RESEARCH ARTICLE

Transcriptome Changes Induced by Epstein-Barr Virus LMP1 and LMP2A in Transgenic Lymphocytes and Lymphoma

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ABSTRACT **Latent membrane protein 1 (LMP1) and LMP2A affect cell growth in both epithelial cells and lymphocytes. In this study, the effects on cellular gene expression were determined by microarray analysis of transgenic mice expressing LMP1, LMP2A, or both using the immunoglobulin heavy chain promoter and enhancer. Large differential changes were detected, indicating that LMP1 and LMP2A can both potently affect host gene transcription, inducing distinct transcriptional profiles. Seventy percent of the changes detected in LMP1/2A doubly transgenic lymphocytes were also modulated by LMP1 or LMP2A alone. These common and unique expression changes indicate that the combined effects of LMP1 and LMP2A may be additive, synergistic, or inhibitory. Using significant pathway analysis, the expression changes detected in LMP1, LMP2A, and LMP1/2A transgenic B lymphocytes were predicted to commonly target cancer and inflammatory pathways. Additionally, using the correlation coefficient to calculate the regulation of known c-Rel and Stat3 transcriptional targets, both were found to be enhanced in LMP1 lymphocytes and lymphomas, and a selection of Stat3 targets was further evaluated and confirmed using quantitative reverse transcription-PCR (RT-PCR). Analyses of the effects on cell growth and viability revealed that LMP2A transgenic lymphocytes had the greatest enhanced viability *in vitro*; however, doubly transgenic lymphocytes (LMP1/2A) did not have enhanced survival in culture and these mice were similar to negative littermates. These findings indicate that the combined expression of LMP1 and LMP2A has potentially different biological outcomes than when the two proteins are expressed individually.**

IMPORTANCE **The Epstein-Barr virus proteins latent membrane protein 1 (LMP1) and LMP2A have potent effects on cell growth. In transgenic mice that express these proteins in B lymphocytes, the cell growth and survival properties are also affected. LMP1 transgenic mice have increased development of lymphoma, and the LMP1 lymphocytes have increased viability in culture. LMP2A transgenic lymphocytes have altered B cell development and enhanced survival. In this study, analysis of the cellular gene expression changes in transgenic LMP1 and LMP2A lymphocytes and LMP1 lymphomas revealed that both transgenes individually and in combination affected pathways important for the development of cancer and inflammation. Importantly, the combined expression of the two proteins had unique effects on cellular expression and cell viability. This is the first study to look at the combined effects of LMP1 and LMP2A on global changes in host gene expression.**

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Epstein-Barr virus (EBV) is a human gammaherpesvirus that is causally linked to a variety of B cell lymphomas, including Burkitt lymphoma (BL), Hodgkin lymphoma (HL), and post-transplant and AIDS-associated lymphomas, and the epithelial cell cancers nasopharyngeal carcinoma and gastric carcinoma. The viral proteins latent membrane protein 1 (LMP1) and LMP2A have potent effects on cell growth, including oncogenic and pro-survival properties. LMP1 is considered the principal viral oncoprotein as it can transform rodent fibroblasts to tumorigenicity in nude mice and is required for EBV B cell immortalization. Functionally, LMP1 mimics a constitutive member of the tumor necrosis factor receptor (TNFR) family as it interacts with TNFR-associated molecules, including TRAFs, TRADD, and RIP (1–5). LMP1 activates multiple signaling pathways, including NF-κB, Stat3, and mitogen-activated protein kinase (MAPK). LMP2A also affects cell growth, and in B lymphocytes, it alters B cell receptor signaling and can promote the survival of immunoglobulin-negative B cells in transgenic mice (6–11). In epithelial cells, LMP2A inhibits differentiation and promotes cell migration (12–16). The effects of LMP2A are mediated through its direct recruitment of Src and Syk family tyrosine kinases and activation of phosphatidylinositol 3-kinase (PI3-kinase) and Akt (16–18).

Both LMP1 and LMP2A transgenic mouse models with expression regulated under the control of the immunoglobulin heavy chain promoter and enhancer have been generated and character-

ized (8, 19, 20). Expression of LMP1 in B lymphocytes of transgenic mice results in increased development of lymphoma in approximately 50% of mice over 12 months old, and a recent study indicates that suppression of the immune response increases this incidence (19–21). The lymphomas in both studies develop in the $CD5^+$ (B-1a) subset of lymphocytes, a self-renewing population of B cells predisposed to clonal expansion with age and frequently detected in the peritoneal and pleural cavity (20). The LMP1 transgenic lymphocytes have enhanced survival *in vitro*, and this effect requires activation of NF-κB and Stat3 (20). The LMP1 lymphomas have rearranged immunoglobulin genes and have activated Akt, Jun N-terminal protein kinase (JNK), p38, and NF-κB, with specific activation of the NF-κB family member c-Rel (19, 20, 22). In transgenic mice that express high levels of LMP2A in B cells, a survival signal is induced that enables immunoglobulin-negative B cells normally destined to die from apoptosis to bypass developmental checkpoints and survive in the peripheral blood (6, 8). These transgenic systems provide a unique opportunity to investigate the *in vivo* effects of LMP1 and LMP2A on cellular gene expression and to identify specific pathways that may contribute to lymphoma development and survival of primary B cells in culture.

In this study, the effects of LMP1 on cellular gene expression in the transgenic B cells and transgenic lymphomas were determined using expression microarray analysis. Additionally, the effect of LMP2A on B cell gene expression was assessed alone and in combination with LMP1. Interestingly, pathway analysis identified significant effects on cancer and inflammation for LMP1, LMP2A, and LMP1/2A doubly transgenic lymphocytes. The data indicate that LMP1 and LMP2A can potently and distinctly affect host gene transcription and that their combined expression is not necessarily additive but can have unique effects on gene expression with potentially different biological outcomes.

RESULTS

Gene expression profiling of transgenic B lymphocytes and lymphoma cells. To obtain sufficient material for analysis and to obtain pure populations of malignant splenic lymphoma cells, primary lymphomas were inoculated and passaged in SCID mice. The LMP1 lymphomas could be consistently established as xenografts; however, only one spontaneous lymphoma that developed in a negative littermate was also successfully passaged. Interestingly, the doubly transgenic mice did not have lymphoma incidence enhanced above that of LMP1 transgenic mice. For microarray analysis, three distinct LMP1 transgenic lymphomas, one negative lymphoma, and splenic $CD19^+$ B lymphocytes from negative littermates and LMP1, LMP2A, and LMP1/2A transgenic mice were analyzed as test samples. The three separate LMP1 lymphomas were chosen to represent low, medium, and high levels of LMP1 expression and have been previously characterized as having enhanced growth and survival effects relative to the wild-type lymphoma (20). For lymphocytes, transgene expression was verified using reverse transcription-PCR (RT-PCR) and two to five biological replicates were pooled to obtain sufficient RNA for analyses and to reduce variation between single samples. Test samples were analyzed by 2-color hybridization using a pool of splenic $CD19^+$ B lymphocytes from multiple control BALB/c mice as the reference sample. To enable direct comparison between test samples, the same reference was used between different microarray hybridizations. The three LMP1-positive and one spontaneous lymphoma sample were hybridized to one 4x44K microarray chip (Agilent whole-mouse genome microarray). One set of pooled LMP1, LMP2A, LMP1/2A, and negative littermate lymphocyte samples was hybridized in duplicate to two microarray chips which revealed a high degree of technical reproducibility. The microarray platform represents 41,000+ genes and transcripts, mapping to 20,937 genes recognized by ingenuity pathway analysis (IPA). The data set was assessed using hierarchical clustering and analyzed for genes that are ≥2-fold changed compared to the $CD19^+$ splenic B cells of negative littermates.

