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Epstein-Barr Virus Latent Membrane Protein 2A (LMP2A) enhances IL-10 production through the activation of Bruton's tyrosine kinase and STAT3

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ABSTRACT

Previous data demonstrate that Epstein-Barr Virus Latent Membrane Protein 2A (LMP2A) enhances IL-10 to promote the survival of LMP2A-expressing B cell lymphomas. Since STAT3 is an important regulator of IL-10 production, we hypothesized that LMP2A activates a signal transduction cascade that increases STAT3 phosphorylation to enhance IL-10. Using LMP2A-negative and –positive B cell lines, the data indicate that LMP2A requires the early signaling molecules of the Syk/RAS/PI3K pathway to increase IL-10. Additional studies indicate that the PI3K-regulated kinase, BTK, is responsible for phosphorylating STAT3, which ultimately mediates the LMP2A-dependent increase in IL-10. These data are the first to show that LMP2A signaling results in STAT3 phosphorylation in B cells through a PI3K/BTK-dependent pathway. With the use of BTK and STAT3 inhibitors to treat B cell lymphomas in clinical trials, these findings highlight the possibility of using new pharmaceutical approaches to treat EBV-associated lymphomas that express LMP2A.

1. Introduction

Epstein-Barr virus (EBV) is a member of the gamma herpesvirus family that infects B lymphocytes in more than 90% of the world population (Kang and Kieff, 2015; Kempkes and Robertson, 2015). While most infections are asymptomatic or result in infectious mononucleosis (Thorley-Lawson et al., 2013), EBV is associated with the development of multiple autoimmune diseases and lymphomas of the immune system (Ascherio and Munger, 2015; Thorley-Lawson and Gross, 2004). One mechanism by which Epstein-Barr virus could contribute to these diseases is by influencing B cell function and survival.

After initial infection, EBV transitions to a latent state in which few viral genes are expressed. There are multiple latency gene patterns identified in either normal latency and/or EBV-associated pathology (Price and Luftig, 2015). The EBV latency protein Latent membrane protein 2A (LMP2A), which contains 12 transmembrane domains with a long amino terminal domain, is expressed in multiple programs of EBV latency (Babcock et al., 1998; Babcock et al., 2000, 2001; Bell et al., 2006; Decker et al., 1996; Hochberg et al., 2004; Niedobitek et al., 1997), suggesting the importance of this protein in normal latency and EBV-associated diseases. LMP2A acts as a B cell receptor (BCR) mimic to increase the survival of latently-infected B cells (Mancao et al., 2005; Mancao and Hammerschmidt, 2007; Portis and Longnecker, 2004). Previous studies indicate that LMP2A constitutively activates many of the kinases and signal transduction molecules used by the BCR, including Syk, Ras, PI3K, BTK, and AKT (Fruehling and Longnecker, 1997; Merchant and Longnecker, 2001; Portis and Longnecker, 2004) to promote B cell survival (Merchant and Longnecker, 2001; Portis and Longnecker, 2004). Additional studies demonstrate that LMP2A signaling in B cells directly results in an increase in anti-apoptotic factors, such as BCL-2 and BCL-xL (Bultema et al., 2009; Portis and Longnecker, 2004; Swanson-Mungerson et al., 2010). More recently, it has become appreciated that LMP2A indirectly promotes B cell survival by increasing the production of pro-survival cytokines, such as IL-10 (Incrocci, McCormack, and Swanson-Mungerson, 2013). Due to the redundant expression of LMP2A throughout many phases of the EBV life cycle, targeting its pro-survival abilities in EBV-associated tumors may be of therapeutic benefit.

Pharmacological therapies to treat tumors typically induce the death of cells that are rapidly proliferating or by blocking signal transduction pathways that directly increase tumor cell survival (Dominguez-Brauer et al., 2015; Pistritto et al., 2016). However, an alternative approach may be to block the production of pro-survival

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factors, such as IL-10. Inhibiting the LMP2A-dependent increase in IL-10 that promotes tumor survival may provide a potential novel approach to enhance current chemotherapeutic strategies for EBV-associated lymphomas. Therefore, we sought to identify the signals required for LMP2A to increase IL-10 production in B cell lymphomas. Our findings indicate for the first time, that LMP2A activates BTK to phosphorylate STAT3 in B cell tumors, which mediates the LMP2A-dependent increase in IL-10. Due to the identification of new therapeutics that target BTK and STAT3 in clinical trials, these findings have important implications for innovative treatments of LMP2A-expressing B cell tumors.

2. Materials and methods

2.1. Cell lines

All B cell lines used in this study have been described previously (Ikeda and Longnecker, 2007). Briefly, the BJAB B cell lymphoma line was transduced with either the vector backbone alone or the vector backbone with LMP2A. Transduced cells were selected using hygromycin and gentamycin and LMP2A expression was identified in all selected cells by immunofluorescence and found to be similar in levels when compared to lymphoblastoid cell lines (Incrocci et al., 2013). Independent clones were isolated and maintained in cRPMI media supplemented with hygromycin (0.4 μg/ml) (EMD Millipore) and gentamycin (2 μg/ml) (Sigma Aldrich) at 37 °C/5% CO_2. The lymphoblastoid cell lines LCL3 (LMP2A-positive) and ES1 (LMP2A-negative) were generously provided by Richard Longnecker (Northwestern University-Chicago, Illinois) and were maintained in cRPMI at 37 °C/5% CO_2.

