Heliyon

Calcium mobilization is responsible for Thapsigargin induced Epstein Barr virus lytic reactivation in *in vitro* immortalized lymphoblastoid cell lines

Aki Hoji [1], **Susie Xu** [1], **Holly Bilben** [1], **David T. Rowe**[*],[1]

University of Pittsburgh, The Graduate School of Public Health, Department of Infectious Diseases and Microbiology, 130 Desoto St., Pittsburgh, PA, 15261, USA

[*] Corresponding author.

E-mail address: rowe1@pitt.edu (D.T. Rowe).

[1] These authors equally contributed to this work.

Abstract

The latent state is a critical component of all herpesvirus infections, and its regulation remains one of the most active areas of Epstein-Barr Virus (EBV) research. In particular, identifying environmental factors that trigger EBV reactivation into a virus-productive state has become a central goal in EBV latency research. Recently, a category of chemicals known as inducers of the endoplasmic reticulum unfolded protein response (UPR) have been shown to trigger EBV lytic reactivation in various established EBV-associated lymphoma cell lines. This has led to the recent belief that UPR is a universal cellular signaling pathway that directly triggers EBV lytic reactivation irrespective of cell type. We tested the potency of several widely used UPR inducers for EBV lytic reactivation on virus-immortalized primary lymphoblastoid cell lines (LCLs) *in vitro*. We found that, with the exception of Thapsigargin (Tg), UPR inducers did not trigger significant increases in BZLF1 transcripts or changes in the numbers of EBV genomic copies/cell in our panel of primary LCLs. Further

investigation revealed that induction of lytic reactivation by Tg appeared to be due to its ability to trigger intracellular Ca^{2+} mobilization rather than its ability to induce UPR, based on our observations in which UPR induction alone was not sufficient to trigger the EBV lytic cycle in our LCLs. EBV immortalized LCLs have rarely been included in the majority of the lytic reactivation studies yet the characteristics of latent infection in LCLs should resemble those of proliferating B cells in clinically encountered lymphoproliferative diseases. Based on these observations, we propose an alternative mechanism of action for Tg in triggering EBV lytic reactivation in LCLs, and suggest that the proposed use of any chemical inducers of UPR for a purpose of oncolytic/lytic induction therapy needs to be fully evaluated pre-clinically in a panel of LCLs.

Keyword: Virology

1. Introduction

EBV is an oncogenic gamma-herpes virus. Infection of humans is highly prevalent, reaching nearly 90% of entire human population [1]. EBV establishes latent infection in B cells soon after resolution of primary infection and although it is well understood that some forms of EBV latency are maintained by the action of a set of latency associated viral proteins, the biological mechanism(s) underlying switch (es) from latent to lytic infection still remains an area of intense investigation. This is in part due to lack of a clear understanding of how environmental conditions and endogenous cellular factors interact with the viral latency program in regulating expression of viral regulatory switch genes such as BZLF1 and BRLF1.

Latent EBV can be triggered to undergo lytic reactivation *in vitro* by a number of chemicals known to potently trigger various cellular signaling pathways. These inducers have been used to model mechanisms by which EBV latency switches to lytic virus production *in vivo* and have become the theoretical basis for proposals of experimental therapeutic intervention for EBV associated diseases. Such a therapeutic approach, known as 'lytic induction therapy' or 'oncolytic therapy' aims to selectively eliminate EBV positive tumor cells by pharmacological induction of lytic reactivation and subsequent pyroptosis. Success of this therapeutic approach critically depends on the level of lytic induction achieved. While several classes of chemicals have been shown to induce robust EBV lytic reactivation in EBV positive cell lines such as Akata-Burkitt's lymphoma (BL) *in vitro*, lytic induction by a particular inducer often appears to be cell type specific and just as often only a small fraction of latently infected cells appear to respond to the chemical inducer at physiologically relevant concentrations [2, 3, 4]. Moreover, very few chemical inducers are analyzed on EBV immortalized large primary

lymphoblastoid B cell lines which are arguably the most relevant target for lytic induction therapy. The potential for significant differences in cellular responses to chemical inducers between model cell lines and primary LCLs begs further investigation.

Among these chemical lytic inducers, a class of molecules known to induce unfolded protein responses (UPR), including Thapsigargin (Tg), Bortesomib (BZ) and Tunicamycin (Tm) has been extensively characterized for lytic induction therapy. Tg is an alkaloid extracted from a plant, Thapsia garganica and is a noncompetitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and blocking SERCA impede the uptake of cytosolic Ca^{2+} by ER, which results in rapid depletion of ER luminal Ca^{2+} storage and concurrent rise in cytosolic Ca^{2+} levels [5, 6]. Insufficient Ca^{2+} level in the ER lumen causes the accumulation of misfolded proteins, triggering ER stress and leading ultimately to UPR [7]. BZ is a proteasome inhibitor which blocks the proteosomal digestion of unwanted misfolded protein, accumulation of which triggers ER stress and subsequently leads to UPR. Alternatively, BZ treatment results in up-regulation of CCAAT/enhancer-binding proteinβ (C/EBPβ) which directly activates BZLF1 gene (the viral immediately early protein that initiates lytic reactivation), leading to lytic reactivation in Burkitt lymphoma lines [8]. Tumicamycin (Tm), an inhibitor of protein glycosylation causes ER stress through accumulation of unglycoslated proteins in ER-Golgi membrane network and has also been reported to induce EBV lytic reactivation [9]. Importantly, UPR is thought to be the natural cellular trigger of EBV lytic reactivation in human B cells [10] and in fact, when B cells undergo plasma cells differentiation, UPR is typically initiated as key UPR associated transcription factors are being expressed; one of which, a spliced variant of X-box binding protein-1 (XBP-1s) is known to directly activate transcription of BZLF1 [11]. As many of these chemical inducers have only been characterized *in vitro* by using model EBV tumor cell lines, their mechanisms of action need to be more broadly examined if they are to be proposed for the purpose of therapeutic application. Another important issue is that the perceived magnitude of the response to chemical inducers of lytic reactivation may largely depend on the type of cells, EBV latency program, and the assays used.

In the present study, we used a panel of LCLs to characterize EBV lytic reactivation by UPR inducers, and in particular Tg, in order to examine the mechanism by which these drugs induced lytic reactivation in LCLs. Overall, the results indicate that the triggering mechanism of EBV lytic reactivation in LCLs differs significantly from the model EBV-positive tumor-derived B cell line with respect to the involvement of the UPR. The ramifications of our findings are discussed in the context of EBV lytic reactivation and the potential of lytic induction therapy.

