to 100%. The results shown are based on the mean of six technical replicates per data point.

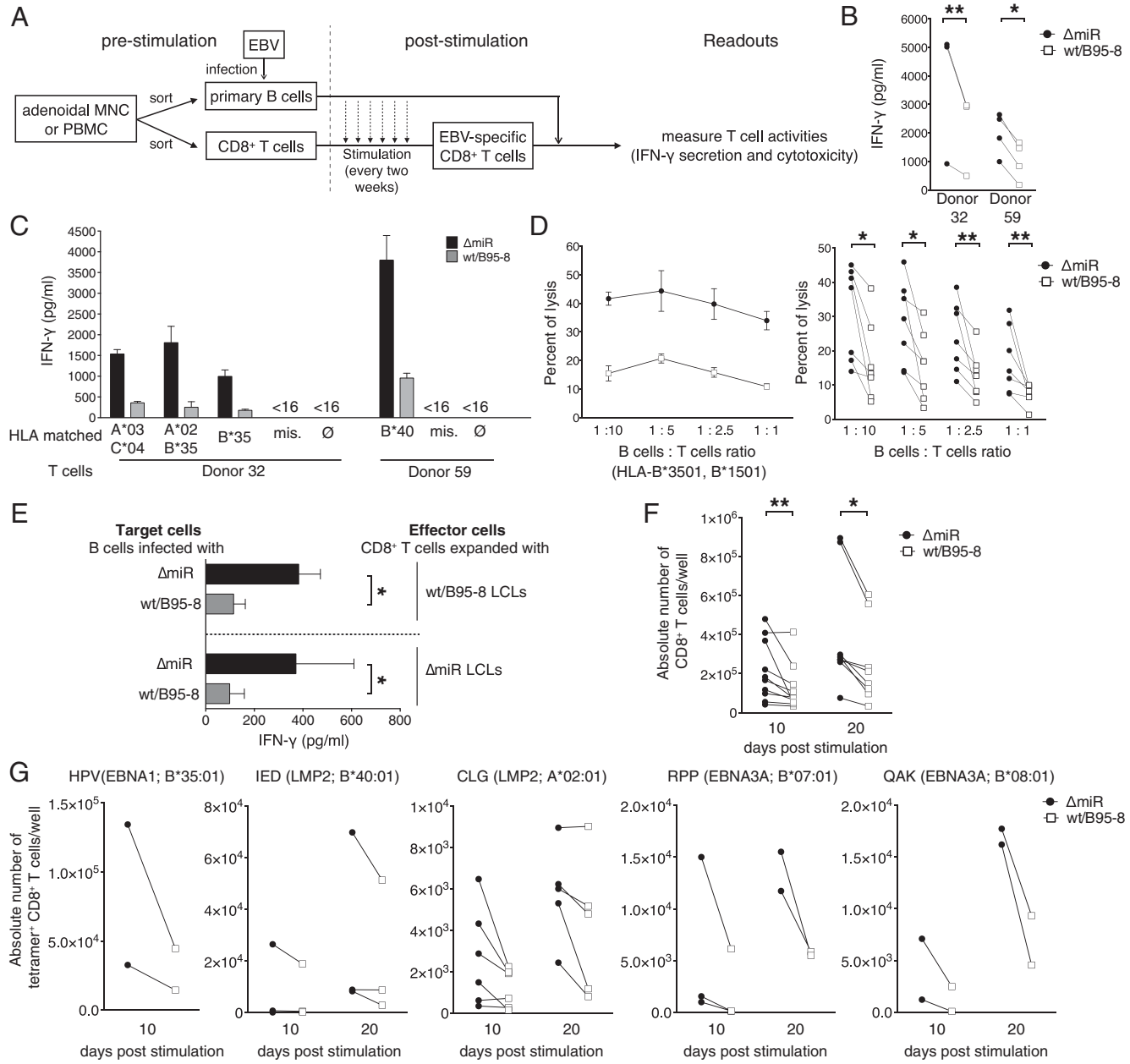

Fig. 2. EBV miRNAs inhibit recognition and killing of infected B cells as well as expansion of EBV-specific CD8$^+$ T cells. (*A*) Schematic overview of the experiments shown in the remaining panels of this figure. Polyclonal EBV-specific CD8$^+$ T cells were obtained by repeated stimulation (every two weeks) with autologous irradiated WT/B95-8 EBV-infected LCLs. The T-cell activities of the EBV-stimulated CD8$^+$ T cell were subsequently analyzed with target B cells infected with WT/B95-8 EBV or ΔmiR EBV stocks. (*B*) Equal numbers of polyclonal EBV-specific CD8$^+$ T cells and autologous B cells infected for 15 d with the indicated EBV strains were cocultured. After 16 h, IFN-γ released from T cells was measured by ELISA. Results of three to four biological replicates are shown for each donor. (*C*) Polyclonal EBV-specific CD8$^+$ T cells were cocultured with HLA-matched or mismatched EBV-infected B cells and tested as in *B*. Matched HLA class I alleles are indicated; mis., mismatched; ∅, only T cells; <16, below the threshold of detection (16 pg/mL). HLA allotypes of the donors are listed in Table S1. Data are shown as mean values. Error bars indicate SD of three replicates. (*D*) Cytotoxic activities of EBV-specific CD8$^+$ T cells directed against HLA-matched infected B cells were analyzed at various B:T cells ratios in calcein release assays after 4 h of coculture as shown in *A*. A representative experiment with mean values and SD of four replicates (*Left*) and the overview of seven donors (*Right*) are shown. (*E*) CD8$^+$ T cells of donor 115 were repetitively stimulated for 2 mo with irradiated autologous B cells infected with WT/B95-8 or ΔmiR EBV as indicated. The expanded effector T cells were assayed with autologous infected B cells as targets in coculture experiments and tested as in *B* measuring the IFN-γ released by ELISA. Error bars indicate SD of three biological replicates. (*F* and *G*) CD8$^+$ T cells isolated from PBMCs were stimulated on day 0 and 10 with irradiated B cells infected with the indicated EBV strains. Absolute numbers of T cells were determined by flow cytometry 10 d later (on day 10 and 20). (*F*) Expansion of total CD3$^+$ CD8$^+$ T cells. (*G*) Expansion of EBV epitope-specific CD8$^+$ T cells, stained with HLA/peptide pentamers as indicated. Results from 3 to 10 different donors are shown. The significance of difference in the T-cell expansion experiments was calculated by Wilcoxon matched-pairs signed rank test. *$P < 0.05$, **$P < 0.01$.

EBV-specific (Fig. 2*C*). In cytotoxicity assays, we found that the viral miRNAs inhibited killing of infected B cells by EBV-specific CD8$^+$ T cells at all B:T cell ratios and at any HLA-matched conditions tested (Fig. 2*D*). It did not matter whether EBV-specific CD8$^+$ T-cell cultures had been generated by expansion with ΔmiR or WT/B95-8 EBV-infected B cells; in each case, ΔmiR

EBV-infected B cells were better recognized than B cells infected with WT/B95-8 EBV (Fig. 2*E*).

Because clonal expansion is essential for effective antiviral T-cell responses, we also investigated the selective expansion of EBV-specific $CD8^+$ T cells in response to autologous B cells infected with either WT/B95-8 or ΔmiR EBV (Fig. 2*F*). From PBMCs of 10 donors, we sorted $CD8^+$ T cells and stimulated them twice on day 0 and day 10 with irradiated autologous B cells, which had been infected for 15 d with WT/B95-8 or ΔmiR EBV. Total numbers of $CD8^+$ T cells obtained at days 10 and 20 (after one or two stimulations) were significantly higher after expansion with ΔmiR EBV-infected cells compared with WT/B95-8 EBV-infected cells (Fig. 2*F*). In the same setting, we also analyzed the expansion of EBV-specific $CD8^+$ T cells that were specific for five different epitopes from EBV proteins latent membrane protein (LMP)2A, EBV nuclear antigen (EBNA)1, and EBNA3A (Fig. 2*G*). We consistently found increased expansion in response to ΔmiR EBV-infected cells for each of these specificities (Fig. 2*G*). Together, our data suggest that viral miRNAs in EBV-infected B cells reduce clonal expansion of a wide range of antiviral $CD8^+$ effector T cells.

EBV MiRNAs Inhibit MHC Class I Antigen Processing and Presentation Pathways. We screened cellular transcripts targeted by EBV miRNAs and likely critical in fending off antiviral $CD8^+$ T cells. To identify potential targets, we performed high-throughput screening with primary B lymphocytes infected with the different EBV strains and a combination of RNA and RNA induced silencing complexes-immunoprecipitation (RISC-IP) sequencing (35). With this approach, we identified *IL12B* and three genes (*IFI30*, the IFN-γ-regulated thiol reductase *GILT*; *LGMN*, the asparagine endopeptidase *AEP* alias legumain; and *CTSB*, the peptidase cathepsin B) encoding lysosomal enzymes and important for $CD4^+$ T-cell differentiation and antigen processing as direct targets of viral miRNAs (35). Here, we focused on genes consistently inhibited by EBV miRNAs and known to play a role in antigen processing and presentation and cytokine–cytokine receptor interactions or are considered cell adhesion molecules according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway categories (Table 1). A subset of corresponding mRNAs was also enriched in our RISC-IP sequencing analysis (Table 1), indicating that these mRNAs were most likely direct targets of EBV miRNAs. Interestingly, *TAP1* and *TAP2* were significantly down-regulated in RNA-sequencing (RNA-Seq) experiments, and *TAP2* was also found enriched in RISC-IP sequencing (Table 1). The TAP1/TAP2 heterodimer mediates transport of antigenic peptides into the ER lumen, where they are loaded onto MHC class I molecules. The presentation of many EBV epitopes depends on TAP (39), and thus we delineated the mechanisms by which EBV miRNAs regulate it.

First, we verified the regulation of *TAP1/2* expression by viral miRNAs. Fifteen days post infection expression of *TAP1* and *TAP2* was reduced in B cells infected with WT/B95-8 EBV compared with ΔmiR EBV both at the level of transcript (Fig. 3*A*) and protein (Fig. 3*B*). As a control, we verified that *IPO7* (Importin-7), a known target of EBV miR-BART3 (40), was also down-regulated (Fig. 3 *A* and *B*). Because RISC-IP (Table 1) in combination with the in silico target algorithm TargetScan (41) predicted that the 3′UTR of *TAP2* was directly targeted by EBV miRNAs, we performed dual luciferase reporter assays to test this assumption. We cotransfected HEK293T cells with a luciferase reporter plasmid containing the 3′UTR of *TAP2* and single expression plasmids, each of which encoded one viral primary miRNA.

The expression of exogenous miR-BHRF1–3 significantly decreased the luciferase activity of the *TAP2* reporter (Fig. 3*C*, *Left*). A mutation within the 3′UTR in the seed-matching region abolished this inhibition completely, demonstrating that *TAP2* is a direct target of miR-BHRF1–3 (Fig. 3*C*). Similarly, miR-BART17, which is expressed in EBV field strains but not in the WT/B95-8 strain, targeted the 3′UTR of the *TAP2* directly at one of two predicted sites (Fig. 3*C*; predicted seed sequences are provided in Fig. S2*A*). In contrast, we did not observe a regulation of the *TAP1* 3′UTR by any viral miRNA present in WT/B95-8 EBV (Fig. 3*D*). This result suggested that *TAP1* may not be a direct target of EBV miRNAs, consistent with our RISC-IP data (Table 1). A parallel dual luciferase reporter assay performed for *IPO7* served as a positive control together with miR-BART3 in these assays (Fig. S2*B*).

Table 1. Selected genes (cytokine–cytokine receptor interaction, antigen processing and presentation, and cell adhesion molecules) and their regulation by EBV miRNAs in RNA-Seq and RISC-IP experiments

Gene symbol	Mean of log-twofold change	*z* score	RISC-IP
IL12B	−2.3108	−2.5002	Yes
CD274	−0.6644	−2.3705	No
CD80	−0.9250	−2.3533	No
ICAM1	−0.6553	−2.3351	No
TAP2	−0.4012	−2.1261	Yes
TNF	−0.6389	−2.0936	No
CD40	−0.4912	−2.0739	Yes
CD58	−0.5088	−2.0568	No
RFX5	−0.3576	−1.9605	No
PSME1	−0.3338	−1.9373	No
CTSB	−0.4520	−1.9370	Yes
PSME2	−0.4680	−1.9335	No
CD86	−0.4486	−1.8001	No
TAP1	−0.3426	−1.7800	No
ALCAM	−0.4300	−1.6637	No
ICAM3	−0.5567	−1.6273	No
ERAP2	−0.2923	−1.6048	No
IPO7	−0.2826	−1.6870	Yes

Genes were identified by mRNA sequencing of WT/B95-8 vs. ΔmiR-infected B cells, ranked by *z* score, and where indicated, confirmed by RISC-IPs as described in Tagawa and coworkers (35). *IPO7* served as a positive control.

Next, we quantified the levels of classical HLA class I (HLA-A, -B, and -C) cell-surface expression on WT/B95-8 or ΔmiR EBV-infected B cells during the course of infection. Steady-state surface levels of HLA class I molecules are a function of TAP activities, as HLA class I molecules lacking peptides are unstable. We consistently observed a slight reduction by 10–20% of overall surface MHC class I molecules in cells infected with WT/B95-8 relative to ΔmiR EBV during the entire observation period (Fig. 3*E*). By assaying individual HLA class I alleles, we found that HLA-B*07, B*08, and B*40 allotypes were reduced by 20–30%, whereas HLA-A*02 levels were not reduced (Fig. 3*F*). This finding is consistent with the known preference of HLA-A*02 (but not of the other allotypes investigated here) to bind highly hydrophobic peptides, some of which reach the ER independently of TAP (42). Dual luciferase reporter assays were performed for HLA-B*07 and B*08, but direct targeting by miRNAs could not be demonstrated (Fig. S2*C*).

