Metabolic stress is a barrier to Epstein–Barr virus-mediated B-cell immortalization

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EBV–Barr virus (EBV) is an oncogenic herpesvirus that has been causally linked to the development of B-cell and epithelial malignancies. Early after infection, EBV induces a transient period of hyperproliferation that is suppressed by the activation of the DNA damage response and a G1/S-phase growth arrest. This growth arrest prevents long-term outgrowth of the majority of infected cells. We developed a method to isolate and characterize infected cells that arrest after this early burst of proliferation and integrated gene expression and metabolic profiling to gain a better understanding of the pathways that attenuate immortalization. We found that the arrested cells have a reduced level of mitochondrial respiration and a decrease in the expression of genes involved in the TCA cycle and oxidative phosphorylation. Indeed, the growth arrest in early infected cells could be rescued by supplementing the TCA cycle. Arrested cells were characterized by an increase in the expression of p53 pathway gene targets, including sestrins leading to activation of AMPK, a reduction in mTOR signaling, and, consequently, elevated autophagy that was important for cell survival. Autophagy was also critical to maintain early hyperproliferation during metabolic stress. Finally, in assessing the metabolic changes from early infection to long-term outgrowth, we found concomitant increases in glucose import and surface glucose transporter 1 (GLUT1) levels, leading to elevated glycolysis, oxidative phosphorylation, and suppression of basal autophagy. Our study demonstrates that oncogene-induced senescence triggered by a combination of metabolic and genotoxic stress acts as an intrinsic barrier to EBV-mediated transformation.

Epstein–Barr virus | oncogene-induced senescence | autophagy | B cell | metabolism

Epstein–Barr virus (EBV) is a gamma herpesvirus that establishes a lifelong, latent infection in >90% of adults worldwide. EBV is associated with a number of malignancies, including African endemic Burkitt’s lymphoma, posttransplant lymphoproliferative disease, nasopharyngeal carcinoma (NPC), and HIV-associated lymphomas (1). These malignancies primarily develop in immunocompromised patients, pointing to the critical role that the immune system plays in controlling infection. However, it has recently become appreciated that additional intrinsic responses limit the ability of EBV to transform cells.

In vitro stimulation of B cells either through EBV infection or mitogen treatment results in a transient period of hyperproliferation reminiscent of a germinal center reaction. EBV elicits entry into the cell cycle through the EBV latency proteins, EBNA2 and EBNA-LP, which up-regulate the expression of progresion genes (2–4). This period of rapid proliferation leads to the activation of the DNA damage response (DDR), which can signal through p53 to induce either apoptosis or senescence (5). In contrast to mitogen-stimulated cells, EBV-infected cells are able to escape apoptosis and, instead, a subset undergo a G1/S-phase growth arrest (6). The specific cellular pathways that contribute to this growth arrest are poorly understood.

Oncogene-induced senescence (OIS) is a premature form of senescence in which cells undergo an irreversible growth arrest after chronic oncogene expression or the inactivation of tumor suppressors (7, 8). Current models suggest that the onset of OIS is a consequence of a persistent DDR resulting from replicative stress induced during oncogene-driven hyperproliferation (9–11). It is now appreciated that OIS plays an important role in suppressing tumorigenesis in a wide range of cell types (7). Additionally, studies suggest that OIS can suppress proliferation driven by the overexpression of viral proteins or after oncogenic virus infection (12, 13).

Increasing evidence suggests that there is a link between senescence and macroautophagy (hereafter referred to as autophagy) (7). Autophagy is a catabolic process in which organelles or proteins are targeted for lysosomal degradation and recycling (14, 15). Studies have demonstrated that autophagy promotes cell-cycle arrest and the production of senescence-associated interleukins (16). However, autophagy has also been linked to the progression of tumorigenesis by providing metabolic intermediates to fuel proliferation (14). Oncogene activation leads to a substantial increase in the need for ATP, biosynthetic intermediates, and reducing equivalents to maintain proliferation, thereby creating metabolic stress (17). Cancer cells have been shown to mitigate this stress by up-regulating the basal level of autophagy and by transitioning their metabolic profile from oxidative phosphorylation (OXPHOS) toward aerobic glycolysis, also known as the Warburg effect (18, 19).