2. Materials and methods

2.1. Cell lines and chemicals

14 EBV (B95-8) transformed lymphoblostoid cell lines (LCLs) previously established [12] were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) or Iscove's Modified Dublbeccos's Media (IMDM) (Life Technologies) supplemented with 10% Fetal Calf Serum (Hyclone) and Glutamax (Life Technologies). Other cell line including EBV-negative and positive Aktata cell lines (a human Burkitt's lymphoma (BL)-derived cell line) [13] obtained from Dr. Shair with a permission from Dr. Hutt-Fletcher and Raji (a human EBV-positive BL cell line) [14] were also maintained in RPMI 1640 or IMDM with 10% FCS. EBV positive Akata cell line carries latent a recombinant Akata EBV EGFP [15]. We used following chemicals; Thapsigargin (Sigma, St. Louis, MO), Ionomycin (Sgima), Tunicamycin (Sigma), BTP2 (Santa Cruz Biotech, Dallas, TX), and Botezomib (Santa Cruz Biotech, San Diego, CA).

2.2. Induction of EBV lytic cycle and UPR

Induction of UPR and EBV lytic cycle by Tg and TM has been described preciously [9]. Briefly, $2-4 \times 10^5$ LCLs were treated with 500 nM Tg (unless otherwise indicated), 5 µg/ml TM for 6 hrs and then cells were washed with complete medium to remove Tg or TM and continued in the fresh medium until they were harvested at indicated time points. As for a flow cytometric analysis, $1-2 \times 10^6$ LCLs were incubated in phenol red free IMDM complete medium and treated with Tg as described and then cells were harvested for flow cytometric analysis.

2.3. Relative quantitation of EBV BZLF1 and endogenous gene expressions by quantitative Real Time PCR

A method for relative quantitation of EBV and endogenous gene expressions by quantitative Real Time PCR (qPCR) was previously described [12, 16]. Briefly, total RNA was extracted by the TRIzol (ThermoFisher/Life Technologies) and reverse-transcribed by using the High-Capacity Reverse Transctiption kit (Invitogen); cDNA was mixed with TaqMan Gene Expression Master Mix with TaqMan probe and primer sets (ThermoFisher/Life Technologies) for PCR targets, CHOP10 (Hs00358796_g1), and C/EBPβ (Hs00270923_m1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts from the known cell counts of IB4 cell line was measured by the Taqman probe primer set (Hs02758991_g1), and used to generate a qPCR standard. Relative abundance of target transcripts is displayed as equivalent of GAPDH or transcript copies per given numbers of IB4 cell line. For normalization of differential input cell numbers among samples, the number of GAPDH gene were used as preciously described [17].

2.4. Absolute quantitation of EBV genomic copy numbers by qPCR

A cell-associated EBV genomic copy number was determined by the previously published qPCR method [12]. A qPCR target was EBV BLLF1 gene encoding gp350 and was detected by forward 5′-GTATCCACCGCGGATGTCA-3′, reverse primer 5′-GGCCTTACTTTCTGTGCCGTT-3′, and probe 5′ FAM-TGGACTTGGTGTCACCGGTGATGC-TAMARA 3′ (IDT). Lysate of the Namalwa cell line was used to generate the PCR standard for absolute estimation of EBV copy and the number of GAPDH genes was used to normalize differential cell numbers among samples as described above.

2.5. Intracellular Ca^{2+} influx blocking

For Ca^{2+} mobilization inhibition experiments, cells in complete medium were first treated with 1uM BTP2 for 2 hours prior to Tg treatment. Cells were continued incubated in the presence of Tg for additional 3 hours, and then were washed and maintained in fresh medium until they were harvested at 3hours after the wash for CHOP10 (**C**/EBP **HO**mologous **P**rotein10/GADD153) transcript, 48 hrs for BZLF1 transcript, and day 6 for EBV copy measurements by qPCR. We also employed a calcium free RPMI 1640 medium (US Biological, Salem, MA) with 10% dialyzed FCS (Atlanta Biologicals. Flowery Branch, GA) to demonstrate the null extracellular $Ca2^+$ influx. Cells were maintained in calcium free medium 2 hours prior to Tg treatment and assays were performed as described.

2.6. Flow cytometric analysis

Cells were harvested at day 2, 3, and 4 after treatment with Tg, washed, counted, and labeled with Aqua, live dead discrimination dye (ThermoFisher/Life Technologies) for 30 min at 37 °C in serum free IMDM. After the incubation, 2×10^6 cells were washed with IMDM with 10% FCS and subsequently fixed and permeabilized by a FoxP3 staining kit (eBioscience, San Diego, CA) or BD Phosflow buffer II (BD biosciences, San Jose, CA). Cells were stained with pre-titrated amount of mouse monoclonal antibodies (mabs) specific for EBV BZLF1 (clone BZ1) and for EBV gB100 (clone 5B2) (Santa Cruz Biotechnology, San Diego, CA) for 40min in PBS supplemented with 10% fish gelatin (Biotium, Hayward, CA) and subsequently fixed in 1% paraformaldehyde (Polysciences, Warrington, PA). Stained samples were run on a BD Fortessa cytometer (Becton-Dickinson, San Jose, CA). Typically, over 1 million total events were collected, and subsequently analyzed and visualized by FlowJo v10 (Treestar, Portland, OR). The mabs against EBV proteins and isotope matching control antibodies were labeled indirectly with Zenon antibody labeling kits (ThermoFisher/Life Technologies), or directly with Mix-n-Stain (Biotium) according to the manufacturer's protocols.

The level of cytosolic Ca2+ was determined by using Ca2+ sensitive dye, Cal520^{TM}-AM (AAT Bioquest, Sunnyvale, CA). Briefly, approximately 2×10^6 cells were prelabeled with Cal520^{TM}-AM in IMDM with no phenol red (Thermo-Fisher/Life Technologies) for 30 min at 37 °C. After the incubation, cells were washed twice and resuspended in complete medium. Labeled cells cells were then run on BD FACS Aria II+ cytometer (Becton-Dickinson) for initial 3 minutes. Then, data acquisition was briefly paused to allow administration of chemicals directly into the tube. Immediately after the brief vortex, the tube was put back on the cytometer and data were acquired for additional 3 minutes. FlowJo v9 (TreeStar, Eugene, OR) was used to analyze and to visualize the cytometric data.

2.7. Statistical analysis

All the data were reported as a mean ± S.E.M unless otherwise noted. Statistical significance of two group means and multiple group means was determined by a nonparametric Mann-Whitney U test and ANOVA respectively. The least square method was used for linear regression analysis of correlative data. The data were considered statistically significant when p value was less than 0.05. Statistical analysis was performed by JMP Pro v11 (SAS, Cary, NC).

