EBV LMP2A provides a surrogate pre-B cell receptor signal through constitutive activation of the ERK/MAPK pathway

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Latent membrane protein 2A (LMP2A) of Epstein–Barr virus (EBV) provides developmental and survival signals that mimic those of a B-cell receptor (BCR). Expression of LMP2A during B-cell development results in the ability of B cells to exit the bone marrow in the absence of a BCR and persist in the periphery, where they would normally undergo apoptosis. This study extends the current knowledge of LMP2A function by examining the growth properties of bone marrow B cells from TgE LMP2A mice. Despite the lack of pre-BCR expression, bone marrow B cells from TgE LMP2A mice proliferate and survive in low concentrations of interleukin 7, similar to wild-type cells. Constitutive phosphorylation of ERK/MAPK and PI3K/Akt in TgE LMP2A bone marrow B cells is also reminiscent of signalling through the pre-BCR, altogether demonstrating that LMP2A provides a pre-BCR-like signal to developing B cells.

Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus that infects >90% of the adult population (Kieff & Rickinson, 2007). While EBV infections are typically asymptomatic, disease in adolescents presents as infectious mononucleosis and immunocompromised patients are susceptible to lymphoproliferative disorders (Rickinson & Kieff, 2007; Thorley-Lawson, 2005; Thorley-Lawson & Gross, 2004). EBV is associated with malignancies of lymphoid and epithelial origin, including Hodgkin’s lymphoma, Burkitt’s lymphoma and nasopharyngeal carcinoma (Rickinson & Kieff, 2007). As is characteristic of herpesviruses, EBV is able to persist in the human host through the establishment of a lifelong latent infection. EBV establishes latency in vitro in B lymphocytes by limiting viral gene expression to a subset of genes which includes EBV nuclear antigens 1, 2, 3A, 3B, 3C and LP (EBNAs), latent membrane protein 1 (LMP1) and latent membrane protein 2 (LMP2A) (Kieff & Rickinson, 2007). Early studies indicated LMP2A functions in viral latency by altering normal B-cell receptor (BCR) signalling (Miller et al., 1993, 1994, 1995).

The transgenic mouse model used by our laboratory has been invaluable in elucidating the function of LMP2A in vivo. LMP2A expression in vivo interferes with normal B-cell development and allows BCR-negative cells to exit the bone marrow and colonize peripheral lymphoid organs (Caldwell et al., 1998, 2000). Normally, successful immunoglobulin heavy-chain (IgH) rearrangement is necessary for the transition from the CD19+/CD43+ pre-BI stage to the CD19+/CD43− pre-BII stage of development in the bone marrow. Subsequently, the light-chain genes are rearranged and the complex between the light-chain and heavy-chain forms the BCR expressed on the cell surface. Expression of a BCR allows the B cell to transition to the IgM+ immature B-cell stage and migrate out of the bone marrow into the periphery. Unsuccessful rearrangement of the immunoglobulin genes causes the B cell to undergo apoptosis (Era et al., 1991; Hardy et al., 1991). The striking phenotype of the TgE LMP2A transgenic mice is the lack of expression of surface IgM on B cells in the bone marrow and spleen. Additionally, although TgE LMP2A mice are unable to rearrange the heavy-chain genes necessary for expression of a pre-BCR or a BCR, they are able to transition to a CD43− stage, albeit less efficiently than a wild-type B cell (Caldwell et al., 1998, 2000). These data indicate that LMP2A functions as a surrogate BCR to allow for survival of IgM− cells in the periphery.

For a more thorough understanding of B-cell biology in TgE LMP2A mice, the growth properties of the bone marrow B cells were examined. Bone marrow from TgE LMP2A mice and wild-type (WT) controls was grown in methylcellulose containing interleukin 7 (IL-7) for 7 days (Ikeda & Longnecker, 2005). Cells were harvested and stained with fluorophore-conjugated antibodies for analysis by flow cytometry. For comparison, Rag2−/− IL-7-dependent cell cultures were used, as these cells are pre-BCR−/IgM−/CD43+ (Corfe et al., 2007). Cell cultures derived from the bone marrow of TgE LMP2A and WT mice are predominantly B cells that express CD43 (Fig. 1, 84 and 85%, respectively). To extend the finding that the B...
cells of TgE LMP2A mice bypass developmental checkpoints, an antibody that recognizes the pre-BCR (μ IgH, λ5 and V preB) was used (Fig. 1). Forty percent of the bone marrow cells from wild-type mice express a pre-BCR, in contrast to barely detectable levels in TgE LMP2A and Rag2−/− cells. These data show that, not only do TgE LMP2A mice lack a BCR; they are also lacking a pre-BCR. Therefore, LMP2A may provide signals that mimic those normally provided by the pre-BCR.

