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Induction of lytic cycle sensitizes Epstein-Barr virus infected B cells to NK cell killing that is counteracted by virus-mediated NK cell evasion mechanisms in late lytic cycle

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Abstract (208/250 words)

Epstein-Barr Virus (EBV) persists for the lifetime of the infected host despite eliciting strong immune responses. This persistence requires a fine balance between the host immune system and EBV immune evasion. Accumulating evidence suggests an important role for natural killer (NK) cells in this balance. NK cells can kill EBV infected cells undergoing lytic replication *in-vitro* and studies in both humans, and mice with reconstituted human immune systems have shown NK cells can limit EBV replication and prevent infectious mononucleosis. We now show that NK cells, via NKG2D and DNAM-1 interactions, recognize and kill EBV infected cells undergoing lytic replication, and that expression of a single EBV lytic gene, BZLF1, is sufficient to trigger sensitization to NK cell killing. We also present evidence suggesting the possibility of the existence of an as yet unidentified DNAM-1 ligand which may be particularly important for killing lytically infected normal B cells. Furthermore, whilst cells entering lytic cycle become sensitized to NK cell killing, we observed that cells in late lytic cycle are highly resistant. We identified expression of the vBcl-2 protein, BHRF1, as one effective mechanism by which EBV mediates this protection. Thus, contrary to the view expressed in some reports, EBV has evolved the ability to evade NK cell responses.

Importance (98/150 words)

This report extends our understanding of the interaction between EBV and host innate responses. It provides the first evidence that the susceptibility to NK cell lysis of EBV infected B cells undergoing lytic replication is dependent upon the phase of lytic cycle. Induction of lytic cycle is associated with acquired sensitization to NK cell killing, while progress through late lytic cycle is associated with acquired resistance to killing. We

provide mechanistic explanations for this novel observation, implicating important roles for the BZLF1 immediate-early transactivator, the BHRF1 vBcl-2 homologue, and a novel ligand for the DNAM-1 NK cell receptor.

Introduction

Epstein-Barr Virus (EBV), one of eight human herpesviruses, is carried by over 90% of the world's adult population. Primary EBV infection occurs in the oropharynx, leading to infection of B lymphocytes (1, 2). These infected B cells can support lytic cycle, in which more than 80 viral genes are expressed to generate new infectious virus, but they more frequently host non-productive infections through expression of a limited number of so-called latent EBV genes (Latency III genes) that drive lymphoproliferation as an alternative mechanism of expanding the infected cell pool. *In-vitro,* this growth transformation is demonstrated by the ready establishment of lymphoblastoid cell lines (LCLs) following infection of resting B cells. Following initial infection *in-vivo*, EBV downregulates the expression of all viral proteins and enters a true latent phase (Latency 0) in the memory B-cell population where it establishes a lifelong infection (1). Periodically the virus reactivates and undergoes full lytic replication, which both aids the expansion of the virus within the host and enables transmission to new hosts (2).

A major component of the immune control of EBV is considered to be the strong and persistent T cell responses both to the transformation-associated Latency III EBV gene products and to several lytic-cycle-associated EBV proteins (3). However, an increasing body of evidence suggests that natural killer (NK) cells have an important role to play in the virus host balance. NK cells expand following primary infection with EBV (4, 5), and patients with genetic defects leading to loss or impairment of NK cell differentiation or function are prone to complications associated with EBV infection (6). Furthermore, mice with reconstituted human immune system components experimentally infected with EBV, experience enhanced symptoms resembling infectious mononucleosis and EBV-associated lymphomagenesis when depleted of NK cells; these pathogenic outcomes of NK cell-depletion were shown to be due to loss of control over EBV lytic replication (7).

Successful persistence of viruses in the infected host requires some degree of evasion of the various potent immune responses. Like other herpesviruses, in addition to establishing antigenically silent latent infections, EBV has multiple mechanisms to evade both $CD8^+$ and $CD4^+$ T cell responses to viral proteins expressed following reactivation of lytic cycle or growth-transformation (8). However, the possible existence of EBV evasion mechanisms against NK cells is unclear.

Other human herpesviruses, most notably Human cytomegalovirus (CMV) but also Kaposi's Sarcoma-associated virus (KSHV), Herpes simplex virus 1 (HSV-1) and 2, Varicella Zoster Virus (VZV) and human-herpes virus 7 (HHV-7), all possess some NK cell evasion mechanism; most frequently, but not exclusively, involving modulation of NKG2D ligands (9-12). In one respect it could be argued that EBV evades NK cell responses through infecting B lymphocytes and, in growth-transformed cells, maintaining high levels of MHC class I molecules that ligate inhibitory receptors on NK cells. Certainly, EBV-transformed latently-infected LCLs are not killed unless they are experimentally defective for HLA expression (13). With regards to B cells lytically infected with EBV, however, there is only evidence that EBV sensitizes them to NK cell recognition and killing. This evidence was derived entirely from studies with malignant cell lines, principally the AKBM line derived from Burkitt's lymphoma cells engineered to express two selection markers, green fluorescent protein (GFP) and truncated CD2, when induced into lytic cycle through ligation of surface immunoglobulin (14). The switch from latent to lytic infection in AKBM cells triggers an upregulation of NKG2D ligands that is at least partly responsible for the sensitization to NK cell killing. However the mechanism of NKG2D ligand upregulation in lytic cycle was not determined and, due to technical limitations of these earlier experiments, the possibility of counteracting evasion mechanisms was not investigated. Importantly, the generality and relevance of the AKBM observations to normal B cell infection has not been demonstrated.

In the present study we identified the immediate-early protein, BZLF1, as being able to sensitize cells to NK cell killing through upregulating the ULBP NKG2D ligands. We also identified the vBcl-2 homologue, BHRF1, as a potential NK evasion gene that could protect BZLF1-sensitized cells from NK cell killing. Consistent with these findings, we demonstrated that whereas AKBM cells in the early stages of lytic cycle were killed by NK cells, AKBM cells at the late stages of lytic cycle were resistant. Importantly, this phenomenon was also observed in lytically infected LCLs, even though these non-malignant cells were primarily killed through NK cell receptor/ligand combinations that differed from those utilized in NK cell killing of lytic AKBM cells.

Materials and Methods

Cell lines

The NK cell line NKL (15) was maintained in RPMI 1640 supplemented with 10% foetal calf serum (FCS) and 200 IU/ml IL-2. The NK cell line NK-92 (16) was maintained in RPMI 1640 supplemented with 10% FCS, 10% horse serum, 5% human serum and 400 IU/ml IL-2. Both NKL and NK-92 were obtained from the American Tissue Culture Collection, and their activating receptor profiles were determined for this study (Figure 1). AKBM cells are a derivate of the Akata Burkitt lymphoma cell line engineered to carry a reporter plasmid that expresses GFP when the cells enter the lytic cycle. These cells were maintained in RPMI 1640 supplemented with 8% FCS, and were induced into lytic cycle by cross-linking surface IgG molecules as previously described (14). The EBV negative Burkitt lymphoma cell line DG75 (17) and EBV-transformed LCLs (18) were maintained in RPMI 1640 supplemented with 8% FCS. DG75-control and DG75-BHRF1 were generated through transduction and NGFR-sorting as described above and maintained in RPMI 1640 supplemented with 8% FCS. A doxycycline (DOX)-inducible BZLF1 expression vector, pRTS-CD2-BZLF1, or control vector with the reverse BZLF1 sequence (pRTS-CD2-control) (27) were introduced into DG75 by electroporation and rCD2 selection. BZLF1 expression was induced by addition of DOX, and the induced cells were positively selected by magnetic cell sorting with anti-NGFR Microbeads and LS columns (Miltenyi Biotech). Human embryonic kidney (HEK) 293 cells (19) were maintained in DMEM supplemented with 10% FCS.

Plasmids

The BZLF1 and BRLF1 genes from the B95.8 prototype EBV (GenBank accession numbers CAA24861.1 and CAA24814.1) were subcloned into the pCDNA3-IRES-nls-GFP plasmid vector (20), and were verified by restriction digest and sequence analysis. BHRF1, also from the B95.8 prototype EBV, was cloned into the pLZRS-IRES-ΔNGFR vector (21) to generate retroviruses expressing BHRF1 and the truncated nerve growth factor receptor (ΔNGFR) for selection of infected cells.

Transfection and electroporation

Transient transfection of HEK 293 cells was performed using lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Plasmid DNA was transfected into DG75 cells by electroporating cells at 270V and 950μF in 4mm curvettes. Cells transduced with PLZRS-NGFR vectors were positively selected for the expression of NGFR using MACSelect NGFR-Transfected cell selection kit (Miltenyi Biotec) according to the manufacturer's instructions to establish stably transduced cell lines.

Isolation of NK cells

Blood was taken from healthy donors with ethical consent according to the human tissue act. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lympholyte cell separation media (Cedarlane Labs) and untouched NK cells were isolated from PBMCs using the NK cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol.

Antibodies

For flow cytometry experiments, FITC-conjugated, PE- conjugated and unconjugated antibodies to CD19 (HIB19), NGFR (ME20.4) and CD155 (TX24) were purchased from Biolegend. The FITC-conjugated anti-DNAM-1 (11A8), APC-conjugated anti-

NKp30 (P30-15) and APC-conjugated anti-human IgG Fc (HP6017) were also purchased from Biolegend. The APC-conjugated anti-NKp46 (9E2) was purchased from Ebioscience. The APC-conjugated anti-NKG2D (1D11), anti-CD112 antibody (R2.525) and the Alx647-conjugated antibody to active-caspase-3 (C92-605) were purchased from BD Biosciences. APC-conjugated and PE- conjugated antibodies to ULBP2/5/6 (165903) and MICA/B (159207) were purchased from R&D biosystems. Recombinant Human DNAM-1/CD226 Fc Chimera Protein (666-DN-050) was also purchased from R&D biosystems. The BZLF1 (BZ.1) antibody (22) was generated by our investigators, and the BcLF1 (V3) antibody (23) was a kind gift from Dr Gary Pearson, previously of Georgetown University, Washington DC. To detect unconjugated antibodies PerCP-Cy5.5-conjugated or Alx647-conjugated secondary antibodies against mouse IgG_1 (RMG1-1) or IgG_{2a} (RMG2a-62) were purchased from Biolegend. For blocking experiments, antibodies to NKG2D (1D11), DNAM-1 (DX-11) and NKp46 (9E2) were purchased from BD biosciences. For western blotting the anti-calregulin antibody was purchased from Santa Cruz Biotechnology, the BZLF1 antibody (BZ.1) is described above, and the BHRF1 antibody was purified from cultures of the 5B11 hybridoma (24) obtained from Dr Elliott Kieff, Harvard.

Flow cytometry analysis

Stained cell samples were detected on BDbiosciences Accuri C6 Flow Cytometer. Data were analyzed using FlowJo software (TreeStar).

