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Regulation of Virus-Associated Lymphoma Growth and Gene Expression by Bacterial Quorum-Sensing Molecules

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ABSTRACT Kaposi's sarcoma-associated herpesvirus (KSHV) can cause several human cancers, including primary effusion lymphoma (PEL), which frequently occur in immunocompromised patients. KSHV-infected patients often suffer from polymicrobial infections caused by opportunistic bacterial pathogens. Therefore, it is crucial to understand how these coinfecting microorganisms or their secreted metabolites may affect KSHV infection and the pathogenesis of virus-associated malignancies. Quorum sensing (QS), a cell density-based intercellular communication system, employs extracellular diffusible signaling molecules to regulate bacterial virulence mechanisms in a wide range of bacterial pathogens, such as *Pseudomonas aeruginosa*, which is one of the most common opportunistic microorganisms found in immunocompromised individuals. In this study, we evaluated and compared the influence on PEL growth and the host/viral interactome of the major QS signaling molecules [*N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), *N*-butyrylhomoserine lactone (BHL), and 2-heptyl-3-hydroxy-4-quinolone (PQS)] in conditioned medium from wild-type (wt) and QS mutant laboratory strains as well as clinical isolates of *P. aeruginosa*. Our data indicate that *P. aeruginosa* coinfection may facilitate virus dissemination and establishment of new infection and further promote tumor development through effectively inducing viral lytic gene expression by its QS systems.

IMPORTANCE Currently, most studies about KSHV infection and/or virus-associated malignancies depend on pure culture systems or immunodeficient animal models. However, the real situation should be much more complicated in KSHV-infected immunocompromised patients due to frequent polymicrobial infections. It is important to understand the interaction of KSHV and coinfecting microorganisms, especially opportunistic bacterial pathogens. Here we report for the first time that *P. aeruginosa* and its quorum-sensing signaling molecules display a complicated impact on KSHV-associated lymphoma growth as well as the intracellular host/viral gene expression profile. Our data imply that targeting of coinfecting pathogens is probably necessary during treatment of virus-associated malignancies in these immunocompromised patients.

KEYWORDS KSHV, primary effusion lymphoma, *Pseudomonas aeruginosa*, quorum sensing

Kaposi's sarcoma-associated herpesvirus (KSHV) represents a principal causative agent of several cancers arising in immunocompromised patients, including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman disease

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(MCD) (1–3). Among these malignancies, PEL, formerly known as body cavity lymphoma, usually comprises transformed B cells harboring viral episomes and presents as pleural, peritoneal, and pericardial neoplastic effusions. PEL is a rare but aggressive B-cell-derived lymphoma, with patients having a median survival time of approximately 6 months even under conventional chemotherapy (4). The exact mechanism by which KSHV promotes oncogenesis in PEL is an area under active investigation. Most infected cells express a latent pattern of viral gene expression, while a very small percentage expresses viral lytic genes (5). Even with the expression of latent genes, infected cells can undergo clonal expansion, eventually leading to neoplastic transformation through mechanisms including increased proliferation and impaired apoptosis (4).

It is well-known that KSHV-related malignancies, including PEL, usually occur in the setting of an immunocompromised subpopulation, especially HIV-positive (HIV^+) patients, who always suffer from polymicrobial infections, including those caused by opportunistic bacterial pathogens. Therefore, it is necessary and interesting to understand how these coinfecting microorganisms or their products may affect KSHV infection and the pathogenesis of virus-associated malignancies. One recent study has reported that the short-chain fatty acids produced by periodontal pathogens, including *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, can induce KSHV lytic reactivation and promote virus replication (6). Our previous study reported that pretreatment of primary human oral fibroblasts with two prototypical pathogen-associated molecular patterns (PAMPs) produced by periodontal pathogenic bacteria, lipoteichoic acid (LTA) from *Staphylococcus aureus* and lipopolysaccharide (LPS) from *P. gingivalis*, increased KSHV entry and subsequent viral latent gene expression (7). We also demonstrated that *S. aureus* LTA and/or *P. gingivalis* LPS increased the level of several cellular receptors for KSHV entry (in particular, heparan sulfate proteoglycan [HSPG]) and increased the production of reactive oxygen species (ROS) as a cofactor facilitating virus entry, as well as the activation of intracellular signaling pathways, such as mitogen-activated protein kinase and NF-κB, which are required for KSHV latency establishment within oral cells (7).