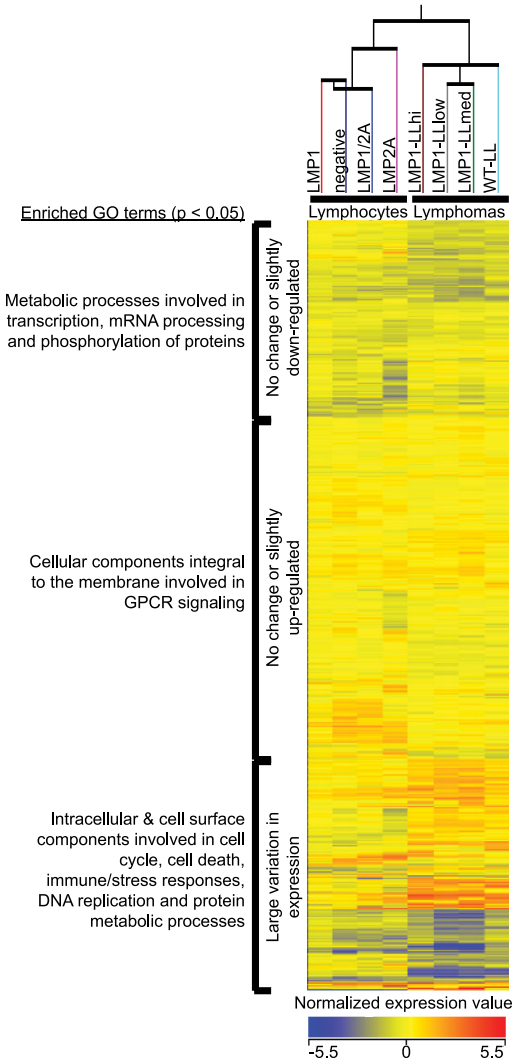

FIG 1 Heat map of all detected probes for transgenic lymphocytes and lymphoma cells. Hierarchical clustering and expression relationships were analyzed for enriched GO terms using GeneSpring GX. The bracket tree indicates similarity between transgenic samples. Normalized expression value relative to the reference sample (control BALB/c mice) is indicated in the legend: upregulation (red), downregulation (blue), and no change (yellow).

Using hierarchical clustering to display the differences in normalized expression values, the lymphoma cells clustered separately from the normal lymphocytes (Fig. 1). The LMP1 lymphoma cells were more similar to the negative wild-type lymphoma than to the normal LMP1 transgenic lymphocytes. Previ-

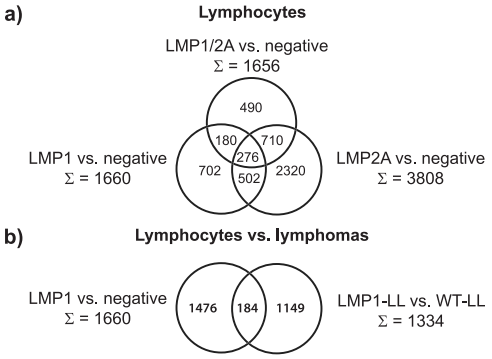

FIG 2 Venn diagrams of differentially regulated genes from transgenic lymphocytes and lymphoma cells. Shown are differential genes from transgenic lymphocytes (LMP1, LMP2A, and LMP1/2A) (a) and LMP1 transgenic lymphocytes and lymphoma cells (b). Differential lists represent ≥2-fold changes relative to the negative lymphocyte or lymphoma.

ous analyses of the LMP1 and wild-type lymphomas have also shown that the lymphomas are similar with regard to extended survival *in vitro* and for the requirement for the same activated signaling pathways (20). The LMP2A transgenic lymphocytes clustered separately from the other genotypes (negative, LMP1, and LMP1/2A), indicating the most distinct gene expression profile (Fig. 1). Gene expression profiles were also clustered based on expression trends relative to the reference sample, generating three recognizable categories: no change or slightly downregulated, no change or slightly upregulated, and large variation in expression in any direction (Fig. 1). The genes with the greatest changes in expression levels were enriched for gene ontology (GO) terms in intracellular and cell surface components involved in processes such as cell cycle, cell death, immune/stress responses, DNA replication, and protein metabolism. This suggests that transgene expression is most likely to affect cell growth, survival, and immune response processes.

LMP2A lymphocytes had the greatest number of genes changed at least 2-fold (3,808 genes) relative to the negative littermates, which was nearly twice as many changes as were detected for LMP1 (1,660 genes) or LMP1/2A (1,656 genes) (Fig. 2A). Approximately 70% (1,166/1,656) of the entities changed in the doubly transgenic LMP1/2A lymphocytes were commonly changed by LMP1 or LMP2A expression alone (Fig. 2A). The remaining 30% (490/1,656) may represent unique effects of LMP1 and LMP2A coexpression.

Expression in the LMP1 lymphomas was compared to the wild-type lymphoma by identifying genes consistently changed ≥2-fold in the same direction (up/down) in every LMP1 lymphoma (high/medium/low). This comparison identified 1,334 genes changed greater than 2-fold in the same direction in all three LMP1 lymphomas. Comparison with the 1,660 genes changed in the LMP1 transgenic lymphocytes relative to negative littermates indicated that approximately 1/10 (184/1,334) of the genes changed ≥2-fold in LMP1 lymphomas were also commonly changed in LMP1 lymphocytes (Fig. 2B). The common genes were further filtered to consider the direction of change (up/down) and to retain only genes that were changed in the same direction in every LMP1 lymphoma sample and in the LMP1 lymphocytes. Out of 184 common genes (Fig. 2B), this retained 93 genes changed in the same direction (60 genes up, 33 genes down). These genes are displayed by GO terms in their respective functional categories (see Table S1 in the supplemental material). These findings indicate that LMP1 has distinct effects in normal versus malignant cells.

Identification of significantly changed pathways and validation of targets by quantitative RT-PCR (qRT-PCR). To predict pathways that are significantly represented in the gene list generated from 2-fold change comparisons, "significant pathway analysis" from GeneSpring GX was performed. The pathways chosen for comparison were derived from the BioPAX database and include the Cancer Cell Map and Integrating Network Objects with Hierarchies databases. These represent curated signaling pathways involved in processes such as cell growth, death, attachment, immune recognition, and cytokine signaling. Pathways were considered significant with a *P* value cutoff of 0.05, taking into account the representation of the pathway entities on the array platform used.

Consistent with the greater number of genes that were affected in the LMP2A lymphocytes, many more significant pathways were identified for LMP2A lymphocytes than for the LMP1 or LMP1/2A genotypes (Table 1). These pathways included Notch, Wnt, transforming growth factor β (TGF-β), and integrin signaling, which have been experimentally linked to the function of LMP2A in B lymphocytes and epithelial cells, lending support to the validity of the predicted pathways (17, 23–26). Interestingly, the c-Kit receptor signaling pathway, a receptor tyrosine kinase and proto-oncogene involved in a variety of hematological malignancies and solid cancers, was identified as a significant pathway in LMP2A lymphocytes with 23 genes affected from the 63 c-Kit involved genes represented on the array. The LMP2A transgenic mice do not have increased lymphoma incidence, and so the effects on the c-Kit pathway may reflect the effects of LMP2A on cell survival or B cell signaling.

The expression analyses identified two significant pathways for LMP1 lymphocytes which were enriched for genes in JAK/STAT and tumor necrosis factor alpha (TNF-α)/NF-κB signaling pathways. In the JAK/STAT pathway, the negative regulators *Sirpa* and *Ptpn6* were downregulated. *Sirpa* encodes a receptor-type transmembrane glycoprotein that recruits protein tyrosine phosphatases such as Ptpn6 to dephosphorylate activated protein tyrosine kinases such as JAK1 and JAK2. The loss of *Sirpa* and *Ptpn6* could contribute to the previously described independence from interleukin-4 (IL-4) stimulation in LMP1 transgenic lymphocytes and lymphoma cells and the constitutive activation of Stat3 that is required for their enhanced survival in culture (20). These pathways were not identified in the expression arrays of the doubly transgenic LMP1/2A lymphocytes. However, multiple pathways significantly affected in the doubly transgenic lymphocytes were in common with the LMP2A lymphocytes, including TGF-β, Notch, and Wnt signaling. Pathways uniquely represented in LMP1/2A lymphocytes include IL-1, toll-like receptor, ID, and Hedgehog signaling, which could reflect the combined effects of LMP1 and LMP2A (Table 1).