EBV MiRNAs Control Multiple Facets of Viral Immune Evasion. These results suggested that EBV miRNAs impose allele-specific controls of HLA molecules, namely affecting HLA-B allotypes. We therefore asked if HLA-B allotype-restricted antigen presentation is directly controlled by viral miRNAs (Fig. 4). We cocultured infected B cells and $CD8^+$ T-cell clones specific for the IED or the FLY epitope, both of which are derived from the viral LMP2 protein (Fig. 4*A*). Presentation of the B*40:01-restricted IED epitope is dependent on active TAP transportation (39), whereas the HLA-A*02:01–restricted epitope FLY is highly hydrophobic and presented TAP-independently (26). In a time course experiment

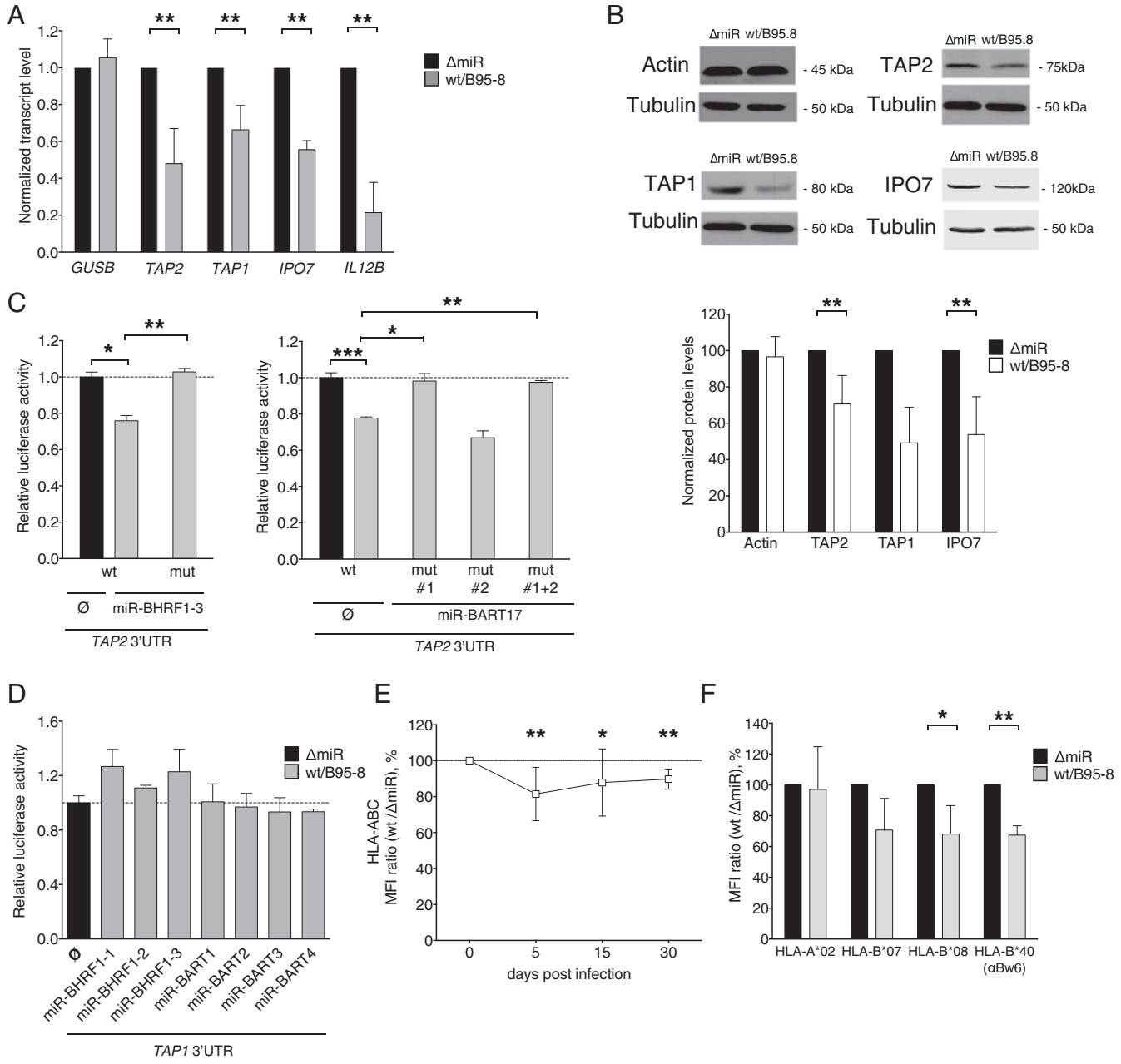

Fig. 3. EBV miRNAs reduce TAP and MHC class I levels in infected B cells. (*A*) Transcript levels of *TAP2*, *TAP1*, *IPO7*, and *IL12B* were assessed by quantitative RT-PCR in EBV-infected B cells 15 d post infection (dpi). *IPO7* is a known target of viral miRNAs and is used here as positive control. *GUSB* was used as negative control. Transcript levels were quantified relative to the mean of the housekeeping genes *HPRT1* and *HMBS* (35) and were normalized to the transcript level of ΔmiR EBV-infected cells. Data are shown as mean values and SD of seven donors. (*B*) Protein levels of TAP1 and TAP2 were assessed by Western blot analyses in EBV-infected B cells 15 dpi. β-Actin served as negative and IPO7 as positive controls. Representative examples (*Top*) and protein levels relative to Tubulin (*Bottom*) are shown. The results were normalized to the protein levels of ΔmiR EBV-infected cells, set to 100%. Data are shown as mean values and SD of three to seven donors. (*C* and *D*) EBV miRNAs directly regulate *TAP2* but not *TAP1*. HEK293T cells were cotransfected with miRNA expression vectors and dual luciferase reporter plasmids carrying a wild-type or mutated 3′UTR of *TAP2* (*C*). For the analysis of *TAP1* (*D*), all viral miRNAs present in WT/B95-8 EBV were tested with the exception of miR-BART15, which is barely expressed in our infection model (35). Sequence details of the 3′UTRs are contained in Fig. S2. The luciferase activities were normalized to lysates from cells cotransfected with the wild-type 3′UTR reporter and an empty plasmid in place of the miRNA expression plasmid. Data are shown as mean values and SD of three to four replicates. mut, mutated 3′UTR; WT, wild-type 3′UTR; ∅, empty plasmid. (*E* and *F*) Cell surface expression levels of total HLA class I (*Left*) and specific HLA class I allotypes (*Right*) of B cells infected with the indicated EBV strains for 15 d were measured by flow cytometry. Ratios (%) of WT/B95-8 divided by ΔmiR EBV-infected B cells are shown. Data are shown as mean values and SD of experiments with 5 to 10 different donors. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

with B cells infected with either WT/B95-8 or ΔmiR EBV, we observed reactivity of the clonal T cells as early as 5–7 d post infection (Fig. 4 *B* and *C*). EBV miRNAs significantly reduced the activation of the IED-specific T-cell clone (Fig. 4*B*) as expected from the down-regulation of the TAP complex and subsequent reduction of HLA-B molecules. Surprisingly, the activation of the FLY-specific T-cell clone was also strongly reduced (Fig. 4*C*) even though FLY is a TAP-independent peptide and presented via HLA-A*02:01, which

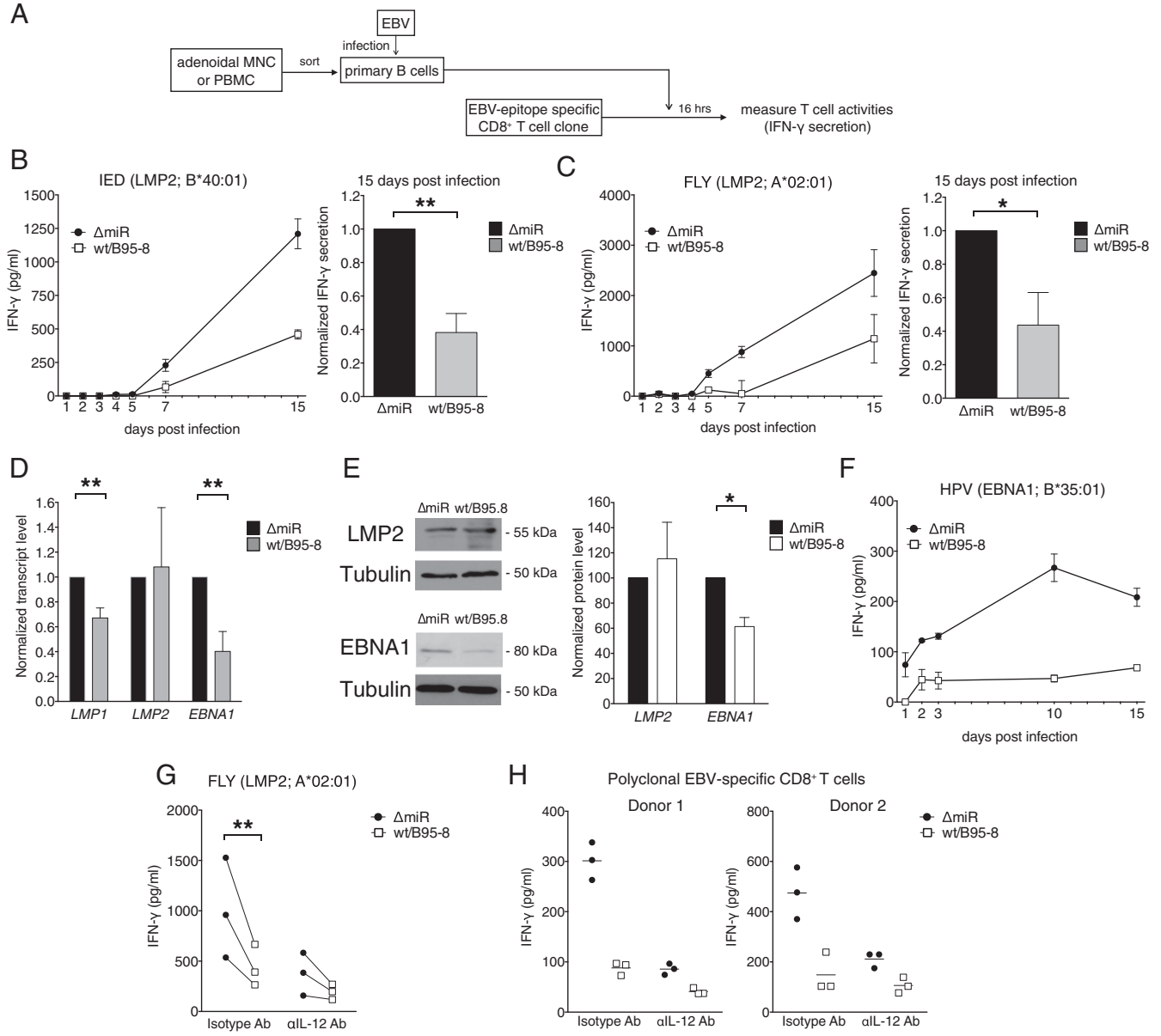

Fig. 4. EBV miRNAs control recognition of diverse types of CD8$^+$ T-cell epitopes. (*A*) Schematic overview of the experiments shown in *B*, *C*, *F*, and *G* of this figure. The presentation of viral epitopes from B cells infected with WT/B95-8 or ΔmiR EBV was analyzed with epitope-specific CD8$^+$ T-cell clones or polyclonal lines. Equal numbers of B and T cells were cocultured for 16 h, and IFN-γ release was measured by ELISA at the indicated time points. (*B* and *C*) The presentation of two LMP2 epitopes by infected B cells was analyzed with CD8$^+$ T-cell clones specific for the HLA-B*40:01–restricted IED epitope (*B*) or the HLA-A*02:01–restricted FLY (*C*) epitope. A representative time course experiment with mean values and SD of three replicates (*Left* in *B* and *C*) and the summary of all experiments performed 15 dpi with B cells from five different donors (*Right* in *B* and *C*) are shown. Results are normalized to values of ΔmiR EBV-infected cells. (*D*) Relative transcript levels of LMP1, LMP2, and EBNA1 were assessed by quantitative RT-PCR in B cells infected with WT/B95-8 or ΔmiR EBV at day 15 post infection. Transcript levels are normalized as in Fig. 3*A*. LMP1 is a known target of viral miRNAs (43, 44) and was used here as a positive control. Data are shown as mean values and SD of five donors. (*E*) Western blot analysis of EBNA1 and LMP2 in B cells infected with WT/B95-8 or ΔmiR EBV 15 dpi. Representative examples (*Left*) and protein levels relative to Tubulin (*Right*) are shown. The result from ΔmiR EBV-infected cells was set to 100%. Data are shown as mean values and SD of four different donors. (*F*) Recognition of the HLA-B*35:01–restricted EBNA1 epitope HPV presented by infected B cells as in *B* and *C*. (*G*) IL-12 neutralization reduces the activation of an epitope-specific CD8$^+$ T-cell clone. Infected B cells (15 dpi) were cocultured as in *C* (*Right*) with the FLY-specific T-cell clone together with an anti-IL12B antibody or a control antibody of the same isotype (2.5 μg/mL). After 16 h, IFN-γ release was measured by ELISA. An overview of three experiments with different donors is shown. (*H*) Equal numbers of infected B cells and polyclonal EBV-specific CD8$^+$ T cells were cocultured together with an anti-IL12B antibody or a control antibody of the same isotype (2.5 μg/mL). After 16 h, IFN-γ release was measured by ELISA. Results from two different donors are shown. $*P < 0.05$. $**P < 0.01$.

was not affected by the expression of EBV miRNAs (Fig. 3*F*). These experiments strongly supported the presence of additional immunoevasive mechanisms that affect recognition of the FLY epitope. To address the possibility that LMP2A/B gene expression may be regulated by EBV miRNAs, we evaluated the gene transcript levels by quantitative RT-PCR in infected B cells 15 d post infection and found LMP2A/B unaffected by viral miRNAs (Fig. 4*D*). In contrast, LMP1, a known target of EBV miRNAs (43, 44), was down-regulated as expected (Fig. 4*D*). Similarly, LMP2 protein levels did not depend on viral miRNAs (Fig. 4*E*), substantiating the conclusion

that EBV miRNAs regulate the activation of LMP2A/B epitope-specific T-cell clones without affecting the viral source of the epitopes they recognize.