3. Results

1. **Among UPR inducers surveyed, only Thapsigargin triggered the robust lytic reactivation in LCLs.**

Studies of the chemical induction of the EBV lytic cycle often involve responses measured in well-defined tumor-derived cell lines. When studied at the individual cell level with anti-BZLF1 antibodies as opposed to the population level with RNA analyses, it has always been observed that, regardless of the nature of the chemical treatment, only a portion of the treated cells respond by entering the lytic cycle (Fig. 1A). For example, Akata-EBV cell cultures, which contain a sizable fraction of spontaneously virus productive cells, can be further induced by a variety of chemical inducers that either mimic B Cell Receptor signaling (TPA), or induce cellular unfolded protein responses (thapsigargan and tunicamycin). Of the latter, thapsigargan (Tg) appears to drive the largest number of cells into lytic virus production (Fig. 1A). We started our investigation into the potency of chemical UPR inducers in LCLs with an initial experiment done only with Tg and Tunicamycin (Tm) and Tg. It was then repeated as new inducers were added to the panel to try and find a stress inducer (other than Tg) that would trigger EBV reactivation. The final gallery shown in Fig. 1 has a total of 6 inducers (Thapsigargin, Tunicamycin (TM), Brefeldin A (BFA), Bortizomib (BZ), Cyclosporine (CSN), and Dithiothreitol
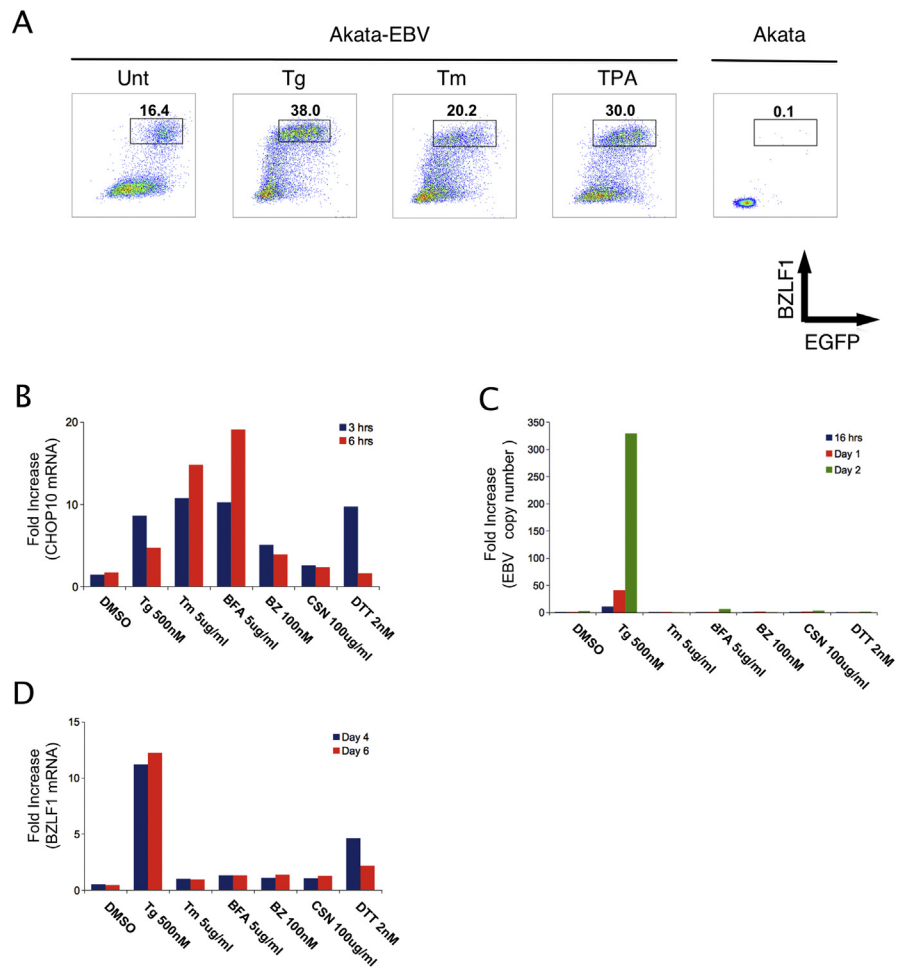
Fig. 1. Several UPR inducers triggered EBV lytic reactivation in Akata cells, while only Tg, triggered robust EBV lytic reactivation in LCLs. (A) Akata cells latently infected with EBV-GFP (Akata-EBV) were treated with Tg (500nM), BZ (100nM), TPA (20nM) or untreated (Uns) for 6 hours, cells were harvested 1 day post-Tg treatment, and stained for BZLF1 as described in the materials and methods. Plots show the pattern of BZLF1 staining (y-axis) vs EGFP (x-axis) as indicated. A rectangular box shown in the plot indicates the gate for BZLF1$^+$ EGFP$^+$ populations. Numbers above the gate box indicate percent positive events that fall in the corresponding gate. Untreated Akata cells (Akata) were also stained and used as a negative control. (B), (C) and (D) LCL clone3 was treated with Tg (500nM), Tm (5 μg/ml), BFA (5 μg/ml), BZ (100nM), CSN (100 μg/ml), DTT (2nM) or DMSO for 6 hours and cells were harvested at indicated time post treatment after which the CHOP10 (C/EBP Homologous Protein10/GADD153) (B), and the BZLF1 (D) message and cell associated EBV copy numbers (D) was measured. The relative copy of the CHOP10, BZLF1 message and cell associated EBV copy numbers at time 0 hr was used to generate the fold increase.

(DTT)), each had been tested at least 3 times and the results are representative several drugs with varying mechanisms of action, all of which have been shown to induce UPR and induce lytic cycle reactivation in tumor cell lines. The effects of the drugs was assessed by measuring changes in the levels of CHOP 10 (C/EBP Homologous Protein) mRNA which is an inducible ER response factor. All the drug treatments induced varying degrees of elevation in CHOP 10 RNA

expression levels indicative of a UPR activation (Fig. 1B). Increases in RNA expression levels of the BZLF1 immediate early transcriptional activator gene are the usual indicator of a switch from latent to lytic virus replication. Only Tg was observed to cause an increase in BZLF1 RNA measured at 48 hrs post-induction (Fig. 1C) and a subsequent increase in EBV DNA concentration 4−6 days later (Fig. 1D). We then constructed a detailed time course analysis of EBV lytic reactivation after treatment with Tg comparing the response to Tm treatment which is reported to induce UPR and EBV lytic reactivation in the study involving similar LCLs [9]. 6hr pulse-Tg treatment produced a higher cell-associated EBV DNA copy number (at 1800 ± 100 copies/cell) on day 4 post-treatment (Fig. 2A).

Tg treatment of LCL cell lines produced an increase in cell-associated EBV DNA copy numbers that peaked (at 1031 ± 217 copies/cell) after approximately 2−6 days (Fig. 2B) with varying degrees of fold lytic induction determined on day 4 and/or day 6 (3.51 ± 0.54 fold induction). In contrast, no increase in cell-associated EBV DNA copy number was detected after a Tm treatment (0.48 ± 0.06 fold induction), indicating that Tm treatment did not induce lytic reactivation in any of our 14 LCLs examined. As reported previously [12], we found a significant correlation (R^2 adjusted = 0.36, p = 0.00134, n = 14) between the number of cell-associated EBV genome before and after induction of lytic reactivation (Fig. 2C) and thus, magnitude of EBV lytic reactivation in LCLs appeared to be in part dependent on the pre-treatment load of latent EBV genomes on a per cell basis.