2.2. Analysis of IL-10 production

5×10^4 LMP2A-negative or LMP2A-positive B cell lines described above were grown in a 96-well plate in the absence or presence of an optimized concentration of the following pharmacological inhibitors: Syk (R788-EMD Millipore, 5 μm), Ras (Manumycin A-EMD Millipore, 0.5 μm), PI3K (Wortmannin-EMD Millipore, 10 μm), STAT3 (Stattic-EMD Millipore, up to 1.75 μm), or BTK (Ibrutinib-Selleckchem, up to 10 μm). All inhibitors were initially diluted in DMSO and final dilutions were reached in cRPMI. The cells that were not exposed to inhibitor were exposed to an equivalent amount of DMSO to control for the potential effects of DMSO alone in all experiments. After 24 h, 20 μl of supernatants were isolated for use in an LDH assay (Sigma-Aldrich, St. Louis MO) to analyze inhibitor toxicity and 100 μl of supernatants were isolated for analysis using the Human IL-10 Ready, Set, Go® ELISA kit (Ebioscience). All of the inhibitors used in the study did not induce toxicity at 24 h as determined by LDH assay (data not shown), confirming that any differences seen in IL-10 levels was not due to toxicity of the assay.

2.3. Western Blot analysis for STAT3

All B cell lines (10×10^6) were incubated for 24 h in the absence or presence of Syk (5 μm), Ras (Manumycin A- 0.5 μm), Wortmannin (10 μm), Ibrutinib (10 μm) or Stattic (1.75 μm) and then lysed in RIPA buffer (Pierce, Rockford IL) containing 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS, along with Halt™ Protease and Phosphatase inhibitors (Pierce, Rockford IL). Protein concentrations from cell lysates were quantified using a BCA assay (Pierce, Rockford IL) and equal amounts of protein were analyzed by Western blot. Antibodies against phosphorylated- and total-STAT3 (Cell Signaling Technology, Massachusetts) or GAPDH (Biolegend, San Diego, CA) were diluted 1:1000 in blocking buffer containing milk, followed by washes in TBST. A secondary anti-rabbit IgG-HRP conjugated Ab (Pierce, Rockford IL) diluted in blocking buffer (1:5000) was added to the blot followed by ECL imaging (GE Healthcare, Buckinghamshire UK) using a Bio-Rad imager. Image analysis and quantification was done using ImageJ software (National Institutes of Health). The ratio of phosphorylated STAT3 to Total STAT3 was determined by dividing the value of the band for phosphorylated STAT3 according to ImageJ software by the value of the band for total STAT3 under different culture conditions.

2.4. Statistics

Each experiment was performed at least three times. All ELISA experiments were initially analyzed by a two-way analysis of variance (ANOVA), followed by a Bonferonni post hoc test to compare individual groups using Prism Graphpad software. All experiments that demonstrated a $p < 0.05$ by ANOVA and only findings that reached $p < 0.05$ by Bonferroni comparisons were considered significant.

3. Results

LMP2A is a BCR mimic that directly promotes B cell survival through a RAS/PI3K dependent pathway (Portis and Longnecker, 2004) and indirectly by inducing IL-10 production in both LMP2A-expressing primary murine transgenic B cells and in B cell lymphomas (Incrocci et al., 2013). An additional study at the time indicated that the Syk inhibitor, R406, blocked the enhancement of IL-10 production in EBV-positive tumor cells from post-transplant lymphoproliferative disease (PTLD) patients (Hatton et al., 2011). These studies suggested that LMP2A uses Syk to increase IL-10. However, PTLD cells express numerous EBV latency proteins and therefore we sought to confirm that LMP2A uses Syk to increase IL-10 by using multiple B cell lines that only express LMP2A. Two independently-derived B cell lines that express LMP2A (LMP2A1.1 and LMP2A1.2) and two LMP2A-negative control cell lines (Vector.1 and Vector.2) were exposed to the Syk inhibitor (R788, Fostamatinib) for 24 h and IL-10 production was analyzed using ELISA. As shown in Fig. 1A, the addition of the Syk inhibitor significantly decreased the LMP2A-dependent increase in IL-10 production. Additionally, the Syk inhibitor did not significantly affect IL-10 production by LMP2A-negative B cell lines, indicating that this effect is specific for LMP2A. Since Syk activation leads to Ras stimulation during BCR signaling (Beitz et al., 1999; Mocsai et al., 2010), we next tested if LMP2A required Ras to increase IL-10 production. As shown in Fig. 1B, the addition of the Ras inhibitor, Manumycin A, also decreased the LMP2A-dependent increase in IL-10 production, without significantly affecting IL-10 production in the LMP2A-negative cell lines. One downstream target of Ras activation is p38K phosphorylation and signaling (Shin et al., 2005). Since p38K controls IL-10 regulation in monocytes, macrophages, and dendritic cells (Chi et al., 2006; Foey et al., 1998; Jarnicki et al., 2008; Kim et al., 2005), we initially tested if LMP2A activates p38K in the LMP2A-expressing B cells used in this study. However, Western blot analysis of protein lysates from LMP2A-negative and –positive B cell lines indicate that LMP2A does not increase p38K phosphorylation (data not shown). Therefore, the Ras-mediated increase in IL-10 production must be via an alternative pathway downstream of Ras.

Ras leads to the activation and signaling via PI3K in B cells. Our previous work using the PI3K inhibitor, Ly294002, suggested that LMP2A utilizes PI3K to increase IL-10 production (Incrocci et al., 2013). However, due to the identification that Ly294002 can also affect NF-kB activation (Avni et al., 2012), we wanted to confirm our previous findings through the use of the more PI3K-specific inhibitor Wortmannin (Avni et al., 2012). Therefore, LMP2A-expressing and non-expressing B cell lines were incubated in the absence or presence of Wortmannin for 24 h and IL-10 levels were once again analyzed by ELISA. As shown in Fig. 1C, Wortmannin decreased the LMP2A-dependent increase in IL-10 production, confirming that LMP2A requires PI3K activation to increase IL-10 levels.

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