Epstein–Barr virus (EBV) latently infected B-cells are the precursors of EBV-associated malignancies. EBV-infection induces the production of pro-survival and anti-inflammatory cytokines that may be important in the transition between latency and malignancy. One EBV protein, LMP2A, can be detected in both latently infected resting B-cells and in EBV-associated malignancies. Therefore, we tested the ability of LMP2A to influence cytokine production using both LMP2A-Tg primary B-cells and LMP2A-expressing B-cell lines. Our data demonstrate that LMP2A does not globally alter B-cell-produced cytokine levels, but specifically targets IL-10.

Additional studies using ELISA and real-time-RT-PCR confirm that LMP2A utilizes PI3-kinase to increase IL-10 levels. Finally, the data demonstrate that LMP2A-expressing B-cell lines are more dependent on IL-10 for survival in comparison to LMP2A-negative B-cell lines. These data identify a novel function of LMP2A in the alteration of a cytokine that is important for both tumour survival and anti-tumour responses.

Epstein–Barr virus (EBV) maintains a latent state with the expression of very few proteins in memory B-cells and EBV-associated lymphomas. The EBV protein, latent membrane protein 2A (LMP2A), can be detected in both latent and pathological states (Decker et al. 1996; Niedobitek et al. 1997; Babcock et al. 1998, 2000, 2001; Hochberg et al. 2004; Bell et al. 2006). The expression of LMP2A at multiple stages of EBV infection suggests that LMP2A plays an important role in both EBV latency and EBV-associated pathology. Previous studies demonstrate that LMP2A promotes B-cell survival in resting B-cells and pre-tumour B-cells through increases in Bcl family members (Bechtel et al. 2005; Mancao & Hammerschmidt 2007; Bultema et al. 2009; Swanson-Mungerson et al. 2010). The survival of B-cells and B-cell lymphomas is also regulated in part by cytokines (Banchereau et al. 1993; Brennan, 2001) and it is under-appreciated that B-cells are capable of producing cytokines to promote their own survival and proliferation in an autocrine manner (O’Garra & Vieira, 2007). Previous studies indicate that EBV infection modulates B-cell cytokine production (Samanta et al., 2008; Konforte & Paige, 2009; Tsai et al., 2009). However, to date there is no published data testing the possibility that LMP2A directly regulates B-cell cytokine production to modulate B-cell proliferation, survival, lymphoma generation, and/or pathology. Therefore, we initially tested if LMP2A influences cytokine production using B-cells from LMP2A-Tg mice (Caldwell et al., 1998). Lipopolysaccharide (LPS) or an activating anti-CD40 mAb was used in these experiments, since both stimuli induce cytokine production by B-cells (O’Garra et al., 1990; O’Garra & Howard, 1992; Burdin et al., 1995). Purified CD19+ B-cells from both non-transgenic (non-Tg) and LMP2A-Tg mice were stimulated with either LPS or anti-CD40 mAb to induce cytokine production for 48 h and supernatants were analysed using a mouse ELISArray (SA Biosciences). The results suggest that LMP2A does not globally influence B-cell cytokine production (Table S1, available in JGV Online), but rather, that LMP2A increases IL-10 production approximately twofold in response to both LPS- and CD40-stimulation. The finding that IL-10 is specifically enhanced by LMP2A is intriguing due to the role of IL-10 in both dampening antiviral immune responses and promoting the survival of B-cell lymphomas (Cortes & Kurzrock, 1997). To confirm that LMP2A increases mitogen-induced IL-10 production using the LMP2A-Tg mouse model, purified non-Tg and LMP2A-Tg B-cells were cultured in the absence or presence of either LPS or anti-CD40 mAb and IL-10 levels were determined by either ELISA or quantitative real-time-RT-PCR. These data
confirm that LMP2A-Tg B-cells produce more IL-10 protein (Fig. 1a) and RNA (Fig. 1b) in response to LPS or anti-CD40 mAb when compared with non-Tg B-cells.

