The Release of Superoxide by Human B cells is Down-Regulated at the Gene Expression Level

Microchimerism after renal transplantation and blood transfusion ouse dust mites in the city of Salvador-BA

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Abstract

Objective: We investigated the NADPH oxidase activity, cytochrome b\textsubscript{558} content, and gene expression of gp91-phox and p47-phox in normal EBV-transformed B lymphocytes, as compared to EBV-transformed B lymphocytes from patients with X-linked chronic granulomatous disease (CGD), normal peripheral blood neutrophils or mononuclear cells, and the A301 or C8166 lymphoblastoid cell lines.

Methods: CGD phenotypes included both "classic" disease with no detectable gp91-phox protein (termed X91\textsuperscript{0}) and "variant" phenotype with reduced but detectable gp91-phox protein (X91\textsuperscript{−}).

Results: Normal EBV-transformed B lymphocytes show a dose dependent PMA-induced superoxide release. Culturing these cells with IFN-g (100 U/ml), TNF-a (1000 U/ml), alone or in combination for se-seven days, caused a modest increase in their NADPH oxidase activity (p>0.05 in all situations). Normal EBV-transformed B lymphocytes have lower NADPH oxidase activity and cytochrome b\textsubscript{558} content than peripheral blood neutrophils or mononuclear cells (p<0.05 in all situations). In contrast they have higher NADPH oxidase activity and cytochrome b\textsubscript{558} content than X91\textsuperscript{−} CGD EBV-transformed B lymphocytes (p<0.05 in all situations). A301 or C8166 lymphoblastoid cell lines, and X91\textsuperscript{0} CGD EBV-transformed B lymphocytes have barely detectable NADPH oxidase activity or cytochrome b\textsubscript{558} content (p<0.05 in all situations). Gene expression studies also show a modest increase in expression and transcription rates of gp91-phox and p47-phox genes in normal EBV-transformed B lymphocytes cultured with IFN-g (100 U/ml), TNF-a (1000 U/ml) during seven days.

Conclusion: The NADPH oxidase activity and cytochrome b\textsubscript{558} content of EBV-transformed B lymphocytes are limited at the transcriptional level of genes encoding components of the NADPH oxidase system.

We further investigated the transcription rates of the genes encoding gp91-phox and p47-phox in nuclei obtained from EBV-transformed B lymphocytes, as assessed by nuclear run-on assays. As shown in figure 5, culturing B lymphocytes with IFN-g (100 U/ml) and TNF-a (1000 U/ml) for seven days caused a respective 1.3- and 1.2-fold increase in the transcription rates of the genes encoding gp91-phox and p47-phox, in parallel with NADPH oxidase activity, cytochrome b\textsubscript{558} content, and steady state mRNA levels.

Fig. 4 – Gp91-phox and p47-phox gene expression in EBV-transformed B lymphocytes: Each lane contains 10µg total RNA from normal EBV-transformed B lymphocytes, cultured in standard (STD) conditions or with IFN-g (100 U/ml), TNF-a (1000 U/ml), alone or in combination during seven days, or HeLa cells cultured in STD conditions, as indicated. The figure shows a representative northern blot probed with \textsuperscript{32}P-labeled cDNAs for the indicated genes; 18S probing was used as a normalization parameter. Culturing normal EBV-transformed B lymphocytes with IFN-g (100 U/ml), TNF-a (1000 U/ml), alone or in combination during seven days, caused a modest increase in the transcripts of gp91-phox and p47-phox genes (p>0.05 in all situations, n=3).
Resumo

Objetivo: Comparamos a atividade NADPH oxida-se, conteúdo do citocromo b558 e expressão dos genes gp91-phox e p47-phox entre linfócitos B normais imortalizados com vírus Epstein-Barr (linfócitos B EBV); linfócitos B de pacientes com doença granuloma-tosa crônica (DGC) imortalizados com vírus Eps-tein-Barr; neutrófilos e células mononucleares do sangue periférico normal; e as linhagens linfoblas-tóides A301 e C8166.

Métodos: Os fenótipos de DGC incluíram a doença “clássica”, caracterizada pela ausência da proteína gp91-phox (denominada X910) e seu fenótipo “varian-te”, caracterizado pela quantidade reduzida, porém detectável da proteína gp91-phox (X91-).