Pseudomonas aeruginosa is a ubiquitous, Gram-negative bacterium that thrives in diverse habitats and environments. *P. aeruginosa* can act as an opportunistic pathogen, especially in patients who are intubated over long periods and immunocompromised and elderly individuals (8). More importantly, the infections caused by *P. aeruginosa* and its biofilms are usually resistant to multiple antibiotics, which can lead to severe and persistent infections (9, 10). For example, once established, the eradication of *P. aeruginosa* from the respiratory tract of HIV^+ individuals with advanced immunosuppression is problematic, and a chronic infective state appears to be common (11). Recent research progress has shown that quorum sensing (QS), a widely distributed bacterial population density-dependent cell-to-cell communication mechanism, plays a key role in modulating the expression of virulence genes as well as biofilm formation in bacterial pathogens, including *P. aeruginosa* (12–14). Typically, QS bacteria produce and release small diffusible signaling molecules, and at a high population density, the accumulated signals interact with cognate receptors to induce the transcriptional expression of various target genes, including genes that encode virulence factors. As a model organism for QS research, *P. aeruginosa* possesses three main QS systems (*las*, *rhl*, *pqs*), which regulate their target genes via three distinct QS signaling molecules, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), *N*-butyrylhomoserine lactone (BHL), and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively. In the current study, we comparatively examined the impact of these QS signaling molecules in conditioned medium from wild-type (wt) and QS mutants of laboratory strains as well as clinical isolates of *P. aeruginosa* on PEL growth and the host/viral gene profile.

RESULTS

Regulation of PEL growth and viral gene expression by *P. aeruginosa* QS signaling molecules. By using WST-1 cell proliferation assays, we first tested and compared the regulation of PEL growth by 3 *P. aeruginosa* QS signaling molecules, OdDHL, BHL, and PQS (Fig. 1A). We found that among the 3 QS molecules, PQS

