RESEARCH LETTER

blood advances

TO THE EDITOR:

Immune disparities in the TME of EBV$^+$ and EBV$^-$ BL: role of reactive oxygen species generation pathways and platelet activation

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In a previous issue of this journal, we published an original article on the immune landscape of Burkitt lymphoma (BL) patients with typical "starry sky" pattern or rare "granulomatous reaction."[1] Notably, the Epstein-Barr virus (EBV)–positive BL patients with granulomatous reactions exhibited a unique immune signature with M1-polarized macrophages, upregulation of interferon gamma signaling, increased infiltration of cytotoxic CD8$^+$ T cells, and a favorable clinical outcome with spontaneous regression in 3 of 7 patients. Conversely, the patients with a typical starry sky pattern showed predominantly M2-polarized macrophages, creating an immunosuppressive, tumorigenic niche.

Here, we extended the analysis of our previously published gene expression profiling data set,[1] focusing on differences related to EBV positivity[2] in patients with a typical starry sky pattern. Differential gene expression analysis was conducted using the DESeq2 in R focusing on 8 EBV-positive BL and 8 EBV-negative BL patients. Differentially expressed genes were identified based on a $\log_2$ fold change ($|\log_2 \text{ fold change}| > 0.58$) and a Benjamini-Hochberg–adjusted $P < .05$ (Figure 1).

We were able to confirm differences in the expression of genes involved in NF-κB (*TNSF14* and *TNSF13B*), JAK-STAT (*STAT4* and *IL23R*), B-cell receptor pathways (*SYK* and *LYN*) as well as epigenetic regulation in EBV-positive BL.[1] The latter is remarkable because recent evidence suggests that EBV status strongly correlates with DNA methylation–based subgrouping of BL, although not completely.[3,4] We were also able to confirm the heterogeneity of macrophage polarization in EBV-positive vs EBV-negative patients. Indeed, by applying CIBERSORTx analysis we could demonstrate an enrichment of M2 macrophages (**$P = .0049$) and cytokines (*CCL24*, *CXCL16*, *CCL5*, and *CXCL13* and its receptor *CXCR5*) in EBV-negative cases, whereas enrichment in natural killer–activated cells was demonstrated in EBV-positive patients (*$P = .034$), together with a higher fraction of CD8-positive T cells, mast cells, and dendritic cells. The activated macrophage subset patterns

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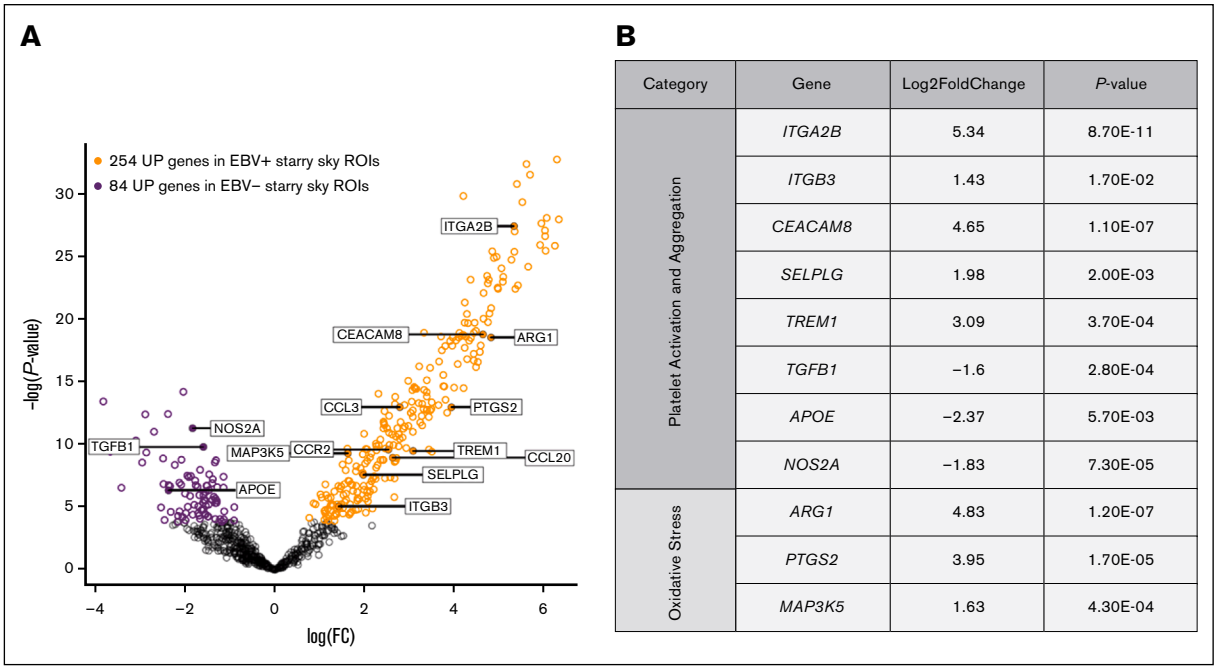
The molecular and clinical data from the patients with Burkitt lymphoma used in this publication can be found on the National Cancer Institute's Genome Data Commons publication page (https://gdc.cancer.gov/about-data/publications/CGCI-BLGSP-2022-1).