Resultados: Linfócitos B transformados pelo EBV liberam superóxido de maneira dose-dependente quando estimulados pelo PMA. A cultura destas células com IFN-g (100 U/ml) e/ou TNF-a (1000 U/ml), durante sete dias, resulta em modesto aumento de sua atividade NADPH oxidase (p>0.05 em todas situações). Linfócitos B EBV têm maior atividade NADPH oxidase e conteúdo de citocromo b558 do que neutrófilos e células mononucleares do sangue periférico normal (p<0,05 em todas situações). Em contrapartida, linfócitos B transformados pelo EBV têm maior atividade NADPH oxidase e conteúdo de citocromo b558 do que linfócitos B EBV X91- (p<0,05 em todas situações). As linhagens de células linfoblastóides A301, C8166 e linfócitos B EBV X910 têm atividade NADPH oxidase e conteúdo de citocromo b558 quase indetectáveis (p<0,05 em todas situações). Nossos estudos de expressão gênica revelam um modesto aumento na expressão e atividade transcripcional dos genes gp91-phox e p47-phox genes em linfócitos B EBV cultivados com IFN-g (100 U/ml) e/ou TNF-a (1000 U/ml) durante sete dias.

Conclusão: A limitação da atividade NADPH oxi-dase e conteúdo do citocromo b558 em linfócitos B transformados pelo EBV, ocorre ao nível transcripcional dos genes que codificam componentes do sistema NADPH oxidase.

Introduction

Phagocytes, such as macrophages and granulo-cytes, contain a membrane associated nicotinami-de adenine dinucleotide phosphate (NADPH)2 o-xidase that produces superoxide and other reacti Ve oxygen intermediates responsible for microbi-cidal, tumoricidal, and inflammatory activities1,2. Defects in oxidase activity in chronic granuloma-tous disease (CGD) lead to severe, life-threat-ening infections that demonstrate the prime impor-tance of the oxygen-dependent microbicidal sys-tem in host defense3,4. The enzyme system res-ponsible for superoxide generation forms a small trans-membrane

Discussion

Our results show that PMA induces dose-de-pendent NADPH oxidase activity in normal EBV-transformed B lymphocytes. This activity was approximately 10% of that observed in fresh peripheral blood neutrophils or mononuclear cells and significantly higher than that observed in X-linked CGD B lymphocytes or other lymphoblastoid cell lines. Furthermore, culturing the EBV-transformed cells with IFN-g or TNF-a, alone or in combination, did not significantly affect the capacity of these cells to release super-oxide. In parallel, the cytochrome b558 content in these cells correlated with their NADPH oxidase activity.

Our gene expression studies have shown that EBV-transformed B lymphocytes transcribe the genes encoding the oxidase components gp91-phox and p47-phox, but to a much lower extent than phagocytic cells lines22, 23, 51. In addition, IFN-g , TNF-a , alone or in combination did not significantly affect transcription or steady-state mRNA levels of the oxidase genes, although these cytokines have well characterized receptors and transduction mechanisms in B lymphocy-tes52, 53. This low level of expression is in proba-
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electron transport system that results in the oxidation of NADPH on the cytoplasmic surface and the generation of superoxide on the outer surface of the membrane. Individual protein constituents and their genes have been identified and cloned. The terminal electron donor to oxygen is a unique low-midpoint-potential flavocytochrome, termed cytochrome b$_{558}$, located primarily in the plasma membrane. Cytochrome b$_{558}$ is a heterodimer composed of a 91 kDa glycoprotein (termed gp91-phox, for glyco-protein, 91 kDa, of phagocyte oxidase) and a 22 kDa nonglycosylated polypeptide (p22-phox). Activation of the NADPH oxidase complex from a resting state to full superoxide-generating activity requires the chemical modification and trans-location of additional subunits from the cytosol to the oxidase complex on the cell membrane. Two such polypeptides with Mr 47 kDa and 67 kDa (p47-phox and p67-phox) have been identified and their genes cloned. Low molecular weight G proteins associated with the oxidase include rac2, which translocates with the cytosolic oxidase proteins, and rap1, which closely associates with the p22-phox component in the membrane. A newly-identified and cloned cytosolic component of the oxidase p40-phox, associates with p67-phox, but its role in oxidase activity remains unknown.