To identify pathways modified by LMP1 in lymphoma cells, significant pathway analysis was performed on differential genes consistently changed ≥2-fold in the same direction in every comparison of LMP1 lymphoma (low/medium/high expression) to wild-type lymphoma. The c-Kit receptor pathway was identified as significant ($P \leq 0.05$); interestingly, it was also identified as significant in LMP2A lymphocytes (Table 1). This may indicate

TABLE 1 Significant pathway analysis on genes changed ≥2-fold in transgenic lymphocytes and lymphomas[c]

Significant pathways identified ($P \leq 0.05$)[a]	No. of genes			P value[b]
	Defined in pathway	Represented on microarray platform	From pathway matched with gene list (≥2-fold change)	
For LMP1 lymphocytes				
Negative regulation of (phosphorylation of cytokine receptor) in JAK STAT pathway	5	3	2	0.015784
TNF-α/NF-κB	220	193	21	0.051242
For LMP2A lymphocytes				
EGF-R1	180	174	66	<E−04
Kit receptor	71	63	23	2.35E−04
TNF-α/NF-κB	220	193	53	3.40E−04
TGF-β-R	197	131	39	3.52E−04
Canonical Wnt signaling pathway	74	17	9	7.96E−04
Mammalian Wnt signaling pathway	70	17	9	7.96E−04
Signaling with Wnt (canonical)	45	11	7	8.36E−04
Signaling with Wnt (mammal)	44	11	7	8.36E−04
Androgen receptor	101	93	29	8.50E−04
Xenopus axis formation Wnt signaling pathway	58	15	7	0.007772
Stabilization and accumulation of cytoplasmic β-catenin (canonical)	18	6	4	0.010281
Stabilization and accumulation of cytoplasmic β-catenin (mammal)	17	6	4	0.010281
β-Catenin degradation signaling (canonical)	10	4	3	0.014187
β-Catenin degradation signaling (mammal)	10	4	3	0.014187
Signaling without Wnt (canonical)	15	4	3	0.014187
Signaling without Wnt (mammal)	15	4	3	0.014187
Notch	94	69	19	0.024539
JNK cascade	9	2	2	0.025317
α6β4 integrin	59	54	15	0.039648
VEGF signaling pathway	50	12	5	0.04288
TGF-β signaling pathway (through TAK1)	33	12	5	0.043328
For LMP1/2A lymphocytes				
TGF-β-R	197	131	18	0.009599
Notch	94	69	10	0.033524
IL-1 signaling (through JNK cascade) (canonical)	48	6	2	0.058911
Toll-like receptor signaling pathway (through JNK cascade) (canonical)	45	6	2	0.058911
ID	30	30	5	0.071556
Hedgehog	25	22	4	0.07939
Xenopus axis formation Wnt signaling pathway	58	15	3	0.092978
For LMP1 lymphomas				
Kit receptor	71	63	8	0.038117

[a] BioPAX Cancer Cell Map and Integrating Network Objects with Hierarchies databases were analyzed for enriched pathways in GeneSpring GX.
[b] Statistically significant ($P \leq 0.05$), calculated in GeneSpring GX.
[c] Abbreviations: EGF, epidermal growth factor; VEGF, vascular endothelial growth factor.

that LMP2A can enhance pathways commonly induced by LMP1. Previous characterization of the biological properties and activated signaling pathways has revealed that the LMP1 transgenic lymphomas and the spontaneous lymphoma are quite similar (20, 22). This similarity is reflected in the few changes that distinguish the LMP1 lymphomas. The JAK/STAT pathway which was identified for LMP1 lymphocytes was not significantly changed by LMP1 in lymphoma cells. This likely reflects the constitutive activation of Stat3 in both the LMP1 transgenic and spontaneous lymphomas, indicating that alterations in the JAK/STAT pathway are commonly affected in the lymphoma cell state (20). However, the activation of this pathway in LMP1 lymphocytes may be one of the contributing factors to the enhanced development of lymphoma from this precancerous population.

In order to validate the changes detected by microarray, 13 targets identified from the c-Kit and Stat3 pathways were chosen for further analysis by qRT-PCR (Table 2). These 13 targets ranged from small to large changes (1.2- to 28-fold), enabling an assessment of reproducibility over a wide range. Two independently passaged biological replicas of a wild-type and a high-LMP1-expression lymphoma were analyzed in triplicate reactions. Values were normalized to the housekeeping gene β2-microglobulin, whose expression did not change across samples as measured by the coefficient of variation from the microarray data. To facilitate direct comparison, the first replica utilized the same preparation of RNA used for microarray hybridizations. In this first replica, 12/13 targets were confirmed to change in the same direction by qRT-PCR, of which 10 were further confirmed in the biological replica (Table 2). Within the confirmed targets, the overall degree of change was also reproducible, with the largest

TABLE 2 qRT-PCR of c-Kit and Stat3 transcriptional targets

Gene symbol	Associated pathway	Fold change by: Microarray (LMP1-LL high relative to WT-LL)	qRT-PCR, 1st replica (LMP1-LL high relative to WT-LL)	qRT-PCR, 2nd replica (LMP1-LL high relative to WT-LL)
Kitl	c-Kit	−10.13	1.18	1.44
Kit	c-Kit	3.48	2.08[a]	1.68[a]
Csf2rb	c-Kit	−2.52	−5.34[a]	−2.02[a]
Tec	c-Kit	−3.25	−5.84[a]	−4.24[a]
Yes1	c-Kit	−3.93	−3.67[a]	−3.55[a]
Epor	c-Kit	28.00	12.67[a]	23.48[a]
Grb10	c-Kit	6.57	5.79[a]	2.31[a]
Cblb	c-Kit	−2.49	−2.98[a]	−1.36[a]
Birc5	Stat3	1.55	1.28[a]	−3.59
Ccne1	Stat3	1.26	1.25[a]	−3.21
Cdkn1a	Stat3	1.47	1.64[a]	1.65[a]
Adm	Stat3	−1.25	−2.7[a]	−2.72[a]
Myb	Stat3	1.76	1.54[a]	1.4[a]

[a] Targets confirmed to change in the same direction as in microarray analysis.

changes detected for *Epor*, *Grb10*, *Tec*, and *Yes1*. Despite inherent differences in the two methodologies and in normalization methods, qRT-PCR indicated a high correlation with the microarray data. Overall, these correlative qRT-PCR results confirm the microarray data and indicate that the targets identified by microarray are reproducible.

Comparison analysis of biological functions and canonical signaling pathways in transgenic lymphocytes and lymphoma cells. Biological functions and signaling pathways that were potentially altered by transgene expression were also identified using IPA. Biological functions with a P value of ≤ 0.05 by the Fisher exact test were considered significant and displayed as "diseases and disorders" to help categorize gene changes by disease associations (Table 3). In the lymphocyte comparison, the common biological functions identified for all transgenic lymphocytes were cancer and inflammatory response. Interestingly, both LMP1 and LMP2A transgenic mice have been shown to hyperactivate B cells, leading to enhanced antibody production and autoimmunity (21, 27–29). IPA can also associate gene expression changes to predict an increasing (promoting) or decreasing (suppression) effect on a biological function by using curated information from published literature. Using this predictive method, most of the changes increased the functions of cancer and inflammatory response pathways, indicating that LMP1 and LMP2A can independently activate cancer and inflammatory mechanisms (Table 3). For example, AICDA has no predicted function for cancer but is predicted to increase inflammatory responses. AICDA-deficient mice develop enlarged germinal centers (GCs) with spontaneously hyperactivated IgM^+ nonswitched B cells (30). The decrease in AICDA expression in LMP1/2A mice (−4.154-fold) would thus predict an increase in inflammatory responses by activating B cells (Table 3). In contrast, there were more decreased predictions for metabolic disorders linked to LMP1/2A lymphocyte function, suggesting that LMP1 and LMP2A alter gene expression signatures that oppose metabolic defects and may thus function to more efficiently utilize metabolic pathways.