Cytotoxicity assays

NKL and NK92 cells and freshly isolated NK cells were used as effectors in cytotoxicity assays. AKBM cells were used as targets 24h post-induction with anti-IgG. DG75 cells were used as targets 24h post transfection with control-GFP, BZLF1-GFP, or BRLF1-GFP expression plasmids. DG75 cells stably expressing control-NGFR or BHRF1-NGFR vectors were used as targets 24h post transfection with control- or BZLF1-GFP expression plasmids. LCLs were screened for levels of spontaneous lytic cycle, and those containing suitable proportions of BZ.1$^+$ cells (≥1%) were selected for use as targets in NK cell assays. Effector and target cells were combined at different ratios and incubated for 4-16h. In 4h assays, cytotoxicity was determined by caspase-3 staining by flow cytometry. Specific cytotoxicity was calculated as: *% caspase-3 positive target cells after co-incubating with NK cells for 4h – % caspase-3 positive target cells after 4h incubation alone*. For blocking experiments NK cells were incubated with saturating amounts of blocking antibody (30μg/ml) for 1h at 37°C, then washed three times before use as effectors in cytotoxicity assays.

In 16h cytotoxicity assays, killing was measured by determining the decline in numbers of target cells against a control population of target cells not killed by NK cells. Killing was calculated by the following the equation: *Killing (%) = 100 – ((experimental GFP% / control GFP%) x 100)*

In the degranulation assay, DG75 target cells and NKL cell line were co-cultured with FITC conjugated anti-CD107a antibody for 5 hours. Then the cells were washed and stained with combinations of APC conjugated anti-NKG2D with PE conjugated anti-CD19 to separate the NKL population from DG75 population. Stained cells were analyzed by flow cytometry.

Western blotting

Total cell lysates were denatured in reducing sample buffer and then sonicated and heated to 100°C for 5 min. Solubilised proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on to 4-12% acrylamide gradient bis-

Tris NuPage minigels with morpholinepropanesulfonic acid running buffer (Invitrogen). Separated proteins were electroblotted to polyvinylidene difluoride membranes and probed with specific antibodies. Samples were then subjected to chemiluminescent detection using the Millipore ECL detection kit (Millipore).

Q-PCR assay

Total RNA isolated from cultured cell lines using the QIAGEN RNeasy kit, was treated with DNase I (Turbo DNA-free kit; Ambion) and then reverse transcribed using qScript™ cDNA SuperMix(Quanta Biosciences). Quantitative, reverse-transcription, polymerase chain reaction (qRT-PCR) assays for MICA, MICB, ULBP2, ULBP5, ULBP6, CD112 and CD155 were performed with TaqMan® Gene Expression Assays (Applied Biosystems), duplexed with b2m assays for normalization.

Statistical analysis

Where statistical analysis was performed, data were analysed with student *t* tests or one-way ANOVA as described in the figure legends. Analysis was performed using Prism 5 software (Graphpad Software).

Results

The switch from latent to lytic infection sensitizes B cells to NK cell killing

We previously reported that the switch from latent to lytic cycle in AKBM cells induced sensitivity to NK cell killing (14). Those experiments were conducted by sorting induced AKBM cells for the expression of rCD2/GFP to isolate homogeneous populations of cells in lytic cycle. Whilst that methodology provided valuable information, it was not suitable for the additional investigations planned in the present study. We therefore designed a novel method of measuring NK cell killing in mixed populations of target cells using flow cytometry.

To validate this new assay, target AKBM cells were induced into lytic cycle by treatment for 1h with anti-IgG. At 24h post-induction cells were incubated with NKL effector cells at varying effector to target ratios. After 4h co-incubation, cells were harvested and stained for cell surface CD19 to differentiate effector and target cells, and for intracellular activated-caspase-3 as a marker of NK cell induced killing. Figure 2A shows CD19 staining to differentiate NK cells from the target population, AKBM cells. Within the target population, cells undergoing latent or lytic cycle were differentiated by GFP expression (latent infection, GFP^-; lytic infection, GFP^+), and activated-caspase-3 was measured in each target population to determine levels of cytotoxicity.

In healthy cells, caspase-3 exists as an inactive pro-enzyme; cleavage of this protein produces the active form of the enzyme activated-caspase-3 (hereafter referred to simply as caspase-3) that plays a central role in the execution phase of apoptosis (25). Cytotoxic lymphocytes such as NK cells and $CD8^+$ T cells are able to kill target cells through two main mechanisms, Fas/FasL interaction and the release of cytotoxic granules containing perforin and granzyme. Killing mediated through either mechanism will initiate a caspase cascade in target cells resulting in conversion of pre-caspase-3 to activated caspase-3 in a target cell; immunostaining and flow-cytometry for activated caspase-3 can therefore be used an early marker of target cell killing by effector cells.

As shown in Figure 2B, with increasing effector: target ratios, the levels of caspase-3 increased in lytic cells but not in the latent cells; this reflects the increased cytotoxicity to lytic cells. At the highest effector to target ratio (4:1) levels of caspase-3 positive cells in the lytic population reached 23%, compared to just 3% in latent cells. This confirms the previous finding of our lab that AKBM cells in lytic cycle are susceptible to killing by NK cells and shows that caspase-3 induction can be used as a marker for NK cell killing in this setting.

NK cells are a highly polymorphic population of cells controlled by different activating and inhibitory receptor ligand combinations. To show that the previous result is not unique to the NKL effectors, the experiment was repeated with two alternative sources of NK cells: the NK cell line NK-92, and polyclonal NK cells freshly isolated from peripheral blood. Figure 2C shows that NK-92 cells activated caspase-3 in 55% of lytic AKBM cells, compared to less than 1% of latent cells, at an effector:target ratio of 4:1. Similarly, figure 2D shows that freshly isolated blood NK cells activated caspase-3 in 50% of lytic cells and just 2% of latent cells. Thus, the same observation was made with the three different sources of NK cells.

NK cell killing of lytically infected AKBM cells was shown previously to be mediated through the activating receptor NKG2D, expressed on NK cells. This observation was confirmed in the present study by performing caspase-3 cytotoxicity assays in the presence of blocking antibodies directed against activating receptors expressed on NK cells (Figure 2E). The inclusion of either a control antibody or a blocking antibody against the NKp46 natural cytotoxicity receptor (NCR) did not decrease the level of caspase-3 induced in target cells. A DNAM-1 blocking antibody showed a small decrease in caspase-3 induction, though this result did not reach significance. When a blocking antibody directed against NKG2D was added to cytotoxicity assays a significant decrease in caspase-3 induction was observed. These results exactly match those previously reported (14) with conventional ^{52}Cr-release assays on purified lytic AKBM populations.

EBV infected cells in late stage lytic cycle are protected from NK cell killing

A major advantage of the flow cytometry based cytotoxicity assay is that it allows simultaneous *in situ* analysis of different target cell populations that might be refractory to physical separation methods. We therefore repeated the NKL cytotoxicity assays on AKBM target cells, which were then immunostained intracellularly for BZLF1 and BcLF1 expression as markers of early and late lytic cycle. Figure 3A shows that this staining protocol allowed us to differentiate three populations of cells; latently infected cells expressing neither BZLF1 nor BcLF1, early lytic cells expressing BZLF1 but not BcLF1, and late lytic cells expressing BZLF1 and BcLF1. Caspase-3 was measured in all three populations of cells and cytotoxicity calculated. The results in Figure 3B show that, as expected, latently infected AKBM cells were resistant to NK cell killing. However, the analysis of different lytic populations revealed a remarkable result; whereas cells in early lytic cycle were highly sensitive to NK cell killing, with activation of caspase-3 observed in around 40% of the BZLF1^{+}/BcLF1^{-} population at an effector to target ratio of 4:1, the BZLF1$^+$/BcLF1$^+$ cells in late stage lytic cycle were completely protected from NK cell killing.

This novel observation suggested to us that sensitization of AKBM cells to NK cells was a very early event following activation of the lytic cycle and that EBV may have active mechanisms for evading the NK cell response.

BZLF1 can induce expression of NKG2D ligands and sensitize B cells to NK cell killing

We hypothesized that the EBV immediate early genes BZLF1 or BRLF1 might cause the sensitization seen in previous experiments as sensitization appears to be an early event and because the HCMV counterpart of EBV BZLF1, IE-1, has been shown to activate transcription of NKG2D ligands (26). We therefore investigated the two immediate-early genes of EBV for their effect on the expression of NKG2D ligands in EBV-negative cells. In the first instance, BZLF1 and BRLF1 were transiently expressed in HEK 293 cells using bicistronic plasmid vectors that co-express the gene of interest along with GFP, which allows identification of transfected cells using flow cytometry. Using an antibody that detects ULBP 2, 5 and 6, the levels of the ULBP ligands of the NKG2D receptor were measured on GFP$^+$ cells by flow cytometry at 24h post-transfection. Whilst cells transfected with BRLF1-GFP showed no significant change in ULBP expression compared to cells transfected with control-GFP (Figure 4A), increased ULBP expression was detected in those cells transfected with BZLF1-GFP (Figure 4B).

As B cells are the natural reservoir for EBV, and the original NK cell sensitivity data were obtained in the Burkitt lymphoma cell line, AKBM, we next investigated the effect of BZLF1 on NK cell ligand expression in an EBV-negative Burkitt lymphoma cell line, DG75. Following electroporation to introduce BZLF1-GFP or control-GFP vectors into DG75, the levels of NK cell ligands were measured by flow cytometry. Expression of BZLF1 in DG75 B cells, at levels comparable to but not exceeding BZLF1 levels in lytic cycle (27), had similar effects to that seen in 293 cells, in that ULBP expression significantly increased (Figure 4C). Expression of two additional NKG2D ligands, the MHC class I-chain related proteins, MICA and MICB, was unaffected by expression of BZLF1 (Figure 4D). As discussed previously, NK cells may be activated by many different receptors. With this in mind, the effect of BLZF1 on the two known DNAM-1 ligands was also tested, but BZLF1 caused no increase in the expression of either CD155 (Figure 4E) or CD112 (data not shown) or binding of DNAM-1-Fc fusion protein (Figure 4F).

To confirm the previous result and further investigate the effect of BZLF1 on the expression of NK cell activating ligands, mRNA expression levels were measured in the absence and presence of BZLF1 protein. As the antibody used in the previous experiment recognises ULBP2, 5 and 6 protein, the transcription levels of these three genes was measured. DG75 cells expressing inducible BZLF1 (27) were enriched and total RNA was then isolated and reverse transcribed to generate cDNA. The relative transcription level of each ULBP gene was then measured using Q-PCR. The level of ULBP2 transcript was increased two-fold in BZLF1 expressing DG75 cells when compared to control cells ($P<0.05$) (Figure 4G). No up-regulation of ULBP6 transcription level was observed (Figure 4H) and no ULBP5 transcription was detected in either control DG75 or BZLF1 expressing DG75 (data not shown). Transcription levels of DNAM-1 ligand were also measured in the same assay but no CD112 or CD155 transcripts were detected in either DG75 or BZLF1 expressing DG75 (data not shown).