In studies examining tissue specificity of expression for the genes encoding the two chains of the cytochrome b$_{558}$ heterodimer, the gp91-phox gene was expressed mainly in differentiating phagocytes, yet that for the p22-phox was constitutively expressed in a variety of cell lineages. Two such genes undergo parallel induction by various cytokines, including interferon-gamma (IFN-g), in monocyte-derived macrophages and granulocytes. Despite being non-phagocytic cells, B lymphocytes also express the cytochrome b$_{558}$, and possess NADPH oxidase activity, they accurately reproduce the biochemical and molecular defects of CGD in patient-derived lymphoblastoid cell lines. Additionally, Epstein-Barr-virus-transformed B lymphocytes (EBV) have been routinely used to induce superoxide release in either B lymphocytes isolated from tonsils or Epstein-Barr-virus-transformed B lymphocytes. Additional stimuli, such as lipopolysaccharide (LPS), aluminum fluoride, ionomycin, arachidonic acid tumor necrosis factor-alpha (TNF-a), interleukin (IL)-1, IL-6, and cross-linking of surface antigens such as IgM, IgD, IgG, HLA-DR, and CD19 also stimulate B lymphocytes to release superoxide.

A major drawback in the use of B lymphocytes as a model system for the study of the NADPH oxidase system is their low oxidase activity. Our aims were to investigate whether oxidase activity and the expression of its components could be up-regulated by inflammatory cytokines in B cell lines, and to determine the biochemical and molecular basis of this low NADPH oxidase activity, previously attributed to a post-transcriptional block in cytochrome b$_{558}$ expression.

Materials and Method

It is noteworthy that IFN-g and TNF-a did not synergise to stimulate the NADPH oxidase activity or gene expression in EBV-transformed B lymphocytes, as usually occurs in phagocytic cell lines. The lack of significant stimulation of the transcription rates for the genes encoding gp91-phox and p47-phox by IFN-g and TNF-a in EBV-transformed B lymphocytes, in parallel with the biochemical and gene expression studies, suggests that the NADPH oxidase activity in B lymphocytes is not constitutive. The NADPH oxidase system is constitutively expressed in B lymphocytes. However, superoxide might have a direct modulatory effect on immune responses in CGD patients. For example, serum immunoglobulin levels in CGD patients are generally very high, and attributed to chronic respiratory burst in phagocytes. In fact, serum immunoglobulin levels in CGD patients are generally very high, and a mechanism to chronic respiratory burst in phagocytes is related to the EBV-transformation process. However, superoxide perhaps can be involved in antigen processing or presentation.

We conclude that expression of the genes encoding gp91-phox and p47-phox in EBV-transformed B lymphocytes correlates with their NADPH oxidase activity and cytochrome b$_{558}$ content. However, despite the parallels we have established between B cells and phagocytes in terms of reproducibility of molecular defects in CGD patients, inflammatory cytokines do not up-regulate oxidase gene expression in the B cell lines. Thus, results from investigations about the NADPH oxidase using EBV-transformed B lymphocytes require careful interpretation.
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**Cell culture.** EBV-transformed B lymphocytes were developed from peripheral blood mono-nuclear cells of healthy individuals and were compared with EBV-transformed B lymphocytes from previously characterized CGD patients, normal fresh isolated peripheral blood mononuclear cells or neutrophils, and the A301 and C8166 lymphoblastoid cell lines.

The CGD EBV-transformed B lymphocytes were developed from two patients with X-linked CGD. The first patient has a splice site mutation in the first intron of CYBB gene encoding gp91-**phox** and the phenotype of variant X-linked CGD (X91**−**). In this nomenclature for CGD phenotype, "X" represents the X-linked mode of inheritance, "91" indicates that the gp91-**phox** component of the phagocyte oxidase is affected and the subscript symbols indicate an undetectable (0), diminished (-), or normal (+) level of gp91-**phox**.