In the lymphoma comparison, LMP1 lymphomas also affected inflammatory response, similarly to LMP1 lymphocytes, although distinct genes belonging to inflammatory response functions were affected (Table 3). Interestingly, LMP1 lymphomas were also distinguished by changes in infectious diseases, including reactivation of EBV. Multiple genes were affected in activation of inflammatory response, with increases in *CD40* and *ZEB*, which negatively regulate the expression of the viral *BZLF1* lytic-switch gene (31–33). The LMP1- and/or LMP2A-differentially regulated genes were placed on the "molecular mechanisms of cancer" pathway map and highlighted for genes associated with lymphomagenesis using IPA. The changes detected for LMP1/2A lymphocytes (see Fig. S1 in the supplemental material) differed from the changes detected from individually expressed transgenes (LMP2A, see Fig. S2; LMP1, see Fig. S3), indicating that the transgenes have unique effects on cancer signaling pathways. This analysis highlights the differences in the effects on key pathways with LMP1/2A coexpression and single gene expression.

Identifying c-Rel transcriptional targets from the lymphoma gene list. It is known that activation of the canonical NF-κB pathway is required for survival of EBV-transformed lymphocytes and the survival of LMP1 transgenic and wild-type lymphoma cells (20, 34–36). Additionally, the NF-κB member c-Rel has been shown to be specifically activated in LMP1 transgenic and wild-type lymphomas, suggesting that c-Rel is the required NF-κB member for maintaining survival (22). The 175 experimentally determined c-Rel transcriptional targets identified from the Ingenuity Knowledge Base were compared with ≥2-fold changes in the lymphoma samples. Targets both commonly affected and uniquely affected in the wild-type or LMP1-expressing lymphomas were identified (see Table S2 in the supplemental material). Interestingly, several of the genes previously identified to be affected by LMP1 in cell lines are confirmed c-Rel targets, including *ICAM1*, *MMP9*, and *IL-6*.

The correlation coefficient (r) was calculated for the expression of all queried c-Rel targets in lymphoma and lymphocyte cells expressing LMP1 relative to their corresponding expression in the negative genotype (Fig. 3). An r value of +1 indicates a perfect linear relationship, indicating no difference in the expression of c-Rel targets, whereas a value of −1 indicates no linear relationship and a definitive change in expression. Comparison between the same cell states, e.g., between lymphocytes (LMP1 versus negative) or between lymphoma cells (LMP1-LL high/medium/low versus WT-LL), indicated the least change or most similar expression profiles as indicated by the highest r values (r range, 0.73 to 0.78). In contrast, comparison between the lymphoma cells

TABLE 3 Predicted outcome of genes changed in transgenic lymphocytes and lymphomas listed by "diseases and disorders"[a]

Biological function grouped by "diseases and disorders"	Predicted outcome of function (based on expression direction)								
	Promotion of function			Suppression of function			No prediction		
	Probe ID	Gene symbol	Fold change	Probe ID	Gene symbol	Fold change	Probe ID	Gene symbol	Fold change
LMP1/2A lymphocyte									
Cancer	A_52_P348214	BCL2L11	3.131	A_52_P432647	ICOSLG	−3.227	A_51_P229664	CD27	5.955
	A_52_P278549	MYC	2.993	A_52_P11174	HIP1	−3.715	A_52_P339495	PDIA6	3.2491
	A_52_P309376	JAK2	2.549	A_52_P116298	MYBL1	−4.215	A_51_P137111	CHEK2	2.925
	A_52_P638895	VEGFA	2.34				A_51_P208240	TNFSF14	2.78
	A_51_P365757	FIP1L1	2.005				A_52_P244956	AICDA	−4.154
	A_51_P400453	PTEN	−2.192						
Inflammatory response—activation	A_51_P430766	IL-10	13.836	A_52_P162967	CD28	5.484	A_51_P270807	TNFRSF17	4.285
	A_51_P196925	CX3CL1	10.707	A_52_P15461	IL-15	5.138	A_52_P507214	MMP9	2.836
	A_51_P318104	APP	6.99	A_51_P181286	CD69	4.391	A_52_P427024	LDLR	−2.533
	A_52_P165705	CD1D	4.606	A_51_P260683	RGS1	4.238	A_52_P490788	CR2	−3.186
	A_51_P418295	CD80	4.48	A_52_P98698	Klra4	2.96			
	A_51_P387591	NFKBIZ	3.834	A_51_P338746	PRDM1	2.933			
	A_51_P432641	CXCL10	2.456	A_52_P161495	BCL6	−2.963			
	A_52_P68893	IFNG	2.341	A_52_P432647	ICOSLG	−3.227			
	A_52_P408757	FCGR2A	2.184						
	A_51_P196695	IL7R	2.122						
	A_51_P201480	Stat3	−2.022						
	A_52_P601383	Lilrb3	−2.091						
	A_52_P466268	CD22	−2.421						
	A_52_P244956	AICDA	−4.154						
Metabolic disorder									
Diabetes mellitus				A_51_P418295	CD80	4.48			
				A_51_P285736	PDCD1	4.065			
Light chain-associated amyloidosis							A_51_P229664	CD27	5.955
							A_51_P473888	IL6ST	3.466
							A_52_P117576	CASP3	2.455
							A_51_P325651	CD47	−2.157
							A_52_P161495	BCL6	−2.963
LMP1 lymphocyte									
Cancer	A_52_P116298	MYBL1	3.413				A_51_P137111	CHEK2	3.482
	A_52_P309376	JAK2	2.301				A_52_P339495	PDIA6	2.469
							A_51_P172266	IL9R	2.187
							A_51_P229664	CD27	2.004
Inflammatory response—activation	A_52_P165705	CD1D	3.049	A_52_P15461	IL-15	2.821			
	A_52_P484194	IL1RL1	−2.025						
LMP2A lymphocyte									
Cancer	A_52_P348214	BCL2L11	4.4	A_51_P400453	PTEN	3.278	A_51_P229664	CD27	5.161
	A_51_P102096	MYC	4.297	A_52_P781398	TP53	2.736	A_51_P482508	MSMO1	2.172
	A_52_P309376	JAK2	3.181	A_52_P469842	MDM2	−2.396	A_52_P70810	PDIA6	2.063
	A_52_P638895	VEGFA	2.31	A_52_P116298	MYBL1	−2.919	A_51_P137111	CHEK2	−2.504
	A_51_P483059	DICER1	2.077				A_52_P244956	AICDA	−2.614
Inflammatory response—activation	A_51_P430766	IL-10	15.521	A_52_P162967	CD28	3.855	A_51_P171999	APOE	2.247
	A_52_P244005	CD80	4.247	A_51_P181286	CD69	2.778	A_52_P222026	CHRNB2	−2.132
	A_52_P420792	TXNIP	2.507	A_51_P252103	SLA2	2.544	A_52_P427024	LDLR	−2.871
	A_52_P566867	BCL10	2.243	A_51_P201480	Stat3	2.079	A_51_P142320	PIK3CD	−3.008
	A_51_P257934	TNFSF13B	2.065	A_51_P146320	SEMA4D	−2.054	A_52_P426049	LAT2	−3.033
	A_52_P73385	FAS	−2.019	A_52_P667098	TPT1	−2.115			
	A_52_P451929	Lilrb3	−2.023	A_51_P154780	VAV1	−2.395			
	A_52_P466268	CD22	−2.192	A_52_P179785	RIPK2	−4.238			
	A_52_P244956	AICDA	−2.614	A_51_P291906	TLR3	−4.439			
	A_52_P194250	ETS1	−2.813						
	A_52_P248513	Klra4	−5.579						
LMP1 lymphomas[b]									
Inflammatory response—activation	A_51_P424338	NQO1	6.4	A_51_P255853	TAL1	11.222	A_52_P661044	CCR3	3.267
	A_51_P475995	THRA	5.325	A_51_P339793	IL1RL1	6.861	A_52_P222026	CHRNB2	−2.422
	A_51_P420229	CCR7	3.716	A_51_P489192	POSTN	6.693	A_52_P472888	MALT1	−4.164
	A_51_P366138	MERTK	2.342	A_51_P428708	C4B	3.777	A_51_P105887	LAX1	−5.184
	A_51_P210956	VCAM1	2.253	A_52_P409601	CD40	−2.475			