The essential role of metabolism in driving virus replication has been hinted at since the 1950s and is now becoming fully appreciated with the advent of new technologies (20). It is now appreciated that many eukaryotic viruses alter host metabolism

Significance

Epstein–Barr virus (EBV) was the first human tumor virus discovered. Although nearly all adults are infected with EBV, very few go on to develop disease, for reasons that we are only beginning to understand. Infection with EBV induces a period of very rapid cell division, which requires an increased supply of metabolites, such as nucleotides, amino acids, and lipids. We found that EBV-infected cells that are unable to meet this increased metabolic demand are forced to stop proliferating and undergo a permanent growth arrest called senescence.


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to provide the energetic and biosynthetic resources necessary to drive virus replication and virion production. Less intuitive is the observation that viruses also alter host cellular metabolism during latent infection despite the lack of need for biosynthetic intermediates to produce viral progeny. Kaposi’s sarcoma-associated herpesvirus (KSHV) induces a Warburg effect during latent infection of endothelial cells, which is necessary for the survival of infected cells (21). A detailed metabolomics study of cells latently infected with KSHV further confirmed the increased production of glycolytic metabolites and also found an up-regulation of long-chain fatty acids (21). Additionally, glycolysis and fatty acid synthesis were found to be up-regulated in KSHV-associated primary effusion lymphoma compared with uninfected B cells (22). EBV latency is also associated with an altered metabolic state. EBV-infected nasopharyngeal carcinoma (NPC) cells exhibit high levels of glycolysis, an effect that can be recapitulated by the expression of EBV latency protein, latent infection membrane protein 1 (LMP1), alone (23). This increased level of glycolysis could be attributed to the increased surface expression of GLUT1 that was shown to be associated with LMP1-mediated NF-κB signaling in B cells (24). However, LMP1 expression is low during EBV-induced B-cell hyperproliferation—a period in which the cell should have the greatest need for increased metabolic flux.

In the present study, we have developed a method to identify and isolate EBV-infected primary human B cells that initially undergo a period of hyperproliferation and then arrest. We have used this approach to define the metabolic demands of hyperproliferation that drives the majority of EBV-infected cells into permanent growth arrest.

Results

EBV Infection of Primary B Cells Induces a Senescence-Like Growth Arrest. Early after EBV infection, B cells undergo a transient period of hyperproliferation that induces a G1/S-phase growth arrest in a subset of the population (5, 6). To functionally characterize this population, we have devised a protocol that allows us to identify and isolate cells that initially proliferate and then arrest. Peripheral blood mononuclear cells (PBMCs) were isolated from human blood and stained with CellTrace Violet followed by infection with the B95-8 strain of EBV. The cells were then stained with a second proliferation tracking dye, 6-carboxyfluorescein succinimidyl ester (CFSE), at day 4 after infection to coincide with the initial burst of hyperproliferation. The cells were then monitored over time, with those that were low for the Violet stain but high for the CFSE stain termed proliferated–arrested (PA) and those that were low for both stains designated proliferated–proliferated (PP) (Fig. 1A and B).