The second patient has a deletion mutation between exons 11-13 of gp91-**phox** and the phenotype of "clas-sic" X-linked CGD with no oxidase activity or detectable gp91-**phox** protein (X91**−**). This patient's phagocytes show NADPH oxidase activity in a range of 10% normal, contain equivalent levels of cytochrome **c**558, and show an unusually dramatic response to IFN-**γ** both in vitro and in vivo.

To initiate B lymphocyte cultures, peripheral blood was fractionated by Ficoll-Hypaque cen-trifugation and the mononuclear cells cultured with supernatants from B95-8, an EBV-producer cell line. After EBV-transformation, B lymphocytes from healthy individuals or from CGD patients were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, at 37ºC in a humid atmosphere saturated with 5% CO2. To examine the effects of cytokines, B lymphocytes were cul-tured for seven days with human recombinant IFN-γ (100 U/ml), TNF-α (1000 U/ml), alone or in combination. Normal peripheral blood mono-nuclear cells or neutrophils, and the A301 and C8166 lymphoblastoid cell lines were not expo-sed to cytokines. Cytokines counts and viability, mo-nitored on a daily basis, were always above 90%.

**NADPH oxidase activity.** Superoxide release was assessed by a modified superoxide dismutase (SOD) inhibitable cytochrome **c** reduction assay. Briefly, cells were transferred to six well polystyrene plates (2 x 10⁶ cells per well); the plates were centrifuged and the supernatant was replaced by Hanks’ balanced salt solution (HBSS) without phenol red, containing cytochrome-**c** (50 µM) and the indicated concentration of 4b-phorbol 12-myristate 13-acetate (PMA). Normal EBV-transformed B lymphocytes were incu-bated with PMA in a range of 3-300 nM. The other

**References**

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cells received 30 nM PMA. Half of the wells received SOD (60 U/ml) at the beginning of the experiment. After one hour incubation, the plates were placed on ice and the other half of the wells received SOD (60 U/ml). The absorbance of the supernatants was monitored at 550 nm and the amount of superoxide released was calculated using an extinction coefficient of 0.21 nM⁻¹ cm⁻¹. The results were expressed as pmol of superoxide released per 10⁶ cells per hour.

Cytochrome b⁵⁵⁸ content. Cytochrome b⁵⁵⁸ was measured by a spectroscopic method designed to avoid the interference of mitochondrial cytochrome-ox or hemoglobin⁴¹. On the day of the experiment, 1 x 10⁷ cells were harvested, washed 3 times with PBS and lysed with 2% Triton X-100 in 0.1 M KH₂PO₄ buffer at pH 7.25, for 30 min-utes on ice. The lysate was centrifuged at 27,000 x g 30 minutes at 4ºC and the supernatant assayed by spectrophotometric scanning (400-600 nm, 750 nm/min). The test sample received 10 μM KCN, 10 μM NaN₃, a few grains of sodium di-thionite and was then aerated by dropwise pipe-ting over 3 minutes. The spectrum of the aerated sample was stored in the spectrophotometer me-mory. The sample was reduced again with a sec-ond addition of dithionite and rescanned. The resulting difference spectrum, representing (reduced second time)-minus-(aerated after first reduc-tion), was obtained. The amount of cytochrome b⁵⁵⁸ was estimated from the height of the band at 558 nm, using an extinction coefficient of 21.6 cm⁻¹ nM⁻¹. The results were expressed as pmol of cytochrome b⁵⁵⁸ per 10⁷ cells.

Gene expression studies. Total cell RNA was extracted by guanidine HCl method⁴² and analy-zed by Northern blots performed according to standard procedures⁴³. Hybridization probes were full-length cDNAs for the human gp91-phox, and p47-phox genes⁵, ¹⁷. Procedures for sequential cy-cles of filter stripping and re-probing were per-formed as described by Gatti et al⁴⁴. Equal loa-ding of lanes was demonstrated by examination of gels after ethidium bromide staining and by re-hybridization with a 5.8-kilobase HindIII res-triction fragment of rat 18S ribosomal cDNA⁴⁵. Positive control RNA was obtained from HL-60 cells differentiated with IFN-γ (100 U/ml) and negative control RNA from HeLa cells²²,⁴⁶. Mes-sage was measured quantitatively by computer analysis of phosphorimager data.