In the bone marrow, pro-B cells proliferate in response to high concentrations of IL-7, whereas once a B cell acquires a pre-BCR the concentration of IL-7 required for proliferation is decreased (Fleming & Paige, 2002). B cells from the bone marrow of Rag-deficient mice are unable to rearrange their immunoglobulin genes, and therefore do not express a pre-BCR on the cell surface and require high concentrations of IL-7 to survive and proliferate (Fleming & Paige, 2001, 2002; Marshall et al., 1998). To determine whether LMP2A provides a pre-BCR signal to bone marrow B cells, proliferation in varying concentrations of IL-7 was examined. TgE LMP2A and WT bone marrow B cells were expanded for 7 days in methylcellulose, as above. Rag2−/− cells were maintained in liquid culture containing IL-7 after CD19 selection as described previously (Corfe et al., 2007). Equal numbers of cells were plated in varying dilutions of IL-7 supernatant (from J558 hybridoma cells) and incubated at 37 °C. After 3 days, proliferation was measured by tritiated thymidine incorporation (Swanson-Mungerson et al., 2005). As shown in Fig. 2(a), Rag2−/− cells proliferated in response to high concentrations of IL-7 (0.1 and 0.05 dilutions), whereas the cells were unable to proliferate in low concentrations of IL-7 (0.01–0.001 dilutions). In contrast, both TgE LMP2A and WT bone marrow B cells proliferated in low concentrations of IL-7 at

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**Fig. 1.** Phenotype of bone marrow cells. Bone marrow B cells from WT, TgE LMP2A and Rag2−/− mice were stained with fluorophore-conjugated antibodies specific for B-cell development markers, as indicated on the x and y-axes, and analysed by flow cytometry. Plots show the live cell-gated population and relative percentages for double positive cells. Data shown are representative of three independent experiments.

**Fig. 2.** TgE LMP2A bone marrow B cells proliferate and survive as well as pre-BCR+ wild-type cells in low concentrations of IL-7. (a) Proliferation of bone marrow B cells was measured by tritiated thymidine incorporation. Relative proliferation was calculated compared with the proliferation in the highest concentration of IL-7 for wild-type cells and plotted against IL-7 dilution on a log scale. The average of two independent experiments is shown ± SD. (b and c) Cells plated in high (black bars) or low concentrations (white bars) of IL-7 were stained with Annexin V and analysed by flow cytometry. The percentage of cells in the live cell gate, based on forward and side scatter properties, was recorded and plotted as relative survival after 48 h (b). The percentage of Annexin V-positive cells was recorded after 48 h (c). Results are the average of three independent experiments ± SD.
similar levels and had increased proliferation at all IL-7 concentrations compared with Rag2\(^{-/-}\) cells. Approximately half the B cells in the WT cultures are pre-BCR\(^{+}\), while B cells in TgE LMP2A cultures are essentially pre-BCR\(^{+}\), indicating that LMP2A provides the necessary pre-BCR signals to allow for proliferation in low IL-7. As expected, incubating WT and TgE LMP2A cells in the presence of 15 \(\mu\)M PD98059 MEK inhibitor resulted in decreased proliferation for both cell types at all concentrations of IL-7, and treatment with LY294002 completely inhibited proliferation, as PI3K is required for IL-7-dependent mitogenic responses (data not shown) (Corcoran et al., 1996).

Rag2\(^{-/-}\) cells are unable to survive in low concentrations of IL-7, as demonstrated by measuring cell recovery (Fleming & Paige, 2001; Marshall et al., 1998). We compared the survival capacity of methylcellulose-derived bone marrow B cells from TgE LMP2A and WT mice with Rag2\(^{-/-}\) cells. In Fig. 2(b, c), equal numbers of cells were plated in high or low IL-7 and assayed after 48 h for survival. Relative survival (Fig. 2b) represents the recovery of live cells based on forward and side-scatter properties on the flow cytometer. For each cell type, the number of events in the live gate for high IL-7 was set to 100\% relative survival and the live gate for low IL-7 was normalized to this value. For TgE LMP2A and WT cells, approximately 80\% of cells remained alive after 48 h in low IL-7 compared with high IL-7. This is in contrast with the results from Rag2\(^{-/-}\) cells in low IL-7, where only approximately 40\% of cells were alive. Cells were also stained with Annexin V (according to the manufacturer’s protocol) and analysed by flow cytometry to determine the number of apoptotic cells in each culture. In agreement with the relative survival data, Fig. 2(c) shows approximately 15\% (WT) and 10\% (TgE LMP2A) of the cells in low IL-7 bind Annexin V, demonstrating that very few cells are apoptotic. In contrast, approximately 50\% of the Rag2\(^{-/-}\) cells bind Annexin V when cultured in low IL-7; therefore half of the cells are undergoing apoptosis. That TgE LMP2A B-cells are able to survive in low IL-7 without expression of a pre-BCR further supports that LMP2A provides signals that mimic those normally provided by the pre-BCR.