2. Thapsigargin and Tunicamycin induced similar kinetics and levels of CHOP10 and C/EBPβ mRNA.

We next compared the degree to which the two drugs induced the UPR response in LCLs over time, by examining the level of the CHOP10 gene, an early UPR responsive gene [18] following drug treatment. Time course studies of CHOP10 expression in LCL6 showed a rapid rise in the expression level that peaked approximately 3 hours post-treatment for both Tg and Tm and was followed by a similar rate of decline to baseline levels by 12 hrs. Levels of CHOP10 in Tm treated cells declined with slightly slower rate and plateaued at a slightly higher level (15 relative copies/cell) over the 72 hour observation period (Fig. 3A).

We also investigated expression of another gene normally activated during a UPR response, the beta subunit of CCAAT/enhancer-binding protein (C/EBPβ). C/EBPβ has recently been reported to be involved in EBV lytic reactivation through induction of UPR by BZ and Tg [8]. As with CHOP10, the rate and magnitude of C/EBPβ expression induced by Tg and Tm treatment (Fig. 3B) were similar except that Tm treatment resulted in a slow rise in C/EBPβ transcription level which continued until the last time point at 72 hr. EBV DNA levels did not change significantly after 6 days in Tm treated cells.
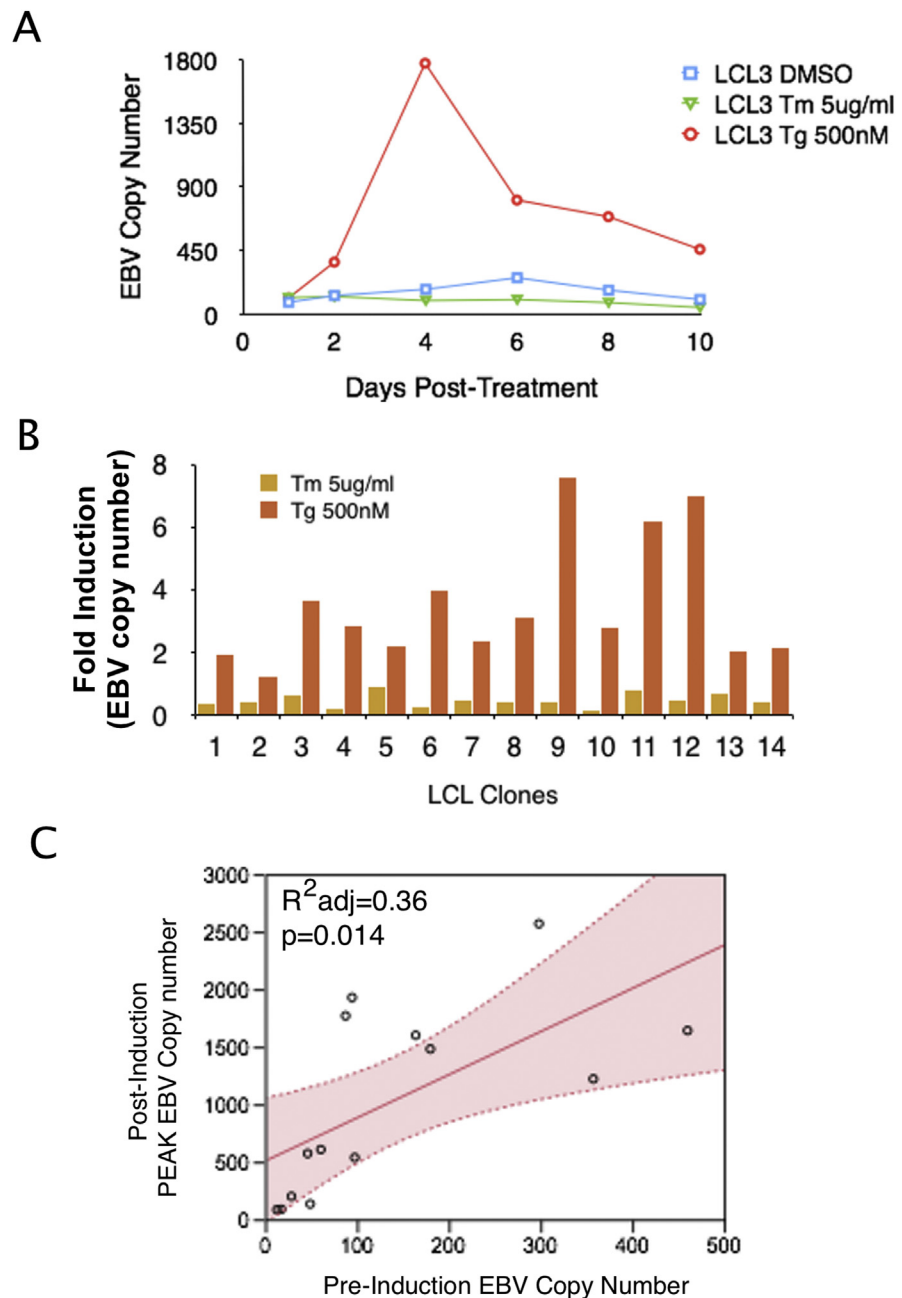
Fig. 2. On day 4 post Tg treatment, all the 14 LCL clones had the increased production of cell associated EBV while Tm induced did not. LCLs (n = 14) were treated with (A) Tg (500nM) and (B) Tm (5 μg/ml) and cell associated EBV copies were determined at indicated days post-treatment as described in Fig. 1 and in the materials and methods. (C) LCL clone3 was treated with Tg (500nM), Tm (5 μg/ml), or DMSO for 6 hours, washed and cell associated EBV copy number was measured at indicated time in post-treatment. This is a representative of time course for experiments done in 14 distinct LCL clones. (D) Maximum fold induction for all 14 cell lines was determined at peak EBV DNA accumulation in Tg and TM treated LCLs demonstrating a failure of Tm to induce EBV lytic reactivation. (D) Pre-induction EBV copy number was positively correlated with the level of peak EBV copy numbers post-Tg treatment. Pre-induction EBV copy number is equivalent to day 0 EBV copy numbers from DMSO treated LCLs. A mean regression correlation line is represented by a red line. The upper and lower 95% confident limits of the expected regression lines from the mean are represented by a red shaded area under dotted lines.
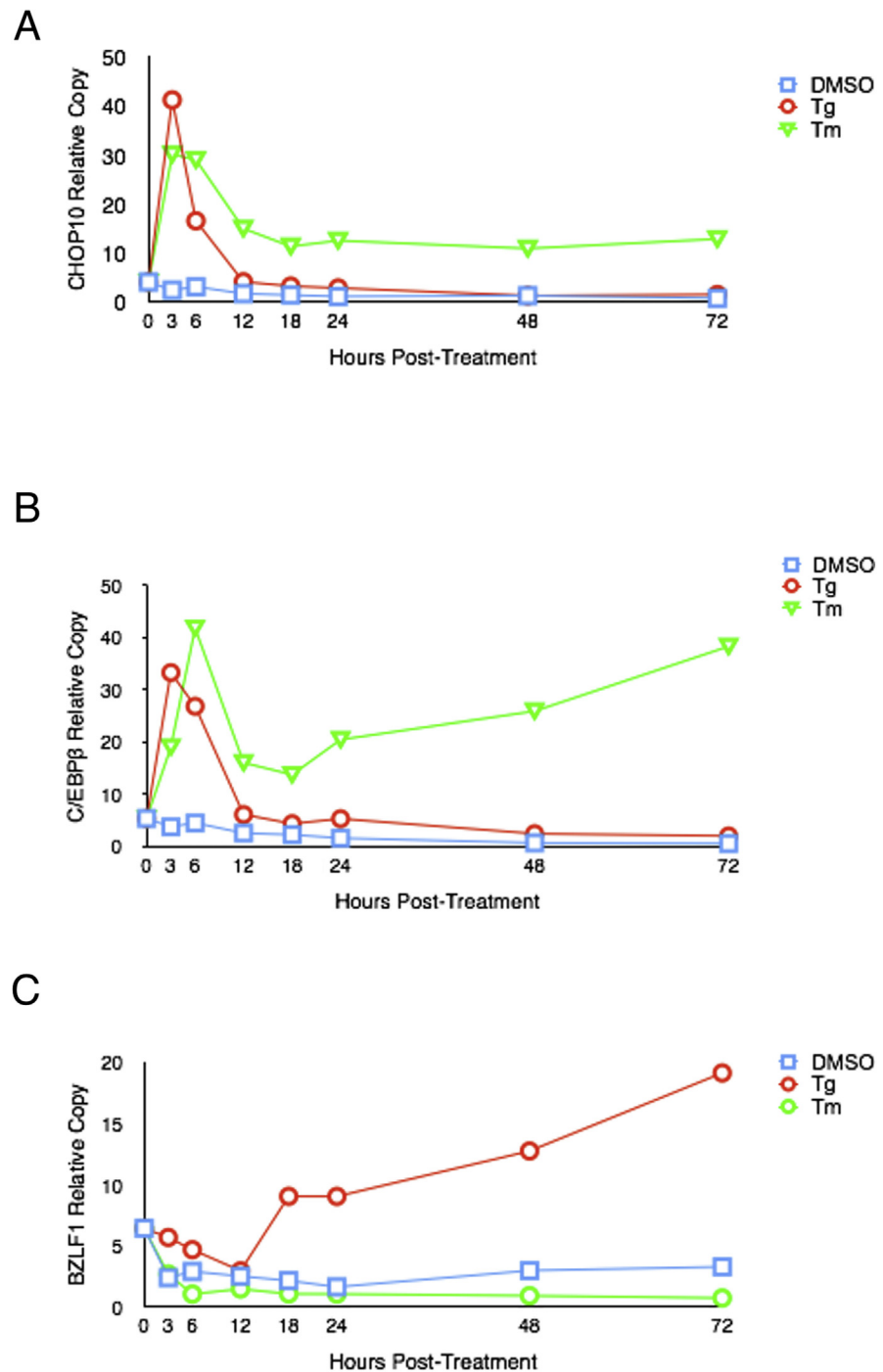
Fig. 3. Both Tg and Tm induced expressions of two UPR marker genes but only Tg induced expression of BZLF1. LCL 6 was treated with Tg (500nM), Tm (5 μg/ml), or DMSO for 6 hrs, washed and harvested at indicated time after which relative copies of (A) CHOP10, (B) C/EBPβ, and (C) BZLF1 were measured. Relative copy of the target message was determined by a GAPDH standard generated from a lysate of known counts of IB4 cell line.