Transcription rates of genes encoding gp91-phox, and p47-phox were assessed by nuclear run-on assays with minor modifications of pre-viously published procedures⁴⁷. Briefly, EBV-transformed B lymphocytes nuclei were isolated by cell lysis in 0.05% Nonidet P-40. Freshly pre-pared nuclei were incubated 30 minutes at 30°C in a reaction mixture containing [³²P]UTP (250 μCi, 3000 Ci/mmol) in buffer modified from Greenberg et al⁴⁷ by addition of 0.8 mM MnCl₂. Newly synthesized RNA was prepared by extract-ion in guanidine thiocianate and ethanol precipi-tation. Equal amounts of incorporated label from each group (1-2 x 10⁷ for inherent phagocytic di-seases: superoxide generation in chronic granulomatous disease and granules in Chediak-Higashi syndrome. J Immunol 1984;133:3006-3009.


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cpm) were then hybridized to saturating amounts of cDNA probes, immobi-lized on filters by slot blotting. The probes used in these experiments included cDNAs for gp91-iphox and p47-iphox genes5,17, ahybridization ne-gative control (plasmid without insert), and cons-titutively expressed genes (b -actin and a -tubu-lin)48. We calculated relative rates of transcript- tion by computer analysis of phosphorimager data. The calculations of relative transcription rates were normalized to negative control and to rates for the constitutively- expressed genes a -tu-bulin and b -actin.

Statistics. Descriptive statistical calculations were performed and the results were represented either as bar/line charts or box plots showing the minimum, 25th percentile, median, 75th percenti-le, and maximum values49. The Mann-Whitney U test was used for comparison between groups; a p value <0.05 was considered significant50.

Results

NADPH oxidase activity of EBV-transformed B lymphocytes. Normal EBV-transformed B lymphocytes show dose-dependent superoxide release in response to PMA over the range of 3-300 nM (fig. 1, p<0.05 in all situations, n=9). Cultur-ring these cells with IFN-g (100 U/ml) for seven days caused a trend to increased NADPH oxidase activity, but without statistical significance (fig. 1, p>0.05 in all situations, n=9). Further experi-ments assessed the PMA (30 nm)-induced supe-roxide release of normal EBV-transformed B lymphocytes cultured in standard (STD) condi-tions or with IFN-g (100 U/ml), TNF-a (1000 U/ml), alone or in combination for seven days. These results were compared to EBV-transfor-med B lymphocyte lines derived from X910 and X910 CGD patients, normal fresh peripheral blood mononuclear cells and neutrophils, and the A301 or C8166 lymphoblastoid cell lines, which were not stimulated with cytokines. These com-parative experiments (summarized in fig. 2) allo-ved us to distinguish four levels of NADPH oxidase activity in the studied cell lines: (respective-ly from highest to lower) 1- Normal peripheral blood mononuclear cells or neutrophils (p<0.05, n=5), 2 - Normal EBV-transformed B lymphocytes (p<0.05, n=5), 3 – X91 CGD EBV-trans-formed B lymphocytes (p<0.05, n=5), and 4 – X910 CGD EBV-transformed B lymphocytes, A301, and C8166 lymphoblastoid cell lines. Cul-turing EBV-transformed B lymphocytes with IFN-g (100 U/ml), TNF-a (1000 U/ml), alone or in combination during seven days, did not cause a statistically significant increase in the NADPH oxidase activity of these cells (fig. 2, p>0.05 in all situations, n=5).

Fig. 1 - NADPH oxidase activity of EBV-transformed B lymphocytes: Dose dependent induction of superoxide (O2-) release by normal EBV-transformed B lymphocytes by 4b -phorbol 12-myristate 13-acetate (PMA), 3-300 nM (p<0.05 in all situations, n=9). Cells were cultured in standard (STD) conditions or in the presence of interferon-gamma (IFN-g , 100 U/ml) for seven days. IFN-g caused a mild increase on the superoxide release by EBV-transfor-med B lymphocytes, however, this increase was not sta-tistically significant (p>0.05 in all situations, n=9).