Previous studies indicate that LPS increases the proliferation of LMP2A-Tg B-cells (Ikeda et al., 2004). Thus, the LMP2A-dependent increase in IL-10 in Fig. 1(a) could be due to the fact that the LMP2A-Tg B-cells are hyperproliferative to LPS, resulting in more B-cells present to produce IL-10. To rule out this interpretation, we tested if LMP2A increases the amount of IL-10 made per B-cell after LPS exposure by flow cytometry. When intracellular IL-10 levels are assessed, LMP2A-Tg B-cells demonstrate a significantly higher mean fluorescence intensity than non-Tg B-cells (Fig. 1c), indicating that LMP2A-Tg B-cells produce more IL-10 per cell. Taken together, these data indicate that LMP2A directly increases IL-10 production in mitogen-stimulated primary B-cells.

Since LMP2A is also identified in EBV-associated tumours (Niedobitek et al., 1997; Bell et al., 2006), we sought to determine if LMP2A increases IL-10 production in human B-cell lymphomas. Since our transgenic B-cells required stimulation to induce cytokine production, we tested if LMP2A affects LPS-induced cytokine production, including IL-10, in a human B-cell lymphoma. For these studies, we used an established LMP2A-expressing BJAB cell line (BJAB-LMP2A.1) and the control cell line that contains only the vector (BJAB-Vector.1) (Miller et al., 1993). In a manner similar to LMP2A-Tg B-cells, LMP2A does not

**Fig. 1.** LMP2A increases IL-10 production by LPS- or CD40-activated B-cells. CD19⁺ B-cells from LMP2A-Tg or non-Tg littermates were isolated (Swanson-Mungerson et al., 2006) and cultured in the absence or presence of 1 μg ml⁻¹ LPS or anti-mouse CD40 mAb. Supernatants were harvested 48 h later and analysed by (a) ELISA (eBioscience) as per manufacturer’s instructions. (b) CD19⁺ B-cells were isolated and cultured as above before RNA isolation, followed by equal amounts of RNA used for reverse transcription (qScript cDNA Supermix; Quanta BioSciences) according to manufacturer’s instructions. cDNA was amplified using murine-specific IL-10 or GAPDH primers as previously described (de Waal Malefyt et al., 1991; Enk & Katz, 1992) with a SybrGreen PCR master mix. Relative gene expression was determined by the ΔΔCt method, and results are expressed as the fold-increase above levels detected in LPS- or CD40-stimulated non-Tg B-cells. (c) Non-Tg and LMP2A-Tg B-cells were cultured as described above with Golgi-stop (BD Biosciences) added for the final 4 h. Intracellular IL-10 staining was performed as suggested by the manufacturer (BD Biosciences), followed by flow cytometric analysis on a FACSCalibur instrument. BLD, Below the level of detection. The data represent three experiments with similar results. All error bars are indicative of SEM. An asterisk (*) indicates a P-value of <0.05 using a two-tailed t-test when compared to non-Tg B-cells stimulated with the same mitogen.
modulate any LPS-induced cytokines, except for LPS-induced IL-10 (Table S1). Subsequent experiments confirm that LMP2A increases LPS-induced IL-10 at both the protein and RNA level (data not shown). Additionally, we tested if LMP2A increases basal cytokine production in LMP2A-expressing B-cell lines. BJAB-Vector.1 and BJAB-LMP2A.1 cells were incubated for 48 h in the absence of any stimuli and supernatants were analysed by ELISA (eBioscience). As shown in Table S1, LMP2A enhances basal IL-10 production in unstimulated BJAB cells. Subsequent experiments using both ELISA and quantitative RT-PCR demonstrate that LMP2A increases basal IL-10 levels at both the protein (Fig. 2a) and RNA levels (Fig. 2c). To confirm that the LMP2A-mediated increase in IL-10 is not specific to BJAB cells, similar experiments were performed using an LMP2A-expressing Ramos cell line (Fukuda & Longnecker, 2004).
LMP2A again increased basal levels of IL-10 at both the protein (Fig. 2b) and RNA levels (Fig. 2d) in the Ramos cell line. Taken together, these data confirm that LMP2A increases basal IL-10 levels in B-cell lymphomas.