As BZLF1 clearly increases the expression of ULBPs in these cells, we next investigated whether BZLF1 expression alone is able to sensitize B cells to killing by NK cells. In order to test this DG75 cells were again transfected with BZFL1 expression vector and used as targets in cytotoxicity assays. A high baseline expression of caspase-3 in viable electroporated DG75 cells precluded the use of the cytotoxicity assay used in Figures 2 and 3, so an alternative method of measuring NK cell killing by flow cytometry was used. Cells were incubated with NK cells for 16h and the percentage of GFP-tagged target B cells remaining after this time was measured at different effector:target ratios. Specific cytotoxicity was calculated by comparing the percentage of GFP positive cells after 16h incubation with NK cells with cultures of transfected cells alone. Figure 4I shows that cells expressing the control-GFP vector were not depleted by NK cells, while expression of BZLF1 sensitized cells to NK cell killing as there was a significant depletion of BZLF1-GFP target cells at all effector: target ratios.

BHRF1 protects B cells from BZLF1 induced NK killing

As BZLF1 is the master transactivator of EBV lytic cycle, the data in figure 4 provide at least one explanation for why AKBM cells in early lytic cycle are susceptible to NK cell killing. We next sought to explain why AKBM cells in late lytic cycle became resistant to NK cell killing despite the levels of BZLF1 protein being maintained during late lytic cycle (Figure 5A). BHRF1 is an early lytic cycle protein whose maximal levels are not achieved until about 12h post-induction, coincident with the appearance of late lytic cycle antigens (Figures 5A, B). As BHRF1 is a vBcl-2 homologue with powerful anti-apoptotic functions (28, 29), we hypothesised that it might be a contributor to the protection against NK cells.

To test this possibility, BHRF1 was co-expressed with BZLF1 in DG75 cells to determine if BHRF1 could counteract the sensitization caused by BZLF1. DG75 cells were transduced with either control or BHRF1 expressing retroviral vectors co-expressing a truncated NGFR as a selectable marker. Following magnetic selection these cell lines were shown to be 100% NGFR positive (Figure 6A). The two cell lines were then electroporated with either control or BZLF1-GFP expression vectors, as in figure 4, and levels of BHRF1 and BZLF1 protein in these DG75 lines were monitored by immunoblotting (Figure 6B). Finally the four cell lines were used as targets in cytotoxicity assays to measure sensitivity to NK cell killing (Figure 6C). As expected, there was no significant NK cell killing of DG75-control and DG75-BHRF1 cells. As seen before, expression of BZLF1 in control DG75 cells caused the cells to become sensitive to NK cell killing, but expression of BZLF1 in DG75 cells stably expressing BHRF1 resulted in no sensitization. Therefore, BHRF1 is able to completely antagonise BZLF1 and protect B cells from NK cells killing.

From what is known about BHRF1, we anticipated that this vBcl-2 protects B cells from NK cell killing through its anti-apoptotic function rather than by directly reversing the effects of BZLF1 through downregulation of ULBPs. To rule out the latter possibility, we assayed the surface expression of ULBP (Figure 6D). As before, BZLF1-transfected DG75 cells revealed elevated expression of ULBP relative to control-transfected DG75 cells. BZLF1-expressing DG75-BHRF1 cells showed a similar elevated ULBP expression showing that BHRF1 has no effect on ULBP expression.

Despite being resistant to NK cell killing, we hypothesised that due to increased ULBP expression BZLF1-expressing DG75-BHRF1 cells will still be recognised by NK cells causing the NK cells to become activated and degranulate. To confirm this hypothesis, degranulation of NK cells was studied following co-culture with DG75 cells expressing BZLF1 and BHRF1. Figure 6E shows, as expected, an increased degranulation in NKL cells stimulated with BZLF1 expressing DG75 cells compared to control DG75 cells. This increased degranulation was unchanged in NKL cells stimulated with BZLF1 expressing DG75-BHRF1 cells, despite BHRF1 protecting these cells from NKL cytotoxicity. This suggests that BHRF1 is able to protect cells from NK cell killing through its intrinsic anti-apoptotic function despite NK cells still recognising and degranulating in response to such cells.

LCLs in late stage lytic cycle are also protected from NK cell killing

Whilst the AKBM and DG75 cell lines were useful tools for establishing and characterising the phenomena of lytic cycle sensitization and protection from NK cell killing respectively in early and late phases of lytic cycle, it could be argued that they are malignant cell models and that the relevance to normal B cell infection is unclear. Indeed, due to the technical difficulties it has not previously been shown that lytically infected normal B cells can be killed by NK cells. Our new flow cytometry based cytotoxicity assay (Figures 2 and 3) provided an opportunity to address this question in the present study.

EBV naturally infects and transforms B cells *in-vitro*, establishing a continuously growing but non-malignant LCL. EBV infection in LCLs is predominantly non-productive, expressing only a limited number of growth-transforming latent viral genes, and showing resistance to NK cell killing. However, viral gene expression can be quite heterogeneous, and in many LCL cultures a small proportion of cells can spontaneously enter lytic cycle. We assayed a panel of different LCL cultures for the presence of cells undergoing spontaneous lytic cycle and selected suitable lines (i.e. those with >1% BZ.1$^+$ cells) as targets in NK cell cytotoxicity assays. Cell were co-cultured with NKL cells for 4h, harvested and stained for the expression of CD19, BZLF1 and BcLF1 to distinguish CD19$^+$ target cells in latent infection (expressing neither BZLF1 nor BcLF1), early lytic infection (expressing BZLF1 but not BcLF1), and late lytic infection (expressing both BZLF1 and BcLF1). Caspase-3 was measured in all three populations of cells and cytotoxicity calculated. The results obtained using multiple LCL cultures (Figure 7A) were remarkably similar to the earlier results using the AKBM model. Latently infected LCLs were resistant to killing by NK cells; cells in the early stages of lytic cycle were highly sensitive to NK cell killing, whilst cells in late lytic cycle were completely resistant to NK cell killing.

Although NK cell recognition and killing of AKBM cells has been shown to be mediated by NKG2D/ULBP interactions, differing reports exist in the literature as to the expression of NKG2D ligands on LCLs (30-32). We therefore examined whether NK cell killing of LCLs undergoing lytic cycle is mediated through NKG2D, by performing cytotoxicity assays in the presence of blocking antibodies directed against different activating receptors (Figure 7B). In contrast to what we observed previously in experiments with the AKBM cells, blocking NKG2D or NKp46 had no effect on NK cell killing of LCLs expressing BZLF1, but including a blocking antibody against DNAM-1 substantially ablated NK cell killing of target cells. Furthermore, staining of LCLs with antibodies to NKG2D ligands failed to detect expression of either MICA/B or ULBP (Figure 7C, 7D). These data suggest that NK killing of LCLs is predominantly mediated through DNAM-1, and that the precise mechanism(s) of sensitization of lytically-infected B cells to NK cell killing may depend on the cellular origin or phenotype.

DNAM-1 has two known cellular ligands; CD155 and CD112 (33). As with NKG2D ligands, there is some disagreement in the literature as to the expression of DNAM-1 ligands on LCLs. To ascertain if the sensitization of LCLs undergoing early lytic cycle was due to increased expression of known DNAM-1 ligands we stained LCLs from different donors with antibodies against CD155 and CD112. The results showed that neither latent nor lytically infected cells in LCL cultures expressed CD155 (Figure 7E) or CD112 (Figure 7F) despite clear staining on control cells (HeLa for CD155, and K562 for CD112). This experiment was repeated using multiple antibodies to both ligands and multiple LCLs from different donors and in all cases neither CD155 nor CD112 expression was detected. Interestingly, when CD155 or CD112 blocking antibodies were included in cytotoxicity assays, they had no effect on NK cell killing of lytic LCLs (data not shown). These data indicate that whilst NK cell killing of lytically infected LCLs is mediated through the DNAM-1 receptor on NK cells, the LCLs do not express detectable amounts of either of the two known DNAM-1 ligand proteins.

Discussion

In this study we have demonstrated that the acquisition of sensitivity to NK cell killing of EBV infected B cells upon entry into lytic cycle is not an artefact of the unusual malignant cell line model in which the observation was first made. This phenomenon of sensitization to NK cell killing is also observed in independently established, normal LCLs in which a small subpopulation of cells spontaneously enters lytic cycle. The cytotoxicity assay that we developed to be able to investigate NK killing of the minor population of lytically infected cells within LCL cultures has also allowed the discovery of another important finding; that during the late stages of EBV lytic cycle, EBV infected B cells acquire a profound resistance to NK cell killing.

In the AKBM cell model, sensitization of lytically infected cells appears to be predominantly mediated by upregulation of ULBPs, which are ligands for the NKG2D activating receptor on NK cells. Furthermore, we showed that expression of a single EBV gene, the lytic transactivator BZLF1, causes a significant upregulation of these NKG2D ligands in an EBV-negative B cell line and coincidentally sensitize the cells to killing by NK cells. This upregulation of surface ULBP expression correlates with increased transcript level of ULBP2 in BZLF1 transfected DG75 cells (Figure 4G). BZLF1 is a powerful transcription factor that not only initiates a cascade of EBV lytic cycle gene expression but also regulates more than 270 cellular genes in AKBM cells (34). The BZLF1-regulated cellular genes identified by ChIP analysis do not include known NK cell receptor ligands. However, our present analysis indicates that BZLF1 expression leads to a 2-fold increase in ULBP-2 transcripts (Figure 4G). It is therefore likely that BZLF1 indirectly targets ULBP-2 gene transcription and/or that BZLF1 indirectly targets ULBP-2 post-transcriptionally. It is known that BZLF1 binds to DNA damage response proteins, causing their mis-localization and, consequently, increased DNA damage in cells expressing BZLF1 (35). NKG2D ligands are known to be upregulated in response to a number of stress signals including DNA damage (36), raising the possibility that upregulation of NKG2D ligands by BZLF1 may be an indirect result of induced DNA damage.

As mentioned above BZLF1 is the master regulator of EBV lytic virus replication and thus critical for the virus life cycle. The sensitization to NK cell killing initiated by BZLF1 expression and/or by other early lytic genes is therefore a price that the virus must pay. Though seemingly counterintuitive, EBV’s ability to initiate an NK cell response to control viral infection is an evolutionary advantage to the virus since NK cell control of EBV is an important factor in establishing a stable relationship between host and virus thus allowing asymptomatic EBV persistence. An absence of effective NK cell responses in immunodeficiencies such as XLP and X-MEN syndrome is associated EBV-related pathogenic complications (6, 32). In addition, two reports have described patients with CD16 mutation who experienced prolonged EBV infections and complications such as EBV-associated Castleman's disease (37, 38). As well as NK cell deficiencies, NK cell phenotype has been shown to correlate with outcome of EBV infection. Two reports have shown that certain polymorphisms in killer immunoglobulin like receptors (KIRs) can predispose people to infectious mononucleosis or hemophagocytic lymphohistiocytosis (39, 40). Equally, an alternative KIR polymorphism can actually protect from infectious mononucleosis (40).