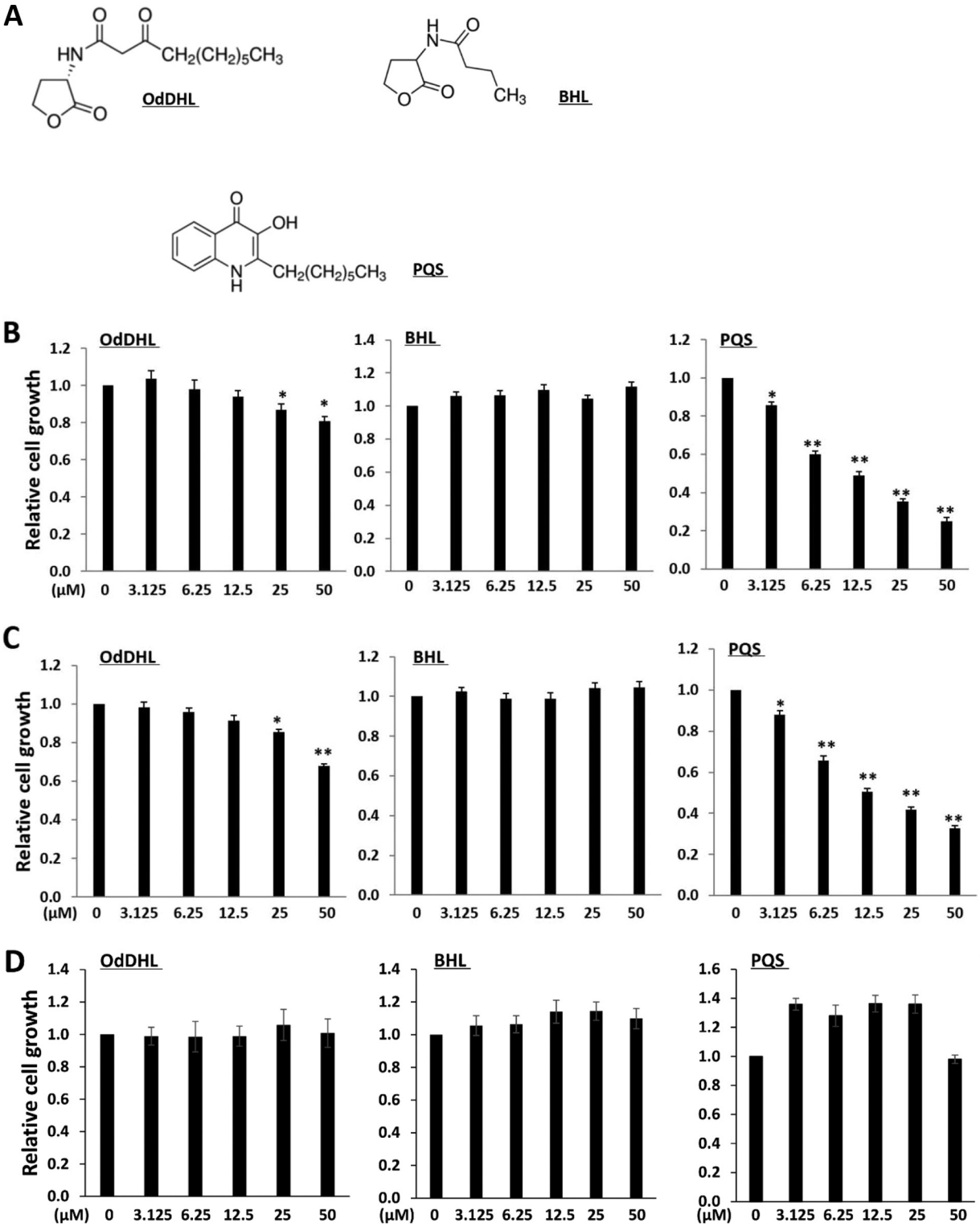

FIG 1 *P. aeruginosa* QS signaling molecules regulate the growth of KSHV$^+$ PEL cells. (A) Structures of major *P. aeruginosa* QS signaling molecules. OdDHL, *N*-(3-oxododecanoyl)-L-homoserine lactone; BHL, *N*-butyrylhomoserine lactone; PQS, 2-heptyl-3-hydroxy-4-quinolone. (B to D) Cells of the KSHV$^+$ PEL cell lines BCBL-1 (B) and BCP-1 (C) or a virus-negative lymphoma cell line, BL-41 (D), were incubated with the indicated concentrations of OdDHL, BHL, or PQS for 48 h. The cell proliferation status was examined using WST-1 cell proliferation assays (Roche). Error bars represent the SD from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