To rule out the possibility that the cell lines used in Fig. 2 express significantly higher LMP2A levels than those found naturally after EBV transformation, as in a lymphoblastoid cell line (LCL), we compared LMP2A levels by immunofluorescence and did not see any significant difference between the LCL, LCL1 (Ikeda & Longnecker, 2007), the BJAB-LMP2A.1 cells or the Ramos-LMP2A cells (Fig. 2e).

Additionally, the ELISArray implicated that LMP2A increases TGF-β production in unstimulated BJAB-LMP2A.1 B-cells (Table S1). However, these findings were not confirmed in later experiments (data not shown). The fact that LMP2A did not increase the levels of TGF-β suggest that LMP2A is not driving the differentiation of these B-cells to a IL-10⁺TGF-β⁺ 'regulatory B-cell' phenotype (Mizoguchi & Bhan, 2006).

IL-10 is a cytokine with pleotropic effects on the immune system. IL-10 not only dampens cellular immune responses in the environment of tumours, but also enhances the survival and proliferation of B-cell tumours in vivo (Beatty et al., 1997; Cortes & Kurzrock, 1997; Khatri & Caligiuri, 1998; Vockerodt et al., 2001). We initially assessed if LMP2A increases proliferation of the BJAB cell line. As shown in Fig. 3(a), our data indicate that the level of proliferation in BJAB-LMP2A.1 cells is equivalent to the level of proliferation in BJAB-Vector.1 cells. These findings are consistent with previous studies, indicating that LMP2A does not significantly affect the proliferation of transformed B-cells in vitro and in vivo (Longnecker et al., 1992; Rochford et al., 1997). Therefore, we tested the possibility that the increase in basal IL-10 levels in BJAB-LMP2A.1 cells significantly enhances the survival of these cells. BJAB-Vector.1 and BJAB-LMP2A.1 cells were incubated in the presence of a neutralizing IL-10 antibody or isotype control antibody for 24 h and the percentage of apoptotic cells was determined by Annexin-V staining. LMP2A-expressing cells demonstrate a significant decrease in the percentage of cells that are Annexin-V-positive (Fig. 3b–c), suggesting that LMP2A increases the survival of these cells. However, the addition of a neutralizing IL-10 antibody to BJAB-LMP2A.1 cells increases the percentage of Annexin-V-positive cells to levels found in BJAB-Vector.1 cells (Fig. 3b–c), suggesting that the LMP2A-mediated increase in IL-10 is important for promoting the survival of these cells.

It initially is surprising that the LMP2A-mediated increase in survival did not translate to increases in the number of cells in Fig. 3(a). However, the percentage of Annexin-V-positive cells are low in our assay; therefore, the decrease in Annexin-V-positive cells probably does not translate into differences in the overall number of cells detected by our proliferation assay at 48 h. However, it is likely that an LMP2A⁺ tumour in vivo expressing IL-10 would have a significant advantage by promoting its own survival and dampening anti-tumour immune responses.

IL-10 production in B-cells is modulated by numerous signal transduction pathways, including PI3-kinase (PI3K) (Lambert & Martinez, 2007; O’Garra & Vieira, 2007). LMP2A constitutively activates the Ras/PI3K pathway (Portis & Longnecker, 2004) and therefore, experiments were designed to analyse if LMP2A utilizes PI3K to modulate basal levels of IL-10 production. The addition of the PI3K inhibitor, LY294002, significantly decreases IL-10 protein levels in both the BJAB-LMP2A.1 and Ramos-LMP2A cell lines (Fig. 3d–e), suggesting that LMP2A requires the activation of PI3K to augment the basal levels of IL-10.

While the PI3K inhibitor decreases IL-10 production in the BJAB-LMP2A.1 cells at both the protein and RNA levels (Fig. 3d, f), the PI3K inhibitor only slightly inhibits IL-10 protein (Fig. 3d) and not IL-10 RNA (Fig. 3f) in the BJAB-Vector.1 cells. IL-10 is regulated at both the transcriptional and post-transcriptional levels (Saraiva & O’Garra, 2010) and there is a paucity of data analysing the regulatory mechanisms responsible for influencing IL-10 production in B-lymphocytes. Our data point to the possibility that LMP2A regulates IL-10 production in a distinct manner from IL-10 regulation in transformed B-cells that lack LMP2A. If this is the case, it is possible that drugs directed at novel regulatory mechanisms employed by LMP2A may help target LMP2A-positive, IL-10-producing tumour cells, such as lymphoma cells from post-transplant lymphoproliferative disease (PTLD).