Finally, we measured the level of transcriptional activation of the viral immediate early activator gene BZLF1. The number of BZLF1 transcripts began rising 12 hours post Tg treatment and increased over the 72 hour observation period, reaching approximately a 6-fold increase from the level at the 12 hour time point. There was no increase in the level of BZLF1 transcription over the course of 72 hours post Tm treatment (Fig. 3C), which is in agreement with the observation that no change in the viral DNA copy numbers had occurred after Tm treatment (Fig. 1D). Taken together, these results suggest that activation of the UPR response alone is not sufficiently to trigger lytic reactivation in LCLs. Furthermore, none of the other UPR inducers (BZ, BFA, CSN or DTT) induced virus production in LCLs (Fig. 1C) or activation of the BZLF1 gene (Fig. 1D) providing additional support for our notion that factors other than UPR induction are necessary for lytic reactivation by thapsigargan in LCLs.

3. Lytic reactivation in LCLs by Thapsigargin is triggered by intracellular Ca^{2+} mobilization

Our observations thus far had indicated that UPR was not fully responsible for induction of EBV lytic reactivation in LCLs by Tg. To explain our rather unexpected observation, we hypothesized that the rise in the cytosolic Ca^{2+} level rather than the UPR induction was responsible for the induction of EBV lytic reactivation by Tg in these LCLs. To directly test this hypothesis, we suppressed the rise in cytosolic Ca^{2+} level by using two different approaches: i) blocking plasma membrane Ca^{2+} channels with BTP2, an inhibitor of calcium release activated calcium channels (CRACs) and ii) applying the Tg treatment to cells suspended in a Ca^{2+} free medium. These conditions were chosen because they could exert potent suppression of the cytosolic Ca^{2+} raise without significantly altering the level of the UPR induced by Tg. As predicted, for two representative cell lines, BTP2 (1μM) and the use of Ca^{2+} free medium abrogated induction of BZLF1 transcription at Day 2 post-induction (Fig. 4A) and subsequent virus production by Day 6 (Fig. 4B). Moreover, both treatments resulted in slightly higher levels of expression of CHOP10 transcription than was observed with Tg alone (Fig. 4C). This suggests that the rise in the cytosolic Ca^{2+} levels triggered by Tg, but not the UPR response, was critical for the EBV lytic reactivation in LCLs.