![Fig. 1. A subset of EBV-induced hyperproliferating cells exhibit characteristics of OIS. (A) Schematic representing the experimental protocol. (B) Histograms showing CD19+ B-cell division measured at 8 d after infection. (B, Left) Proliferation of CD19+ B cells was determined through the dilution of the CellTrace Violet stain. (B, Right) The cells labeled “Prolif” were further analyzed for dilution of the CFSE stain. Cells that dilute the CellTrace Violet stain, but not the CFSE stain, are considered arrested. (C) EBV-infected CD19+ B cells were sorted into PA and PP populations and recultured in fresh medium. Samples were counted by trypan blue exclusion every 48 h (n = 3). (D) The percent of BrdU incorporation in PA or PP cells at day 8 after infection. (E) IF of DAPI (blue) or 53BP1 (red) measured from sorted PA and PP cells (n = 3). (F) The expression level of CDKN2A and CDKN1A mRNA was measured from sorted PA and PP cells. Relative mRNA abundance was normalized to SETDB1. Data are represented as fold change relative to the PP cells (n = 3). (G) IF of DAPI (blue) or H3K9me3 (green) measured from sorted PA and PP cells (n = 3). (H) Representative TEM images of sorted PA or PP cells (n = 2). (M magnification: PA, 3,300×; PP, 4,400×.) (Scale bars: 1 μm.) Insets show heterochromatic DNA. (Scale bar: 0.5 μm.) Error bars represent SEM. (I, Left) Representative immunoblot of sorted PA and PP cells or LCLs stained with the indicated antibody (n = 3). (I, Right) Quantitation of immunoblot normalized to MAGOH loading control (n = 3). Error bars represent SEM. *P < 0.05; **P < 0.01; ***P < 0.001 as determined by a paired t test. n, the number of independent donors tested.]
Precursor cohort analysis (25) of the double-stained cells on day 8 after infection demonstrated that the PA cells had a mean division number of 2, similar to the day 4 cell population and in contrast to the PP cells, which had a mean division number of 4 (Fig. S1A). Additionally, the double-stain experiment allows us to determine the percentage of cells that arrest after each initial division. The majority of the day 8 cells that divided three times or less, population doublings 1–3 (PD1–3), arrested (PA), whereas the cells that had divided more than four times were predominately in the PP population (Fig. S1B). Of note, the PD1–3 population was previously found to have elevated markers of the DDR, which is known to attenuate EBV-mediated B-cell transformation (5).

The observed growth arrest could be a transient quiescence or senescence. To begin to address these possibilities, we sorted PA cells from three independent donors to purity and monitored their growth for 12 d. The number of PA cells remained constant with no new proliferation or cell death as determined by trypan blue exclusion. In contrast, the PP cells continued to divide and ultimately transformed into lymphoblastoid cell lines (LCLs) (Fig. 1C). Additionally, the PA cells had a significant decrease in the expression of the proliferation marker MKI67 (Fig. S1C), as well as decreased BrdU incorporation, further confirming the growth arrest (Fig. 1D). Senescent cells exhibit a number of distinctive characteristics in addition to growth arrest that allow for their identification in vitro (7). Unscheduled oncogene activation, resulting in DNA damage, leads to the activation of the DDR and the downstream p53-p21 and Rb cascade, which can mediate the senescence growth arrest (7). The PA cells had a significant increase in the expression of the DDR marker 53BP1 (Fig. 1E) and the tumor suppressors, CDKN1A (p21) and CDKN2A (p16) (Fig. 1F). Senescent cells are also characterized by altered chromatin structure known as senescence-associated heterochromatic foci, which are enriched for trimethylated lysine 9 of histone H3 (H3K9me3) (7). Immunofluorescence (IF) analysis demonstrated that the PA cells have a significant increase in H3K9me3 staining relative to the PP cells (Fig. 1G), as well as an increase in overall heterochromatic DNA, as evidenced by transmission electron microscopy (TEM) (Fig. 1H and Fig. S1D).

EBV promotes and maintains B-cell proliferation through the concerted action of the EBV latency proteins. EBNA2 and EBNA-LP induce the expression of proproliferative genes (2–4), whereas EBNA3C inhibits the expression of tumor suppressors such as p16 (26). We reasoned that the growth arrest could be due to the reduced expression of one of these proteins. However, we observed that there was a modest increase in the expression of EBNA-LP and only a slight decrease in EBNA3C in the PA cells relative to the PP population (Fig. 1I). This finding is consistent with our previous observations demonstrating that PD1–3 cells have a greater ratio of EBNA-LP to EBNA3C, reflective of an earlier state of viral latency-driven outgrowth (5) and is also consistent with the work of others indicating that proliferating B cells after EBV infection are uniformly EBNA2-positive (27).