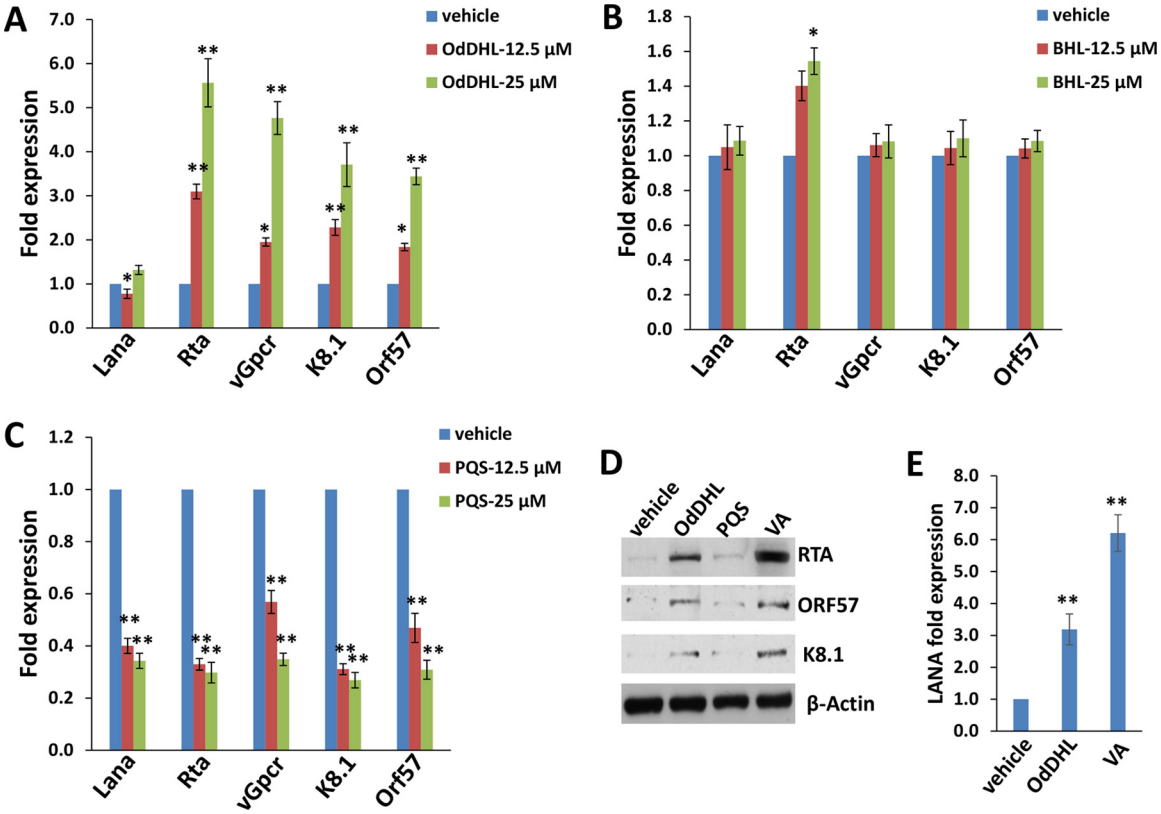

FIG 2 *P. aeruginosa* QS signaling molecules regulate KSHV gene expression from PEL cells. (A to C) BCBL-1 cells were incubated with the indicated concentrations of OdDHL (A), BHL (B), or PQS (C) for 48 h, and then quantitative real-time PCR (qRT-PCR) was used to quantify viral transcripts representing either latent (*Lana*) or lytic (*Rta*, *vGpcr*, *K8.1*, and *Orf57*) genes. Data were normalized to those for vehicle-treated cells, and β-actin was used as a loading control. (D) Protein expression was measured using immunoblots. (E) Released virions were isolated, purified from the supernatant of BCBL-1 cells that had been treated with OdDHL or valproic acid (VA; as the positive control) for 4 days, and then used to infect fresh HUVEC. After 24 h postinfection, *Lana* transcripts were quantified using qRT-PCR. Error bars represent the SD from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

significantly reduced the growth of KSHV-positive (KSHV$^+$) PEL cell lines, BCBL-1 and BCP-1, in a dose-dependent manner (Fig. 1B and C). OdDHL caused an intermediate reduction of PEL cell growth, especially at high concentrations (e.g., 50 μM). In contrast, BHL was almost not able to affect PEL cell growth over the dose range that we tested. Interestingly, all of the 3 QS molecules displayed little effect on the growth of a virus-negative lymphoma cell line, BL-41, over the same dose range (Fig. 1D). Although the concentrations of the QS molecules used here are higher than those reported in some human host samples (e.g., sputum from cystic fibrosis patients) (15, 16), the latter are likely to be underestimates since local OdDHL concentrations of up to 600 μM have been detected in the culture supernatants of *P. aeruginosa* biofilms grown *in vitro* (17).

Next, we measured the viral gene expression from PEL cells after being exposed to *P. aeruginosa* QS molecules using quantitative real-time PCR (qRT-PCR). We found that OdDHL significantly induced the expression of viral lytic genes (e.g., *Rta*, *vGpcr*, *K8.1*, *Orf57*) from PEL cells at concentrations of 12.5 and 25 μM (Fig. 2A). In contrast, PQS greatly reduced both latent (e.g., *Lana*) and lytic gene expression at similar concentrations (Fig. 2C). However, BHL was almost not able to affect viral gene expression within PEL cells (Fig. 2B). Immunoblot analysis confirmed the elevated expression of representative lytic proteins, such as RTA, ORF57, and K8.1, by OdDHL and valproic acid (VA; a positive control) from BCBL-1 cells (Fig. 2D). Furthermore, we found that both OdDHL and valproic acid induced PEL cells to release infectious KSHV particles, as demonstrated by increased LANA expression within fresh human umbilical vein endothelial cells (HUVEC) infected by purified virions isolated from OdDHL- or VA-treated PEL cell supernatants (Fig. 2E).