To further characterize intracellular Ca^{2+} mobilization in LCLs by Tg alone or Tg with a BTP2, we measured the rate of cytosolic Ca^{2+} influx by flow cytometry. Tg (100nM) treatment resulted in a biphasic and relatively slow rate of Ca^{2+} cytoplasmic influx (Fig. 4E and H). As a positive control ionomycin (1μM) induced an expected rapid influx of Ca^{2+} into the cytosol that immediately saturated the maximum binding capacity of the Ca^{2+} detection dye (Cal520) (Fig. 4D and G). BTP2 treatment had no effect on the first phase of Tg-induced cytosolic Ca^{2+} influx (Fig. 4E and H). However, BTP2 treatment abolished the second longer lasting and higher level of Ca^{2+}
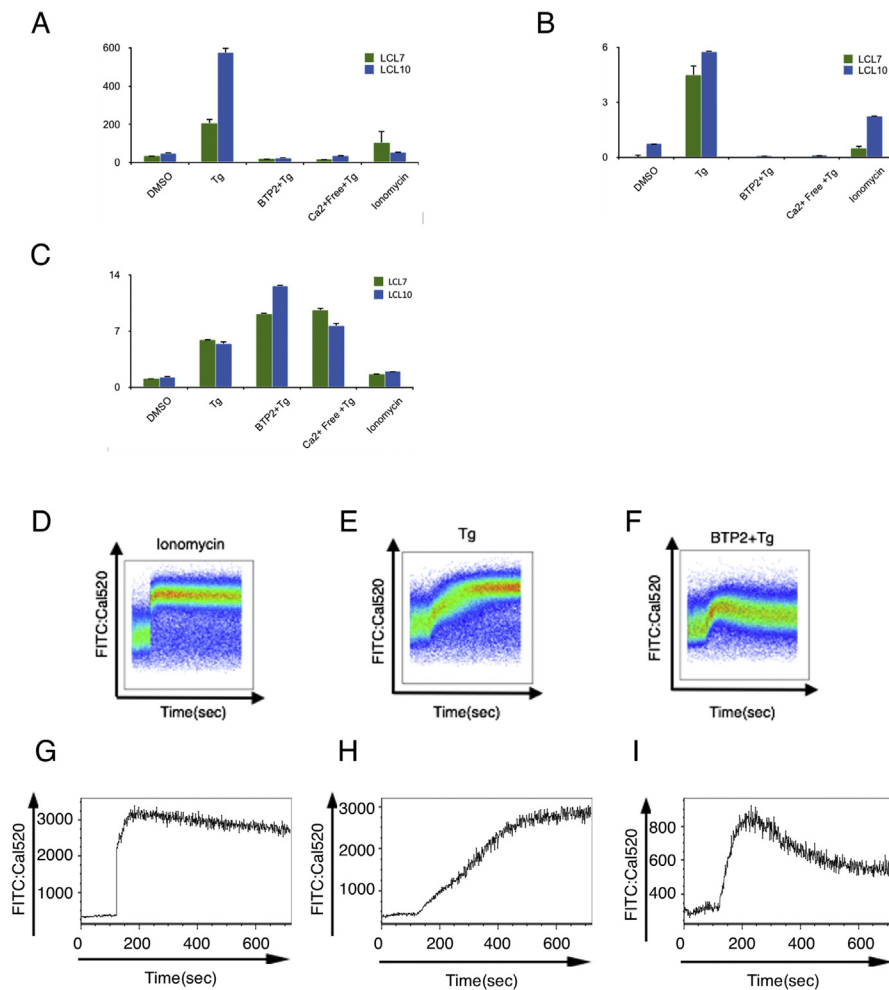
Fig. 4. Intracellular Ca2+ mobilization is required for EBV lytic reactivation. Two LCL lines (LCL 7 and 10) were treated with Tg (500nM), Tg + BTP2 (10nM), Tg + Ca2$^+$ free medium, Ionomycin (1μM), and DMSO and harvested at 6 days, 2 days and 3 hrs for determine EBV copy (A), BZLF1 transcript (B), and CHOP10 transcript (C) respectively. Cytoplasmic Ca2$^+$ influx was visualized by flow cytometry in the LCL preloaded with the Ca2$^+$ indicator dye, Cal520 and treated with Ionomycin (D), Tg (E) and Tg and BTP2 (F) as described. The panel D, E and F show acquisitions of live cytoplasmic Ca2$^+$ levels over the course of measurement (in sec.) while panels G, H and I show the median cytoplasmic Ca2$^+$ levels (Y-axis) of the panel D, E, and F over the duration of acquisition (X-axis).

influx, presumably by inhibiting CRAC, the known molecular target of BTP2 (Fig. 4F and I). Although the magnitude of lytic reactivation in LCLs treated by ionomycin was unexpectedly low (Fig. 4B), these results collectively support the hypothesis that the Tg induced mobilization of cytosolic Ca^{2+} rather than induction of a UPR is the most critical signaling event for inducing lytic reactivation in LCLs.

4. Analysis of EBV lytic reactivation at single cell level by flow cytometry

Based on the qPCR analysis above, it was clear that Tg induced the robust EBV lytic reactivation in our collection of LCLs. However, as pointed out by earlier studies