It has been suggested that the ability of B cells to proliferate depends upon reaching a threshold of ERK activation. This threshold of phosphorylated ERK can be achieved either through high concentrations of IL-7 available for signalling through the IL-7 receptor, or tonic signalling through the pre-BCR (Bannish et al., 2001; Fleming & Paige, 2001; Fuentes-Panana et al., 2004; Marshall et al., 1998; Shaffer & Schlissel, 1997; Teh & Neuberger, 1997). TgE LMP2A bone marrow B cells can proliferate in low IL-7 in the absence of a pre-BCR; therefore we examined the levels of phosphorylated ERK. Cells were washed and starved in the presence of DMSO or inhibitor, and restimulated after 3 h as described previously (Fleming & Paige, 2001). TgE LMP2A cells have constitutively activated ERK (P-ERK) independent of the presence of IL-7, similar to pre-BCR\(^{+}\) WT cells (Fig. 3). P-ERK induction for WT cells appears higher than TgE LMP2A cells due to the unequal loading between cell types, evident by comparing the levels of total ERK for each. Rag2\(^{-/-}\) cells that have been starved do not express P-ERK unless they are restimulated with IL-7. This result is specific, as treatment with an inhibitor of the ERK/MAPK pathway, 15 \(\mu\)M PD98059 MEK inhibitor, decreases the amount of phosphorylated ERK in both TgE LMP2A and WT cells independent of IL-7 stimulation. Additionally, treatment with an inhibitor of the PI3K pathway, 20 \(\mu\)M LY294002, results in decreased phosphorylated ERK and Akt, indicating that TgE LMP2A and WT cells use similar signalling pathways to activate ERK/MAPK. This result is consistent with previous results that demonstrate inhibition of PI3K activity blocks ERK activation, probably through PLC\(_{\gamma}\) (Jacob et al., 2002). These results suggest that LMP2A in TgE cells supplies a signal that mimics signalling by the pre-BCR through activation of the ERK/MAPK and PI3K pathway, and provides a plausible explanation for proliferation in low concentrations of IL-7.

TgE LMP2A bone marrow B cells have a cell surface marker phenotype that resembles B cells derived from Rag2\(^{-/-}\) bone marrow. However, the similarities between the two cell types end here. The proliferation and survival response of TgE LMP2A bone marrow B cells to IL-7 more closely mimics that of WT bone marrow B cells, which have begun

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**Fig. 3.** LMP2A constitutively activates the ERK/MAPK and PI3K/Akt pathways in bone marrow B cells. Bone marrow B cells from WT, TgE LMP2A or Rag2\(^{-/-}\) mice were starved in the presence of inhibitor or DMSO. Following starvation, cells were left untreated (−) or activated with IL-7 or F(ab\(^{-}\))\(^{2}\) anti-mouse \(\mu\)HC (Ig) for 20 min. ‘Unt’ indicates cells were not treated in any way during the experiment. Western blot analysis was performed on whole-cell lysates; data are representative of four independent experiments.
the pre-B cell transition and express a pre-BCR. Additionally, signalling cascades that are activated by LMP2A in bone marrow B cells are similar to those activated by pre-BCR signalling as well as IL-7 stimulation, in contrast to the Rag-deficient cells that are dependent on IL-7 for signalling. Altogether, these results indicate that LMP2A provides a surrogate pre-BCR signal that contributes to its ability to promote aberrant B-cell development in the TgE LMP2A mice.