In contrast to LMP2A, EBNA1 transcripts appeared to be under the control of viral miRNAs (Fig. 4*D*) (35), also resulting in decreased levels of EBNA1 protein 15 d post infection (Fig. 4*E*). We therefore tested the recognition of the HPV epitope of EBNA1 presented by the HLA-B*35:01 allele in a time course experiment (Fig. 4*F*). After only 1 day post infection, the HPV-specific T-cell clone was clearly activated but only when challenged with ΔmiR EBV-infected B cells. Later on, B cells infected with either virus presented the HPV peptide, but ΔmiR EBV-infected B cells were preferentially recognized (Fig. 4*F*). Regulation of TAP (Fig. 3 *A*–*C*); lower surface levels of the presenting HLA-B allele, which might be down-regulated similar to other HLA-B allotypes (Fig. 3*F*); and reduced levels of EBNA1 gene expression (Fig. 4*E*) may all have contributed to this result.

The analysis of EBNA1 epitope presentation did not reveal why TAP-independent LMP2A-derived peptides presented via HLA-A*0201 were under the control of viral miRNAs (Fig. 4*C*). We speculated that other costimulatory molecules or proinflammatory cytokines (35) may be responsible for this TAP-independent immunoevasive function. In particular, IL-12, which contributes to the activation of effector T-cell functions (45), was a possible candidate because it is a direct and prominent target of at least five different EBV miRNAs 5 d post infection (35) and was also down-regulated at the transcript level 15 d post infection (Fig. 3*A*). To address this possibility, we neutralized IL-12 secreted from EBV-infected B cells with a suitable antibody and measured IFN-γ secretion by the FLY-specific T-cell clone (Fig. 4*G*). IL-12 neutralization dramatically reduced the activation of the T cells cocultured with ΔmiR EBV-infected cells. T-cell activation with WT/B95-8 EBV-infected target cells was also affected but to a lesser extent. Very similar results were observed with polyclonal EBV-specific $CD8^+$ T cells cocultured with HLA-matched infected B cells (Fig. 4*H*), showing that miRNA-mediated regulation of IL-12 was globally decreasing recognition by $CD8^+$ T cells. This effect, which was clearly evident for EBV-specific $CD8^+$ T cells, was mild with EBV-specific $CD4^+$ T cells (Fig. S3).

Discussion

In this study, we show that EBV miRNAs inhibit surveillance of EBV by $CD8^+$ T cells. Viral miRNAs reduce virus-specific proliferation, cytokine production, and killing of infected cells by $CD8^+$ T cells with various EBV latent epitope specificities. We identified several mechanisms for this inhibition. First, miRNAs target *TAP2* directly, down-regulate the entire TAP complex, and reduce HLA allotypes that preferentially present TAP-dependent epitopes. Second, miRNAs repress EBNA1, which limits the level of a protein but is essential during most forms of EBV latency. Third, miRNAs diminish IL-12 release by infected B cells, reducing the virus-specific activity of EBV-specific $CD8^+$ T cells. Thus, EBV miRNAs limit surveillance by $CD8^+$ T cells through multiple mechanisms, likely contributing to the maintenance of lifelong infection.

It is an attractive hypothesis (35) that T-cell immunoevasion in latency would be most economically achieved by miRNAs due to their nonantigenicity. This hypothesis is now fully substantiated by our present findings that EBV miRNAs interfere with several steps of antigen presentation preventing $CD8^+$ T-cell recognition of latently infected B cells. These results are complementary to our previous findings documenting that EBV miRNAs regulate multiple pathways important for differentiation and activation of antiviral $CD4^+$ T cells in the first days of infection (35). That study also provided some evidence that $CD4^+$ T-cell recognition is also regulated later, as the structural protein gp350 could be detected by $CD4^+$ T cells in cells 15 d post infection but only when miRNAs were absent (35). The mechanisms of regulation we identified in that context—that is, regulation of MHC II, lysosomal enzymes, and IL-12—are likely relevant in latency as well and may explain why EBNA-specific $CD4^+$ T cells are generally impaired in recognizing LCLs (46). The present study focused on established latency, but because we observed a strong miRNA regulation of EBNA1 recognition by $CD8^+$ T cells already on days 1, 2, and 3 after infection, the hypothesis that EBV miRNAs generally suppress $CD8^+$ T-cell recognition already in the first days of infection during prelatency (13, 22, 47) deserves closer investigation in the future.

An overview of EBV miRNAs that directly target pathways involved in $CD8^+$ and/or $CD4^+$ T-cell recognition of infected B cells is provided in Table 2. As large subsets of viral miRNAs are expressed in all phases of EBV's life cycle (48), it appears plausible that viral miRNAs inhibit these target molecules globally. For example, miRNAs miR-BART1, miR-BART2, and miR-BART22, which target IL-12 (35), are all highly expressed not only in latency III but also in EBV-infected germinal center B cells (latency II) and memory B cells (latency 0/I) from healthy donors as well as different types of EBV-associated cancer cells (34, 48, 49). Therefore, miRNA-mediated reduction of IL-12 could lead to decreased T-cell activation and recognition at different stages of infection and malignant disease. Among TAP-regulating miRNAs, miR-BHRF1–3 is predominantly expressed initially upon infection and in latency III in vitro (36), but miR-BART17 also shows expression in memory B cells and cancer cells (48), suggesting that EBV miRNA-mediated TAP regulation could likewise be important in vivo.

Although EBV-specific immunity is likely to operate at different stages of latency and lytic replication to control viral infection, the question of interest is whether latency III, in its own right, is a target of EBV-specific immunosurveillance by T cells. For immunosuppressed patients, it appears clear that T-cell deficiency favors appearance of latency III malignancies (3), that adoptive T-cell therapy can prevent this (4, 5), and that T-cell therapy fails if the EBV strain in question does not express crucial $CD8^+$ T-cell epitopes in a latency III protein (50). Regarding infection in immunocompetent carriers, there were early arguments against a T-cell surveillance of latency III (51), but later studies showed that latency III-associated $CD8^+$ T-cell epitopes are in fact under a selective pressure that depends on the frequency of HLA class I allotypes in a population (52, 53). Cumulatively, these reports suggest that EBV-specific $CD8^+$ T-cell surveillance of latency III is an important aspect of infection control in vivo.

In this work, we have analyzed expansion of EBV-specific T cells, cytokine secretion, and cytolysis to study the interference of EBV miRNAs with $CD8^+$ T-cell functions. We found such

Table 2. Direct targets of EBV miRNAs with immune functions

Function	Gene (protein)	EBV miRNAs
Antigen processing	*CTSB* (Cathepsin B)	BHRF1–2
		BART2–5p
	LGMN (AEP)	BART2–5p
	IFI30 (GILT)	BART1–5p
		BART1–3p
Peptide transport	*TAP2* (TAP)	BHRF1–3
		BART17
Cytokines	*IL12B* (IL-12p40)*	BHRF1–2
		BART1–3p
		BART2–5p
		BART10-3p
		BART22

*Component of IL-12 (p35/p40) and IL-23 (p19/p40).

interference for T cells specific for five out of five epitopes from three different antigens (LMP2A/B, EBNA1, and EBNA3A). Because this collection contained epitopes with different HLA class I restrictions, derived from different categories of antigen (nuclear vs. transmembrane proteins) and with different processing requirements (TAP-dependent or -independent, proteasome- or immunoproteasome-dependent), our data indicate that the cumulative functional impact of miRNAs allows latently infected B cells to hide from $CD8^+$ T cells in general. Our findings with polyclonal $CD8^+$ T cells of complex composition corroborated our view.

miRNAs mediate their effects through direct binding to their target transcripts such as *TAP2*, for example (Fig. 3*C*). Because the recognition of TAP-independent epitopes is also inhibited by miRNAs, other mechanisms must contribute to the reduced presentation of the TAP-independent LMP2 epitopes CLG (Fig. 2*G*) and FLY (Fig. 4). Only a minority of all $CD8^+$ T-cell epitopes (including those of viral origin) are expected to be TAP-independent (42), and the importance of TAP is reflected by the many herpesviruses that have evolved their own TAP-inhibitory proteins (54, 55). A broad impact of TAP regulation on the immunological status in latency is also suggested by our observation that viral miRNAs do not affect global levels of HLA-A2, which is capable of presenting highly hydrophobic peptides that are more likely to be TAP-independent (39, 56), but do reduce levels of the HLA class I allotypes tested (HLA-B7, B8, and B40). These and most HLA class I allotypes are less likely to present TAP-independent epitopes, because they require the presence of polar or charged anchor residues in the peptide (57, 58).

Another effect of EBV miRNAs, suppression of IL-12, seems to act globally on the function of antigen-specific T cells. Although IL-12 was originally identified as a product of EBV-infected LCLs (59), its role in EBV-specific $CD8^+$ T-cell immunity has remained obscure. In addition to its well-known function in promoting Th1 differentiation (60), IL-12 was shown in mouse and human studies to promote $CD8^+$ T-cell functions such as proliferation, cytolysis, and IFN-γ production (60, 61) through STAT4 signaling, up-regulating T-bet, and increasing IL-2 sensitivity (62, 63). In our experiments, blockade of IL-12 fully reverted the effect of the miRNA deletion for polyclonal EBV-specific $CD8^+$ T cells (Fig. 4 *G* and *H*) but unexpectedly had only a minor effect on polyclonal EBV-specific $CD4^+$ T cells (Fig. S3). The reason for this difference is not clear yet, but one possibility may be a differential requirement for costimulatory signals (64). However, as we have shown (35), EBV miRNAs affect $CD4^+$ T-cell responses through IL-12 regulation already at the level of differentiation from naive T cells and thus act on both major classes of T cells. Because we found IL-12 to be the gene product most strongly down-regulated by EBV miRNAs, control of this cytokine appears to be central to the maintenance of EBV infection.

Materials and Methods

Patient Samples. PBMCs and surgically removed adenoid biopsies were obtained from volunteer blood donors and patients from the Department of Otorhinolaryngology of the Universitätsklinikum der Ludwig-Maximilians-Universität München, respectively. The local ethics committee (Ethikkommission bei der Ludwig-Maximilians-Universität München) approved the use of this human material. Informed consent was not required because the biopsies originated from disposed tissues from anonymous donors who underwent routine surgery.

Human Primary Cells, Cell Lines, and Cell Culture. Human primary B and T cells were prepared from adenoidal mononuclear cells (MNCs) or PBMCs as described (35). The EBV-positive Burkitt's lymphoma cell line Raji, HEK293-based EBV producer cell lines, infected human primary B cells, LCLs, and isolated T cells were cultivated as described in *SI Materials and Methods*.

Preparation of EBV Stocks and Infection of Human Primary B Cells. Stocks of recombinant EBV strains were essentially prepared and quantitated as described (65). Details can be found in *SI Materials and Methods*.

In Vitro Model of EBV Infection (B-Cell Outgrowth Assay). B cells ($CD19^+$) were isolated from PBMCs of EBV-positive donors and infected with WT/B95-8 or ΔmiR EBV strains. After 12 h, the infected B cells were extensively washed to remove free virions. $CD8^+$ T cells isolated from the same donors were cocultured at B:T cell ratios ranging from 8:1–1:2 (seeding 32,000 B cells per well). B cells cultivated without T cells served as control. Cells were refed weekly. After 4 wk, the cultures were analyzed for viable cells in MTT assays as previously described (22).

Establishment of EBV-Specific Effector T Cells and T-Cell Clones. EBV-specific $CD8^+$ T-cell clones were established by limiting dilution from polyclonal T-cell lines that were generated by stimulating PBMCs with LCLs infected with WT/B95-8 or from specific T cells directly obtained from peripheral blood cells by peptide stimulation, IFN-γ capture (Miltenyi Biotec), and magnetic isolation (66, 67). Likewise, EBV-specific $CD4^+$ T cells were generated by repetitive stimulation of sorted $CD4^+$ T cells with autologous LCLs infected with WT/B95-8 EBV as described previously (66).

EBV-Specific T-Cell Recognition. EBV-specific effector T-cell activities were measured with IFN-γ ELISA and calcein release assays. For IFN-γ detection from T cells, effector and target cells were cocultured at a 1:1 ratio (5×10^4 cell per well) for 16 h in a 96-well plate (V bottom). IFN-γ levels were detected with ELISA following the manufacturer's protocol (Mabtech). IFN-γ concentrations below 16 pg/mL were regarded negative. Neutralization of IL-12 was performed with an antibody (2.5 μg/mL), which was added directly to the coculture and is directed against the p40 subunit of IL-12 (C8.6; BioLegend). An analogous isotype control antibody (MOPC-21; BioLegend) was used as a control.

T-Cell Cytotoxicity Assays. EBV-infected B cells were purified by Ficoll-Hypaque (PAN-Biotech) gradient centrifugation, and 5×10^5 target cells were labeled with calcein (Invitrogen) at 0.5 μg/mL. After three washing steps with PBS, target and effector cells were cocultured in V bottom 96-well plates with different ratios in RPMI without Phenol Red (PAN-Biotech). After 4 h of coculture, fluorescence intensities in supernatants were measured by the Infinite F200 PRO fluorometer (Tecan). As controls, spontaneous calcein release of target cells cultivated without effector cells and cells lysed with 0.5% Triton-X100 (Carl Roth) were used to define the levels of no and fully lysed target cells, respectively.