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Cytochrome b<sub>558</sub> content of EBV-transformed B lymphocytes.
We further assessed the cytochrome b<sub>558</sub> content of normal EBV-transformed B lymphocytes, compared to X91<sup>-</sup> or X91<sup>0</sup> CGD EBV-transformed B lymphocytes, normal fresh peripheral blood mononuclear cells or neutrophils, and the A301 or C8166 lymphoblastoid cell lines. In parallel with the NADPH oxidase activity, these experiments (presented in fig. 3) allowed us to distinguish the neutrophils (p<0.05, n=5), 2 – Normal EBV-transformed B lymphocytes (p<0.05, n=5), 3 – X91<sup>-</sup> CGD EBV-transformed B lymphocytes (p<0.05, n=5), and 4 – X91<sup>0</sup> CGD EBV-transformed B lymphocytes, A301, and C8166 lymphoblastoid cell lines.

Fig. 2 - NADPH oxidase activity of EBV-transformed B lymphocytes compared to other cell types: 4b - phorbol 12-myristate 13 acetate (PMA, 30 nM) induced more superoxide (O<sub>2</sub><sup>-</sup>) release in normal peripheral blood mononuclear cells (MON) or neutrophils (NEU) than in the other cell lines (*p<0.05, n=5). Under the same circumstances, normal EBV-transformed B lymphocytes released more superoxide than EBV-transformed B lymphocytes derived from a patient with variant X-linked (X91<sup>-</sup>) chronic granulomatous disease (CGD) (**p<0.05, n=5). These in turn, release more superoxide than EBV-transformed B lymphocytes derived from a patient with classic X-linked (X91<sup>0</sup>) CGD or lymphoblastoid cell lines A301, and C8166, all cultured in standard (STD) conditions (†p<0.05, n=5). Culturing EBV-transformed B lymphocytes with IFN-γ (100 U/ml) or TNF-α (1000 U/ml), alone or in combination, for seven days did not cause a statistically significant increase on the NADPH oxidase activity of these cells (P>0.05 in all situations, n=5).
Expression of genes encoding components of the NADPH oxidase in EBV-transformed B lymphocytes. Considering the results showing the lower NADPH oxidase activity and cytochrome b$_{558}$ content of EBV-transformed B lymphocytes when compared to peripheral blood mononuclear cells or neutrophils, we extended our investigation to the gene expression level, as assessed by northern blot hybridization. Figure 4 shows that expression of the genes encoding gp91-phaox and p47-phaox in EBV-transformed B lymphocytes correlates with the cells' NADPH oxidase activity and cytochrome b$_{558}$ content. Furthermore, culturing EBV-transformed B lymphocytes with IFN-g (100 U/ml) alone for seven days caused a respective median 1.7- and 1.3-fold increase in gp91-phaox and p47-phaox gene expression (p > 0.05 in all situations, n=3). TNF-a (1000 U/ml) alone caused a respective median 1.4- and 1.1-fold increase in gp91-phaox and p47-phaox gene expression (p > 0.05 in all situations, n=3). IFN-g (100 U/ml) plus TNF-a (1000 U/ml) caused a respective median 1.8- and 1.2-fold increase in gp91-phaox and p47-phaox gene expression (p > 0.05 in all situations, n=3). 

Figure 4 also shows the strong induction on the expression of gp91-phaox and p47-phaox genes in HL-60 cells differentiated with IFN-g (100 U/ml) for two days. It is noteworthy that no significant synergism between IFN-g and TNF-a occurred in the induction of these genes in EBV-transformed B lymphocytes.

Fig. 3 – Cytochrome b$_{558}$ content of EBV-transformed B lymphocytes compared to the other cell types: normal peripheral blood mononuclear cells (MON) or neutrophils (NEU) have a higher cytochrome b$_{558}$ content than all the other cell lines (＊p<0.05, n=5). Normal EBV-transformed B lymphocytes have a higher cytochrome b$_{558}$ content than EBV-transformed B lymphocytes derived from a patient with variant X-linked (X91*) chronic granulomatous disease (CGD) (†p<0.05, n=5). These in turn, have a higher cytochrome b$_{558}$ content than EBV-transformed B lymphocytes derived from a patient with classic X-linked (X910) CGD, A301, and C8166 lymphoblastoid cell lines (‡p<0.05, n=5).