Data are available from the coauthor, Maria Chiara Siciliano (siciliano10@student.unisi.it), on request.



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Category	Gene	Log2FoldChange	*P*-value
Platelet Activation and Aggregation	*ITGA2B*	5.34	8.70E-11
	ITGB3	1.43	1.70E-02
	CEACAM8	4.65	1.10E-07
	SELPLG	1.98	2.00E-03
	TREM1	3.09	3.70E-04
	TGFB1	−1.6	2.80E-04
	APOE	−2.37	5.70E-03
	NOS2A	−1.83	7.30E-05
Oxidative Stress	*ARG1*	4.83	1.20E-07
	PTGS2	3.95	1.70E-05
	MAP3K5	1.63	4.30E-04

Figure 1. Differentially expressed genes (DEGs) between EBV-positive and EBV-negative BL. (A) Volcano plot showing DEGs between EBV-positive and EBV-negative starry sky BL. Upregulated genes in EBV positive are shown in orange, whereas upregulated genes in EBV negative are shown in purple. Selected significant genes are highlighted. (B) Table summarizing representative DEGs enriched in platelet activation/aggregation and oxidative stress pathways, with corresponding $\log_2$ fold change and adjusted *P* values.

and the upregulation of their cytokine profile (*CCL3*, *CCL20*, and *CCR2*) observed in the microenvironment of our EBV-positive BL patients may be relevant for understanding the systemic immune activation in other EBV-related conditions that were not evaluated in our study, such as hemophagocytic lymphohistiocytosis with macrophage activation syndrome.[5] In addition, our global analysis showed a significantly different expression of genes related to oxidative stress (*PTGS2*, *ARG1*, and *MAPK3K5*) and platelet activation and functions (*ITGA2B*, *ITGB3*, *TREM1*, *CEACAM8*, *SELPLG*, *TGFB1*, *APOE*, and *NOS2A*)[6-8] (Figure 1B). However, the upregulation of genes related to platelet function were detected in both EBV-positive BL (*ITGA2B*, *ITGB3*, *TREM1*, *CEACAM8*, and *SELPLG*) and EBV-negative BL (*TGFB1*, *APOE*, and *NOS2A*), suggesting a distinct pattern of platelet functions by EBV status.

To increase the number of patient specimens, a set of additional 30 primary BL patients was used to validate the differential expression of *SELPLG* (encoding P-selectin glycoprotein ligand 1 [PSGL-1]), its receptor *SELP* (encoding P-selectin) as well as BZLF1 on protein level. To this end, we performed immunohistochemistry on the Ventana BenchMark Ultra (Roche Diagnostics, Monza, Italy), according to the manufacturer's instructions. Focal BZLF1 expression was detected in EBV-positive cases (7/15). Focal PSGL-1 (*SELPLG*) protein expression within perivascular lymphoid infiltrates and distinct P-selectin (*SELP*) staining on endothelial cells and platelets adherent to the endothelium was found in EBV-positive BL (9/15) but not in EBV-negative BL patients (0/15) (Fisher test $P = .0015$). These results were further confirmed by comparison with a published data set, which confirmed that both genes were significantly higher expressed in EBV-positive than in EBV-negative BL, although with some heterogeneity.[9] Figure 2 depicts the association between the activation of the virus's lytic cycle, increased angiogenesis, and the platelet activation, both at immunohistochemistry and gene expression profiling levels.