Whilst NK cell control, along with $CD4^+$ and $CD8^+$ immune T cell responses, is clearly important for limiting the pathogenic potential of EBV, the successful persistence of the virus for the life of the infected host implies some viral immune evasion mechanisms to evade elimination. For $CD4^+$ and $CD8^+$ responses, active mechanisms for evasion during lytic cycle are well-documented (3, 8, 41). However, evasion of NK cell responses in lytic cycle is poorly understood. It has been suggested that EBV micoRNAs, notably miR-BART2, may transcriptionally regulate NK cell ligands (42). However, expression of miR-BART2 is only weakly upregulated, by less than 2-fold, in AKBM cells upon induction of lytic cycle, which argues against a significant evasion function accounting for our observed resistance of late-lytic cycle cells to NK cell killing. A more recent study of a relatively complex experimental model of primary infection of PBMCs, indicated a clear role for the vIL-10 (BCRF1) in modulating NK cell activity (43). This effect appears to be due to vIL10 and huIL10 acting on the NK cells, rather than affecting the sensitivity of the EBV-infected cells. Whilst not devaluing the importance of the published data, it is unlikely that BCRF1 contributes to our observed resistance of late lytic cells to NK cells since early lytic cells in the same culture are highly sensitive to the same NK cells.

Against this background, our novel finding that BHRF1 can afford substantial protection to NK cell lysis is important as it offers a plausible mechanism for the resistance of late lytic cycle cells. However, the lessons from other herpesviruses would suggest that BHRF1 is unlikely to be the only mechanism that EBV has evolved to counteract NK cell responses and enable some virus replication to occur *in-vivo*. Human cytomegalovirus (HCMV) is the most well-studied in the context of NK cell evasion, and has multiple different mechanisms that act in synergy (44). CMV is able to reduce expression of multiple NKG2D ligands: UL16 reduces expression of ULBP1, ULBP2 and MICB; while US142, US18 and US20 reduce expression of MICA (45-48). UL141 blocks surface expression of DNAM-1 ligands, CD112 and CD155 (49, 50). CMV also ligates NK inhibitory receptors through expression of HLA homologues such as UL18 that binds LIR-1 or stabilising HLA-C through the action of UL40 (11, 51).

The value of extending our work beyond the AKBM model to non-malignant LCLs extends beyond showing the generality of the basic observations that cells in early lytic cycle are sensitized to NK cell killing whilst cells in late lytic cycle acquire resistance. The results revealed another interesting point that the same end result might be achieved through slightly different mechanisms in different cells. Whereas NK cell recognition of lytic AKBM cells is predominantly through upregulation of NKG2D ligands, recognition of LCLs is mediated not through NKG2D but through DNAM-1. Paradoxically, in all the LCLs we tested neither of the two known DNAM-1 ligands was detected, whether on latent or lytic infected cells. Interestingly, a small but significant increase in CD155 transcripts was observed in lytic LCLs (Figure 7G), but the magnitude of the elevated transcripts was such that the biological significance is questionable. Preliminary attempts to identify the DNAM-1 ligand responsible for sensitization to NK cell killing were hampered by the inability to obtain significant binding of DNAM-1-Fc fusion protein to LCLs (Figure 7H); a result that we attribute to the insensitivity of the fusion protein reagent. We hypothesize that LCLs in lytic cycle express a third as yet undiscovered DNAM-1 ligand. This ligand may be cellular, as is the case with CD155 and CD112. Alternatively, this ligand may be of viral origin; a number of NK receptors recognise pathogenic proteins, so it is possible that EBV expresses an uncharacterised DNAM-1 ligand in lytic cycle.

This study makes a significant contribution to the knowledge of the basic immunology of EBV infection by greatly extending our knowledge of the interaction of innate responses to virus-infected cells. The discovery of BHRF1 as a *bona fide* immune evasion gene capable of protecting cells from NK cell killing may also have wider implications. Although not examined, the mechanism of action implies that BHRF1 might also afford significant protection against EBV-specific cytotoxic $CD8^+$ and $CD4^+$ T cells.

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References

1. **Longnecker, R., E. Kieff, and J. Cohen.** 2013. Epstein-Barr Virus, p. 1898-1959. *In* D. Knipe and P. Howley (ed.), Fields Virology. Lippincott Williams & Wilkins.

2. **Thorley-Lawson, D. A., J. B. Hawkins, S. I. Tracy, and M. Shapiro.** 2013. The pathogenesis of Epstein-Barr virus persistent infection. Current opinion in virology **3:**227-232.

3. **Rickinson, A. B., H. M. Long, U. Palendira, C. Munz, and A. D. Hislop.** 2014. Cellular immune controls over Epstein-Barr virus infection: new lessons from the clinic and the laboratory. Trends in immunology **35:**159-169.

4. **Balfour, H. H., Jr., O. A. Odumade, D. O. Schmeling, B. D. Mullan, J. A. Ed, J. A. Knight, H. E. Vezina, W. Thomas, and K. A. Hogquist.** 2013. Behavioral, virologic, and immunologic factors associated with acquisition and severity of primary Epstein-Barr virus infection in university students. The Journal of infectious diseases **207:**80-88.

5. **Williams, H., K. McAulay, K. F. Macsween, N. J. Gallacher, C. D. Higgins, N. Harrison, A. J. Swerdlow, and D. H. Crawford.** 2005. The immune response to primary EBV infection: a role for natural killer cells. British journal of haematology **129:**266-274.

6. **Parvaneh, N., A. H. Filipovich, and A. Borkhardt.** 2013. Primary immunodeficiencies predisposed to Epstein-Barr virus-driven haematological diseases. British journal of haematology **162:**573-586.

7. **Chijioke, O., A. Muller, R. Feederle, M. H. Barros, C. Krieg, V. Emmel, E. Marcenaro, C. S. Leung, O. Antsiferova, V. Landtwing, W. Bossart, A. Moretta, R. Hassan, O. Boyman, G. Niedobitek, H. J. Delecluse, R. Capaul,**

and C. Munz. 2013. Human natural killer cells prevent infectious mononucleosis features by targeting lytic Epstein-Barr virus infection. Cell reports **5:**1489-1498.

8. **Ressing, M. E., D. Horst, B. D. Griffin, J. Tellam, J. Zuo, R. Khanna, M. Rowe, and E. J. Wiertz.** 2008. Epstein-Barr virus evasion of CD8(+) and CD4(+) T cell immunity via concerted actions of multiple gene products. Seminars in cancer biology **18:**397-408.

9. **Campbell, T. M., B. P. McSharry, M. Steain, B. Slobedman, and A. Abendroth.** 2015. Varicella Zoster Virus and Herpes Simplex Virus-1 Differentially Modulate NKG2D Ligand Expression During Productive Infection. J Virol.

10. **Grauwet, K., C. Cantoni, M. Parodi, A. De Maria, B. Devriendt, D. Pende, L. Moretta, M. Vitale, and H. W. Favoreel.** 2014. Modulation of CD112 by the alphaherpesvirus gD protein suppresses DNAM-1-dependent NK cell-mediated lysis of infected cells. Proc Natl Acad Sci U S A **111:**16118-16123.

11. **Prod'homme, V., P. Tomasec, C. Cunningham, M. K. Lemberg, R. J. Stanton, B. P. McSharry, E. C. Wang, S. Cuff, B. Martoglio, A. J. Davison, V. M. Braud, and G. W. Wilkinson.** 2012. Human cytomegalovirus UL40 signal peptide regulates cell surface expression of the NK cell ligands HLA-E and gpUL18. J Immunol **188:**2794-2804.

12. **Thomas, M., J. M. Boname, S. Field, S. Nejentsev, M. Salio, V. Cerundolo, M. Wills, and P. J. Lehner.** 2008. Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. Proc Natl Acad Sci U S A **105:**1656-1661.

13. **Chen, X., P. P. Trivedi, B. Ge, K. Krzewski, and J. L. Strominger.** 2007. Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity. Proceedings of the National Academy of Sciences **104:**6329-6334.

14. **Pappworth, I. Y., E. C. Wang, and M. Rowe.** 2007. The switch from latent to productive infection in epstein-barr virus-infected B cells is associated with sensitization to NK cell killing. J Virol **81:**474-482.

15. **Robertson, M. J., K. J. Cochran, C. Cameron, J. M. Le, R. Tantravahi, and J. Ritz.** 1996. Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. Experimental hematology **24:**406-415.

16. **Gong, J. H., G. Maki, and H. G. Klingemann.** 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia **8:**652-658.

17. **Ben-Bassat, H., N. Goldblum, S. Mitrani, T. Goldblum, J. M. Yoffey, M. M. Cohen, Z. Bentwich, B. Ramot, E. Klein, and G. Klein.** 1977. Establishment in continuous culture of a new type of lymphocyte from a "Burkitt like" malignant lymphoma (line D.G.-75). International journal of cancer. Journal international du cancer **19:**27-33.

18. **Leibold, W., T. D. Flanagan, J. Menezes, and G. Klein.** 1975. Induction of Epstein-Barr virus-associated nuclear antigen during in vitro transformation of human lymphoid cells. Journal of the National Cancer Institute **54:**65-68.

19. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol **36:**59-74.

20. **Zuo, J., A. Currin, B. D. Griffin, C. Shannon-Lowe, W. A. Thomas, M. E. Ressing, E. J. Wiertz, and M. Rowe.** 2009. The Epstein-Barr virus G-protein-coupled receptor contributes to immune evasion by targeting MHC class I molecules for degradation. PLoS Pathog **5:**e1000255.

21. **Heemskerk, M. H., M. Hoogeboom, R. Hagedoorn, M. G. Kester, R. Willemze, and J. H. Falkenburg.** 2004. Reprogramming of virus-specific T cells into leukemia-reactive T cells using T cell receptor gene transfer. J Exp Med **199:**885-894.

22. **Young, L. S., R. Lau, M. Rowe, G. Niedobitek, G. Packham, F. Shanahan, D. T. Rowe, D. Greenspan, J. S. Greenspan, A. B. Rickinson, and et al.** 1991. Differentiation-associated expression of the Epstein-Barr virus BZLF1 transactivator protein in oral hairy leukoplakia. J Virol **65:**2868-2874.

23. **Vroman, B., J. Luka, M. Rodriguez, and G. R. Pearson.** 1985. Characterization of a major protein with a molecular weight of 160,000 associated with the viral capsid of Epstein-Barr virus. J Virol **53:**107-113.

24. **Pearson, G. R., J. Luka, L. Petti, J. Sample, M. Birkenbach, D. Braun, and E. Kieff.** 1987. Identification of an Epstein-Barr virus early gene encoding a second component of the restricted early antigen complex. Virology **160:**151-161.

25. **Slee, E. A., C. Adrain, and S. J. Martin.** 2001. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J Biol Chem **276:**7320-7326.

26. **Venkataraman, G. M., D. Suciu, V. Groh, J. M. Boss, and T. Spies.** 2007. Promoter region architecture and transcriptional regulation of the genes for the MHC class I-related chain A and B ligands of NKG2D. J Immunol **178:**961-969.