T-Cell Expansion Assay. $CD8^+$ T cells were isolated from PBMCs of EBV-positive donors. We stimulated 1×10^6 $CD8^+$ T cells with 1×10^5 autologous irradiated B cells (infected for 15 d) and 20 U/mL IL-2. Cells were restimulated every 10 d. At 10 and 20 d after the first stimulation, T cells were stained with unlabeled HLA/peptide pentamers (Proimmune) for 20 min at 37 °C. Counterstaining was done with CD8 and CD3-specific antibodies and Pro5 fluorotag (Proimmune) on ice for 30 min. T-cell numbers were determined using calibrated APC-beads as volume standard by flow cytometry (68).

Luciferase Reporter Assays. Details of the reporter plasmids and the technical aspects of the dual luciferase reporter assays can be found in *SI Materials and Methods*.

Quantitative RT-PCR. Isolation of RNAs and their analyses by PCR are described in *SI Materials and Methods*.

Western Blotting. Cell lysis and antibodies used to detect viral and cellular proteins of interest can be found in *SI Materials and Methods*.

Flow Cytometry and Antibodies. Techniques and antibodies used to detect various surface molecules are described in detail in *SI Materials and Methods*.

Statistical Analysis. We used Prism 6.0 software (GraphPad) for statistical analysis, and the two-tailed ratio *t* test was applied unless otherwise mentioned.

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Supporting Information

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SI Materials and Methods

Cell Lines and Cell Culture. The EBV-positive Burkitt's lymphoma cell line Raji, HEK293-based EBV producer cell lines (36), infected human primary B cells, and isolated T cells were maintained in RPMI medium 1640 (Life Technologies). HEK293T cells were maintained in DMEM. All media were supplemented with 10% (vol/vol) FBS (Life Technologies), penicillin (100 U/mL; Life Technologies), and streptomycin (100 mg/mL; Life Technologies). Cells were cultivated at 37 °C in a 5% (vol/vol) CO_2 incubator.

Separation of Human Primary Lymphocytes. Human primary B and T cells were prepared from adenoidal MNCs or PBMCs by Ficoll-Hypaque gradient centrifugation. B cells and $CD8^+$ T cells were isolated using MACS separation columns (Miltenyi Biotec) with CD19 or CD8 MicroBeads, respectively.

Preparation of EBV Stocks. The recombinant EBV genome designated WT/B95-8 in this study is identical to plasmid 2089, which contains the complete EBV strain B95-8 genome, the F factor origin of replication, the chloramphenicol resistance gene, the gene for the green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) promoter, and the hygromycin resistance gene as a selectable marker in eukaryotic cells (69). Inactivation of all miRNA genes from this construct resulted in plasmid 4027, also called ΔmirALL or ΔmiR (36). Infectious virus was produced by lytic induction of producer cell lines stably carrying these recombinant EBV genomes in episomal form.

To induce EBV's lytic cycle in the ΔmiR (4027) or WT/B95-8 (2089) HEK 293 producer cell lines, plasmids coding for BZLF1 and BALF4 were transiently transfected. Supernatants were collected after 3 d. Virus stocks were titrated using Raji cells as described in detail recently (65). Isolated primary B cells were infected with the virus stocks at a multiplicity of infection of 0.1 GRU (Green Raji Units, see ref. 65). Eighteen hours later, the infected B cells were cultivated in fresh medium at an initial density of 5×10^5 cells per milliliter.

Luciferase Reporter Assays. The 3′UTRs of *TAP2* (ENST00000374897), *TAP1* (ENST00000354258), *IPO7* (ENST00000379719), and *HLA-B7/B8* (ENST00000412585/ENST00000425848) were cloned downstream of Renilla luciferase (*Rluc*) into the expression plasmid psiCHECK-2 (Promega). The pCDH vectors expressing single viral miRNA were used as previously described (35). The psiCHECK-2 reporter and pCDH-EF1-MCS plasmid DNAs (System Biosciences) with a viral miRNA of interest were cotransfected into HEK293T cells using Metafectene Pro (Biontex). miR-BHRF1–3 was expressed from a modified pLSP plasmid vector (70). It was digested with BamHI and EcoRI and ligated with miR-BHRF1–3 sequences obtained from p2089 (36). The resulting pLSP-BHRF1–3 plasmid was digested with SfiI and XbaI, and the *Cerulean* gene was inserted as a phenotypic marker. Twenty-four hours after DNA transfection, we measured luciferase activities with the Dual-Luciferase Assay Kit (Promega) and the Orion II Microplate Luminometer (Titertek-Berthold). The activity of Rluc was normalized to the activity of Firefly luciferase (Fluc) encoded by the psiCHECK-2 reporter. Site-specific mutagenesis was performed as previously described (35). We performed in silico prediction of EBV miRNA binding sites on 3′UTRs with TargetScan (www.targetscan.org) (41).

Quantitative RT-PCR. RNA was isolated using the Direct-zol RNA MiniPrep columns (Zymo Research). RNA was treated with DNase I (Thermo Fisher Scientific) and reverse transcribed with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), and quantitative PCR was performed using the LightCycler 480 SYBR Green I Mix (Roche) and the LightCycler 480 Instrument II (Roche) according to the manufacturer's instructions. The following primers were used:

HPRT1 for 5′-tgaccttgatttattttgcatacc-3′ and rev 5′-cgagcaagacgttcagtcct-3′,

HMBS for 5′-ctgaaagggccttcctgag-3′ and rev 5′-cagactcctccagtcaggtaca-3′,

GUSB for 5′-cgccctgcctatctgtattcattggaggtg-3′ and rev 5′-gatgaggaactcttggtgacagcc-3′,

IPO7 for 5′-tcgccattgtattcgagaaa-3′ and rev 5′-gaatgcatgtagtaagctgtaccc-3′,

IL12B for 5′-ccctgacattctgcgttca-3′ and rev 5′-aggtcttgtccgtgaagactcta-3′,

TAP1 for 5′-agtgccctggatgcaaac-3′ and rev 5′-agaaagaggatgtggtcacg-3′,

TAP2 for 5′-tgcgggacagaaacaacgtc-3′ and rev 5′-agcctgtgagcaatcaccag-3′,

EBNA1 for 5′-aagcatcgtggtcaaggagg-3′ and rev 5′-gcgaccaagttccttcgtc-3′,

LMP1 for 5′-aggctaggaagaaggccaaa-3′ and rev 5′-ctgttcatcttcgggtgctt-3′.3gtt

LMP2 for 5′-atcgctggtggcagtatttt-3′ and rev 5′-gagtatgccagcgacaatca-3′.

Western Blotting. We lysed cells with RIPA buffer [50 mM Tris·HCl (pH 8), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% DOC] and boiled the extracts with Laemmli buffer. Proteins were separated on 10% (vol/vol) SDS/PAGE gels (Carl Roth) and transferred to nitrocellulose membranes (GE Healthcare Life Science) using the Mini-PROTEAN Tetra Cell apparatus (Bio-Rad). Membranes were blocked for 30 min with Roti-Block (Carl Roth) followed by antibody incubation. Secondary antibodies conjugated with horseradish peroxidase were used (Cell Signaling) and exposed to CEA films (Agfa HealthCare). Protein levels were quantified with the software ImageJ. The following primary antibodies reactive to human proteins were used: anti-human Tubulin (B-5–1-2; Santa Cruz), anti-human Actin (AC-74; Sigma), anti-human IPO7 (ab88339; Abcam), anti-human TAP1 (1.28; Acris), and anti-human TAP2 (2.17, Acris). Elisabeth Kremmer, Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Molecular Immunology, Munich, provided the antibodies specific for the EBV proteins LMP2, LMP1, and EBNA1.

Flow Cytometry and Antibodies. Stained cell suspensions were measured with the LSRFortessa or FACSCanto (BD Biosciences) flow cytometers and the FACSDiva software (BD Biosciences). Acquired data were analyzed with FlowJo software Ver. 9.8 (FlowJo). The following human-specific antibodies were used:

anti–HLA-ABC APC (W6/32, IgG2a; BioLegend),

anti–HLA-Bw6 PE (REA143, IgG1; Miltenyi Biotec),

anti–HLA-A2 PE (BB7.2, IgG2b; BioLegend),
anti–HLA-B7 PE (BB7.2, IgG2b; Santa Cruz),
anti–HLA-B8 unlabeled mAb (8.L.215; USbiological),
anti-mouse IgG PE (poly4053; BioLegend),
isotype IgG1 PE (MOPC-21; BioLegend),
isotype IgG2b PE (MPC-11; BioLegend),
isotype IgG2a APC (MOPC-173; BioLegend),
anti-CD8 Pacific Blue (RPA-T8; BioLegend),
anti-CD8 PerCP/Cy5.5 (RPA-T8; BioLegend),
anti-CD4 PE (RPA-T4; BioLegend),
anti-CD3 APC (HIT3a; BioLegend),
anti-CD3 APC/Cy7 (HIT3a; BioLegend),
anti-CD19 FITC (HIB19; BioLegend),
HLA-A*0201/CLG pentamer (CLGGLLTMV, LMP2; Proimmune),
HLA-B*0702/RPP pentamer (RPPIFIRRL, EBNA3A; Proimmune), and
HLA-B*0801/QAK pentamer (QAKWRLQTL, EBNA3A; Proimmune).

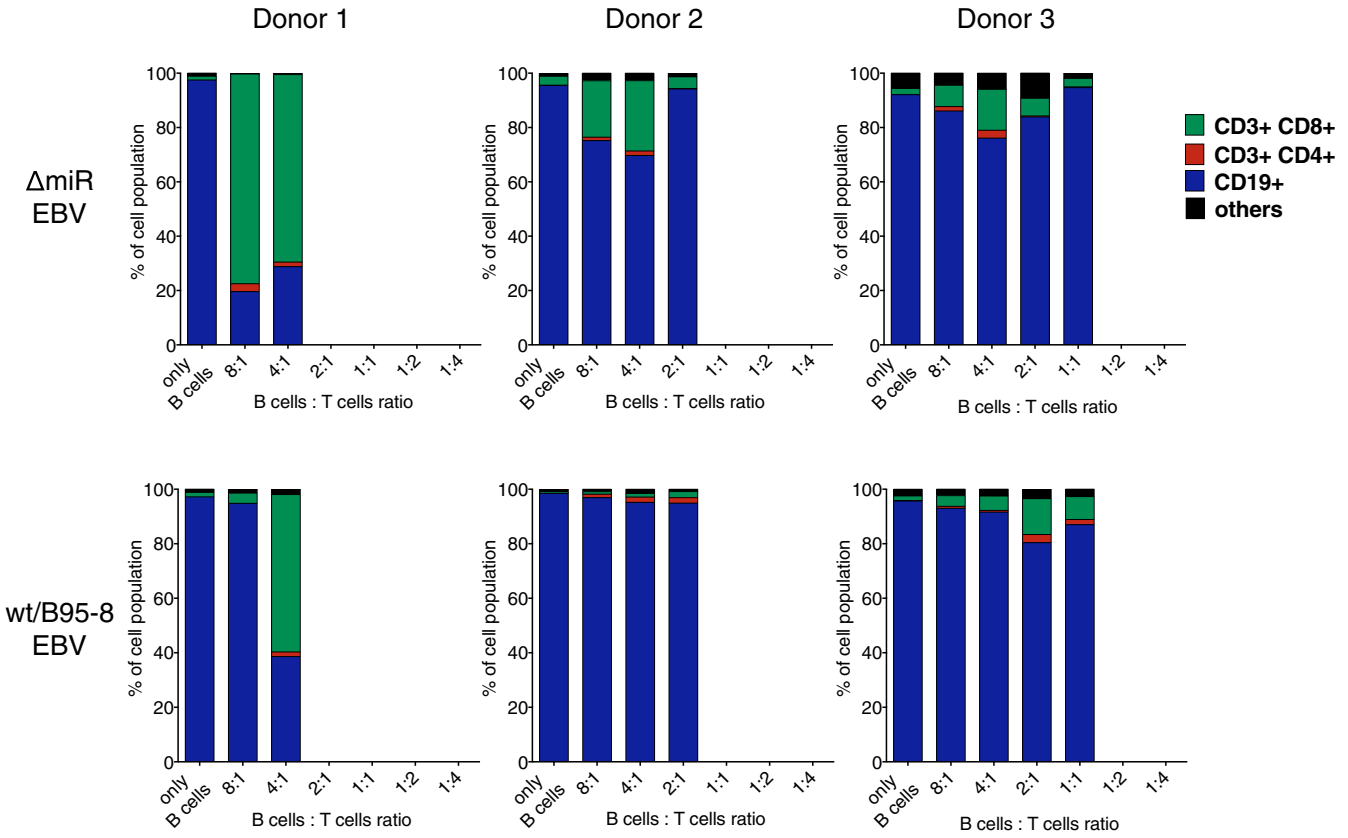

Fig. S1. Composition of long-term cocultures of WT/B95-8 or ΔmiR EBV-infected B cells with autologous $CD8^+$ T cells from three different EBV-positive donors. Coculture experiments of $CD19^+$ B cells infected with WT/B95-8 EBV or ΔmiR EBV stocks and autologous $CD8^+$ T cells were performed as shown in Fig. 1. After 4 wk, the compositions of the outgrowing cells were analyzed by FACS: B cells ($CD19^+$), $CD8^+$ T cells ($CD8^+/CD3^+$), $CD4^+$ T cells ($CD4^+/CD3^+$), or cells negative for all of the four markers are indicated.