Transcriptomic analysis of the host gene profile altered within PEL cell lines exposed to QS signaling molecules. To determine the overall host gene profile affected by QS signaling molecules (especially OdDHL and PQS), we used a HumanHT-12 (v4) Expression BeadChip system (Illumina), which contains more than 47,000 probes derived from the NCBI Reference Sequence (RefSeq) database, release 38, and other sources, to analyze the gene profile altered between vehicle- and OdDHL- or PQS-treated BCBL-1 and BCP-1 cell lines. Intersection analysis indicated that there were 314 common genes significantly upregulated and 162 common genes downregulated ($\geq$2-fold and $P < 0.05$) within both PEL cell lines exposed to OdDHL; 256 were uniquely upregulated genes and 304 were uniquely downregulated in BCBL-1 cells, and 63 were uniquely upregulated genes and 133 were uniquely downregulated in BCP-1 cells (Fig. 3A). Within the common gene set, the top 20 upregulated genes and the top 20 downregulated genes in the OdDHL-treated PEL cell lines are listed in Tables 1 and 2, respectively. We also found that there were a total of 37 common genes whose expression was significantly altered (24 upregulated and 13 downregulated genes) within both PEL cell lines exposed to PQS; 29 genes were uniquely upregulated and 25 genes were uniquely downregulated in BCBL-1 cells, and 3 genes were uniquely upregulated and 6 genes were uniquely downregulated in BCP-1 cells (Fig. 3D). Within the common gene set, the top 10 upregulated and downregulated genes in PQS-treated PEL cell lines are listed in Table 3.

We also performed enrichment analysis of these significantly altered candidates by using the Gene Ontology (GO) Processes and Process Networks modules from Metacore software (Thompson Reuters). Notably, our analysis showed that both OdDHL and PQS treatment regulated cell growth-related functional categories in PEL, including varied phases of cell cycle and regulation, cytoskeleton_spindle microtubules, DNA damage, etc. (Fig. 3B, C, E, and F). Moreover, PQS treatment also regulated many cellular metabolism functional categories, such as glycolysis, fructose metabolism, glycogen metabolism, amino sugar metabolism, and iron metabolism (Fig. 3E and F). The top 2 scored pathway/network maps based on enrichment analysis of the common gene set are listed in Fig. S1 and S2 in the supplemental material, respectively. Since many cell cycle checkpoint or regulatory proteins were altered within OdDHL- or PQS-treated PEL cells, for functional validation, we demonstrated that both OdDHL and PQS treatment significantly caused G_1 cell cycle arrest for the BCBL-1 and BCP-1 cell lines by using flow cytometry analysis (Fig. 4A and B). Interestingly, only PQS dramatically induced PEL cell apoptosis, while OdDHL slightly increased cell apoptosis (with no statistical significance) (Fig. 4C).

Regulation of PEL growth and viral gene expression by conditioned medium from *P. aeruginosa* PAO1 wt and QS mutants. Since these QS signaling molecules are produced and secreted by *P. aeruginosa*, we next tested and compared the impact on PEL growth and viral gene expression of filtered conditioned medium from the *P. aeruginosa* PAO1 wild-type (wt) laboratory strain and its QS mutants (a *lasI* mutant deficient in OdDHL, an *rhlI* mutant deficient in BHL, and a *pqsC* mutant deficient in PQS). We found that conditioned medium from the PAO1 wt effectively inhibited PEL cell growth in a dose-dependent manner compared to the Luria-Bertani (LB) medium control (Fig. 5A and B). The conditioned medium from the various PAO1 QS mutants (especially the *lasI* and *pqsC* mutants) displayed a partially impaired ability to inhibit PEL growth compared to that from the PAO1 wt. However, we noticed that the conditioned medium from none of these single-QS-system mutants completely lost the ability to have an inhibitory effect on PEL growth. These data indicate that multiple QS systems may coordinate and/or some QS-independent factors of *P. aeruginosa* are able to regulate PEL cell growth. In contrast, the conditioned medium from the PAO1 wt and QS mutants displayed much less of an inhibitory effect on the growth of the virus-negative lymphoma cell line BL-41 (Fig. 5C). Instead, the conditioned medium from the PAO1 *pqsC* mutant increased BL-41 cell growth.