TABLE 3 (Continued)

Biological function grouped by "diseases and disorders"	Predicted outcome of function (based on expression direction)								
	Promotion of function			Suppression of function			No prediction		
	Probe ID	Gene symbol	Fold change	Probe ID	Gene symbol	Fold change	Probe ID	Gene symbol	Fold change
	A_52_P247424	SH2B3	−2.938	A_51_P316042	CARD11	−2.483			
	A_52_P237087	CD180	−3.443	A_52_P566867	BCL10	−2.754			
	A_51_P349341	NPC1	−3.494	A_52_P502577	S1PR3	−2.844			
	A_52_P466268	CD22	−4.135	A_52_P77106	FASLG	−3.063			
	A_52_P68893	IFNG	−4.535	A_51_P495242	LAT	−3.471			
	A_51_P261517	TYROBP	−5.066	A_51_P345121	CD8A	−3.793			
	A_52_P601383	Lilrb3	−7.225	A_52_P442031	KLF2	−4.05			
				A_52_P682768	ICOS	−4.17			
				A_52_P618569	IKZF1	−4.199			
				A_52_P165705	CD1D	−4.409			
				A_51_P281734	PLXNB1	−4.592			
				A_51_P342688	ITK	−4.756			
				A_51_P105078	S100A4	−4.896			
				A_51_P351015	LTA	−18.439			
Inflammatory disease—colitis				A_52_P409601	CD40	−2.475	A_51_P358765	SPP1	99.857
Infectious disease									
Reactivation of virus	A_52_P409601	CD40	−2.475				A_52_P32733	NF-κB1	−7.723
	A_52_P1083741	ZEB1	−2.828						
Quantity of virus	A_51_P261517	TYROBP	−5.066						
	A_51_P284608	CD74	−5.67						
Infection by bacteria	A_51_P351015	LTA	−18.439	A_52_P68893	IFNG	−4.535	A_52_P32733	NF-κB1	−7.723

[a] Pathways grouped by "diseases and disorders" were analyzed for enriched pathways by using ingenuity pathway analysis. Statistically significant ($P \leq 0.05$, Fisher's exact test).
[b] Fold changes for LMP1 lymphomas are averaged values.

(LMP1-LL high/medium/low, WT-LL) and the negative lymphocytes revealed the greatest differences in expression values (*r* range, 0.2 to 0.39). Additionally, the number of upregulated genes that satisfied the ≥2-fold cutoff was used to predict whether c-Rel targets were affected by up (≥+2) or down (≤−2) regulation. There were more genes changed by upregulation in LMP1 lymphocytes and in all lymphoma cells (Fig. 3). Overall, the pathway overlay and correlation coefficient analysis support the experimental observation that c-Rel is activated in both LMP1 transgenic and wild-type lymphoma cells and that this activation leads to transcriptional upregulation of c-Rel targets (22).

Identifying Stat3 transcriptional targets from LMP1 lymphocytes and the lymphoma gene list. Stat3 is constitutively activated in the nucleus of LMP1 lymphocytes and lymphoma cells, and inhibition of Stat3 using the drug cucurbitacin I decreased viability in primary B cell cultures (20). To identify important Stat3 transcriptional targets induced by LMP1 or by lymphoma development, pathway

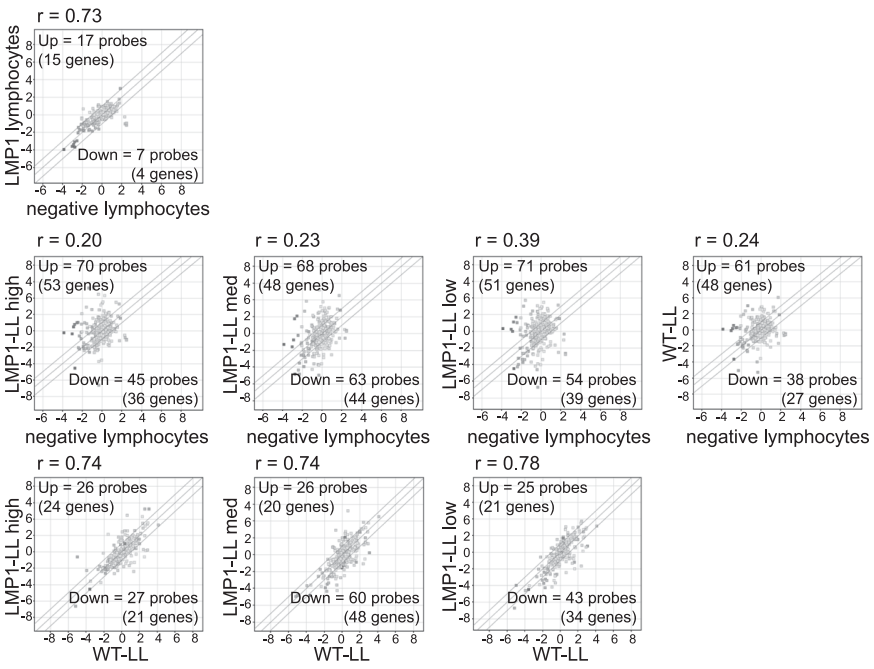

FIG 3 Correlation coefficient analysis comparing the regulations of c-Rel transcriptional targets from $CD19^+$ splenic LMP1-positive and -negative lymphocytes and lymphoma cells. The Pearson correlation coefficient *r* of normalized expression values is displayed for c-Rel transcriptional targets defined by IPA. A linear regression is shown to indicate a 2-fold change cutoff. Each point indicates a probe detected on the microarray, and the number of corresponding genes that are up-/downregulated is indicated in parentheses.

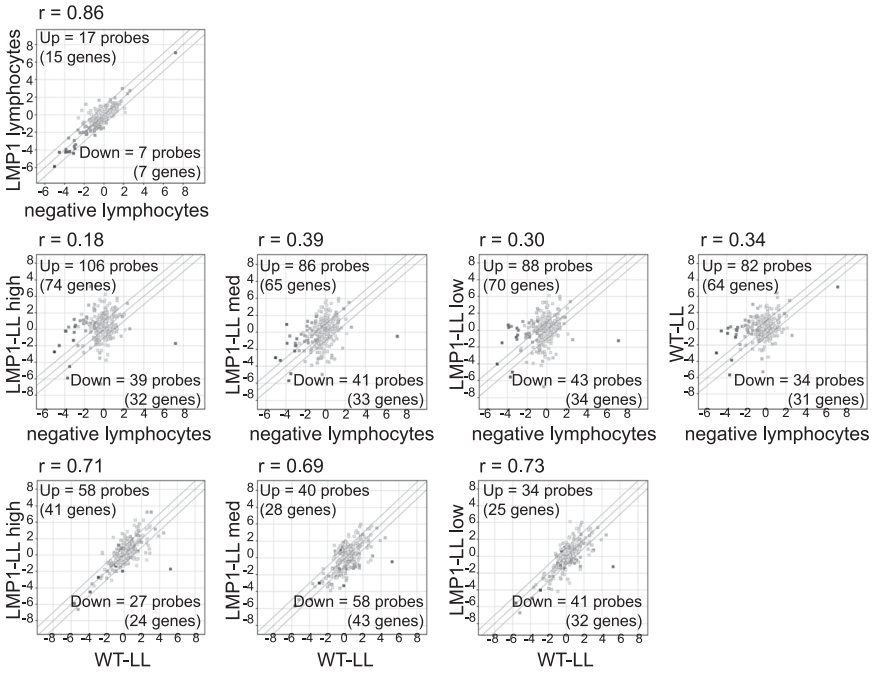

FIG 4 Correlation coefficient analysis comparing the regulation of Stat3 transcriptional cancer targets from CD19$^+$ splenic LMP1-positive and -negative lymphocytes and lymphoma cells. The Pearson correlation coefficient *r* of normalized expression values is displayed for Stat3 transcriptional cancer targets defined by IPA. A linear regression is shown to indicate a 2-fold change cutoff. Each point indicates a probe detected on the microarray, and the number of corresponding genes that are up-/downregulated is indicated in parentheses.