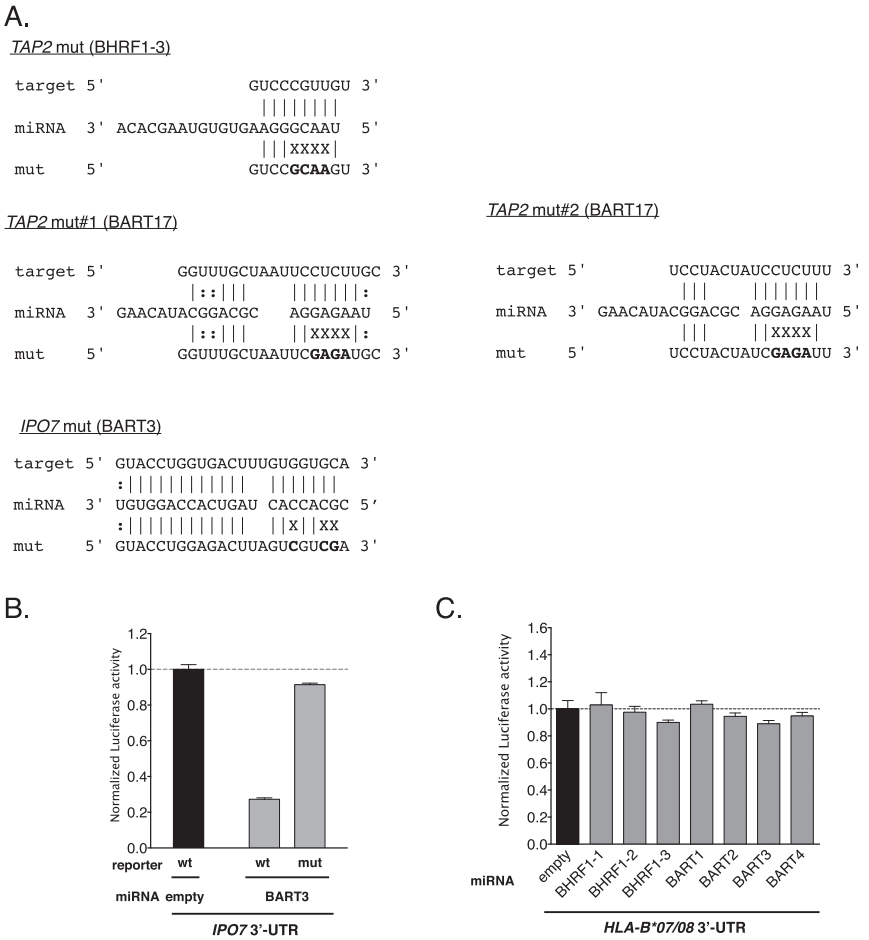

Fig. S2. Predicted miRNA target sites, their mutations, and luciferase assays of selected targets. (*A*) Partial sequences of 3′UTRs of selected transcripts analyzed in Fig. 3*C* and in *B* below are shown with corresponding miRNAs and mutations within the 3′UTRs in the reporter vectors. Complementarities are based on in silico predictions according to the RNAhybrid algorithm and depicted as Watson–Crick ('|') or G:U (':') pairs. Nonmatching nucleotide residues are indicated (X). (*B* and *C*) HEK293T cells were cotransfected with miRNA expression plasmids and luciferase reporter plasmids carrying either a wild-type or mutated 3′UTR of *IPO7* (*B*) or the 3′UTR of HLA-B*07/B*08 (*C*). The luciferase activities were normalized to lysates from cells cotransfected with the wild-type 3′UTR reporter and an empty plasmid in place of a miRNA plasmid. Data are shown as mean values and SD of three replicates. Mut, mutated 3′UTR; WT, wild-type 3′UTR; ∅, empty plasmid.

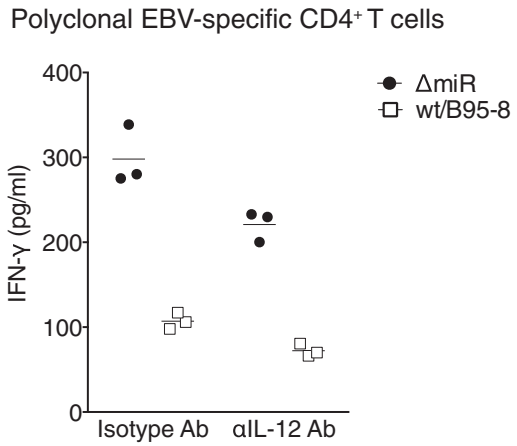

Fig. S3. IL-12 neutralization barely reduces the activation of EBV-specific $CD4^+$ T cells. Equal numbers of infected B cells (5 dpi) were cocultured with polyclonal EBV-specific $CD4^+$ T cells together with an anti–IL-12B antibody or a control antibody of the same isotype (2.5 μg/mL). After 16 h, IFN-γ release was measured by ELISA. One representative experiment out of four with different donors is shown.

Table S1. HLA class I types of the donors

Donor	*HLA-A*	HLA-B	HLA-C
32	*0201, *0301	*3501, *1501	*0102, *0401
59	*11, *24	*35, *40	n.a.
115	*02, *23	*49, *40	n.a.

DISCUSSION

1. *In vitro* model of EBV infection

Prior to establishing a stable, latent phase, many viral lytic genes are expressed for a short period of time in newly infected B cells during the pre-latent phase, which lasts seven to ten days (Kalla and Hammerschmidt, 2012). Among these lytic genes, at least two immunoevasins of the lytic cycle, BNLF2a and BCRF1 (viral IL-10), are also expressed immediately upon B-cell infection (Jochum et al., 2012a). BNLF2a and viral IL-10 interfere with the recognition of infected cells by EBV-specific effector T cells and natural killer cells, respectively, but the two gene products are insufficient to abolish T cell recognition completely (Jochum et al., 2012a).

The lack of miRNAs after infection and within the pre-latent phase leads the infected B cells to be highly activated, thus presenting a plethora of viral epitopes from latent and lytic gene products. Thus, the cells are perfect targets of immune cells and presumably rapidly recognized and killed by them (Fig. 1A). Therefore, we decided to use an *in vitro* model of infection to characterize the role of miRNAs in immune evasion within the first two weeks after infection.

1.1 Screening and validation of direct targets of viral miRNAs

Prior to our study, many putative targets of EBV's miRNAs were identified, but only few were experimentally confirmed to be direct targets of EBV's miRNAs. Several groups performed high-throughput screenings, mainly based on RISC-IP, to identify direct targets of EBV's miRNAs. These approaches generated catalogues of putative targets but the lists of genes published by several groups show only a small overlap (Klinke et al., 2014). The authors used different established EBV-positive B cell lines, which often were not related to appropriate negative controls, like

corresponding cell lines infected with EBV mutants lacking the miRNAs. Moreover, because these cell lines have been culture for many years, they could have accumulated several mutations and have undergone clonal selection for *in vitro* survival that might include additional biases.

For our approach, we wished to steer away from such problems. Consequently, we infected primary human B lymphocytes with wt/B95.8 or ΔmiR viruses and performed high-throughput screening five days after infection. This model allowed us to directly compare B cells infected with wt/B95.8 or ΔmiR virus, which differ with respect to their miRNAs coding capacity, only (Seto et al., 2010). We performed both RNA-seq and RISC-IP-seq experiments and combined the obtained data to reduce false positive candidates. The comparison of RNA-seq data between wt/B95.8 and ΔmiR EBV-infected cells provided us with a list of candidate genes that were consistently downregulated in the presence of viral miRNAs in B cells of six donors tested (Publication 2 Fig. 1). The RISC-IP-seq experiments revealed mRNA targets that were enriched in the presence of EBV's miRNAs. Combining the results of the two high-throughput screening approaches, we generated a list of genes that were both downregulated in RNA-seq and enriched in RISC-IP-seq (Publication 1 Supplementary Table 1). These genes were considered to be directly targeted by one or multiple viral miRNAs. We are aware that this approach has its limitations. The majority of the miRNAs interactions cause a downregulation of the target mRNA (Guo et al., 2010), but a small fraction of miRNAs rather blocks translation of the target mRNA than promoting its degradation. As a consequence, our approach will not identify these targets.

Because our approach is based on next generation sequencing, we introduced additional criteria of target validation: mRNA levels were confirmed by qPCR and

protein levels of the candidates were analyzed by either WB, ELISA, or Flow cytometry. Furthermore, the functional interaction between target and single miRNAs had to be demonstrated in "dual luciferase assays". Towards this end, we cloned the 3'UTR of the target transcript downstream of a renilla luciferase gene in a reporter plasmid and confirmed that the expression of individual viral miRNA led to the downregulation of the luciferase activity. To identify the predicted miRNA binding site we used bioinformatics tools such as TargetScan 6 (Garcia et al., 2011). In case a 3'UTR was identified as potential target, the putative miRNA-binding site was mutated to abrogate the miRNA-mRNA interaction. A de-repression of the luciferase activity would thus indicate a bona fide miRNA-driven target expression regulation.

2. IL-12 downregulation is important for viral immune evasion

Our RNA-seq experiments generated a catalogue of downregulated transcripts in the presence of viral miRNAs. Performing a KEGG enrichment analysis we checked which pathways were predominantly affected by EBV's miRNAs (Publication 1 Fig. 1B). As expected, apoptosis, cell cycle, and p53 signaling pathways were found to be among the top downregulated pathways, confirming previous reports. Interestingly, the top pathways regulated by viral miRNAs included those involved in innate and adaptive immune responses (Publication 1 Fig. 1B), strengthening our hypothesis that EBV's miRNAs are acting as immunoevasins.

IL12B was the most prominent target gene constitutively repressed by EBV's miRNAs among the six donors (Publication 1 Fig. 2C). *IL12B*, also known as IL-12p40, is part of a family of heterodimeric cytokines that encompasses IL-12, IL-23, IL-27, and IL-35 (Collison and Vignali, 2008). The subunit p40 is the α-chain that can bind two different β chains p19 or p35, forming IL-23 or IL-12 respectively. IL-12 is a

proinflammatory cytokine produced by dendritic cells, B cells, and macrophages in response to pathogens' infection (Vignali and Kuchroo, 2012). IL-12 promotes the differentiation of naive $CD4^+$ T cells to antiviral Th1 cells and supports the production of IFN-γ from activated T cells (O'Shea and Paul, 2002). Like IL-12, IL-23 is a proinflammatory cytokine mainly produced by macrophages and dendritic cells upon CD40 activation (Kastelein et al., 2007). In contrast to IL-12, IL-23 is reported to enhance the differentiation to $CD4^+$ Th17 cells in mice (Bettelli et al., 2006), stabilizing IL-17 production, but it is not an essential differentiation factor of Th17 cells.

We could confirm the downregulation of IL-12p40 by EBV's miRNAs at transcript and protein levels (Fig. 2A and B), which leads to the reduction of the functional heterodimeric cytokines IL-12 and IL-23 (Publication 1 Fig. 2A). As a consequence, when co-cultured with wt/B95.8 EBV-infected cells, naive $CD4^+$ T cells showed a reduced Th1 differentiation compared to cells infected with ΔmiR EBV (Publication 1 Fig. 3). The data indicated that the miRNAs prevent the antiviral response of Th1 cells limiting the antiviral activity of T cells. Interestingly, we confirmed that five different EBV's miRNAs directly repressed *IL12B* 3'UTR at seven different positions (Fig. 2C). Two of these viral miRNAs are not expressed in wt/B95.8 EBV-infected cells (Seto et al., 2010). Our findings suggest that the downregulation of this mRNA targets is extremely important for the virus. To our knowledge, this is the first report of a single mRNA targeted by that many viral miRNAs.

As described in the introduction of this thesis, we generated polyclonal EBV-specific T cell lines from both $CD4^+$ and $CD8^+$ T cells, which were used as tools to quantify the antigen presentation of EBV-infected cells. Polyclonal EBV-specific T cells co-cultured with B cells infected with wt/B95.8 EBV always showed a lower activation compared to the T cells co-cultured with ΔmiR EBV-infected B cells ($CD4^+$

T cells in Publication 1 Fig. 6 and $CD8^+$ T cells in Publication 2) indicating that miRNAs prevent recognition of infected cells (explained below in more details). Since IL-12 is also reported to be important for T cells activation (Vignali and Kuchroo, 2012), we tested the contribution of IL-12 in our co-culture system with polyclonal EBV-specific T cells. We found that neutralizing IL-12 with a specific antibody drastically reduced the activation of EBV-specific $CD8^+$ T cells when co-cultured with ΔmiR EBV-infected B cells to a level similar to wt/B95.8 EBV-infected cells (Fig. 2D). This result indicated that IL-12 also plays an important role in $CD8^+$ T cells activation. Surprisingly, this was not the case with EBV-specific $CD4^+$ T cells because IL-12 neutralization did not affect their activation (Fig. 2E). To my knowledge, this finding has not been reported previously. It emphasizes the critical role of IL-12 with respect to $CD4^+$ Th1 cell differentiation, which is independent of T cell activation upon antigen encounter.

IL-12 downregulation was already proposed to be a possible viral strategy to evade the host immune response (Smith et al., 2003). In our study, we corroborated this hypothesis identifying IL-12 as a key target for viral immune evasion by EBV's miRNAs, suggesting that probably also other viruses may regulate this cytokine for this purpose.