We next found that conditioned medium from the PAO1 wt significantly induced viral lytic gene expression from PEL cells compared to the LB medium control (Fig. 5D

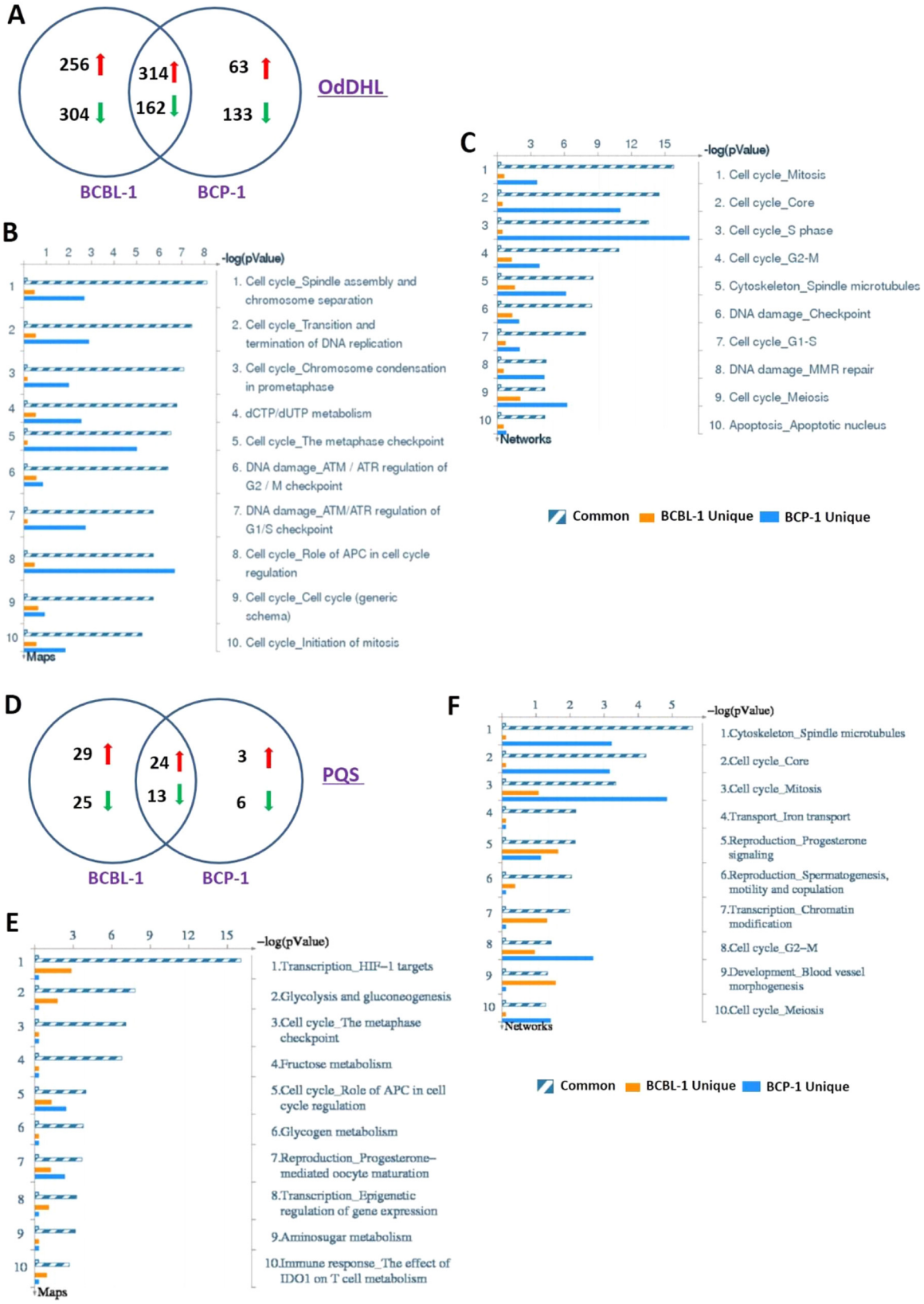

FIG 3 Transcriptome analysis of cells of OdDHL- or PQS-treated PEL cell lines. (A and D) BCBL-1 and BCP-1 cells were incubated with 25 μM OdDHL (A) or PQS (D) for 48 h, and then the HumanHT-12 (v4) Expression BeadChip system (Illumina) was used to detect the gene profile altered within OdDHL- or PQS-treated PEL cells compared to that in vehicle-treated cells. (B, C, E, and F) Enrichment analysis of the gene profile altered by OdDHL or PQS in PEL cell lines was performed using the MetaCore software modules of Gene Ontology Processes and Process Networks. APC, anaphase-promoting complex; ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3 related; MMR, mismatch repair.

TABLE 1 Top 20 common candidate genes upregulated in OdDHL-treated BCBL-1 and BCP-1 cell lines

Gene symbol	Description	Fold change in expression	
		BCBL-1 cells	BCP-1 cells
HSPA6	Heat shock 70-kDa protein 6 (HSP70B)	93.13	61.73
HSPA7	Putative heat shock 70-kDa protein 7	90.2	58.98
RN7SK	RNA, 7SK small nuclear	45.65	35.76
FOSB	Protein FosB	44.19	21.02
SNORD3C	Small nucleolar RNA, C/D box 3C	40.59	49.2
FOS	Proto-oncogene protein c-*fos*	37.3	37.56
SNORD3A	Small nucleolar RNA, C/D box 3A	34.66	39.87
RNU1-4	RNA, U1 small nuclear 4	30.77	36.16
RNU1-5	RNA, U1 small nuclear 5	26.12	34.75
RNU1-1	RNA, U1 small nuclear 1	22.29	28.62
IEX1	Radiation-inducible immediate early gene IEX-1	21.51	18
RGS2	Regulator of G-protein signaling 2	20.8	12.88
GADD45B	Growth arrest and DNA-damage-inducible protein GADD45 beta	19.77	12.94
PTGS2	Prostaglandin G/H synthase 2	19.75	16.78
HBEGF	Heparin-binding EGF[a]-like growth factor	18.57	14.98
DUSP12	Dual-specificity protein phosphatase 12	16.41	13.84