overlay and correlation coefficient calculation were performed. To more specifically elucidate cancer-relevant transcriptional targets, Stat3 direct targets as defined by the Ingenuity Knowledge Base were filtered to retain genes that have been experimentally shown to be transcriptionally regulated by Stat3 and linked to cancer pathways, resulting in a total of 182 potential targets. The majority of Stat3-responsive genes induced in lymphoma cells were affected in both wild-type and LMP1 lymphomas; however, there was also a subset of unique targets upregulated by each genotype (see Table S3 in the supplemental material). The large number of Stat3-responsive genes induced in lymphoma cells compared to LMP1 lymphocytes (66 versus 8 genes [see Table S3]) indicates that Stat3 transcriptional activation is increased in lymphoma cells. The correlation coefficients support this prediction that Stat3 targets are most dramatically affected in lymphoma cells as assessed by the lowest correlation coefficients ($r = 0.18$ to 0.39) compared to negative lymphocytes (Fig. 4). In the same comparisons to negative lymphocytes, more genes were upregulated than downregulated in all lymphoma cells (wild type and LMP1) as well as in LMP1 lymphocytes, indicating an overall induction of Stat3-responsive genes. These observations suggest that the elevated phosphorylation of Stat3 in lymphoma cells and LMP1 lymphocytes results in the transcriptional activation of Stat3 and the increased expression of Stat3 targets (20).

Cell survival analysis of transgenic B lymphocytes. It has been previously shown that LMP1 can partially substitute for CD40 signaling and that the LMP1 transgenic lymphocytes have enhanced B cell survival in culture which is increased by IL-4 treatment while IL-4 alone does not affect the viability of control lymphocytes or LMP2A lymphocytes (20, 37). To compare the viabilities of the transgenic lymphocytes *in vitro* and to determine the response to IL-4 treatment, LMP1, LMP2A, and doubly transgenic LMP1/2A mice were analyzed in splenocyte cultures and compared to negative littermates. Live B cell populations were analyzed by flow cytometry and enumerated by gating for CD19$^+$ B cells and for annexin V-/propidium iodide-negative live cells. In freshly isolated splenocytes, the majority (80%) of negative-littermate B cells were alive (Fig. 5a). This was significantly higher than the live cell populations in any of the transgenic lymphocyte populations (LMP1, LMP2A, and LMP1/2A, $P < 0.05$), indicating that *in vivo* transgenic lymphocytes are susceptible to cell death. However, after 1 day in culture, only 25% of the negative-littermate B cells survived and IL-4 supplementation did not increase viability (Fig. 5b and c). LMP2A transgenic B cells had the highest level of survival, with approximately 60% viability, which was significantly higher than that of the negative-littermate cells and other transgenic lymphocytes (LMP1 and LMP1/2A) ($P < 0.05$) (Fig. 5b). The viability was not enhanced by IL-4 treatment, confirming similar studies using LMP2 transgenic mice that expressed lower levels of LMP2A (Tg6 lineage) than did the TgE lineage used in this study (Fig. 5c) (6, 37). LMP1 transgenic B cells had increased survival compared to negative littermates (Fig. 5b), and this effect was slightly enhanced by IL-4 treatment (Fig. 5c, $P < 0.05$). Interestingly, doubly transgenic lymphocytes (LMP1/2A) did not have enhanced survival in culture and were not significantly different from cells from negative littermates (Fig. 5b). Treatment with IL-4 did not affect their viability (Fig. 5c). This survival analysis indicates that coexpression of LMP1 and LMP2A is not necessarily synergistic and that LMP2A expression may negate the effects of LMP1.

Comparison of microarray gene expression data to published data sets. The data in this study were compared to other published data sets for expression of LMP1 and/or LMP2A in B lymphocytes. In the gene expression profiling from the original LMP2A transgenic line, the majority (96%, 8,583/8,908 IPA-mapped genes) of the array elements are also represented on the Agilent array used in the current study. Given the high degree of overlap in coverage, the differential gene list for CD19$^+$ splenic LMP2A lymphocytes in this study was directly compared to the differential gene list from the same population of lymphocytes isolated from the original transgenic line. Comparing the functional categories previously selected for likelihood of EBV disease association (25), approximately 29% (52/184 genes) overlapped in the same direction with the gene list from the current analysis and inflammation/immunity again appeared as the top overlapping function (Fig. 6a). Interestingly, the c-Kit oncogene was also identified as a differentially expressed gene grouped in the signaling category (see Table S4 in the supplemental material). Other top-ranking functions, including cell cycle/apoptosis, DNA/RNA

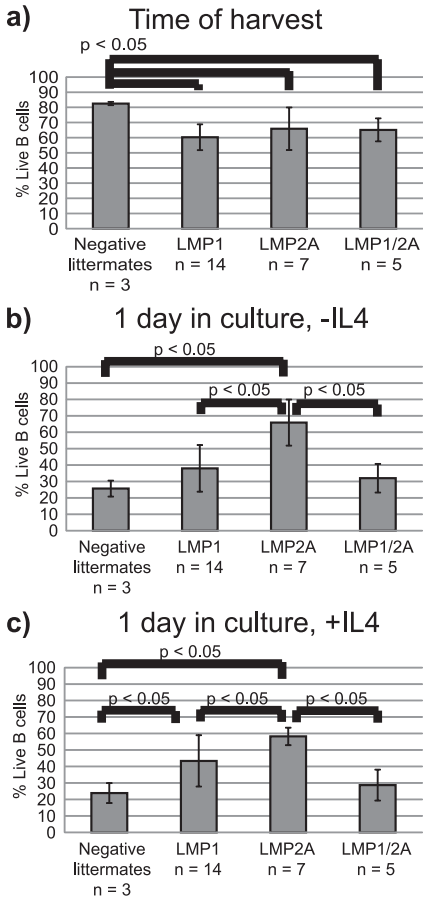

FIG 5 Cell viability analysis of transgenic splenic lymphocytes freshly harvested or from 1-day cultures with and without IL-4 supplementation. (a) Freshly harvested splenic lymphocytes; (b and c) 1-day primary cultures of splenocytes grown without (b) or with (c) IL-4 stimulation. Mean percentages of live B cells ($CD19^+$, annexin V/propidium iodide negative) and the standard deviations are shown. Significant comparisons using the Student *t* test ($P < 0.05$) and the number of mice analyzed per group (*n*) are shown.

metabolism, signaling, transcription factors, and tumor association, also had genes that overlapped with the differential gene list from the current study. The remaining genes that did not overlap may partially be explained by a difference in mouse strains. The original transgenic strain is on a full C57BL/6 background while mice in the current study are the F1 generation of a C57BL/6 and BALB/c cross. Despite these differences, it is striking that genes in inflammatory and tumor-associated pathways are consistently the most affected.