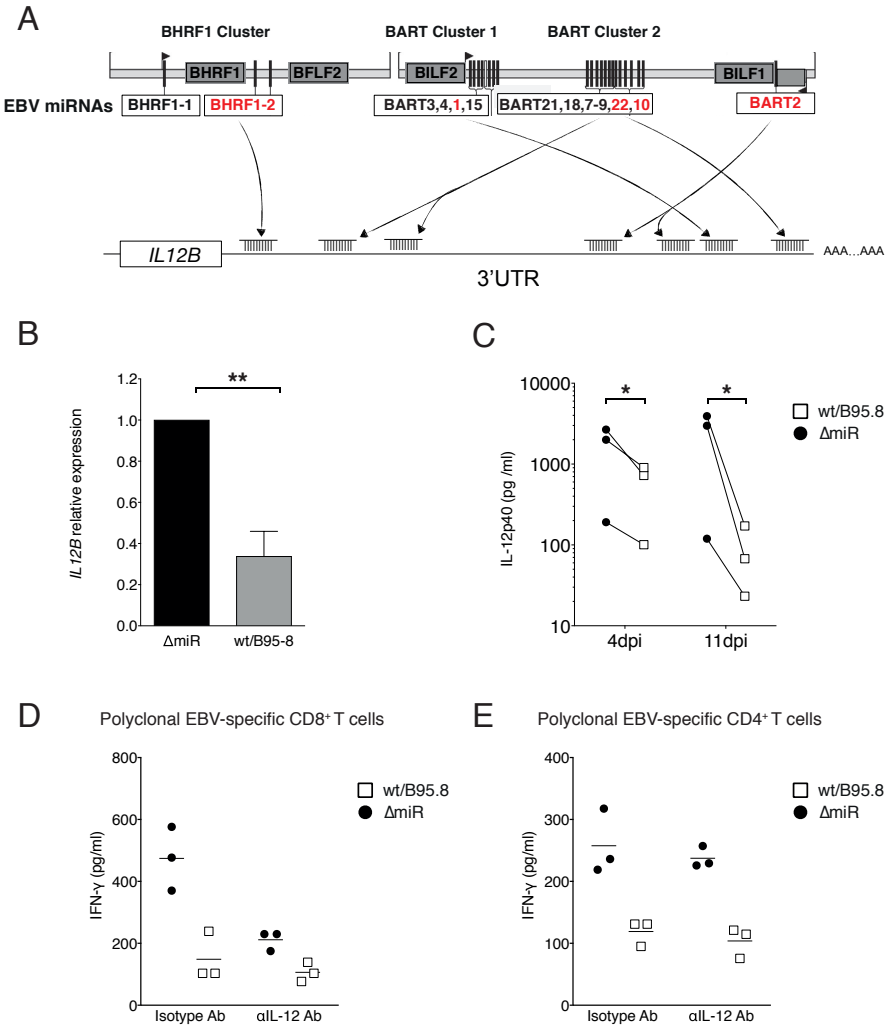

Fig. 2. IL-12 downregulation is important for viral immune evasion
A. *IL12B* 3'UTR scheme together with EBV's miRNAs (top). Five different EBV's miRNAs (in red) bind to seven different sites within the *IL12B* 3'UTR. B. mRNA levels of *IL12B* were measured by quantitative RT-PCR. Total RNA was extracted from B cells infected with wt/B95.8 or ΔmiR EBV for 5 days (n=4). C. B cells infected with wt/B95.8 or ΔmiR EBV for four or 11 days were cultivated for additional four days, when the supernatants were collected to perform ELISA to assess the concentration of IL-12p40 (n=3). D. and E. B cells infected with wt/B95.8 or ΔmiR EBV and polyclonal EBV-specific $CD8^+$ T cells (D) or $CD4^+$ T cells (E) were co-cultured together with an anti-IL12B antibody or a control antibody of the same isotype (2.5 µg/mL). After 16 h, supernatants were collected and the IFN-γ release by T cells was measured by ELISA. Results from two different donors are shown. * P<0.05; ** P<0.01.

3. Role of viral miRNAs in antigen presentation

Specific T cell activation is mediated by interactions of TCRs with the HLA-I or HLA-II molecules in case of $CD8^+$ or $CD4^+$ T cells, respectively. Each TCR recognizes a specific foreign epitope presented by a specific HLA and activates T cells effector functions including production of IFN-γ, perforin, and granzyme (mainly released by $CD8^+$ T cells). In the absence of additional activating signals, the activation of the T cell via its TCR leads to a non-responsive state called "anergy" in which the T cells cannot be activated or stimulated anymore. To become functionally active the T cells need to form an immune synapse with the target cells. The immune synapse is a tight interaction between effector and target cells mediated by the TCR, which confers the specificity, adhesion molecules, which improve the stability of the contact, and costimulatory molecules, which enhance the activation of the TCR. Only when the costimulatory molecules are activated, the TCR activation increases and leads to effective T cells functions (Smith-Garvin et al., 2009). A third signal that enhances the T cell activation requires pro-inflammatory cytokines such as type I interferon (IFN) and IL-12 as already described above.

In our study, we found that EBV's miRNAs impaired or repressed all three signals required for a productive activation of T cells.

3.1 Regulation of molecules involved in antigen processing and presentation

HLA expression on an antigen presenting cell is enhanced when the cells are activated, resulting in a better presentation of foreign antigens to T cells. Upon EBV infection, we found that the infected B cells express high levels of HLA-I on the cell

surface within days post infection, which lasted for approximately two weeks but declined thereafter (Fig. 3A). In this short period of time, infected B cells are clearly susceptible to T cells recognition but they also express certain lytic and latent viral genes together with the viral miRNAs.

We found that HLA-I surface levels were slightly reduced over time in B cells infected with wt/B95.8 EBV compared with cells infected with ΔmiR EBV (Fig. 3B). Not all HLA class I molecules were equally affected (Publication 2 Fig. 3F), but the levels of HLA class II molecules were clearly more repressed in B cells infected wt/B95.8 EBV (Publication 1 Fig. 4B). The stability of HLA molecules on the cell surface is a function of their interaction with peptides. Each HLA can potentially bind several different peptides but some have a better affinity and confer a better stability than others (Geironson et al., 2012). Empty HLA molecules are not stable. Limiting the processing and loading of peptides onto HLA molecules is a well-known strategy of many pathogens to hamper or even avoid T cell activation. For this reason, we investigated if EBV's miRNAs were involved in reducing peptide processing and presentation. Searching our list of predicted targets of EBV's miRNAs (Tagawa et al., 2016), we found several interesting candidates such as *TAP2*, involved in the processing of HLA-I peptides; the lysosomal endopeptidases cathepsin B (*CTSB*), legumain (*LGMN*) and the IFN-γ-inducible lysosomal thiol reductase (*IFI30*); all suggested to be involved in HLA-II peptide processing (Blum et al., 2013).

We validated the candidates as direct targets of different miRNAs: miR-BART-1 for *IFI30*, miR-BART-2 for *LGMN* and miR-BART-2 and miR-BHRF1-2 for *CTSB* (Publication 1 Fig. 5), and miR-BHRF1-3 for *TAP2* (Publication 2 Fig. 3). Using a siRNA knock-down approach, we analysed the functions of the three lysosomal enzymes and confirmed their role in antigen processing and HLA class II presentation

(Publication 1 Fig. 5C and D). It seems plausible that the reduced surface levels of HLA class II (-DR, -DQ, and -DP) observed in B cells infected with wt/B95.8 EBV compared with cells infected with ΔmiR EBV (Publication 1 Fig. 4B) can be, in part, explained with the reduction of the lysosomal enzymes by viral miRNAs.

The central role of the TAP complex with respect to peptide transportation has been characterized already in depth. The importance of this complex is demonstrated by the T2 cell lines, in which the TAP complex is completely lacking. In these cells the MHC-I surface expression is dramatically reduced (Attaya et al., 1992; Spies and DeMars, 1991; Franksson et al., 1993) and, as a consequence, $CD8^+$ T cells recognition is impaired. Interestingly, studies with these cells revealed that certain peptides can be presented via a TAP-independent route. Those peptides are highly hydrophobic and can thus enter the ER directly, using a mechanism independent of the TAP complex (Oliveira and Van Hall, 2013). Due to its molecular characteristics, the HLA-A*02:01 molecule is able to bind hydrophobic peptides with high affinity. As a consequence, peptides presented by this HLA or others with similar characteristics are hardly affected in TAP deficient T2 cell lines (P. Cresswell, 1992; Henderson et al., 1992).

In our model, we observed that viral miRNAs do not reduce surface levels of HLA-A*02 (Fig. 3C), but HLA-B*07, B*08 and B*40, which all require peptides with polar or charged anchor residues are affected (Publication 2 Fig. 3F), probably because their transportation depends on TAP (Sutton et al., 1993).

Because the TAP complex is a common target of several proteins of different viruses and HLA-A*02 is able to present peptides TAP-independently, we speculated that HLA-A*02 evolved to bypass the action of viral immunoevasins. To evaluate the possibility that epitopes presented by HLA-A*02 are not affected by viral miRNAs, we used a T cell clone specific for an epitope derived from the viral protein LMP2 termed

FLY, which is known to be TAP-independent (Lautscham et al., 2003). The steady state levels of LMP2 were not affected by the viral miRNAs (Publication 2 Fig. 4E and F), suggesting that it is a perfect model to assess the immunoevasive functions of viral miRNAs since the source of the antigenic peptides is not downregulated. As a control in these experiments, we used another T cell clone specific for a TAP-dependent epitope, derived from LMP2 (IED, presented by HLA-B*40:01). As expected, the activation of T cells specific for the IED epitope was reduced by viral miRNAs (Fig. 3E), but the presentation of the TAP-independent peptide FLY was also reduced (Fig. 3D). This result strongly suggested that viral miRNAs can also affect additional functions beyond TAP-mediated antigen processing and presentation. We found that the downregulation of IL-12 might contribute to the observed differences (Publication 2 Fig. 4G), suggesting that miRNAs affect several steps involved in T cell activation and beyond antigen presentation.

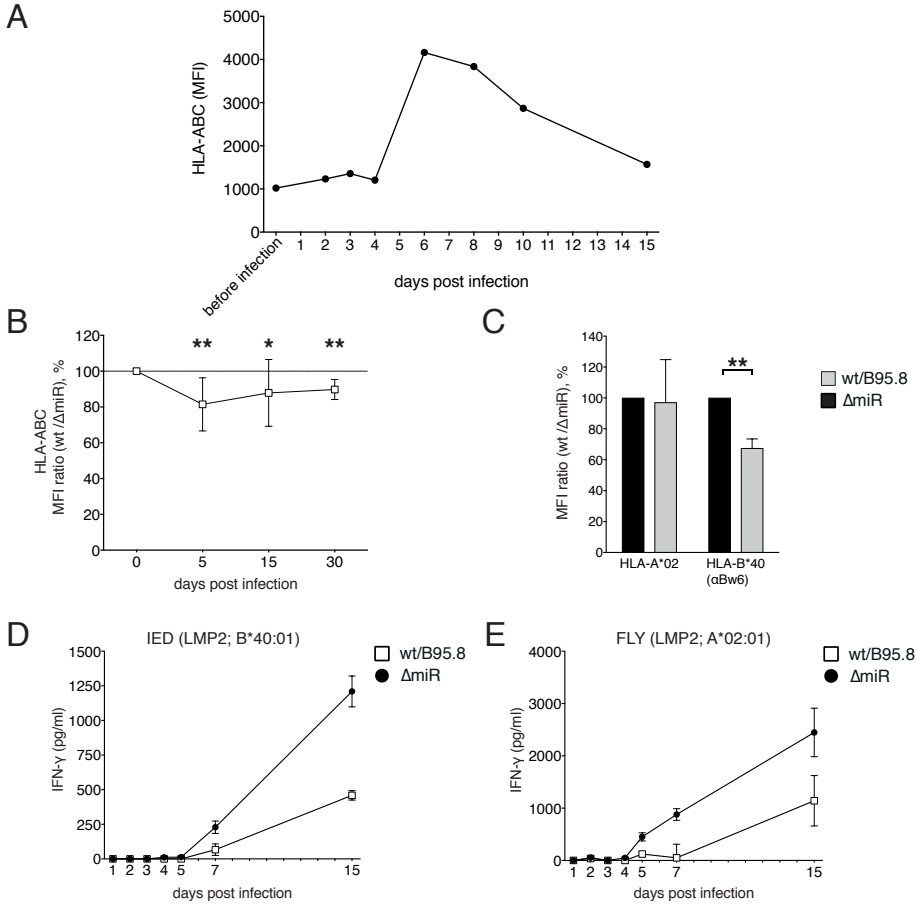

Fig. 3. Role of miRNAs in antigen presentation

A. Median fluorescence intensities (MFIs) of three HLA class I major molecules (ABC) on B cells infected with wt/B95.8 EBV from two different donors were measured by flow cytometry overtime. B. MFIs ratios (%) of HLA class I (ABC) expression of wt/B95.8 versus ΔmiR EBV-infected B cells are shown. C. Cell surface expression of specific HLA class I allotypes 15 days post infection. MFIs ratios (%) of wt/B95.8 versus ΔmiR EBV-infected B cells are shown. Data are shown as mean and SD obtained from experiments with five to ten different donors. D. and E. The presentation of the LMP2 epitope from B cells infected with wt/B95.8 or ΔmiR EBV was analyzed with epitope-specific $CD8^+$ T-cell clones overtime. Equal numbers of B and T cells were co-cultured. After 16 h, cell culture supernatants were collected and IFN-γ release was measured by ELISA at the indicated time points. T cell clones specific for the HLA- A*02:01 restricted epitope FLY (D) or the HLA-B*40:01 restricted epitope IED (E) were used. A representative time course experiment with mean and SD of three replicates are shown. * $P < 0.05$; ** $P < 0.01$.

3.2 LMP1 downregulation is important for immune evasion

As mentioned previously, the TCR needs co-receptors to be successfully activated to trigger a T cell response. Co-receptors contribute to T cells activation in different ways. First, adhesion molecules such as LFA-1 and ICAM-1 on T and APCs, respectively, can enhance the interaction between T and target cells. Second, co-receptors such as CD28 on T cells that binds CD80 and CD86 on APCs can directly enhance the activation of the TCR. Third, costimulatory molecules on T cells can bind receptors on APCs activating them, this results in the upregulation of HLA and costimulatory molecules on APC. For example, CD40L on T cells activates the CD40 receptor on APCs such as B cells, dendritic cells or monocytes.

In our study, B cells infected with wt/B95.8 EBV showed a significant reduction of many co-receptors on their surface compared with ΔmiR EBV-infected cells (Publication 1 Fig. 4C). We scrutinized many co-receptors transcripts for their direct regulation by miRNAs, but only *CD40* gene expression was downregulated in the transcriptome analyses and enriched in the RISC-IP seq experiments. Despite these hints, we could not confirm CD40 to be a direct target of viral miRNAs. The downregulation of CD40 by viral miRNAs would partially explain why costimulatory molecules were similarly affected, because CD40 activation upregulates them (Elgueta et al., 2009).