NR4A2	Nuclear receptor subfamily 4 group A member 2	13.31	9.87
MAFB	Transcription factor MafB	12.88	12.18
PPP1R15A	Protein phosphatase 1 regulatory subunit 15A	12.18	9.84
HSPA1A	Heat shock 70-kDa protein 1	11.42	6.97

[a]EGF, epidermal growth factor.

to F). The conditioned medium from the PAO1 *lasI* or *rhlI* mutant partially reduced such induction abilities, while the conditioned medium from the *pqsC* mutant induced viral lytic gene expression at levels similar to those for the conditioned medium from the wt. Again, the conditioned medium from none of these single-QS-system mutants completely lost the ability to induce viral lytic gene expression. We further confirmed that all the conditioned media from the PAO1 wt and QS mutants effectively induced PEL

TABLE 2 Top 20 common candidate genes downregulated in OdDHL-treated BCBL-1 and BCP-1 cell lines

Gene symbol	Description	Fold change in expression	
		BCBL-1 cells	BCP-1 cells
RRM2	Ribonucleoside diphosphate reductase subunit M2	0.29	0.35
CyclinA2	Cyclin-A2	0.29	0.49
RAB36	Ras-related protein Rab-36	0.29	0.37
TPX2	Targeting protein for Xklp2	0.29	0.46
TNFRSF17	Tumor necrosis factor receptor superfamily member 17	0.3	0.49
FAM81A	Protein FAM81A	0.29	0.35
GPRC5D	G-protein-coupled receptor family C group 5 member D	0.29	0.49
EMP3	Epithelial membrane protein 3	0.29	0.37
HDGF	Hepatoma-derived growth factor	0.29	0.46
DLGAP5	Disks large-associated protein 5	0.3	0.49
SFN	14-3-3 protein sigma	0.29	0.35
ITGA4	Integrin alpha 4	0.29	0.49
TYMS	Thymidylate synthase	0.29	0.37
ACTB	Actin, cytoplasmic 1	0.29	0.46
POLE2	DNA polymerase epsilon subunit 2	0.3	0.49
CCR7	C-C chemokine receptor type 7	0.29	0.35
SPAG5	Sperm-associated antigen 5	0.29	0.49
FOXM1	Forkhead box protein M1	0.29	0.37
IGFBP4	Insulin-like growth factor-binding protein 4	0.29	0.46
APOBEC3B	Probable DNA dC->dU-editing enzyme APOBEC3B	0.3	0.49