### Transcriptomic Analysis of PA vs. PP Indicates Heightened p53 Pathway and Decreased Cell Cycle and DNA Replication.

To delineate the effector pathways that mediate this EBV-induced growth arrest, we performed gene expression analysis using an Affymetrix Human U133 2.0 Plus microarray with three independent donors sorted into PA or PP populations. The resulting hybridization data were robust multi-array average (RMA)-normalized, and a total of 158 genes were expressed higher in the PA cells relative to PP cells (>1.5-fold, P < 0.05), whereas the expression of 160 genes was reduced (>1.5-fold, P < 0.05) (Dataset S1).

The PA cells were depleted for mRNAs in pathways involved in cell-cycle progression and displayed lower levels of transcriptional targets of E2F indicative of G1/S cell-cycle arrest (Fig. 2A and Tables S1–S3). Additionally, the PA cells had elevated levels of p53 pathway transcriptional targets (Fig. 2A and B and Tables S1–S3), which is activated in response to cellular stress and regulates the expression of genes involved in processes such as cell-cycle progression and metabolism (28). Indeed, we confirmed that PA cells displayed activated p53 pathway including p53 phosphorylation on serine 15, accumulation of total p53, and induction of the downstream target p21 (Fig. 2C). In addition, two notable p53 target genes, SESN1 and SESN3, were increased in the PA cells (29). The sestrins are an evolutionarily conserved group of genes induced in response to genotoxic stress and nutrient deprivation, leading to the inhibition of mammalian target...
of rapamycin complex 1 (mTORC1) signaling (29–31). We confirmed increased expression of sestrins in the PA population by quantitative RT-PCR (qRT-PCR) and immunoblot analysis (Fig. 2C and D). These data suggest that EBV-induced hyperproliferation drives a subset of EBV-infected B cells into a state of metabolic or genotoxic stress that is then sensed by pathways that relay signals through the sestrins to induce growth arrest.

**PA Cells Exhibit Reduced Activation of the mTORC1 Pathway and Inefficient Autophagic Flux.** The sestrins inhibit mTOR signaling through activation of the energy sensing protein AMP-activated protein kinase (AMPK) (29, 31). Suppression of mTOR signaling leads to a reduction in energy-consuming pathways, such as protein synthesis, and induces catabolic processes, such as autophagy (32). Consistently, we found that the PA cells had increased activation of AMPK and reduced activation of mTOR pathway components relative to the PP cells (Fig. 3A).

A consequence of decreased mTORC1 activation is the induction of autophagy, which has been linked to the onset of cellular senescence (16). We therefore assayed for markers of autophagy in our PA and PP cells. We observed an increase in the levels of the autophagy marker LC3-II in the PA cells relative to the PP population (Fig. 3A). We also observed an increase in the presence of autophagosomes and phagolysosomes in the PA cells relative to both the PP cells and LCLs as detected by TEM (Fig. 3B and Fig. S2A). Additionally, there was an increase in the colocalization of LC3 with lysosomes in the PA relative to the PP cells (Fig. 3C and Fig. S2B). LCLs exhibited a further reduction in colocalized LC3 with lysosomes relative to all populations (Fig. 3C). LC3-II is subject to autophagic degradation, and its accumulation in the PA cells could be the result of enhanced autophagy or reduced degradation due to a blockage in autophagic flux. To differentiate between these possibilities, we treated sorted PA and PP cells with bafilomycin A, which inhibits lysosomal acidification and autophagic degradation. We observed that there was only a modest increase in LC3-II staining in the PA cells after bafilomycin A treatment, in contrast to the PP cells, which exhibited a substantial increase in the levels of LC3-II (Fig. 3D). These data, combined with the increased colocalization of LC3 with lysosomes and increased number of autophagosomes in the PA cells, indicates that the PA cells have a blockage in autophagic flux that prevents them from efficiently degrading cellular material to drive proliferation.