Interestingly, the viral protein LMP1 mimics an activated CD40 receptor, but LMP1 does not require a ligand to be active (Kieser and Sterz, 2015). Similar to CD40, LMP1 induces the expression of co-receptors, adhesion molecules, and cytokines such as IL-6, for example.

Two groups independently reported LMP1 to be the direct target of different BART miRNAs (Lo et al., 2007; Verhoeven et al., 2016) and we confirmed the direct downregulation by viral miRNAs also in our model early after infection (Publication 1 Fig. 4D, E and F). LMP1 is important for the survival and proliferation of infected B cells, but it also upregulates many molecules that may improve T-cells recognition and thus promote killing of EBV-infected B cells. It appears as if EBV's miRNAs fine tune or limit the levels of LMP1 to reduce the immunogenicity of EBV-infected B cells. This hypothesis explains the reduced levels of many co-receptors in wt/B95.8 EBV-infected cells even though EBV's miRNAs do not target cellular co-receptor directly.

4. Does a single viral miRNA make a difference?

In our studies with infected B cells, we identified many EBV's miRNAs to be important immunoevasins to escape the immune surveillance and support a latent infection. *In vitro* infection of primary B lymphocytes is a valuable model, but it has its limits. We always compared B cells infected with ΔmiR EBV with those infected with the wt/B95.8 EBV strain, which express up to 13 unique viral miRNAs. In this approach, it is not immediately evident which of the many viral miRNAs act as immunoevasin. To address this question, we used DG75 cells, a human EBV-negative B cell line. In this cell line, we ectopically expressed single EBV's miRNAs and used the cells as antigen presenting cells to evaluate the ability of individual miRNAs to interfere with $CD4^+$ or $CD8^+$ T cells recognition (Fig. 4A). We could confirm that miR-BART1, miR-BART2, and miR-BHRF1-3 are able to act as immunoevasins. Surprisingly, the expression of miR-BHRF1-3, which we reported to target TAP2 (Albanese et al., 2016) and which we confirmed to reduce $CD8^+$ T cells activation (Fig. 4B), also caused a reduce recognition of $CD4^+$ T cells (Fig. 4C). This finding suggested

that miR-BHRF1-3 can also regulate other, so far unidentified cellular targets. In contrast, expression of miR-BHRF1-2 that we reported to regulate *CTSB* (Tagawa et al., 2016), which is involved in HLA class II antigen processing, did not result in a reduced activation of antigen specific $CD4^+$ T cells activation (Fig. 4C). This observation does not necessarily indicate that this miRNA is not involved in immune evasion, but rather suggests that it alone is insufficient to produce a noticeable phenotypic effect in this experiment. miR-BART3 showed a repression of $CD8^+$ T cells activation but, in this experiment with DG75 cells, we could not identify any target transcript of this viral miRNA (Fig. 4B).

We investigate the effects of individual viral miRNAs. Expression of single miRNAs is enough to reduce the presentation to HLA class I and II molecules, even if this was not true for all of them (Fig. 4).

This model can also be applied to characterize the cooperative action of two or more viral miRNAs to assess the biological consequences and to identify additional targets of EBV's miRNAs.

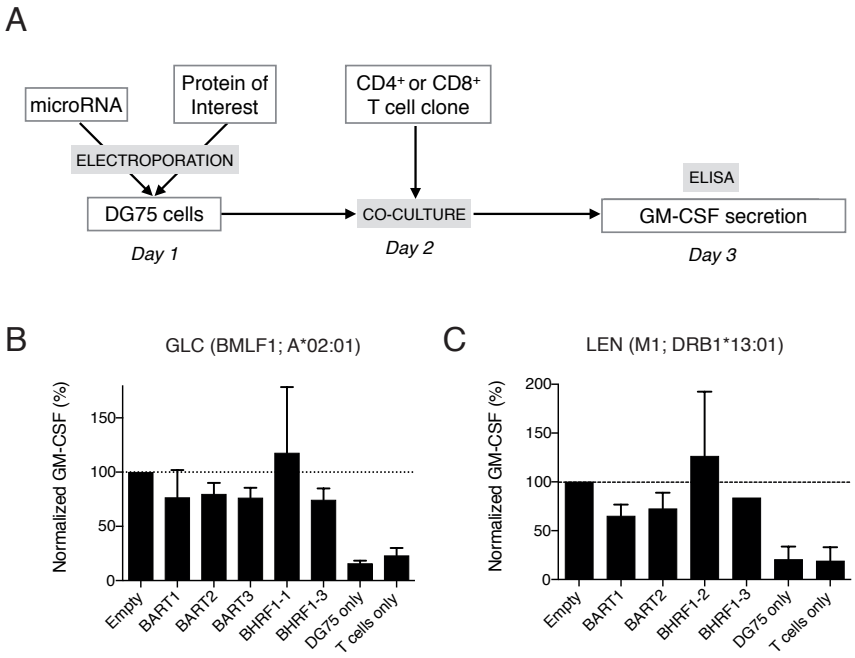

Fig. 4. Does a single miRNA make a difference?
A. Schematic overview of the experimental procedure. Day 1: DG75 cells were electroporated with a single miRNA expressing vector together with a plasmid expressing the protein of interest (EBV-BMLF1 for $CD8^+$ T cells and Influenza-M1 for $CD4^+$ T cells). Day 2: DG75 cells were co-cultured with a $CD8^+$ T cell clone specific for the HLA-A*02 restricted epitope GLC of EBV-BMLF1 (B) or with a $CD4^+$ T cell clone specific for the HLA-DRB1*13 restricted epitope LEN of Influenza-M1 protein (C). Day 3: Supernatants were collected and the levels of GM-CSF secreted by the T cells were determined by ELISA. The results shown are normalized GM-CSF levels obtained with cells transfected with a control plasmid encoding no viral miRNA. Experiments are shown with mean and SD of three replicates.

5. Are EBV's miRNAs important only during the pre-latent phase?

In our *in vitro* model, infection of B cells with EBV leads to latently infected cells, which proliferate and express the typical latency III genes program. Due to its experimental limitation, we cannot directly characterize the immunoevasive function of EBV's miRNAs in cells that maintain the expression profile of latency II, I or 0. Compared with latency III, cells in latency II or I are less immunogenic because they express fewer viral genes. However, during latency I the viral protein EBNA1 is expressed and presented to immune cells. EBNA1 is essential to maintain the viral genome in latently infected cells (Yates et al., 1985; Levitskaya et al., 1997).

To identify whether viral miRNAs can potentially reduce the recognition of EBNA1 epitopes, we used a T cell clone specific for the epitope HPV of EBNA1 (Fig. 5A). Already at one day post infection, activation of this T cell clone was observed, when co-cultured with B cells infected with ΔmiR EBV but not with wt/B95.8 EBV. Starting from day 2 and onwards, B cells infected with wt/B95.8 virus showed reduced T cells activation compared with B cells infected with ΔmiR EBV, indicating that the viral miRNAs limit the presentation of EBNA1 leading to less immunogenic B cells. Interestingly, we also observed reduced levels of EBNA1 in the presence of miRNAs (Fig. 5B), suggesting that lower protein and thus antigen levels together with the repression of TAP2 (Publication 2 Fig. 3), HLA-B (Publication 2 Fig. 3F), costimulatory molecules (Publication 1 Fig. 4C), and pro-inflammatory cytokines (Publication 1 Fig. 2A) all contribute to the reduced activation of EBNA1-specific T cells (Fig. 5A).

Collectively, our data demonstrate that EBV's miRNAs prevent recognition and elimination of infected B cells by $CD4^+$ and $CD8^+$ T cells probably in all types of viral latency.

We did not investigate if viral miRNAs also play an immune evasive role during EBV's lytic phase. However, since viral miRNAs are also highly expressed during the lytic phase, we can assume that they are active together with many others proteinaceous EBV immunoevasins during this phase of EBV's life cycle. Viral miRNAs likely improve the chance of lytically infected cells to release progeny viruses, which spread to other cells supporting lifelong EBV infection of the host.

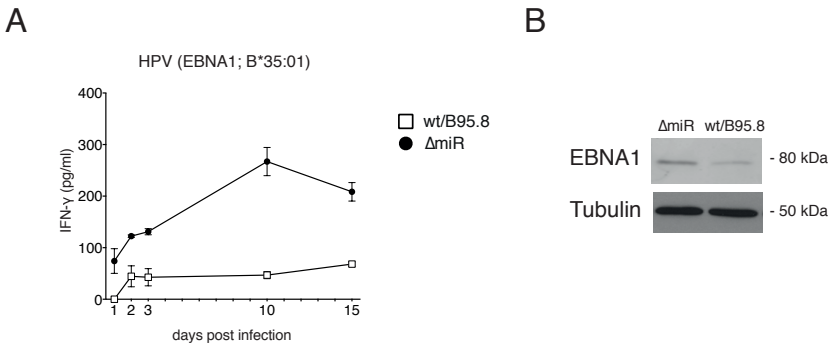

Fig. 5. EBV's miRNAs reduce EBNA1 levels and T cells recognition
A. The presentation of the EBNA1 epitope (HPV) from B cells infected with wt/B95.8 or ΔmiR EBV was analyzed with the HLA-B*35:01 restricted and HPV epitope-specific $CD8^+$ T-cell clone. Equal numbers of B and T cells were co-cultured. After 16 h, supernatants were collected and the IFN-γ release was measured by ELISA at the indicated time points. A representative time course experiment with mean and SD of three replicates are shown. B. Western blot analysis of EBNA1 in B cells infected with wt/B95.8 or ΔmiR EBV 15 dpi. Representative example of one out of four different donors is shown.

6. Circulating microRNAs

In this study, we identified EBV's miRNAs as viral immunoevasins, a previously unknown function. They reduce the immunogenicity of infected B cells and interfere with the host's adaptive immunity. Viral miRNAs affect several nodes of intercellular communication: they reduce cell-cell contact between infected cells and immune cells by reducing the surface levels of HLAs and costimulatory molecules. Viral miRNAs also repress the release of cytokines, in particular the important pro-inflammatory cytokines IL-6 and IL-12.

Viral miRNAs are also secreted in extracellular vesicles, but we have not investigated whether the virus also utilize this route of intercellular communication to its advantage.

miRNAs has been reported to be present in every body fluid, such as blood, urine, and saliva (Gallo et al., 2012; Merchant et al., 2010; Zhou et al., 2012; Lässer et al., 2011). miRNAs can be potentially released from every cell type via extracellular vesicles (EV). Different classes of EVs can be released: exosomes, microvesicles, and apoptotic bodies, which were all reported to contain miRNAs (Valadi et al., 2007; Zernecke et al., 2009). Apoptotic bodies are heterogeneous vesicles ranging from 50 to 5000 nm in diameter. They are produced by cells that undergo programmed cell death, which ensures the dismantling of the cell without causing a strong immune reaction. Apoptotic bodies are recycled by cells with phagocytic activity (Simpson and Mathivanan, 2012; Taylor et al., 2008).

In contrast to apoptotic bodies, exosomes and microvesicles are produced under physiological conditions and are smaller in size, but differ with respect to the secretory

pathways which release them. Microvesicles, also known as ectosomes, directly shed from the plasma membrane, while exosomes are formed by inward budding from large multivesicolar bodies (MVB), which then fuse to the plasma membrane and release the contained exosomes (Kalra et al., 2016). In our experimental model cells do not undergo cell death, thus the term EV refers here to exosomes and microvesicles, only.

Extracellular vesicles can interact with target cells in various ways: proteins on the EV surface can interact with receptors of target cells, EVs can fuse with the plasma membrane of their target cells and release the EVs' content, or, mainly in the case of phagocytic cells, EVs can be internalized to endosomes and the content is either released to the cytoplasm or passes on to lysosomes where the EVs are degraded (Zhang et al., 2015).

Many groups reported that the profiles of cellular miRNAs expression in cells differ from that in EVs, and certain miRNAs appear to be preferentially sorted into EVs, but it is not clear how the miRNAs are actively enriched, different routes were proposed (Guduric-Fuchs et al., 2012; Ohshima et al., 2010; Squadrito et al., 2014; Kosaka et al., 2013; Villarroya-Beltri et al., 2013; Koppers-Lalic et al., 2014).

Interestingly, extracellular miRNAs are not only found in EV, but also in a complex with high density lipoproteins (Tabet et al., 2014; Vickers et al., 2011) or bound to RNA binding proteins such as Ago2 (Arroyo et al., 2011). Surprisingly, all circulating miRNAs seem to be protected from degradation by RNases, which are ubiquitously present in all body fluids (Zhang et al., 2015).

6.1 Circulating EBV's miRNAs

EBV's miRNAs are also reported to be released by LCLs via extracellular vesicles (Canitano et al., 2013; Haneklaus et al., 2012; Pegtel et al., 2010). EVs released by lymphoblastoid cell lines (LCLs) deliver EBV's miRNAs to recipient cells such as monocytes, monocytes derived dendritic cells (moDCs) (Pegtel et al., 2010), plasmacitoid dendritic cells (pDCs) (Baglio et al., 2016), or epithelial cells. EVs appear to be taken up by target cells via a caveola-dependent endocytosis (Nanbo et al., 2013). Once taken up by the cells, viral miRNAs could potentially regulate different cellular targets. Pegtel et al. suggested that EVs with viral miRNAs are transfered from LCLs to moDCs where the released miRNAs repress the expression of known targets of EBV's miRNAs such as *CXCL11* and LMP1. The authors transfected a luciferase reporter vectors with the 3'UTR of these genes into moDCs and treated the cells with EVs released from LCL. In this condition, the luciferase activity was reduced, suggesting that EBV's miRNAs transferred via these EVs can regulate the 3'UTR of the two targets tested in moDCs (Pegtel et al., 2010). Along the same line, Haneklaus et. al. showed that EVs released by LCLs are taken up by THP1 cells, a monocytic cell line, in which they reduced the expression of the inflammasome component NLRP3 limiting a possible pro-inflammatory environment (Haneklaus et al., 2012).