To evaluate how the transgenic lymphocytes from this study compare to the changes in human cell lines, the differential gene list for LMP1 transgenic lymphocytes was compared to a published data set on human $CD10^+$ GC B cells transfected with LMP1 and the differential gene list expressed in HL cell lines (38). The 1,657 genes (562 up, 1,095 down) identified as differentially expressed in LMP1-transfected GC cells were grouped into functional categories according to GO ontology by IPA. The top-ranking functional categories (satisfying a *P* value of ≤ 0.05) were compared to the genes that fall under the same functional categories in the LMP1 transgenic lymphocyte data set from the current study, taking into account the directionality of change (Fig. 6b; see also Table S5 in the supplemental material). The amount of overlap was low, with approximately 5% (107/2,209) of the genes in the tested top-ranking categories also identified in the data set from LMP1 transgenic lymphocytes (Fig. 6b). However, the categories with the most overlap were genes associated with cell death, cancer, and cell cycle functions. Despite a low level of overlap, these genes may be critically relevant to EBV-associated disease. Interestingly, more than half (57%, 61/107) of the LMP1 overlapping genes were also previously defined to be differentially expressed in HL cell lines. To test whether these were significantly enriched for HL signature genes, a χ^2 calculation was applied. The expected overlap was calculated from the published data set, where 45.7% (881/1,926) of the genes differentially regulated by LMP1 were also differentially regulated in HL cell lines (38). The observed overlap of 57% was calculated to be significantly enriched for HL defined genes with a *P* value of 0.02. However, there was no enrichment if the same analysis was applied to all overlapping LMP1 genes without first selecting for significant functional categories (observed overlap with all LMP1- and HL-specific genes was 32%, 24/76). This indicates that the effects of LMP1 in the transgenic mice are in the same functional categories identified in HL cells and affect a significant number of the same genes.

The microarray data were also compared with LMP1-dependent gene signatures in EBV-infected BL cells and lymphoblastoid cell lines (LCLs) that had been identified using a lymphocyte-specific gene array (17,856 cDNA Lymphochip microarray) (34). Approximately 9% (5 up, 1 down) overlapped with the LMP1 transgenic lymphoma gene list, which is comparable to the 4 to 5% overlap calculated from the LMP1-transfected GC B cell comparison to LMP1 transgenic lymphocytes. This low degree of overlap indicates that the effects of LMP1 expression in BL cells and LCLs are different from those in the primary B-1a cells that comprise the transgenic lymphomas.

DISCUSSION

These analyses of cellular expression reveal that LMP1 and LMP2A can both alter host gene expression and that their coexpression is not necessarily additive but can induce distinct transcriptional and functional effects. More than 1,000 genes were identified with a 2-fold expression cutoff for all transgenic lymphocytes and lymphomas. The finding that >94% of these 2-fold changes are changed in the same direction in every LMP1 lymphoma compared to the wild-type lymphoma affirms the consistency of these changes (Fig. 2b). An independent method of validation, qRT-PCR of genes from c-Kit and Stat3 pathways, confirmed the direction and degree of change to levels that were even more subtle than 2-fold (Table 2). By hierarchical clustering and by comparing differential gene lists, cancer and inflammatory response pathways were predicted to be affected in all transgenic lymphocytes (LMP1, LMP2A, and LMP1/2A), with a potentially unique effect on metabolic pathways for LMP1/2A lymphocytes (Table 3). Importantly, expression findings from this study confirmed previous molecular identification of specific activation of c-Rel and the requirement for Stat3-mediated signaling.

The comparison analyses with other EBV expression data sets indicate that the effects of LMP1 and LMP2A can vary substantially between mouse strains and cell lines. The very limited overlap between the LMP1 transgenic lymphocytes and the expression studies of LMP1 in human cell lines is not surprising, considering the very distinct cell types in which LMP1 was expressed. The

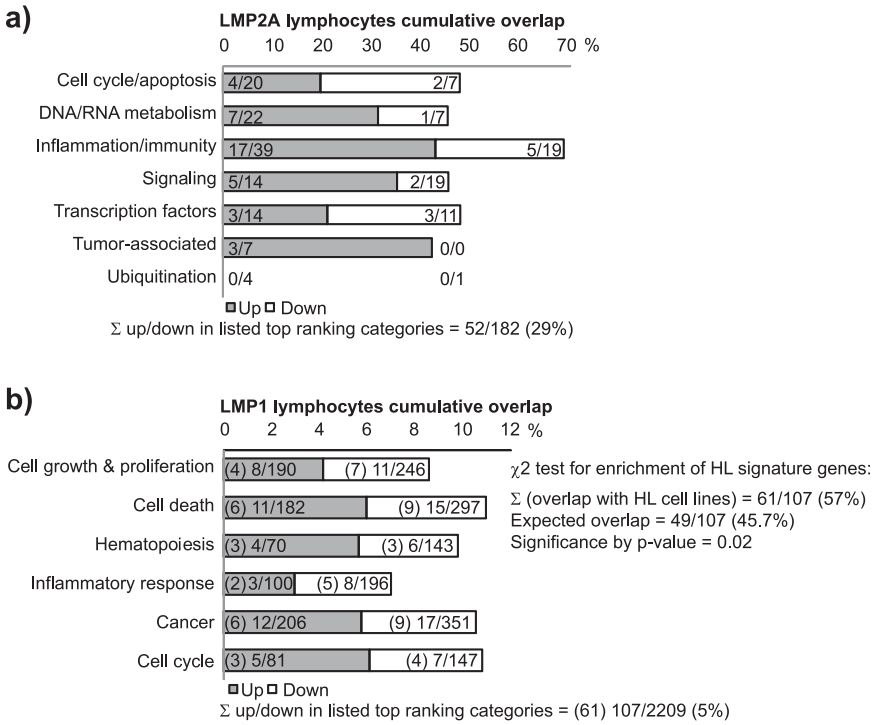

FIG 6 Comparison of differential gene lists from the current transgenic study with published data sets grouped by functional categories. The percent overlap and the numbers of up-/downregulated genes are indicated for each functional category. (a) LMP2A-regulated genes from transgenic splenic CD19$^+$ B cells in the current study compared to the data set from the parental transgenic LMP2A mice (25); (b) LMP1-regulated genes from transgenic splenic CD19$^+$ B cells in the current study compared to the published data set on LMP1-regulated genes in transfected CD10$^+$ germinal center B cells (38). In parentheses are the numbers of genes that are also regulated by Hodgkin lymphoma (HL) cell lines relative to untransfected CD10$^+$ germinal center B cells as previously described (38).

transgenic lymphomas develop in B-1a lymphocytes, a cell type prone to malignant conversion in mice, while the two studies in human cells identified changes in GC, HL, and BL cells or LCLs, each very distinct B cell types that likely impose constraints on the effects of LMP1. However, the overall outcomes of expression have similarities, with major effects on cell growth and proliferation, inflammatory response, cancer, and cell cycle. A similar comparison of the effects of LMP2A in transgenic lymphocytes with EBV-transformed LCLs and with HL cell lines identified commonly affected pathways and biological functions; however, the genes within each category were also unique to the cell type (25).

The genes identified in each of these studies reveal multiple ways through which LMP1 or LMP2A may affect cell function. Potentially significant genes relevant to the promotion of cancer identified for LMP1 lymphocytes included *MYBL1*, *JAK2*, *CHEK2*, *PDIA6*, *IL9R*, and *CD27* (Table 3). Additionally, genes involved in B cell differentiation (*CD27*), cell cycle checkpoint (*CHEK2*), and immune evasion (*PDIA6*) also likely contribute to tumor progression. Overall, these changes in LMP1 lymphocytes predict that LMP1 hyperactivates B cells, inducing cancer control mechanisms such as the induction of cell cycle checkpoint and potentially tumor-immune evasion mechanisms.

Many of the identified pathways for the LMP2A lymphocytes have been previously shown to be affected by LMP2A in biological studies. Activation of Notch signaling by LMP2A has been predicted to be linked to the downregulation of transcription factors involved in B cell development (25, 26). Additionally, LMP2A has been shown to target the Wnt pathway in lymphocytes and epithelial cells through effects on Akt with nuclear accumulation of β-catenin (17, 23, 39), while in this study expression of PTEN was also decreased. Interestingly, the c-Kit receptor signaling pathway, a receptor tyrosine kinase and proto-oncogene involved in a variety of hematological malignancies and solid cancers, most notably chronic myeloid leukemia and gastrointestinal stromal tumor, was identified as a significant pathway in LMP2A lymphocytes and LMP1 lymphomas.