We next wanted to elucidate the functional role of autophagy during hyperproliferation. We treated EBV-infected, CD19+ B cells for 48 h with the autophagy inhibitor 3-methyladenine (3-MA) and observed a substantial decrease in proliferation at 8 d after infection, with treated cells displaying a CellTrace Violet profile reminiscent of cells at the time of treatment (Fig. 3E). We also observed a substantial increase in apoptosis specifically in PA cells relative to both LCLs and the PP population (Fig. 3F). Conversely, treatment with the mTOR inhibitor rapamycin promoted autophagy as determined by an increase in LC3 staining and increased the percentage of arrested B cells (Fig. S2C and D). Overall, these data demonstrate that EBV-infected, hyperproliferating cells need a balanced level of autophagy to promote the biosynthetic intermediates necessary for cell growth and proliferation.

**Metabolic Analysis Reveals Decreased Mitochondrial Respiration in PA Cells.** Autophagy is often triggered as a consequence of nutrient deprivation caused by a cellular metabolic imbalance (33). We therefore wanted to determine whether there was an altered metabolic state between the hyperproliferating cells (PA and PP) and other B-cell populations. Our gene expression analysis indicated that the PA cells had reduced levels of mRNAs in the canonical TCA cycle and respiratory electron transport pathways, as well as transcriptional targets of nuclear respiratory

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**Fig. 3.** The PA cells exhibit suppression of the mTOR pathway and increased autophagy. EBV-infected B cells were stained as described in Fig. 1A and analyzed at day 8 after infection. (A) Representative immunoblot of sorted PA and PP cells stained with the indicated antibody (n = 3). Quantitation was done on three independent donors and normalized to actin. (B, Upper) Representative TEM image of sorted PA and PP cells as well as LCLs. Arrows indicate autolysosomes (n = 2). (Magnification: PA, 7,100×; PP, 8,800×; LCL, 4,400×.) (Scale bars: 2 μm; Inset, 0.5 μm.) (B, Lower) Quantitation of TEM data. The graph represents the percent of cells with greater than two autophagic structures. (C, Left) Imagestream analysis of PA, PP, and LCLs that were stained with Lysotracker and anti-LC3 (n = 3). (C, Right) Quantitation of cells showing high colocalization of Lysotracker with LC3 in double-positive cells. The data are represented as a measure of bright detail similarity as determined by IDEAS software (Version 3.0). (D, Left) Representative immunoblot of sorted PA and PP cells that were treated with Bafilomycin A or mock treated. (D, Right) Quantitation of LC3-II staining after normalization to the actin loading control (n = 3). (E) Histograms showing CD19+ B-cell division measured at 8 d after infection after treatment with 5 mM 3-MA for 48 h. (F) Percentage of Annexin positive PA or PP cells after treatment as in E. Error bars represent SEM. ***P < 0.01; ****P < 0.001 as determined by a paired t test.
factor 1 (NRF1), which activates the expression of genes involved in mitochondrial biogenesis and OXPHOS (Fig. 4A). RT-PCR analysis confirmed that components of complex I of the electron transport chain and TCA cycle enzymes were decreased in the PA cells (Fig. 4B).

EBV infection induces B cells to undergo a period of rapid proliferation combined with a concomitant increase in biomass, processes that require both energy and biosynthetic intermediates. The decreased expression of enzymes important for mitochondrial respiration and the TCA cycle could lead to a metabolic imbalance promoting autophagy and senescence. To look for metabolic changes that occur in B cells before and after primary B-cell infection with EBV, we used the Seahorse XF, which simultaneously measures the basal extracellular acidification rate (ECAR), a marker of glycolysis, and the oxygen consumption rate (OCR), an indicator of OXPHOS.