27. **Zuo, J., W. A. Thomas, T. A. Haigh, L. Fitzsimmons, H. M. Long, A. D. Hislop, G. S. Taylor, and M. Rowe.** 2011. Epstein-Barr virus evades CD4+ T cell responses in lytic cycle through BZLF1-mediated downregulation of CD74 and the cooperation of vBcl-2. PLoS Pathog **7:**e1002455.

28. **Henderson, S., D. Huen, M. Rowe, C. Dawson, G. Johnson, and A. Rickinson.** 1993. Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. Proc Natl Acad Sci U S A **90:**8479-8483.

29. **Kelly, G. L., H. M. Long, J. Stylianou, W. A. Thomas, A. Leese, A. I. Bell, G. W. Bornkamm, J. Mautner, A. B. Rickinson, and M. Rowe.** 2009. An Epstein-Barr virus anti-apoptotic protein constitutively expressed in transformed cells and implicated in burkitt lymphomagenesis: the Wp/BHRF1 link. PLoS Pathog **5:**e1000341.

30. **Azzi, T., A. Lunemann, A. Murer, S. Ueda, V. Beziat, K. J. Malmberg, G. Staubli, C. Gysin, C. Berger, C. Munz, O. Chijioke, and D. Nadal.** 2014. Role for early-differentiated natural killer cells in infectious mononucleosis. Blood **124:**2533-2543.

31. **Rancan, C., L. Schirrmann, C. Huls, R. Zeidler, and A. Moosmann.** 2015. Latent Membrane Protein LMP2A Impairs Recognition of EBV-Infected Cells by CD8+ T Cells. PLoS Pathog **11:**e1004906.

32. **Chaigne-Delalande, B., F. Y. Li, G. M. O'Connor, M. J. Lukacs, P. Jiang, L. Zheng, A. Shatzer, M. Biancalana, S. Pittaluga, H. F. Matthews, T. J. Jancel, J. J. Bleesing, R. A. Marsh, T. W. Kuijpers, K. E. Nichols, C. L. Lucas, S. Nagpal, H. Mehmet, H. C. Su, J. I. Cohen, G. Uzel, and M. J.

Lenardo. 2013. Mg2+ regulates cytotoxic functions of NK and CD8 T cells in chronic EBV infection through NKG2D. Science **341:**186-191.

33. **Bottino, C., R. Castriconi, D. Pende, P. Rivera, M. Nanni, B. Carnemolla, C. Cantoni, J. Grassi, S. Marcenaro, N. Reymond, M. Vitale, L. Moretta, M. Lopez, and A. Moretta.** 2003. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. J Exp Med **198:**557-567.

34. **Ramasubramanyan, S., K. Osborn, R. Al-Mohammad, I. B. Naranjo Perez-Fernandez, J. Zuo, N. Balan, A. Godfrey, H. Patel, G. Peters, M. Rowe, R. G. Jenner, and A. J. Sinclair.** 2015. Epstein-Barr virus transcription factor Zta acts through distal regulatory elements to directly control cellular gene expression. Nucleic acids research **43:**3563-3577.

35. **Yang, J., W. Deng, P. M. Hau, J. Liu, V. M. Lau, A. L. Cheung, M. S. Huen, and S. W. Tsao.** 2015. Epstein-Barr virus BZLF1 protein impairs accumulation of host DNA damage proteins at damage sites in response to DNA damage. Laboratory investigation; a journal of technical methods and pathology.

36. **Gasser, S., S. Orsulic, E. J. Brown, and D. H. Raulet.** 2005. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature **436:**1186-1190.

37. **de Vries, E., H. R. Koene, J. M. Vossen, J. W. Gratama, A. E. von dem Borne, J. L. Waaijer, A. Haraldsson, M. de Haas, and M. J. van Tol.** 1996. Identification of an unusual Fc gamma receptor IIIa (CD16) on natural killer cells in a patient with recurrent infections. Blood **88:**3022-3027.

38. **Grier, J. T., L. R. Forbes, L. Monaco-Shawver, J. Oshinsky, T. P. Atkinson, C. Moody, R. Pandey, K. S. Campbell, and J. S. Orange.** 2012. Human

immunodeficiency-causing mutation defines CD16 in spontaneous NK cell cytotoxicity. J Clin Invest **122:**3769-3780.

39. **Huo, L., M. Y. Jiang, Q. Li, and Y. P. Zhu.** 2015. Novel Association of Killer Cell Immunoglobulin-like Receptor Genes with EBV-infectious Diseases in Children. Biomedical and environmental sciences : BES **28:**303-307.

40. **Qiang, Q., X. Zhengde, L. Chunyan, H. Zhizhuo, X. Junmei, A. Junhong, C. Zheng, J. I. Henter, and S. Kunling.** 2012. Killer cell immunoglobulin-like receptor gene polymorphisms predispose susceptibility to Epstein-Barr virus associated hemophagocytic lymphohistiocytosis in Chinese children. Microbiology and immunology **56:**378-384.

41. **Quinn, L. L., J. Zuo, R. J. Abbott, C. Shannon-Lowe, R. J. Tierney, A. D. Hislop, and M. Rowe.** 2014. Cooperation between Epstein-Barr virus immune evasion proteins spreads protection from CD8+ T cell recognition across all three phases of the lytic cycle. PLoS Pathog **10:**e1004322.

42. **Nachmani, D., N. Stern-Ginossar, R. Sarid, and O. Mandelboim.** 2009. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe **5:**376-385.

43. **Jochum, S., A. Moosmann, S. Lang, W. Hammerschmidt, and R. Zeidler.** 2012. The EBV immunoevasins vIL-10 and BNLF2a protect newly infected B cells from immune recognition and elimination. PLoS Pathog **8:**e1002704.

44. **Wilkinson, G. W., P. Tomasec, R. J. Stanton, M. Armstrong, V. Prod'homme, R. Aicheler, B. P. McSharry, C. R. Rickards, D. Cochrane, S. Llewellyn-Lacey, E. C. Wang, C. A. Griffin, and A. J. Davison.** 2008. Modulation of natural killer cells by human cytomegalovirus. J Clin Virol **41:**206-212.

45. **Ashiru, O., N. J. Bennett, L. H. Boyle, M. Thomas, J. Trowsdale, and M. R. Wills.** 2009. NKG2D ligand MICA is retained in the cis-Golgi apparatus by human cytomegalovirus protein UL142. J Virol **83:**12345-12354.

46. **Fielding, C. A., R. Aicheler, R. J. Stanton, E. C. Wang, S. Han, S. Seirafian, J. Davies, B. P. McSharry, M. P. Weekes, P. R. Antrobus, V. Prod'homme, F. P. Blanchet, D. Sugrue, S. Cuff, D. Roberts, A. J. Davison, P. J. Lehner, G. W. Wilkinson, and P. Tomasec.** 2014. Two novel human cytomegalovirus NK cell evasion functions target MICA for lysosomal degradation. PLoS Pathog **10:**e1004058.

47. **Rolle, A., M. Mousavi-Jazi, M. Eriksson, J. Odeberg, C. Soderberg-Naucler, D. Cosman, K. Karre, and C. Cerboni.** 2003. Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein. J Immunol **171:**902-908.

48. **Spreu, J., T. Stehle, and A. Steinle.** 2006. Human cytomegalovirus-encoded UL16 discriminates MIC molecules by their alpha2 domains. J Immunol **177:**3143-3149.

49. **Prod'homme, V., D. M. Sugrue, R. J. Stanton, A. Nomoto, J. Davies, C. R. Rickards, D. Cochrane, M. Moore, G. W. Wilkinson, and P. Tomasec.** 2010. Human cytomegalovirus UL141 promotes efficient downregulation of the natural killer cell activating ligand CD112. J Gen Virol **91:**2034-2039.

50. **Tomasec, P., E. C. Wang, A. J. Davison, B. Vojtesek, M. Armstrong, C. Griffin, B. P. McSharry, R. J. Morris, S. Llewellyn-Lacey, C. Rickards, A. Nomoto, C. Sinzger, and G. W. Wilkinson.** 2005. Downregulation of natural

killer cell-activating ligand CD155 by human cytomegalovirus UL141. Nat Immunol **6:**181-188.

51. **Prod'homme, V., C. Griffin, R. J. Aicheler, E. C. Wang, B. P. McSharry, C. R. Rickards, R. J. Stanton, L. K. Borysiewicz, M. Lopez-Botet, G. W. Wilkinson, and P. Tomasec.** 2007. The human cytomegalovirus MHC class I homolog UL18 inhibits LIR-1+ but activates LIR-1- NK cells. J Immunol **178:**4473-4481.

Figure Legends

Figure 1. Activating receptors expression profile of NK cell lines and primary NK cells. NKL, NK92, two enriched primary NK cells were stained for NKG2D, DNAM-1, NKp30 and NKp46 surface expression and analyzed using flow cytometry. Solid black lines represent each activating receptor staining and grey-filled histograms represent isotype control.

Figure 2. EBV infected cells undergoing lytic infection are sensitive to NK cell killing. AKBM cells were induced into lytic cycle and used as targets in 4h cytotoxicity assays. (A) Cells were stained for CD19 to differentiate effector and target cells and AKBM cells undergoing lytic infection were identified by GFP expression. Cells were stained for caspase-3 as a marker of NK cell induced killing. NK cell killing was measured in latent and lytic populations at increasing effector target ratios. Effector cells used were: NKL cells (B), NK-92 cells (C) and freshly isolated NK cells (D). NKL cells were incubated with blocking antibodies prior to use in cytotoxicity assays and NK cell killing was measured in the lytic population of AKBM cells at an effector:target ratio of 4:1 (E). Data shown are mean values from three separate experiments, error bars represent standard errors and significance was determined using *t* tests. $P < 0.05$(*) $P < 0.01$(**), $P < 0.001$ (***).

Figure 3. EBV infected cells in late stage lytic cycle are protected from NK cell killing. AKBM cells were induced into lytic cycle and used as targets in 4h cytotoxicity assays using NKL cells. (A) Cells were stained for BZLF1 and BcLF1 to differentiate cells in latent (BZLF1$^-$ BcLF1$^-$), early lytic (BZLF1$^+$ BcLF1$^-$) and late lytic cycle (BZLF1$^+$ BcLF1$^+$). (B) Caspase-3 positivity was assayed in each of the three populations as a

measure of NK cell killing. Data shown are mean values from three separate experiments and error bars represent standard errors.