Although our study is based on a relatively small cohort, the analyses were performed using statistical frameworks specifically designed for high-dimensional transcriptomic data, such as DESeq2, which models count data using shrinkage estimators and borrows information across genes to stabilize dispersion estimates and control false discovery rate, providing stable and reliable estimates of differential expression. This approach supports the validity of the identified molecular and immunological features that distinguish EBV-positive from EBV-negative BL.

Interestingly, in EBV-positive cases, oxidative stress can affect glucose and lipid metabolism, leading to reactive oxygen species production and triggering the MAPK pathway.[10-12] The activation of the MAPK signaling cascade may result in initiating BZLF1 expression, again promoting the EBV lytic cycle as well as stimulating angiogenesis and the production of immunosuppressive cytokines.[13]

Platelets, beyond their classical role in hemostasis, are increasingly recognized as key players in cancer biology. Our findings of the upregulation of different genes related to platelet activation pathways in EBV-positive vs EBV-negative patients suggests that platelet activation may contribute to BL pathogenesis directly by releasing immunomodulatory molecules and interacting with endothelial cells through adhesion molecules.[14] These findings are in line with literature reports that described an elevated platelet

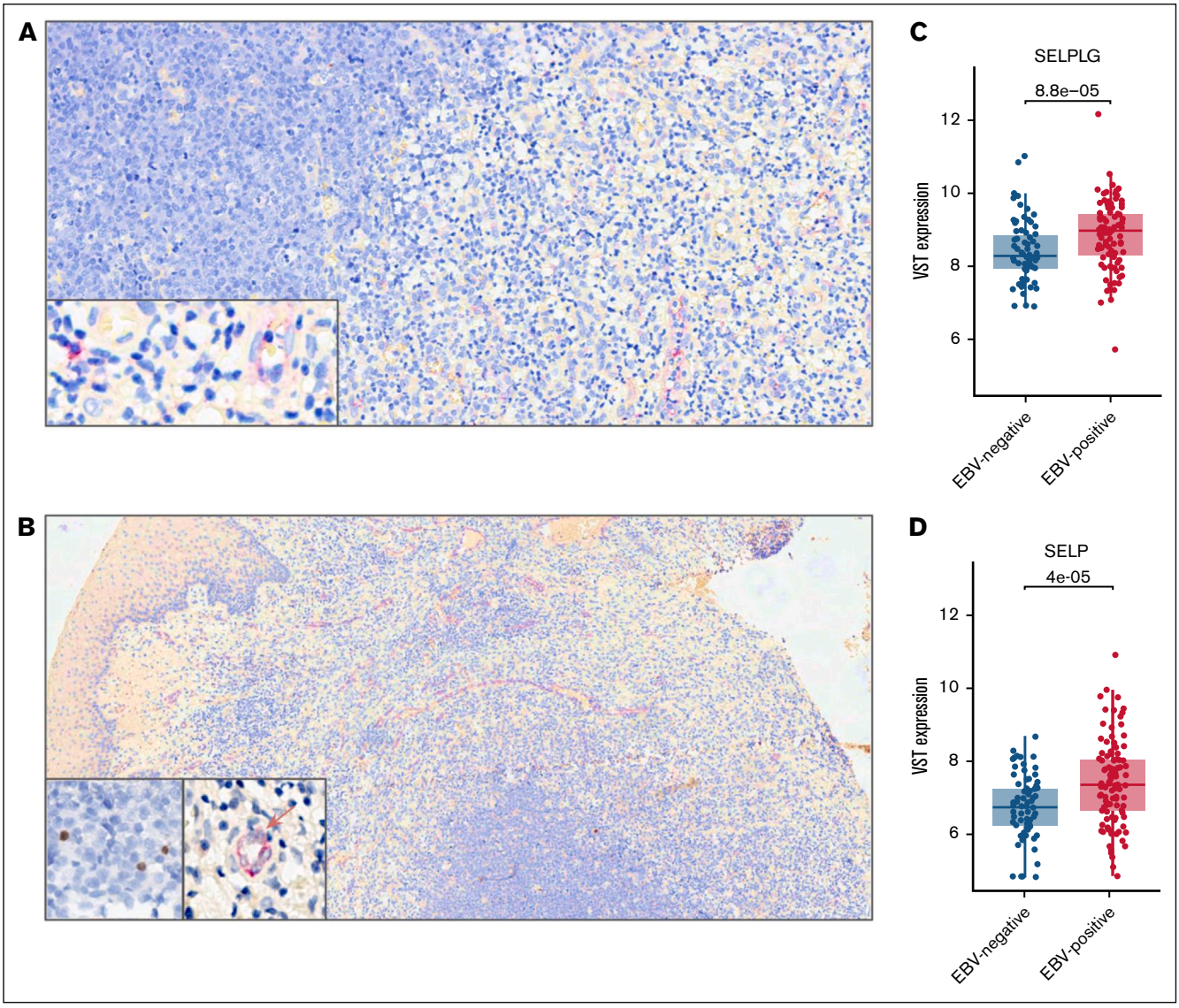

Figure 2. PSGL-1 and P-selectin are increased in EBV-positive BL. (A) PSGL-1 (SELPLG) expression on activated endothelial cells and leukocytes (clone HECA-452, 1:400, pH 6; Abcam) at ×20, with inset at ×40 magnification. (B) BZLF1 expression (in DAB) in scattered neoplastic cells (clone BZ, Santa Cruz; DAB) and P-selectin (SELP; in red) on endothelial cells (clone AK-6, Invitrogen; red) at ×10 magnification, with insets at ×30 magnification. (C-D) Box plots showing the expression levels of SELPLG and SELP in EBV-negative and EBV-positive BL samples (data from Thomas et al[9]). For both genes, expression is significantly higher in EBV-positive cases than in EBV-negative patients. Each dot represents an individual sample. The boxes indicate the interquartile range, with the median shown as a horizontal line, and whiskers represent the range of the data. Statistical significance was assessed using the Wilcoxon rank-sum test, with corresponding *P* values indicated. DAB, 3,3'-diaminobenzidine.