We found that the PA cells are metabolically distinct from the other B-cell populations. Although the PA cells are similar to PP cells in both the basal level and rate of glycolysis (ECAR) (Fig. 4C and Fig. S3A), they have a significantly lower OCR than PP cells, a level that is only slightly higher than that observed in resting B cells (Fig. 4D). Furthermore, the ratio of OCR to ECAR indicates that the PA cells are more heavily reliant on glycolysis to meet their energy needs (Fig. 4E). We also observed that there was a substantial increase in both glycolysis and OXPHOS as cells transition from the hyperproliferating state to transformed LCLs (Fig. 4C–E).

The lower basal level of mitochondrial respiration combined with the lower expression of genes involved in the TCA cycle and the electron transport chain suggests that the PA cells may have a reduced capacity to undergo mitochondrial respiration. To determine whether there was a difference in the potential maximal level of respiration, we uncoupled the electron transport chain from OXPHOS using carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP), which dissipates the proton gradient across the mitochondrial inner membrane. FCCP causes the OCR to increase to the maximum level supported by the electron transport chain and substrate supply. We found that there is a significant decrease in the maximum level of OCR in PA cells relative to the PP cells (Fig. 4F). A comparison of the basal level of OCR relative to the maximal level can be used to calculate the spare respiratory capacity of the cells, which is defined as the amount of additional energy that the cell can make in times of stress. There is only a slight difference between the basal and maximal OCR in both the PA and PP cells (Fig. 4D and F), leading to a significantly lower spare respiratory capacity relative to resting B cells and LCLs (Fig. 4G). These data suggest that the hyperproliferating cells have an overall reduced capacity to mitigate metabolic stress compared with LCLs and that the subset that arrest have a significant reduction in mitochondrial respiration.

**Metabolic Stress and Genotoxic Stress Contribute to the Suppression of Early EBV-Induced B-Cell Proliferation.** We next sought to functionally characterize this metabolic imbalance observed during early EBV infection. We found that early hyperproliferating cells were more sensitive to the nonhydrolyzable glucose analog, 2-DG, relative to LCLs (Fig. 5A). This result was not due to increased glucose import or glucose transporter expression, but rather a reduction in glycolysis (Fig. S3 B and C). As a corollary, we would also expect that increasing the ability of early proliferating cells to promote OXPHOS would stimulate early B-cell proliferation, while not affecting LCLs. Indeed, we found that supplementation of medium with a cell-permeable form of fumarate, dimethyl fumarate (DMF), significantly increased the number of cells that hyperproliferate, but had no effect on uninfected B cells or LCL proliferation (Fig. 5B and C). Additionally, we found that DMF treatment reduced the accumulation of LC3 with lysosomes (Fig. 5D).

We previously demonstrated that the DDR was a major suppressor of EBV-infected B-cell hyperproliferation and that inhibition of the Chk2 kinase would increase the number of...
and 2-DG preferentially inhibits and DMF increases early EBV-infected B-cell proliferation in a manner distinct from Chk2i treatment. (B) Representative histogram showing CD19+ B cells proliferation at day 8 after infection. The cells were treated with either DMSO or 6 μM DMF at the time of infection. (C) The number of proliferating uninfected, CD19+ B cells (B cell); EBV-infected, CD19+ B cells that diluted CTV (Prolif) or LCLs was determined by FACS and graphed as a percentage of the DMSO-treated control. Prolif cells were treated with DMSO or 6 μM DMF at the time of infection and analyzed at day 8 after infection (n = 3). (D) EBV-infected B cells were treated with DMF as in B and analyzed for colocalization of LC3 with Lysotracker by Imagestream analysis. (E–G) The number of proliferating CD19+ B cells was determined for cells that were treated with DMSO, 6 μM DMF, 5 μM Chk2i, or a combination of Chk2i and DMF at the time of infection. The data were analyzed by FACS at day 4 (E), day 6 (F), or day 8 (G) after infection and are presented as a percentage of the DMSO-treated control. A minimum of three independent donors were analyzed for each time point. Error bars represent SEM. *P < 0.05; **P < 0.01 as determined by a paired t test. n, the number of independent donors tested.