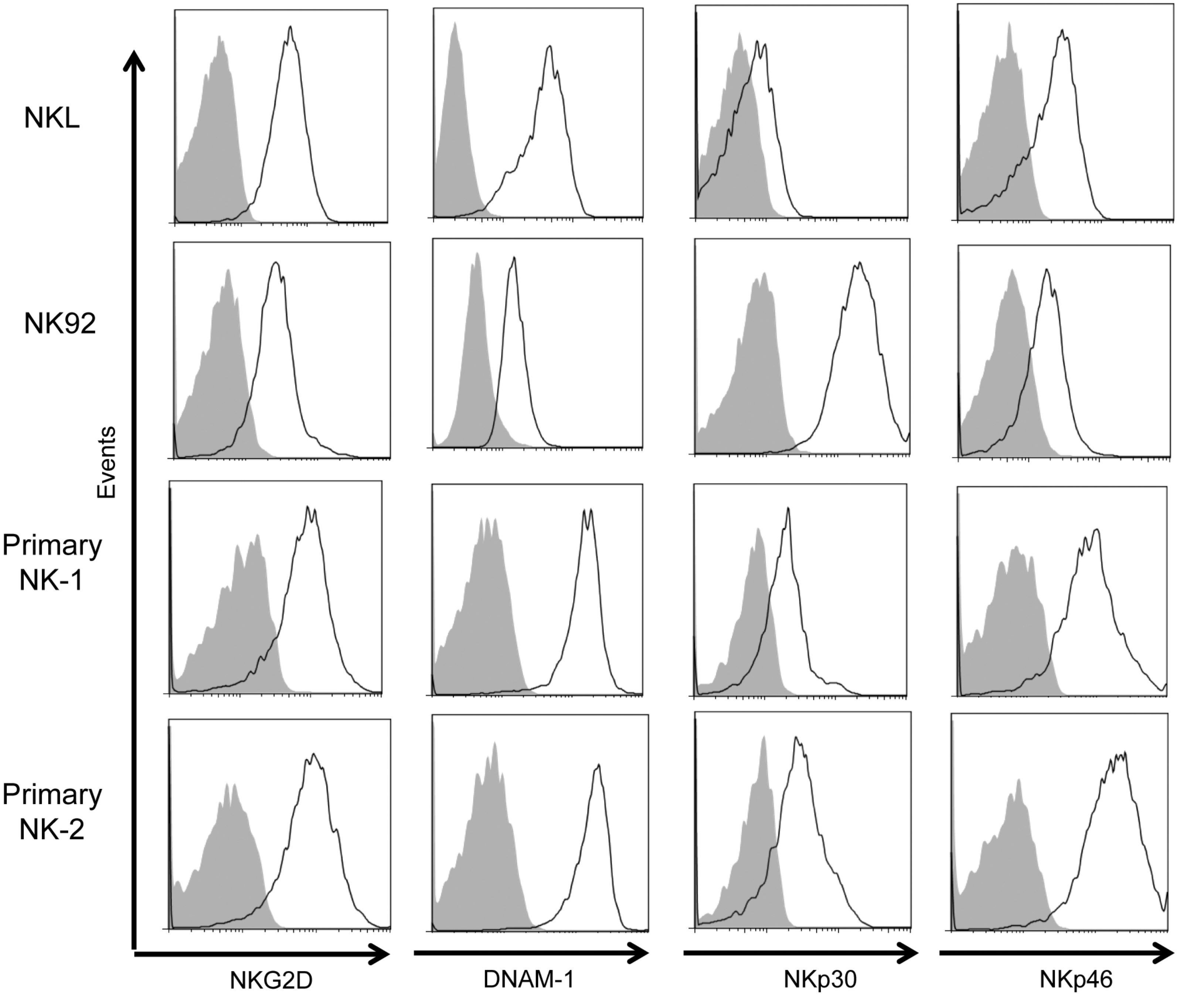
Figure 4. BZLF1 induces expression of NKG2D ligands and sensitizes B cells to NK cell killing. HEK 293 cells (A,B) or DG75 cells (C-F) transiently expressing control-GFP (solid black line), BRLF1-GFP (dashed black line) (A) or BZLF1-GFP (dashed black line) (B-F) were investigated for surface expression of NK cell activating receptor ligands using flow cytometry. Grey-filled histograms represent isotype control staining. Results shown are representative of three separate experiments. (G) (H) Total RNA was isolated from control DG75 and BZLF1 expressing DG75 and then reverse transcribed to cDNA. Relative transcription levels of ULBP2 and ULBP6 were measured by Q-PCR assay, normalized to measured B2m transcripts. Data shown are mean values from three separate experiments, error bars represent standard errors and significance was determined using *t* tests. $P < 0.05$(*) $P < 0.01$(**), $P < 0.001$ (***). (I) DG75 cells transfected with control or BZLF1 expression plasmids were used as targets in NK cell killing assays using NKL cells and specific cytotoxicity was calculated.

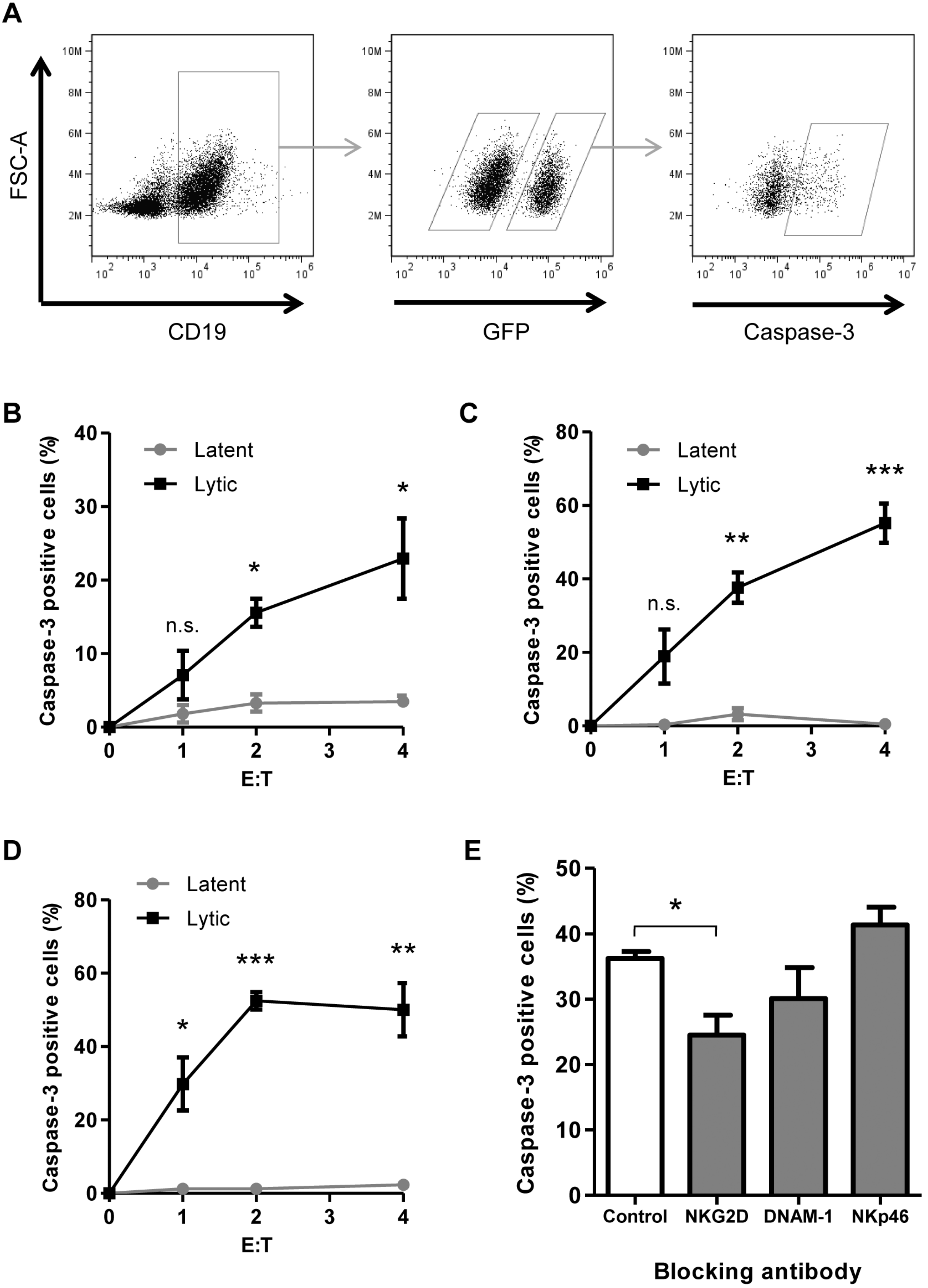
Figure 5. Maximum expression levels of BHRF1 protein occur beyond 12h post-induction of lytic cycle. AKBM cells were induced into lytic cycle by cross-linking of surface immunoglobulin. (A) Levels of BHRF1 (middle) and BZLF1 (upper) protein were measured at time points post-induction (as indicated) using western blot analysis. The level of Calregulin (lower) was detected as a loading control. (B) Relative expression of BHRF1 protein was calculated using Bio-rad Image Lab densitometry software and compared to the Calregulin control at each time point.

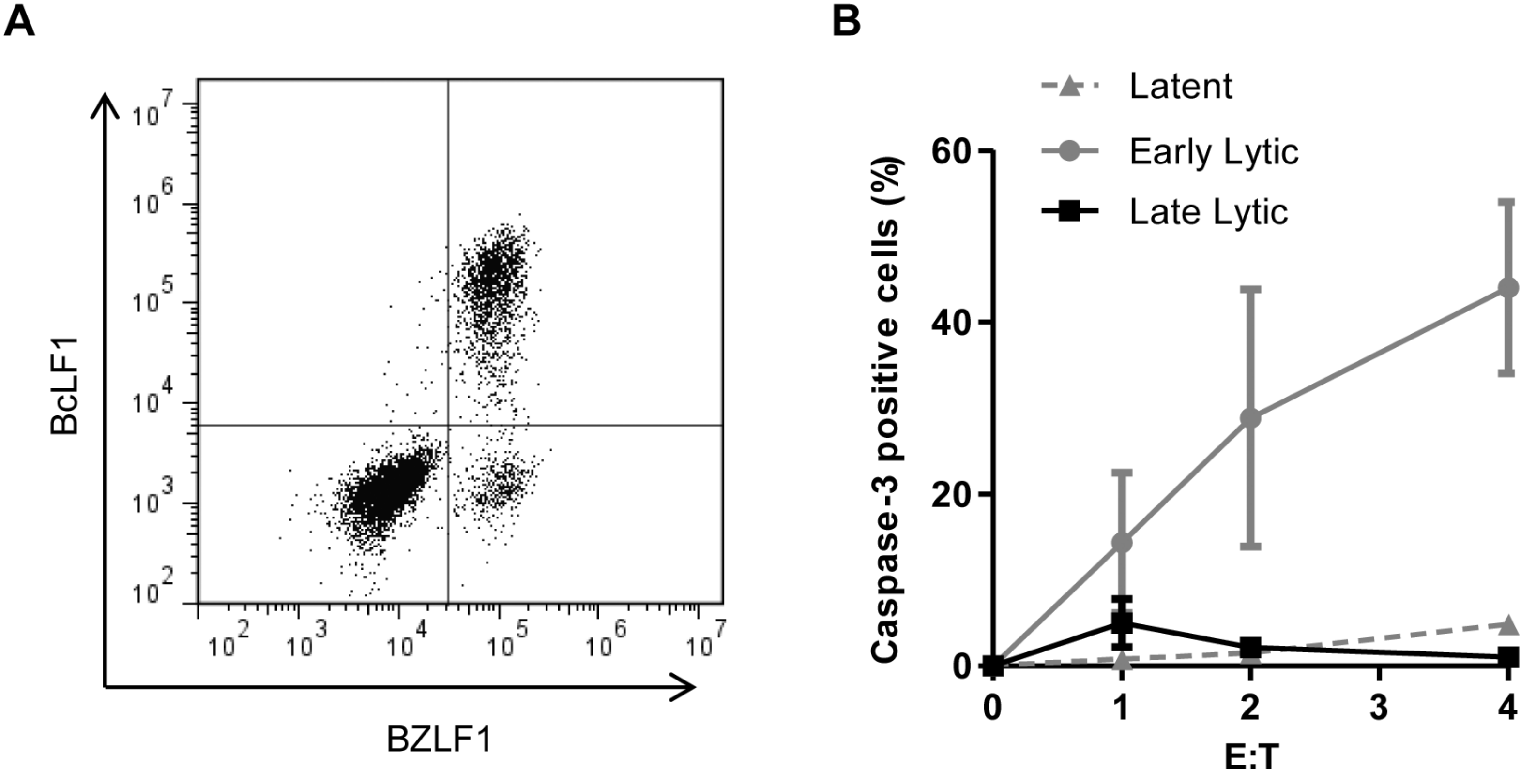
Figure 6. BHRF1 protects B cells from BZLF1 induced NK cell killing. DG75 cells were transduced with control- or BHRF1-NGFR expressing retroviral vectors. (A) Following magnetic enrichment cells were stained for expression of NGFR. Cells were then transfected with control- or BZLF1-GFP expression vectors. (B) Expression of BHRF1 (top) and BZLF1 (middle) protein in the four different cell lines was determined by western blot analysis. Calregulin expression (bottom) was measured as a loading control. The four cell lines were then used as targets in killing assays using NKL cells at increasing effector:target ratios (C), data shown are mean values from three separate experiments and error bars represent standard errors. (D) Surface expression of ULBP was measured on DG75-control cells (grey-filled histograms), DG75-control cells expressing BZLF1 (solid black line) and DG75-BHRF1 cells expressing BZLF1 (dashed black line), data shown is representative of three separate experiments. (E) The four DG75 cell lines mentioned above were co-cultured NKL cells and FITC conjugated anti-CD107a antibody for 5 hours. The surface CD107a expression of NKL cells from four cultures was analyzed by flow cytometry. Data shown are mean values from three separate experiments and error bars represent standard errors. The significance was determined using one way ANOVA tests. $P < 0.05$(*) $P < 0.01$(**).