[2, 3, 4], the robust lytic reactivation (measured by qPCR) could be achieved by relatively a small number of induced cells having substantially high level of lytic reactivation while a majority of drug treated cells remain refractory to EBV lytic reactivation. To address this, we used flow cytometric detections of BZLF1 and gp110 protein and examined the expression of early and late proteins in Tg-treated LCL lines (n = 6) from 2 days to 4 days post Tg treatment (Fig. 5A).
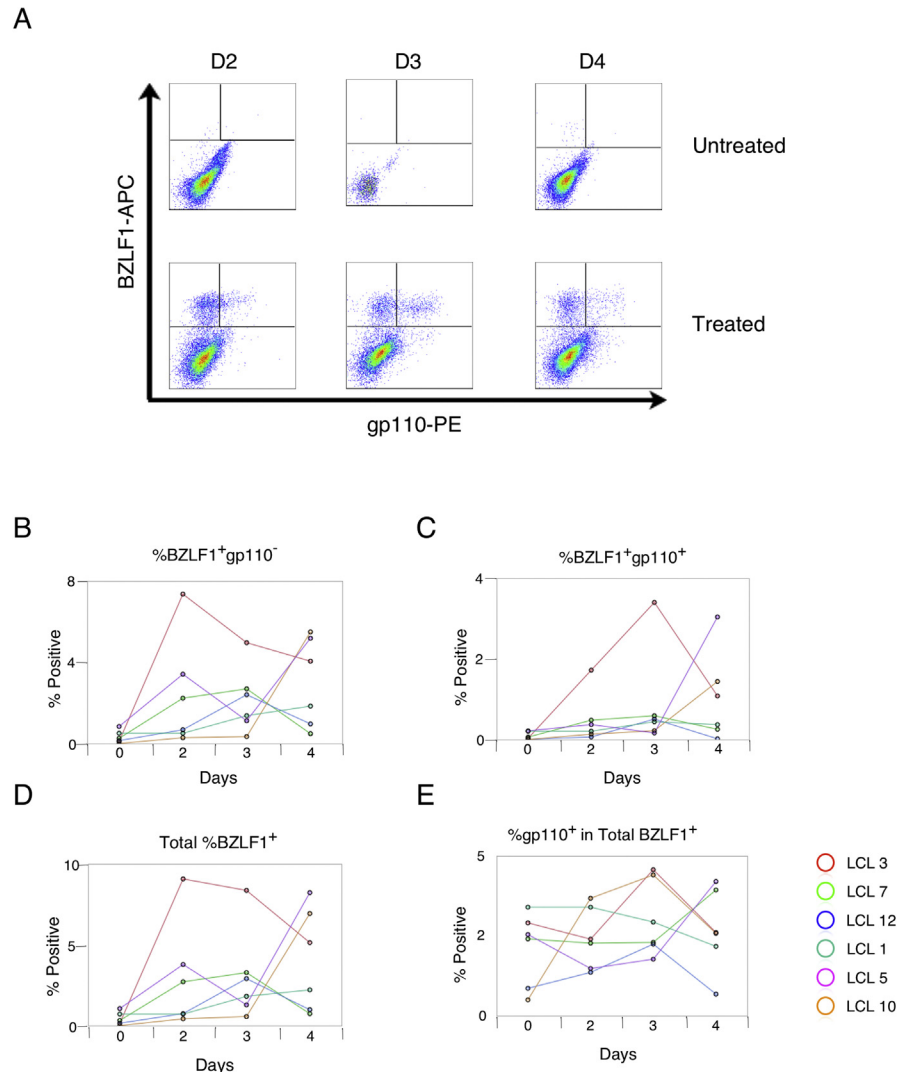
Fig. 5. Flow cytometric detection of Tg induced EBV BZLF1 and gB110 expressions at a single cell level. Representative figures (A) show detection of EBV BZLF1 and gB110 in LCL-1, 2 to 4 (D2-D4) day post treatment with 500 nM Tg as indicated. The single cell analysis also detected a small but clear population of BZLF1$^+$ cells in auntreated group, presumably undergoing the spontaneous lytic reactivation. Summary of the flow cytometric analysis of Tg induced BZLF1 and gB110 expression in selected LCLs at the single cell level (panel B though E). Proportions of (B) BZLF1$^+$gp100$^-$, (C) BZLF1$^+$gB110$^+$, (D) total BZLF1$^+$cells, and (E) gB110$^+$ cells in the total BZLF1$^+$ population was determined by a single cell analysis with flow cytometry at indicted days post Tg treatment. Numbers on the y-axis denote percent expression and color-coded circles indicate distinct LCL samples (n = 6) used in the experiment.

We were able to detect BZLF1$^+$gp110$^{+/-}$ LCLs by day2 and saw the significantly increase in the proportion (p < 0.05) of total BZLF1$^+$ LCLs (including both BZLF1$^+$gp110$^{+/-}$) on day 3 (3.03 ± 1.14%) and day 4 (4.04 ± 1.30%) as compared to day 0 (0.39 ± 0.17%) (Fig. 5B and D). The proportion of total BZLF1$^+$ LCLs in 3 out of 6 LCL lines had peaked during day2 to day 3 with declining trend on day 4, while 2 LCL lines showed evidence of upward trend of % BZLF1$^+$ cells at day 4 (Fig. 5B and D). Over the 4 day period, the highest percentage of total BZLF1$^+$ LCLs was approximately 10% (LCL3 on day 2). Although 2 cell lines had considerable increase in % BZLF1$^+$ cells on day 4 to almost 10%, it appears that a majority of LCLs had less than 10% of cells with a capacity to induce EBV lytic reactivation by Tg at given concentration during 4 day post Tg treatment. Moreover, proportions of gp110$^+$ (BZLF1$^+$) LCLs were consistently low and even smaller than those of total BZLF1$^+$ and comprised up to 50% of the total BZLF1$^+$ population (Table 1). The trend of % gp110$^+$BZLF1$^+$ LCLs over 4 days post Tg treatment was similar among LCLs examined (Fig. 5C and E). Taken all together, these data provide strong evidence indicating that a substantially small pool of Tg treated LCLs are capable of hosting virus production, while the majority of cells remain refractory. This is in agreement with observations of chemical induction experiments from previous reports [2, 3, 4].

4. Discussion

Chemical inducers of UPR are the emerging group of chemical agents that are known to trigger EBV lytic reactivation in leukemic B cells lines and LCLs. Chemicals, including Tm, Tg [9], and BZ [8] have been shown to induce robust UPR in these cells. UPR induction results in production of a spliced variant of XBP-1 (XBP-1(s)) [11] and CCAAT/enhancer-binding proteinβ (C/EBPβ) [8], both of which are known to directly activate expression from the BZLF1 promoter by binding to a ZII region of ZTA. Notably, XBP-1(s) is one of the UPR associated proteins that is known to be expressed at the time of plasma cell differentiation during which EBV lytic reactivation is also known to be triggered [11]. In the biological context, the plasma cell provides an ideal environment for replication and production of the virus because of its increases in protein translational machinery and heightened

Table 1. Proportions of the BZLF+gp110+/- LCL 2−4 days post treatment with Tg (100uM).

	%BZLF1$^+$gp110$^-$	%BZLF1$^+$gp110$^+$	Total %BZLF1$^+$	%gp110$^+$ div %BZLF1$^+$
Day 2	2.41 ± 1.10*	0.45 ± 0.25	2.91 ± 1.35	19.3 ± 9.56
Day 3	2.15 ± 0.66	0.88 ± 0.50	3.03 ± 1.14	25.3 ± 11.8
Day 4	3.00 ± 0.89	1.03 ± 0.46	4.04 ± 1.30	21.9 ± 12.8

*Numbers are presented as mean ± SEM.

metabolic activity. From an evolutionary standpoint, it makes sense that restraining the virus-productive phase of replication cycle until the onset of plasma cell differentiation gives latent virus a significant competitive advantage to its host cell adaptation. It is, therefore, conceivable that EBV genome has evolved in such a way to sense the metabolic change in the host cell through a transcriptional regulation of BZLF1 gene expression by ER stress/UPR signaling.

Despite a large body of evidence documenting UPR induction by Tm and Tg in the various tumor cell lines and primary cells tested so far, it is still not clear whether the UPR response directly, or in conjunction with other factors, triggers EBV lytic reactivation in LCLs. In fact, only two previous reports [8, 9] have provided evidence suggesting UPR is triggering EBV lytic cycle in LCLs, and in particular, Taylor et.al. [9] claimed that Tm induced lytic reactivation in an LCL. In our initial experiments with Tm, we were not able to detect a rise in the levels of EBV copy numbers in 14 LCLs tested during a 10 day post-treatment period of observation by qPCR (Fig. 2B). We determined that it was not for a lack of activating UPR by Tm, sufficiently enough to drive BZLF1 transcription and a subsequent lytic reactivation. In fact, Tm treatment did induce transcriptional activation of two key UPR marker genes CHOP10 [18] and CREB/β [19], with levels comparable to Tg (Fig. 3A and B respectively). Unexpectedly, we saw CREB/β transcriptional activation in Tm treated LCLs in the absence of viral reactivation, which is in contrast to the report showing CREB/β induction of lytic reactivation in a Burkitt's lymphoma cell line [8]. This could be due to several factors; the differences in the resident viruses, the latency states, and/or the patterns of expression of cellular genes between lymphoma cells and *in vitro* immortalized lymphoblastoid cells. Importantly, the difference in cellular responses raises a cautionary note concerning the generalization of observations obtained using so-called well-characterized established cell lines, usually of tumor origin, to all states and conditions in which latent viruses are found. If LCL cells are also more likely to be similar to the sort of EBV infected cells that exist *in situ* in a variety of EBV related lymphoproliferative disorders, then these cells should be included in every study of chemical induction of EBV lytic reactivation.