Fig. 5. 2-DG preferentially inhibits and DMF increases early EBV-infected B-cell proliferation in a manner distinct from Chk2i treatment. (A) EBV-infected cells were treated with the indicated concentration of 2-DG at day 4 after infection, and the number of proliferating CD19+ B cells was determined by FACS on day 8. LCLs were treated for an identical time period (n = 3).
flux that could act as a barrier to the production of the biosynthetic intermediates necessary for proliferation.

The transient period of hyperproliferation requires a substantial increase in nucleotides, amino acids, and lipids for DNA replication and cell division. These needs can be met through a combination of metabolic reprogramming and an increase in the basal level of autophagy (17). We hypothesized that an inability to meet the metabolic demands of hyperproliferation could induce the EBV-infected B cells to undergo senescence. We demonstrate that the PA cells have a reduced ability to undergo OXPHOS and that complementing this defect with a soluble TCA intermediate could increase the number of cells that hyperproliferate early after infection. We believe that the reduced ability to undergo mitochondrial metabolism points to the cause rather than a consequence of OIS, because senescent cells typically favor the more efficient OXPHOS over glycolysis (41).

In the transition from early hyperproliferation to transformation, EBV strongly up-regulates glycolysis and OXPHOS and also suppresses autophagy. LMP1 is an EBV latency protein that has been linked to the regulation of glycolysis and autophagy in LCLs. LMP1 can induce autophagy through the unfolded protein response as a way of regulating its own expression (42, 43). Conversely, LMP1 reduces autophagy in LCLs by up-regulating glucose import via NF-κB signaling (24). Additionally, LMP1 has been shown to up-regulate glycolysis in NPC cells (23). In this study, we found that LCLs have increased expression of GLUT1 as well as increased glucose uptake relative to the hyperproliferating cells, which we have previously shown to express much lower levels of LMP1 (44). We believe that the strong increase in glycolysis and suppression of autophagy that we observed in LCLs relative to the hyperproliferating cells is directly linked to the delayed expression of LMP1.

Our study characterizes the intrinsic pathways that contribute to the arrest of B cells early after EBV infection. Rapid cellular proliferation, as is seen after EBV infection, leads to replicative stress such as stalled or collapsed replication forks (45). Additionally, proliferating cells need to increase the production of biosynthetic intermediates such as nucleotides, amino acids, and lipids to promote cell growth and proliferation (17). An insufficient supply of nucleotides can contribute to the generation of stalled replication forks, linking replicative stress to metabolic stress (46–48). EBV-infected cells meet these metabolic demands through an increase in both glycolysis and mitochondrial respiration. The cells that arrest early after infection have a reduced capacity to undergo OXPHOS, which would lead to a reduction in the intermediates required for faithful DNA replication as well as cell division. Therefore, the combination of genotoxic and metabolic stress activates the p53 tumor suppressor, resulting in sestrin/AMPK-mediated permanent growth arrest of early infected cells, severely limiting EBV-driven B-cell immortalization.

Materials and Methods

Viruses and Cells. B95-8 virus was produced from the B95-8 Z-HT cell line as described (49). Buffy coats were obtained from normal donors through the Viruses and Cells. Primary cells were EBV-infected and stained with CellTrace Violet and CFSE as described above. Proliferation was monitored as described below.

Cell Sorting. CD19+ B cells were sorted for the PA and PP populations based on the CellTrace Violet and CFSE profile by using either a Beckman Coulter Astrors or Beckman Coulter MoFlo XDP sorter.

Imagstream. CFSE and CellTrace Violet labeled and EBV-infected PBMCs or LCLs were stained with Lysotracker as described above. The cells were then washed and incubated with Alexa Fluor 568 goat anti-rabbit IgG (Life Technologies, no. A11036) for 30 min at 4 °C. Images were acquired using an ImageStream multispectral imaging flow cytometer (Amnis Corporation). Data were analyzed by using IDEAS software (Version 3.0; Amnis Corporation) as described (51). Single cells were first gated by size and CellTrace Violet staining to identify the proliferating population. Proliferation was monitored as described below.