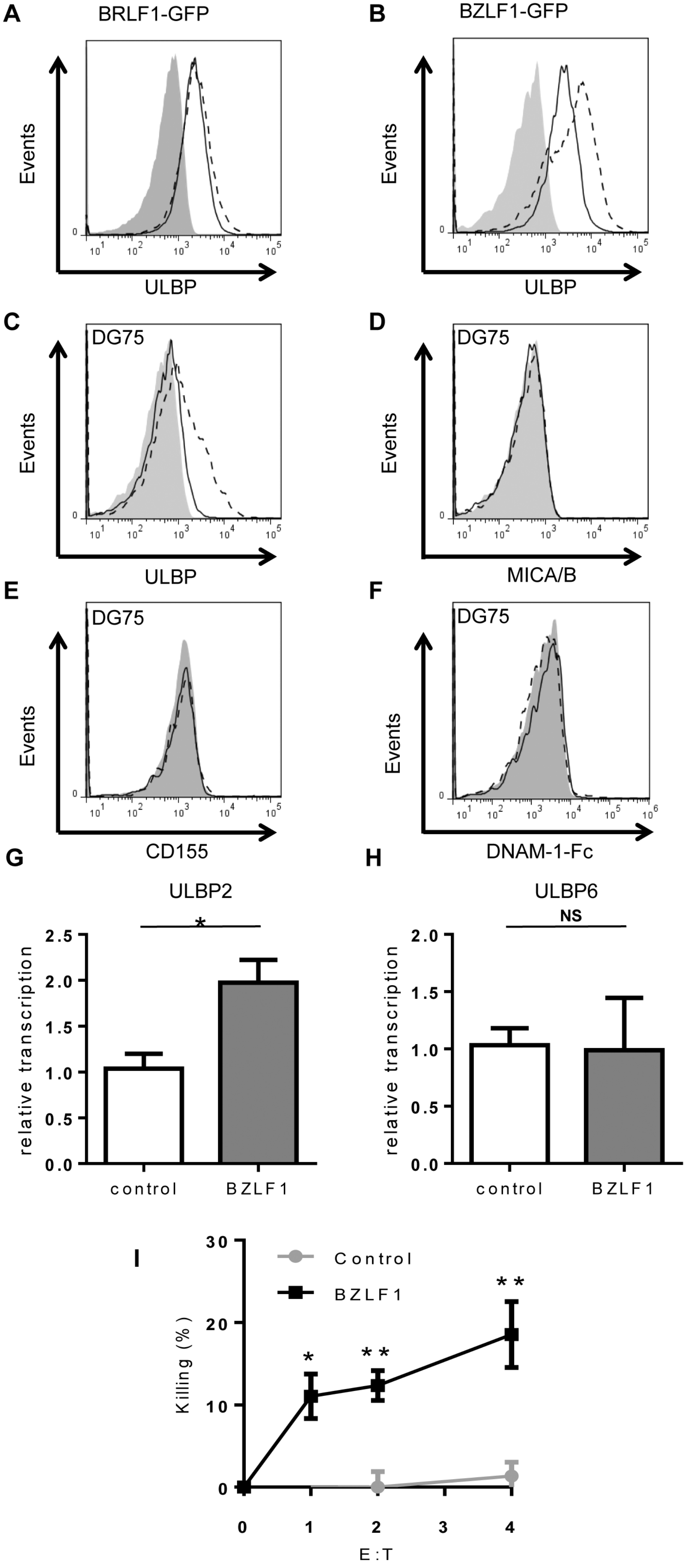
Figure 7. LCLs are also protected from NK cell killing in late stage lytic cycle but killing of cells in early lytic cycle is mediated by DNAM-1. LCLs were screened for the presence of cells undergoing spontaneous lytic cycle and used as targets in 4h cytotoxicity assays using NKL cells. Cells were stained for BZLF1 and BcLF1 to differentiate latent, early lytic and late lytic cells and stained for caspase-3 as a marker

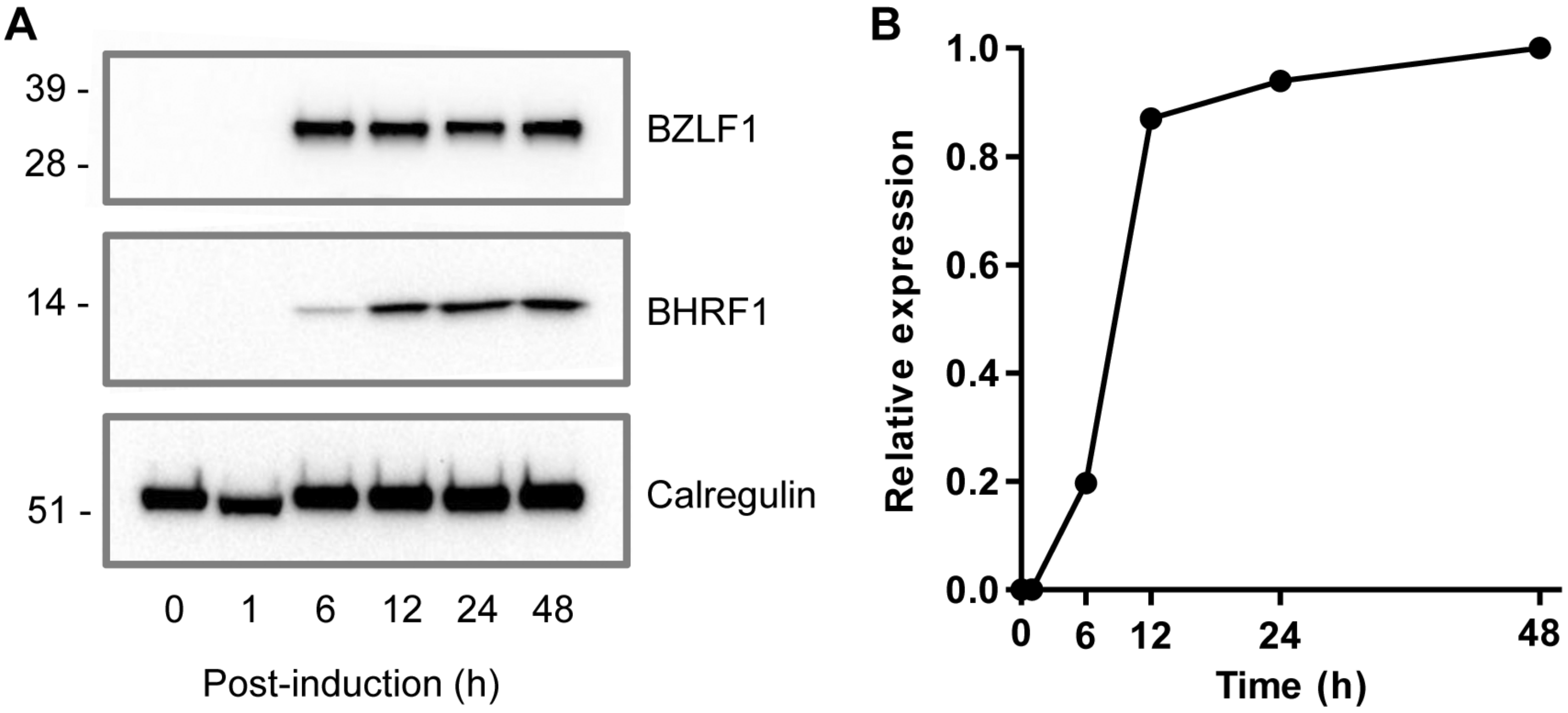
of NK cell induced killing. (A) NK cell killing was measured in the three populations at increasing effector target ratios. (B) NKL cells were incubated with blocking antibodies prior to use in cytotoxicity assays and NK cell killing measured in the early lytic population of LCLs at an effector:target ratio of 4:1. Data shown are mean values from three separate experiments using four different LCLs, error bars represent standard errors and significance was determined using *t* tests. $P < 0.01$(**). LCLs were stained for BZLF1 to detect cells undergoing spontaneous lytic cycle and levels of MICA/B (C), ULBP (D), CD155 (E) and CD112 (F) were measured by flow cytometry. Solid black lines represent BZLF$^-$ (latent cells), dashed black lines represent BZLF1$^+$ (lytic cells) and grey-filled histograms represent isotype control staining of bulk LCLs. HeLa cells were used a positive control for CD155 expression (E) and K562 cells were used as a positive control for MICA/B, ULBP and CD112 expression (C,D,F). Results shown are representative of multiple separate experiments using multiple antibodies to CD155 and CD112. (G) Total RNA was isolated from LCLs lines and then reverse transcripted to cDNA. Relative transcription levels of CD112 and CD155 were measured by Q-PCR assay, normalized to measured B2m transcripts. The error bars represent standard errors of three different LCLs lines. Hela cells were served as a standard for relative transcription in this assay. (H) LCLs were stained for BZLF1 to detect cells undergoing spontaneous lytic cycle and levels of DNAM-1 ligands were measured using DNAM-1-Fc fusion protein by flow cytometry. Solid black lines represent BZLF- (latent cells), dashed black lines represent BZLF1+ (lytic cells) and grey-filled histograms represent isotype control staining of bulk LCLs. K562 cells were used as a positive control.