count in patients with BL compared with that in controls or the general population in both endemic cases (which are overwhelmingly EBV positive) and sporadic cases (which are overwhelmingly EBV negative).[15,16] In this scenario, platelets may be the primary actors in shaping the microenvironment, but the specific genes activated may be influenced by the EBV status. In particular, PSGL-1 (encoded by *SELPLG*) and P-selectin (encoded by *SELP*) are key mediators of platelet–endothelial cell and platelet–immune cell interactions, and together with *ITGA2B*, *ITGB3*, *TREM1*, and *CEACAM8*, induce platelet aggregation and leucocyte adhesion, triggering inflammation.[17-19] Conversely, EBV-negative patients show a higher expression of *TGFB1*, *APOE*, and *NOS2A*, which are related to platelet function and activation. Indeed, transforming growth factor β1 (TGF-β1), stored in platelets, plays an important role in limiting inflammation and inducing an immunosuppressive tumor microenvironment (TME) through the modulation of immune and stromal cell functions.[20] Moreover, TGF-β1 is capable of influencing cancer-associated fibroblasts in producing high amounts of extracellular matrix proteins and proteases involved in the extracellular matrix remodeling, which may create a favorable environment for tumor progression.[21] *APOE* overexpression, in concomitance with the upregulation of the nitric oxide pathway (eg, the *NOS2A* gene), may inhibit platelet aggregation by promoting nitric oxide synthase.[22] The interaction

of platelet factors may also influence the subsequent release of immunoregulatory mediators, the functional state of tumor-associated macrophages and other immune cells within the TME.[23]

The observed upregulation of the oxidative stress pathway together with enhanced platelet activation underscores the multifaceted role of EBV in shaping an immunosuppressive TME.

Our findings provide important insights into potential therapeutic opportunities for EBV-positive BL, and potentially other EBV conditions and lymphomas, which should be considered in future studies. Targeting platelet-tumor interactions or the downstream immunoregulatory effects of platelet activation could represent novel strategies to shape the TME.[24,25] Collectively, these results expand our concepts on EBV-driven lymphomagenesis and may inform both prognostic assessments and future therapeutic interventions.

All procedures performed in this study were in accordance with the ethical standards of the institutional research committee (Comitato Etico Regione Toscana-Area Vasta Sud Est: 29056) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This was a noninterventional study of archived tissue samples.

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