Immunofluorescence. Samples were pelleted, resuspended in 25 μL of PBS, spread on a microscope slide, and dried at 37 °C for 15 min. Cells were fixed in 4% (vol/vol) paraformaldehyde for 15 min at 4 °C, washed in PBS, permeabilized in PBS containing 0.5% Triton X-100 for 10 min and blocked in PBS with 0.2% Triton X-100 containing 5% (vol/vol) normal goat serum for 1 h. Primary antibodies were incubated overnight at 4 °C followed by secondary antibody incubation with Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, no. A11034) for 2 h. Slides were mounted in Vectashield (Vector Laboratories, H-1200) containing DAPI. All IF slides were visualized by using Zeiss 780 upright confocal microscope. The above IF method was modified for visualization of GLUT1. In the case of GLUT1 IF, the cells were not permeabilized.
Protein concentration was determined by BCA assay (Thermo, no. 23225) according to the manufacturer’s protocol. Samples were separated on a 4–12% Bis-Tris gel (NuPAGE, NP no. 0322) run in Mops-SDS running buffer (NuPAGE, NP no. 0001) followed by transfer to PVDF membrane. The PVDF membrane was blocked for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C before washing and staining with a secondary anti-rabbit horse radish peroxidase (HRP)-conjugated antibody (Sigma-Aldrich, no. A0545). Quantification was performed by using Gene Tools software with normalization to β-actin after imaging using the G-box gel imaging system.

RNA isolation and qRT-PCR. Total RNA was isolated by using the RNeasy kit (Qiagen, no. 74106) and reverse-transcribed by using the High Capacity cDNA Reverse Transcription kit (Life Technologies, no. 4368814) according to the manufacturer’s instructions. Relative mRNA abundance was measured by using a SYBR green-based real-time PCR assay with 5 ng of cDNA per reaction. All primers were used at a concentration of 1 μM per reaction. qRT-PCR was carried out by using the Step One Plus Real Time PCR light-cycler (Applied Biosystems), and data were analyzed by using the supplied Step One software. All expression levels were first normalized to SETDB1 as a control and then to PPI. All primers were purchased from Sigma-Aldrich, with the exception of SETDB1, which was purchased from IDT. Primers used in this study are shown in Table 55.

Microarray Analysis. Total mRNA was isolated from sorted PA and PP cells by using an RNeasy kit (Qiagen, no. 74106). The RNA was processed by using an Ambion MessageAmp Premier Package (Life Technologies, no. AM1792) and hybridized to a Human Genome U133 Plus 2.0 Chip (Affymetrix, no. 900466) by the Duke Center for Genomic and Computational Biology Microarray Core. The resultant CEL files were RMA-normalized (Partek), and the data were analyzed with GenePattern (53) and GSEA (Version 2; ref. 54).

Electron Microscopy. B cells were pelleted and washed in serum-free RPMI medium, and 2% (vol/vol) glutaraldehyde was overlaid onto the undisturbed cells. The pellets were scraped into 1- to 1.5-mm piles on paraffin and enameled in 1% molten agar. The agar-embedded pellets were washed three times with 0.1 M phosphate buffer and further fixed and stained in 1% osmium tetroxide in phosphate buffer for 30 min by microwave processing or 1 h in 0.1 M phosphate buffer and further fixed and stained in 1% osmium tetroxide in phosphate buffer for 30 min by microwave processing or 1 h at 4 °C.

Glucose Uptake. Cells were starved in glucose-free medium for 1 h before the addition of 25 μM 2-N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino-2-deoxyglucose (2-NBDG) (Thermo, N13195) for 5–120 min. The mean fluorescence intensity was determined by flow cytometry and fit to linear regression model. The slope of the line was defined as the rate of glucose uptake.

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