The data presented here may have important clinical relevance, since a recent finding highlights the importance of IL-10 in EBV-positive cell lines derived from PTLD patients (Hatton et al., 2011). This report demonstrates that the addition of a Syk inhibitor to PTLD cell lines leads to a decrease in IL-10 levels and the apoptosis of PTLD cells (Hatton et al., 2011). Furthermore, the data demonstrate that Syk directly increases PI3K levels that are important for PTLD cell survival (Hatton et al., 2011). The authors of this study infer that the addition of the Syk inhibitor directly acts on LMP2A signalling and that LMP2A is responsible for increases in IL-10 production in the PTLD cell lines. However, the data in this report are the first to demonstrate that LMP2A directly increases IL-10 (Figs 1 and 2) to promote the survival of B-cell lymphoma lines (Fig. 3).

Since IL-10 promotes the survival of EBV-associated tumours, these data provide an exciting new potential mechanism by which EBV may promote tumour development and survival. It is interesting to note that IL-10 appears to be extremely important throughout EBV infection. During initial infection, EBV expresses the gene BCRF-1 that encodes an EBV viral homologue of human IL-10 (Rode et al., 1994). EBV vIL-10 has a much lower affinity for the IL-10R (Yoon et al., 2012) and demonstrates altered signalling through the IL-10R to increase proliferation (Liu et al., 2011).
Fig. 3. LMP2A-mediated increases in IL-10 influence B-cell survival, but not proliferation. (a) BJAB-Vector.1 and BJAB-LMP2A.1 (1×10⁶) cells were incubated for 48 h and analysed using Cell Titre 96 proliferation assay (Promega). (b–c) BJAB-Vector.1 and BJAB-LMP2A.1 (1×10⁶) cells were incubated in the presence of 1 μg ml⁻¹ of a neutralizing anti-human IL-10 mAb (clone JES3-9D7) or an isotype control (rat IgG1; Biolegend). After 24 h, the cells were stained with Annexin-V (Biolegend) and analysed on a FACSCalibur. The data in (a) and (c) are a combination of three experiments. (d–f) BJAB or Ramos cells were incubated as described in Fig. 2 in the absence or presence of LY294002 (10 μM) for 48 h. ELISA (d–e) and real-time-RT-PCR (f) were performed as described in Fig. 2. The data in (d–f) represent two to three experiments with similar results. All error bars are indicative of SEM. An asterisk (*) indicates a P-value of <0.05 using a two-tailed t-test when compared to control cells lacking LMP2A. A double asterisk (**) indicates a P-value of <0.05 using a two-tailed t-test when compared to cells not exposed to a neutralizing antibody (c) or PI3K inhibitor (d–f).
et al., 1997; Yoon et al., 2012) and to protect infected B-cells from immune recognition (Jochum et al., 2012). However, since both LMP2A and another EBV latency protein, LMP1, increase IL-10 production (Lambert & Martinez, 2007), it suggests that EBV continues to modulate IL-10 production during latency to protect EBV-infected B-cells from immune recognition and promote B-cell survival. This redundancy implies that the modulation of IL-10 could be a critical function of EBV latency proteins in the generation and maintenance of EBV-associated tumours. Future studies that identify downstream targets of LMP2A that are responsible for increasing IL-10 may elucidate potential new therapies against EBV-associated diseases.

Acknowledgements

This work is funded by NIH grant no. 1R15CA149690-01, the Biomedical Sciences Program in the College of Health Sciences, and Midwestern University Intramural Funds.

References


Rode, H. J., Bugert, J. J., Handermann, M., Schnitzler, P., Kehm, R., Janssen, W., Delius, H. & Darai, G. (1994). Molecular characterization and determination of the coding capacity of the genome of equine herpesvirus type 2 between the genome coordinates 0.235 and 0.258 (the EcoRI DNA fragment N; 4.2 kbp). Virus Genes 9, 61–75.