The amino-terminus of LMP2A contains two serine residues, S15 and S102, which are conserved among related γ-herpesviruses (Longnecker, 2000). LMP2A is serine phosphorylated and has been demonstrated to be a substrate for phosphorylation by ERK/MAPK (Longnecker et al., 1991; Panousis & Rowe, 1997). Additionally, a glutathione S-transferase fusion to the amino-terminal 112 amino acids of LMP2A can interact with ERK/MAPK; however, the outcome of this interaction in the context of LMP2A function in B cells has yet to be determined (Panousis & Rowe, 1997). In epithelial cells, LMP2A activates ERK/MAPK and promotes mobility, which may be important for metastasis (Chen et al., 2002). Data demonstrating that LMP2A constitutively activates the ERK/MAPK pathway in bone marrow B cells similar to pre-BCR+ wild-type B cells provide evidence that this pathway may be important for LMP2A-mediated proliferation in vivo. Indirectly, this suggests that LMP2A may use the ERK/MAPK interaction to provide proliferation signals to B cells; however, systems to study LMP2A-mediated proliferation amenable to mutational analysis are currently lacking. With the advent of new culture systems, it will be interesting to investigate whether S15 and S102 are important for LMP2A function.

Pre-BCR-dependent processes include IgH allelic exclusion, IL-7-dependent pre-B cell expansion, maturation through the pre-B to pre-B checkpoint and IgL recombination (Monroe, 2006). Ig-z and Ig-β are both necessary and sufficient for pre-BCR signal transduction, and Src family protein tyrosine kinases (PTK) including Blk, Fyn and Lyn are involved in the pre-BCR signal (Monroe, 2006). It is interesting to note that, like Ig-z and Ig-β, LMP2A contains an ITAM and expression of LMP2A in B cells leads to the constitutive activation of Src family PTKs (Burkhardt et al., 1992; Fruehling et al., 1996, 1998; Fruehling & Longnecker, 1997). Therefore, it is not surprising that, with the exception of IgH allelic exclusion, LMP2A mediates pre-BCR-dependent processes. It has long been predicted that LMP2A may provide a surrogate pre-BCR signal because, in transgenic mice expressing LMP2A, heavy chain rearrangement is bypassed and IgM cells exit the bone marrow (Caldwell et al., 1998).

Additionally, expression of LMP2A during development leads to a downregulation of genes critical for the proper formation of a pre-BCR including V preB, Rag2, mb-1 (Ig-z) and β2, as shown by microarray analysis (Portis et al., 2003). B cells in LMP2A TgE mice also express increased levels of Bcl-xL, an anti-apoptotic Bcl-2 family member that is normally upregulated when the pre-BCR is formed during B-cell development (Fang et al., 1996). It has been proposed that Bcl-xL functions to maintain cell survival during the formation of a pre-BCR, and it is possible that LMP2A maintains Bcl-xL expression levels by providing pre-BCR-like signals (Portis & Longnecker, 2004). Data presented here demonstrate that LMP2A indeed elicits a pre-BCR-like signal to developing B cells, providing signals for proliferation and survival through the ERK/MAPK and PI3K/Akt pathways. These observations allow for the conclusion that LMP2A is sufficient to fulfill or bypass the known processes that are dependent upon a pre-BCR signal.

Several studies have demonstrated that the function of LMP2A and the outcome of expression depend upon the cellular context (Caldwell et al., 1998; Fukuda & Longnecker, 2005; Konishi et al., 2001; Lu et al., 2006; Mancao & Hammerschmidt, 2007; Miller et al., 1993, 1994; Morrison et al., 2003; Portis et al., 2003; Scholle et al., 2000; Swanson-Mungerson et al., 2005, 2006). The common theme among these studies, however, is a role for LMP2A in signalling for aberrant survival and/or proliferation. LMP2A is consistently detected in EBV-associated malignancies, including Hodgkin’s lymphoma, nasopharyngeal carcinoma, post-transplant lymphoproliferative disease and Burkitt’s lymphoma (Bell et al., 2006; Konishi et al., 2001; Rechsteiner et al., 2007; Rickinson & Kieff, 2007; Tao et al., 1998; Xue et al., 2002). Although LMP2A is not considered a true viral oncogene, evidence that LMP2A promotes survival and proliferation in the absence of a growth factor such as IL-7 implicates a role for LMP2A in the maintenance or progression of malignancies. One of the key steps in lymphomagenesis is often to abrogate cytokine dependence. In fact, constitutive activation of MEK, a downstream kinase in the ERK/MAPK pathway, can lead to abrogation of cytokine dependence in cell lines (Steelman et al., 2004). That LMP2A constitutively activates the ERK/MAPK pathway further supports the idea that LMP2A plays a more active role in lymphomagenesis, and is not just an innocent bystander. The constitutive activation of the ERK/MAPK and PI3K/Akt pathways in bone marrow B cells adds to a growing list of signal transduction pathways involved in proliferation and survival that are activated by LMP2A. Altogether, this suggests that, perhaps in functioning during viral latency, LMP2A inadvertently contributes to EBV-associated malignancies.

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