6.2 A possible new role of circulating EBV's miRNAs in recipient cells

Since EBV's miRNAs are reported to be released by EVs, which are taken up by professional antigen presenting cells (APC), it is likely that viral miRNAs act as immunoevasins also in recipient cells, similarly to what we already described in EBV-infected B cells. APCs, in particular DCs, are known to modulate immune responses

during infection. DCs are able to capture vesicles from infected B cells including apoptotic bodies and cross-present their proteinaceous content to both $CD4^+$ and $CD8^+$ T cells. As such, DCs contribute to the immune control of EBV infection and support the priming and activation of innate and adaptive immunity against the virus (Bickham et al., 2003).

It is important for the virus to reduce or even suppress viral antigens presentation and avoid antiviral responses by APCs in the vicinity of EBV-infected or reactivated cells. EBV's miRNAs released by EVs seem to be a perfect way to achieve this goal.

To scrutinize this possibility, we characterized the EVs released from infected B cells. First, we optimized the protocol of EV purification (Fig. 6A). After sequential centrifugation steps, the EV containing fraction is loaded at the bottom of a density gradient using Optiprep, a solution of 60% iodixanol in water with a specific density of 1.32 g/ml to separate EVs from molecular particles with a higher specific weight. EVs float with a density of EVs 1.06 - 1.13 g/ml. We quantified the EVs content of each fraction after the density gradient centrifugation using the Nanoparticle Tracking analysis (NTA; Fig. 6B) and by Western Blot immune detection (WB; Fig. 6C). NTA is a light scatter-based method, which allows the enumeration and sizing of submicroscopic particles (Gardiner et al., 2013; Dragovic et al., 2011). After density gradient centrifugation using the NTA, we could show that the EVs were enriched in fraction two and three (Fig. 6B), which was confirmed by WB (Fig. 6C). The two proteins detected were TSG101 and LMP1, a cellular and a viral protein, respectively, both of which were reported to be enriched in EVs from EBV (Verweij et al., 2015; Baranyai et al., 2015). After validation of the purification method, we continue with the further functional characterization of the purified EVs.

First, we asked if viral miRNAs are included in the EVs as reported previously. We isolated EVs from wt/B95.8 EBV-infected B cells and extracted RNA from each density gradient fraction followed by quantitative RT-PCR using primers specific for different EBV mature miRNAs (Fig. 6D). We could confirm the presence of all the miRNAs tested within the EV fractions two and three. Interestingly, in fraction eight, where no EVs were detected, we could detect viral miRNAs (Fig. 6D) indicating that they do exist in a EV-free form as already described by Arroyo et al. (Arroyo et al., 2011). Certain miRNAs appeared to be more enriched in the EVs fraction (miR-BART1), some appear to be excluded (miR-BART3), and others are equally distributed (miR-BHRF1-2 and human miR-16).

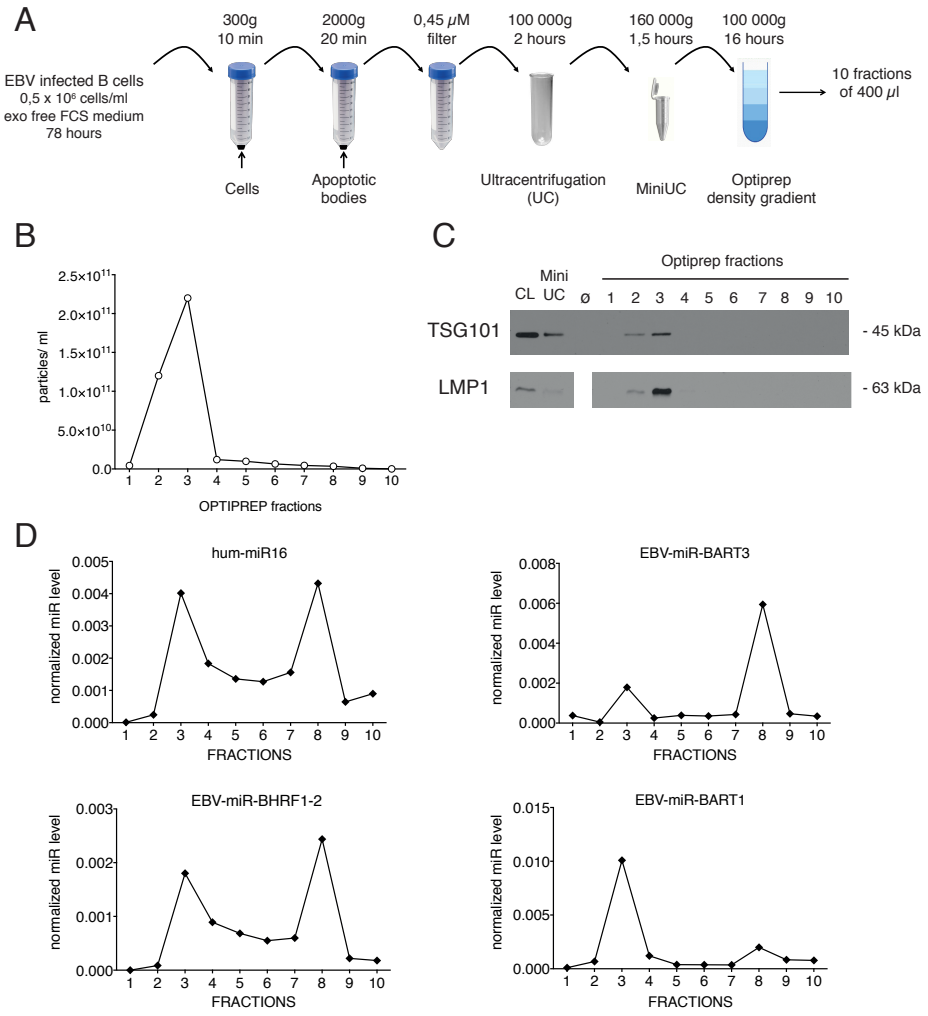

Fig. 6. Circulating EBV's miRNAs

A. Schematic overview of the EVs purification method. EBV-infected cells were seeded at $0.5x10^6$ cells/ml using complete RPMI supplemented with 2% FCS. FCS has been previously depleted from EVs through ultracentrifugation at 100,000g overnight, followed by ultrafiltration using 300 kDa Amicon centrifugal filters. After 78 hours, the supernatant was collected and centrifuged at 300g for 10 min followed by 2000g for 20 min and filtration through a 0.45 µm filter. The cleared supernatant was then ultracentrifugated at 100,000g for 2 hours. The pellet was resuspended in PBS and centrifugated again at 160,000g for 1.5 hours. The pellet was resuspended in PBS and loaded at the bottom of an Optiprep discontinuous gradient, which was centrifugated at 100,000g for 16 hours. Ten fractions of 400 µl each were collected and further characterized. B. Quantification of each Optiprep fraction by NTA. C. WB of the Optiprep fraction using antibodies specific for the human protein TSG101, a marker of EVs, and the viral protein LMP1. Cell lysates (CL) and protein from lysates of EV preparations prior to gradient were used as controls. D. Quantitative RT-PCR of human and viral miRNAs in the Optiprep fractions. Values are normalized using the miRNA levels derived from 200ng total RNA prepared from cell lysates.

Viral miRNAs within EVs can be potentially active once released and transferred to the recipient cells. But which are the target cells that take up EVs released by EBV-infected cells?

To address this question, we performed a simple experiment: highly purified EVs from EBV-infected cells were stained with the dye PKH26, which stains membranes due to its lipophilic tail. PKH26 stained EVs were incubated with isolated PBMCs. Cell-types specific markers were used to identify, which cells "interacts" with higher affinity to EVs from EBV-infected B cells (Fig. 7). We recapitulated the previously described results in different cell types, including DCs, pDCs, and monocytes (Baglio et al., 2016; Pegtel et al., 2010; Haneklaus et al., 2012). Interestingly, B cells, the cells of origin of the EV, interact with EVs only weakly, while no interaction was observed between EVs with T cells (Fig. 7). These finding supported our hypothesis that EVs are taken up by APCs and that they can potentially act as immunoevasins also inside the recipient cells.

It is important to note that the "interaction" of EVs with recipient cells observed in these experiments does not indicate that the content of EVs is taken up and released into the target cells. Further experiments are needed to unequivocally prove the release of the EVs' cargo into target cells and the functionality of the transferred miRNAs to understand the role of viral miRNAs inside the recipient cells.

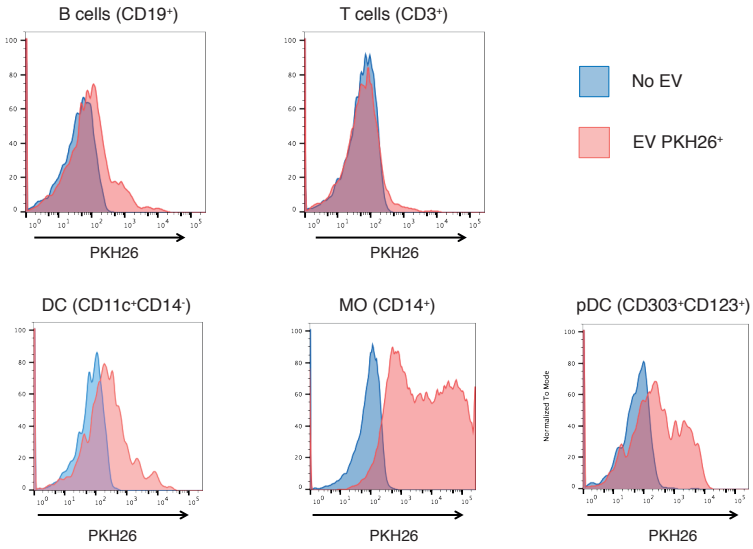

Fig. 7. Identification of target cells of EVs released by EBV-infected cells
EVs stained with the PKH26 dye were incubated with isolated PBMCs for 4 hours (using a ratio 1:1000 cells: EVs). After 4 hours, cells were extensively washed and stained with antibodies specific for cellular markers of interest. Stained cells were analyzed by flow cytometry for the PKH26 signal in the PE gate. One representative experiment of two is shown.

7. Conclusive Remarks

Our data show that the expression of viral miRNAs has a tremendous consequence on the biology of EBV-infected cell. We demonstrated that EBV's miRNAs are essential for the virus to establish its latent phase and for the infected cells to survive. Viral miRNAs promote the cell cycle of infected cells, repress apoptosis and drastically reduce the immunogenicity of the infected cells. The impact of EBV's miRNAs is unconventionally strong and global, since miRNAs usually only fine-tune the expression of their target genes. EBV expresses 44 different miRNAs and our contribution to the field showed that many co-act on the same mRNA transcript causing a considerable reduction of the target expression levels. The downregulation of IL-12 exemplifies this concept the best.

This is the first report uncovering that miRNAs of viral origin play such a key functional role for the virus, but it is likely that other viruses use similar mechanisms. This finding will pave the way for future studies in this direction. Understanding the biology of viruses will facilitate to find ways of fighting pathogen-associated diseases.

ABBREVIATIONS

AGO	argonaute
APC	antigen presenting cell
BART	Bam HI-A region rightward transcript
BCR	B cell receptor
BHRF1	Bam HI fragment H rightward open reading frame 1
BHV	bovine herpes virus
BL	Burkitt's lymphoma
CD	cluster of differentiation
CTL	cytotoxic T cell
CPX	cowpox virus
CR	complement Receptor
CTSB	cathepsin B
DC	dendritic cell
DNA	deoxyribonucleic acid
EA-D	EBV early antigen
EBER	Epstein–Barr virus-encoded small RNAs
EBNA	Epstein Barr Nuclear Antigen
EBV	Epstein Barr virus
EHV	equine herpes virus
ELISA	enzyme-linked immunosorbent assay
IFN	interferon
ERAP1	endoplasmatic reticulum aminopeptidase 1
EV	extracellular vesicles
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony stimulating factor
GP	glycoprotein
HCMV	human cytomegalovirus
HHV-4	human herpesvirus 4
HL	Hodgkin lymphoma
HLA	human leukocyte antigen
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule 1
IFI30	interferon, gamma-inducible protein 30
Ig	immunoglobulin
IL	interleukin
IM	infectious mononucleosis
IP	immunoprecipitation
KHSV	Kaposi's sarcoma-associated herpesvirus
KO	knockout
LCL	lymphoblastoid cell line
LFA	lymphocyte function-associated antigen
LGMN	legumain
LMP	latent membrane protein
MFI	median fluorescent intensity
MHC	major histocompatibility complex
MIC	MHC class I polypeptide-related sequence

miR/miRNA	microRNA
MO	monocyte
moDC	monocytes derived DC
MOI	multiplicity of infection
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
MVB	multivescicular bodies
NK	natural killer
NKG2D	natural killer group 2D
NTA	nanoparticles tracking analysis
OD	optical density
PCR	polymerase chain reaction
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
pDC	plasmacytoid DC
PE	phycoerythrin
PTLD	post-transplant lymphoproliferative disease
qPCR	quantitative PCR
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
RPMI	Roswell park memorial institute
RT-PCR	real time PCR
WB	western blot
SD	standard deviation
Seq	sequencing
snoRNA	small nuclear RNA
SV40	simian virus 40
TAP	transporter associated with antigen processing
TCR	T cell receptor
Th	T helper cells
UTR	untranslated region
VCA	viral capsid antigen

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Appendix

Contributions

Tagawa Takanobu (Research Unit Gene Vectors, Helmholtz Zentrum München) designed Figure 2A and performed experiments shown in Figure 2C.

Liridona Maliqi (Research Unit Gene Vectors, Helmholtz Zentrum München) designed Figure 4A and performed experiments shown in Figures 4B and 4C.

Figures 2B, 2C are already published in Publication 1.

Figures 1C, 2D, 2E, 3B, 3D, 3E, 5A, 5B are already published in Publication 2.

I personally performed all remaining experiments not listed on this page.