Unlike Tm and BZ, the mode of action of Tg is to trigger the UPR by directly inhibiting SERCAs [5, 6], which inhibits the uptake of cytosolic Ca^{2+} into the ER store, where depletion of Ca^{2+} induces ER stress, and subsequently leads to initiation of a robust UPR response [7]. Importantly, this action also leads to the rapid rise in the cytosolic Ca^{2+} level, and conceivably, the rise in the cytosolic Ca^{2+} level rather than UPR induction is responsible for EBV lytic reactivation. In fact, EBV lytic reactivation triggered by rising the cytosolic Ca^{2+} level by cross-linking surface immunoglobulins has been demonstrated in model B cell lines such Akata and Raji [20]. Moreover, the biological link between transcriptional activation of BZLF1 and cytosolic Ca^{2+} is previously established; activation of Calcineurin and Calmodulin

dependent Protein Kinase [21] that activate a family of MEF2 transcription factors known to bind ZTA [22]. Thus, it is entirely plausible that Tg triggers EBV lytic reactivation by the rising cytosolic Ca^{2+} level rather than UPR induction.

We showed that the Tg induced rise in the cytosolic Ca^{2+} level, but not the subsequent UPR induction, was responsible for Tg induction of lytic reactivation by using BTP2, the inhibitor of CRAC [23], and Ca^{2+} free medium, both of which abolished activation of BZLF1 transcription (Fig. 4A) and subsequent reactivation related viral DNA replication (Fig. 4B) by Tg. Thus, our data strongly suggest that intracellular Ca^{2+} mobilization induced by Tg is responsible for EBV lytic reactivation and importantly, triggering of EBV lyric reactivation by intracellular Ca^{2+} mobilization has been known [20]. Apparently, the rise in the intracellular Ca^{2+} level activates Calcineurin and Calmodulin dependent Protein Kinase [21] that then activates a family of MEF2 transcription factors know to bind ZTA and lead to activation of the BZLF1 transcription [22]. Moreover, results of time course study strongly suggest that there was a modality to intracellular Ca^{2+} flux that might be important to fully activate BZLF1 transcription since the BTP2 blockade of extracellular Ca^{2+} influx (from the medium) allowed the initial Ca^{2+} mobilization from intracellular stores and still abrogated BZLF1 gene activation (Fig. 4). It appears that a higher and more sustained Ca^{2+} increase from an extracellular source through CRAC was needed to fully activate BZLF1 transcription in LCLs, however, ionomycin treatment which produced a similar level of prolonged extracellular Ca^{2+} influx (6 hrs) did not activate BZLF1 transcription or lytic reactivation to the same extent as Tg did (Fig. 4A and B). Ionomycin, unlike Tg causes a single burst of $Ca2^{+}$ influx (Fig. 4D and G) which might suppress the function of Calcineurin and Calmodulin dependent Protein Kinase, creating a transient state of functional unresponsiveness to Ca^{2+} influx. More analyses will be required to address how the distinct modality to intracellular Ca^{2+} flux affects BZLF1 transitional activation.

Even though Tg triggers $Ca2^{+}$ influx in the entire population of cultured LCLs, only a small fraction of the cells in a LCL population underwent chemically induced lytic reactivation (Fig. 5), and it appears that the number of pre-induction EBV copies per cell influences a likelihood of cells having chemically induced lytic reactivation (Fig. 2C). These observations suggest that there may be a certain threshold number of latent EBV copies that must be reached in order for Tg to trigger lytic reactivation. This might also explain the fact that latently infected Akata cells which on the average have of higher number of latent EBV genomes than LCLs, display more robust spontaneous and chemically induced lytic reactivation (Fig. 1A).

Conceivably, the latent EBV copy number per cell is highly heterogenous even in clonal LCL lines, and only small fraction of those cells that have over the threshold number of latent EBV genomes may be disposed to the lytic reactivation caused by chemical stimuli. Such heterogeneity with respect to the latent EBV copy numbers

could be a by-product of asymmetric distribution of latent EBV genomes, in part due to the asymmetric distribution of EBNA1 over sister chromatids that has been documented [24]. Thresholds could also be modulated by the level of STAT3 which is shown to negatively regulate cellular sensitivity of EBV lytic reactivation by various stimuli [2, 25], though whether or not the level of STAT3 is directly involved in cellular distribution of the latent EBV genomes and regulation of latent EBV copy numbers remains to be tested.

In summary, we have shown that the lytic reactivation of EBV in LCLs triggered by Tg is mediated by intracellular Ca^{2+} flux rather than activation of the UPR pathway. Our results indicate that there are significant biological differences in the mechanism(s) by which EBV lytic reactivation is initiated in LCLs compared to established EBV positive B cell leukemic/lymphoma cell lines. Moreover, only a minute fraction of LCL cells experience lytic reactivation even with a saturating amount of Tg, suggesting that the modality to EBV lytic reactivation in LCLs is more complex than the model EBV positive cells lines (e.g. Akata-BL) and may well be the source of the apparent heterogeneity of responsiveness to Tg and the other similar class of chemical inducers. Also, these results raise important clinical and therapeutic implications. There is an increasing interest in identifying safe lytic cycle inducing agents for oncolytic induction therapy as an alternative to traditional chemotherapies to treat EBV associated neoplastic diseases. Screening chemical inducers that are ideal for oncolytic therapy will require flow cytometric and/or other imaging-based single cell assays in addition to traditional biochemical measures to determine the degree to which lytic reactivation is occurring at a population level. Using LCL panels as well as the model cell line should be *de rigor* for these types of investigations. Ultimately, it may be unrealistic to have an expectation that chemical agent therapies will be effective at inducing the lytic cycle in a sufficiently large fraction of EBV positive cells *in situ* in lymphoproliferative disease settings. Nevertheless, efforts to develop efficient induction protocols (especially for LCLs) will further our understanding of EBV lytic reactivation and may yet lead to new therapeutic targets.

Declarations

Author contribution statement

Aki Hoji: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Susie Xu, Holly Bilben: Performed the experiments.

David T. Rowe: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Additional information

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