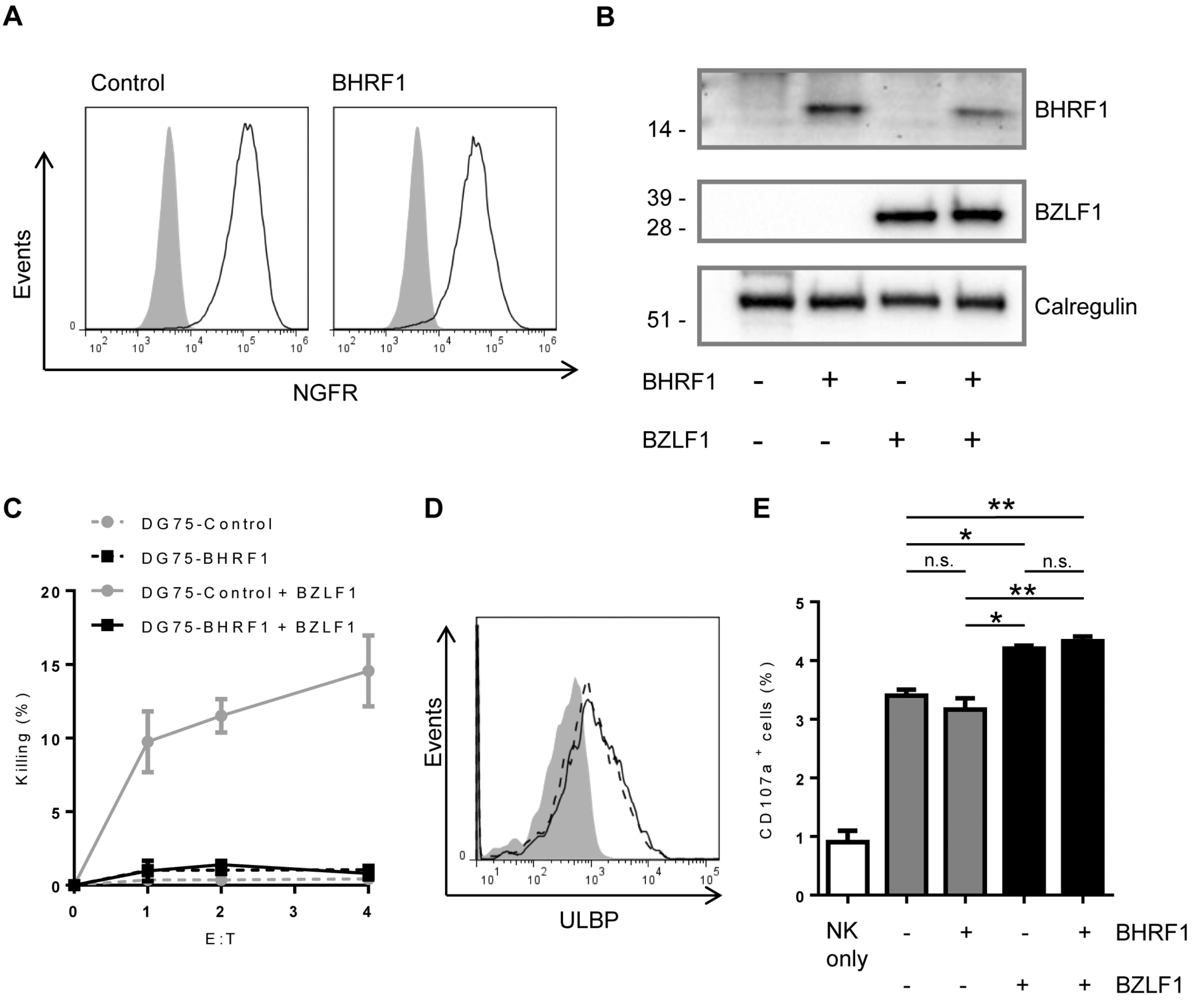

NKL
NK92
Primary NK-1
Primary NK-2
Events
NKG2D
DNAM-1
NKp30
NKp46

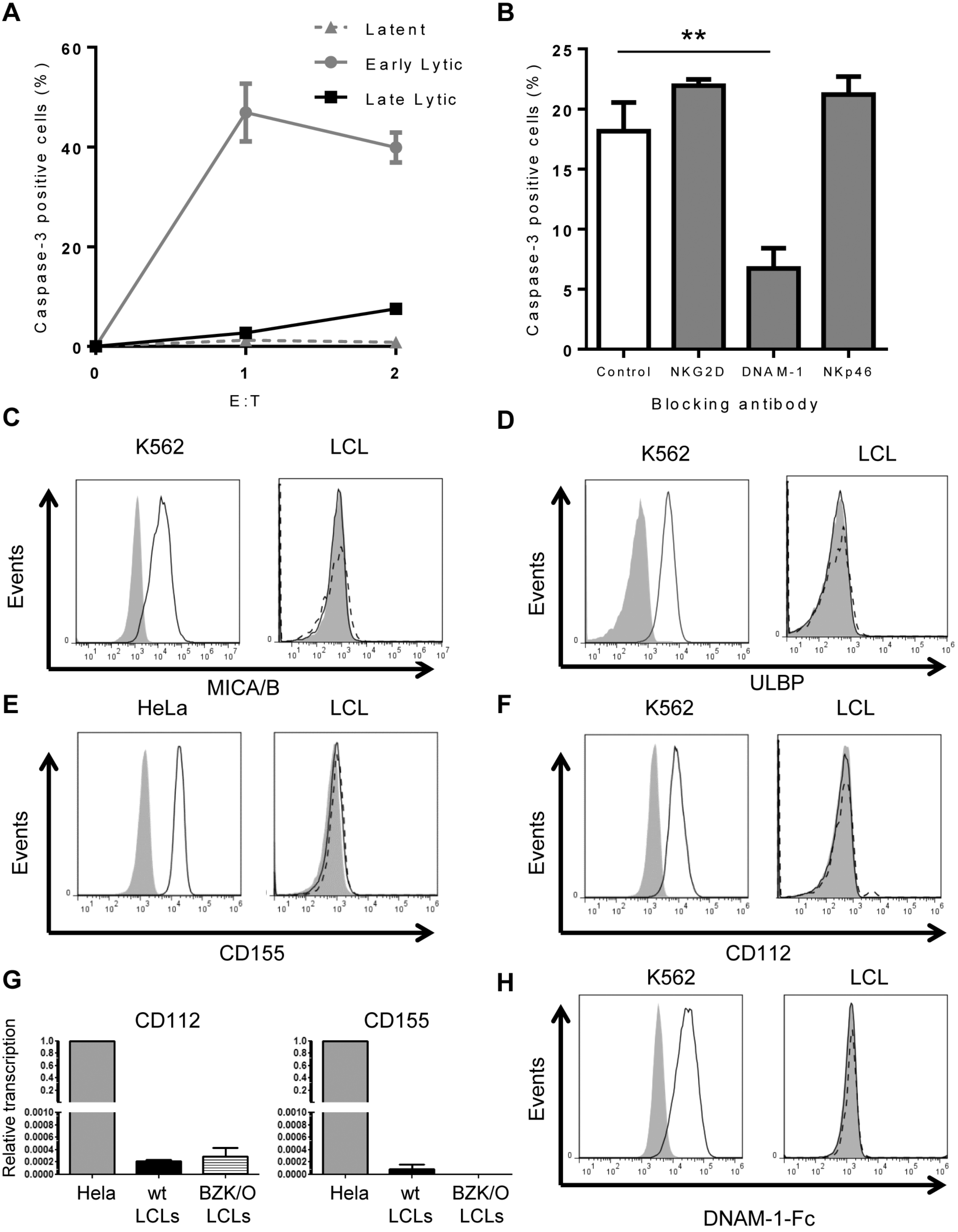

A
FSC-A
10M
8M
6M
4M
2M
CD19
GFP
Caspase-3
B
C
D
Caspase-3 positive cells (%)
Latent
Lytic
n.s.
*
**
***
E:T
0
1
2
3
4
E
Caspase-3 positive cells (%)
*
Control
NKG2D
DNAM-1
NKp46
Blocking antibody


A
BcLF1
BZLF1
10^2
10^3
10^4
10^5
10^6
10^7
B
Latent
Early Lytic
Late Lytic
Caspase-3 positive cells (%)
60
40
20
0
1
2
3
4
E:T


A
BRLF1-GFP
Events
ULBP
B
BZLF1-GFP
Events
ULBP
C
DG75
Events
ULBP
D
DG75
Events
MICA/B
E
DG75
Events
CD155
F
DG75
Events
DNAM-1-Fc
0
10^1
10^2
10^3
10^4
10^5
10^6
G
ULBP2
*
relative transcription
0.0
0.5
1.0
1.5
2.0
2.5
control
BZLF1
H
ULBP6
NS
relative transcription
0.0
0.5
1.0
1.5
2.0
control
BZLF1
I
Control
BZLF1
Killing (%)
0
10
20
30
*
**
**
0
1
2
3
4
E:T


A
39 -
28 -
BZLF1
14 -
BHRF1
Calregulin
51 -
0
1
6
12
24
48
Post-induction (h)
B
Relative expression
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
24
48
Time (h)


A
Control
BHRF1
Events
NGFR
0
10^2
10^3
10^4
10^5
10^6
B
14 -
39 -
28 -
51 -
BHRF1
BZLF1
Calregulin
BHRF1 - + - +
BZLF1 - - + +
C
DG75-Control
DG75-BHRF1
DG75-Control + BZLF1
DG75-BHRF1 + BZLF1
Killing (%)
0
5
10
15
20
E:T
0
1
2
3
4
D
Events
ULBP
0
10^1
10^2
10^3
10^4
10^5
E
CD107a$^+$ cells (%)
0
1
2
3
4
5
**
*
n.s.
n.s.
**
*
NK only
- + - + BHRF1
- - + + BZLF1


A
Latent
Early Lytic
Late Lytic
Caspase-3 positive cells (%)
0
20
40
60
0
1
2
E:T
B
**
Caspase-3 positive cells (%)
0
5
10
15
20
25
Control
NKG2D
DNAM-1
NKp46
Blocking antibody
C
K562
LCL
Events
MICA/B
D
K562
LCL
Events
ULBP
E
HeLa
LCL
Events
CD155
F
K562
LCL
Events
CD112
G
CD112
CD155
Relative transcription
1.0
0.8
0.6
0.4
0.2
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
Hela
wt LCLs
BZK/O LCLs
H
K562
LCL
Events
DNAM-1-Fc