Importantly, 70% of the changes detected in LMP1/2A lymphocytes were also affected by LMP1 or LMP2A expression alone while the remaining one-third of detected changes were unique to their combined expression (Fig. 2a). Additionally, the biological outcome of combined expression resulted in a negation of both phenotypes (Fig. 5b and c). The LMP1 transgenic lymphocytes had a slightly enhanced response to IL-4 while the LMP2A lymphocytes had the highest viability with or without IL-4. However, the doubly transgenic lymphocytes did not respond to IL-4 and had viability similar to that of the negative lymphocytes. It has recently been shown that in coexpressing LMP1/2A transgenic B cells, LMP2A reversed the LMP1-mediated block in GC formation although the reciprocal effects of LMP1 on LMP2A function were not identified (37). These findings from both studies indicate that the combined effects of coexpression may not necessarily be additive and that coexpression can negate their individual effects. Pathway overlap indicated that LMP1 and LMP2A affect a differential set of lymphomagenesis-relevant genes and that LMP1/2A coexpression modifies a subset of these that were found to be commonly changed by LMP2A expression alone (see Fig. S1 to S3 in the supplemental material). This type of analysis highlights key cancer signaling candidates (e.g., c-Jun, Myc, p15, and Bim) modulated by LMP1/2A coexpression and reflects on the differential outcomes by coexpression.

The data presented here reveal very distinct effects on cell expression that will lead to the identification of additional pathways that are distinctly regulated through the coexpression of LMP1 and LMP2A. These distinct and unique effects of LMP1 and LMP2A expression also suggests that during EBV infection, LMP1 and LMP2A expression is likely to be tightly regulated to maximize their molecular properties. The identification of genes uniquely affected by coexpression does suggest potential synergistic effects. A combined effect of LMP1 and LMP2A has recently been shown in LMP1/2A transgenic epithelial cells (40). In this tumor initiation/promotion model of dimethylbenz-α-anthracene (DMBA)/tetradecanoyl phorbol acetate (TPA)-treated skin carcinogenesis, LMP1 functioned as a weak tumor

promoter to induce papilloma formation and coexpression with LMP2A led to increased carcinoma progression. This was the first evidence that coexpression of LMP1 and LMP2A can have an increased oncogenic outcome.

In summary, findings in this study reveal that in different cell types viral genes can alter critical cellular processes in multiple ways through effects on different genes. These differences are evident between different mouse strains and even in the distinct phenotypes of the LMP2A lineages that differ only in their levels of LMP2A expression (6). It is likely that normal cellular expression is very different between GC, BL, and HL cells and LCLs and the B-1a transgenic lymphomas and that these differences contribute to the distinct effects of the viral genes in these cell lines. This interplay between cell and viral genes may also be a factor through which EBV can be a contributing factor in very distinct types of lymphoma and unique types of carcinomas.

MATERIALS AND METHODS

Ethics statement. Animals were housed at the University of North Carolina at Chapel Hill in facilities accredited by the International Association for the Assessment and Accreditation of Laboratory Animal Care. All protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC) (under IACUC protocol identifications 11-089, 09-247, 08-087, and 06-244), compliant with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, the Amended Animal Welfare Act of 1985, and the regulations of the United States Department of Agriculture (USDA) (Animal Welfare Assurance no. A3410-01; USDA registration no. 55-R-004). This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. To minimize suffering, animals were monitored daily and euthanized when showing signs of abdominal distention.

Construction of transgenic mice. Transgenic LMP1 and LMP2A mice driven by the immunoglobulin heavy chain promoter and enhancer have been previously described (8, 19, 20). The lineages with the most clearly defined phenotypes (lineage IL-3 for LMP1 showing 40% lymphoma incidence versus 10% in control mice; lineage TgE for LMP2A showing survival of immunoglobulin-negative cells and expressing the highest level of transgene) were used for breeding (8, 20). LMP1 mice were kept on a BALB/c background, and LMP2A mice were kept on a C57BL/6 background. Adult mice (6 months old) from the F1 generation of the LMP1 and LMP2A breeding (negative littermates and LMP1^{+}, LMP2^{+}, and LMP1/2$^{+/+}$ mice) were used for microarray analysis of normal lymphocytes. Lymphoma cells from LMP1 transgenic and wild-type mice were generated from a full BALB/c background and passaged in SCID mice as previously described (20). Transgenic mice were maintained and lymphoma passages were performed with the assistance of the Animal Studies Core (University of North Carolina at Chapel Hill).

Splenic B cell isolation and analysis of transgene expression by RT-PCR. Splenic lymphocytes and B cells were harvested and isolated as previously described (20). Total RNA was prepared using the RNeasy total RNA isolation kit (Qiagen) with DNase I treatment (Ambion). Expression of the transgenes was confirmed by RT-PCR as previously described for LMP1 and using PCR primers LMP2-251R (5′ GTGCTTACT GCGGTTGTCACT 3′)/LMP2-690L (5′ CACAGTTACAGCTCCAAGGA 3′) for LMP2A. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control (40).

Cell survival analysis. Splenic lymphocytes were analyzed by flow cytometry freshly after harvest and after 1 day in culture. Culture conditions with and without IL-4 supplementation have been previously described (20). Cell viability was assessed using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (Invitrogen). The live B cell population was identified as CD19^{+} and annexin V/propidium iodide negative. Statistical analysis of live cell enumerations was performed using the Student *t* test (2 sided, unpaired) using the *F* test to calculate variance.

Preparation of RNA for microarray hybridization. DNase I-treated total RNA was purified from freshly harvested CD19^{+} splenic B cells. Samples with a high RNA integrity index (RIN), ≥8, determined with an Agilent 2100 Bioanalyzer, were used for microarray hybridization. For comparative analysis, the same RNA pooled from multiple wild-type BALB/c mice was used as the reference sample for all hybridizations. For lymphocyte analysis, RNA was pooled from multiple mice to normalize for anomaly effects and hybridized in duplicate to two Agilent 4-by-44K microarray chips. For lymphoma cell analysis, 3 LMP1 lymphomas (high/medium/low expression) and 1 wild-type lymphoma were separately hybridized to one 4-by-44K microarray chip. The biological properties of these lymphomas have been previously described and refer to lymphoma numbers 2 (high), 6 (medium), and 5 (low) for LMP1 and lymphoma number 1 for the wild-type lymphoma (20).

qRT-PCR. Complementary DNA was synthesized from 50 ng total RNA (Transcriptor; Roche). PCRs were run in triplicate for 2 biological replicates and a negative control without reverse transcriptase (RT). To calculate relative change, the second derivative maximum method was applied. This allows for more precise quantitation by being independent of baseline estimations.

Bioinformatics analysis. GeneSpring GX software (Agilent) was used to process microarray raw data and used for analyses by hierarchical clustering, GO term enrichment, Venn diagrams, and significant pathway analysis. IPA software was used for predicting c-Rel and Stat3 transcriptional targets, for calculating enrichment of differential gene lists to IPA curated biological functions and canonical signaling pathways, and for comparative analysis with specific published data sets. Differential gene lists were generated by applying a 2-fold-change cutoff on normalized data filtered on expression values and flags. The Core Analysis function in IPA was used to calculate enrichment of differential gene lists using Fisher's exact test ($P = 0.05$). To refine the database search, limits were set in Core Analysis to consider only information that has been experimentally observed and in tissues/cell lines relevant to the spleen or of lymphocyte origin. Normalized expression values of Stat3 and c-Rel transcriptional targets were displayed as scatter plots in GeneSpring GX, and the Pearson correlation coefficient *r* was calculated in Excel using the CORREL function.

Microarray data accession number. Microarray data are deposited in Gene Expression Omnibus, accession number GSE38954.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00288-12/-/DCSupplemental.

Figure S1, PDF file, 0.4 MB.
Figure S2, PDF file, 0.4 MB.
Figure S3, PDF file, 0.4 MB.
Table S1, PDF file, 0.3 MB.
Table S2, PDF file, 0.3 MB.
Table S3, PDF file, 0.3 MB.
Table S4, PDF file, 0.2 MB.
Table S5, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

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