TABLE 3 Top 10 common candidate genes up- and downregulated in PQS-treated BCBL-1 and BCP-1 cell lines

Gene symbol	Description	Fold change in expression	
		BCBL-1 cells	BCP-1 cells
PFKFB4	6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4	9.58	8.85
ALDOC	Fructose-bisphosphate aldolase C	5.04	4.97
CA9	Carbonic anhydrase 9	4.24	6.41
BNIP3L	BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like	3.81	3.57
CCDC151	Coiled-coil domain-containing protein 151	3.36	3.64
SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	3.29	3.56
SLC2A3	Solute carrier family 2, facilitated glucose transporter member 3	2.88	2.42
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	2.76	2.55
TXNIP	Thioredoxin-interacting protein	2.75	3.45
PFKFB3	6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3	2.54	2.42
HIST1H3J	Histone H3.1	0.24	0.24
CDC20	Cell division cycle protein 20 homolog	0.38	0.36
HIST1H2BC	Histone H2B type 1-C/E/F/G/I	0.39	0.4
HIST2H2AC	Histone H2A type 2-C	0.4	0.45
AURKA	Aurora kinase A	0.42	0.4
CENPA	Histone H3-like centromeric protein A	0.44	0.43
ZCCHC12	Zinc finger CCHC domain-containing protein 12	0.45	0.36
HMMR	Hyaluronan-mediated motility receptor	0.46	0.46
PLK1	Serine/threonine-protein kinase PLK1	0.47	0.39
KIF20A	Kinesin-like protein KIF20A	0.47	0.47

cell release of infectious KSHV particles, although the conditioned medium from the *lasI* or *rhlI* mutant displayed a partially reduced ability (Fig. 5G).

Regulation of PEL growth and viral gene expression by conditioned medium from *P. aeruginosa* clinical isolates. For further study of clinical relevance for our findings, we tested the regulation of PEL growth and viral gene expression by conditioned medium from several *P. aeruginosa* clinical isolates. PA-CF230 (also named CF57388A) was isolated from the sputum of a cystic fibrosis patient (18); PA-D16 and PA-D23 were both isolated from ventilator-associated pneumonia patients (19). We found that filtered conditioned medium from all 3 of these clinical isolates (especially PA-CF230) dramatically inhibited PEL cell growth compared to the LB medium control (Fig. 6A). In contrast, the conditioned medium from these *P. aeruginosa* clinical isolates displayed much less of an inhibitory effect on the growth of the virus-negative lymphoma cell line BL-41 (Fig. 6B). Furthermore, conditioned medium from these clinical isolates significantly induced viral lytic gene expression and the release of infectious KSHV particles from PEL cells (Fig. 6C to E).

DISCUSSION

KSHV-related malignancies usually occur in immunocompromised individuals, such as HIV$^+$ patients, who frequently suffer polymicrobial infections, including infections caused by opportunistic bacteria. However, there are limited data about how these opportunistic bacteria or their products can regulate KSHV infection as well as virus-related cancer development. QS systems have been found to regulate many virulence factors in both Gram-positive and Gram-negative bacteria. In the current study, we report for the first time the diverse impacts on KSHV$^+$ PEL cell growth and host/viral gene expression of 3 major QS molecules (OdDHL, BHL, and PQS) from *P. aeruginosa*, an opportunistic pathogen commonly seen in immunocompromised individuals. Recently, a fourth QS system, 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde, also named an integrated quorum-sensing system (IQS), has been identified in *P. aeruginosa* (20). IQS synthesis depends on a nonribosomal

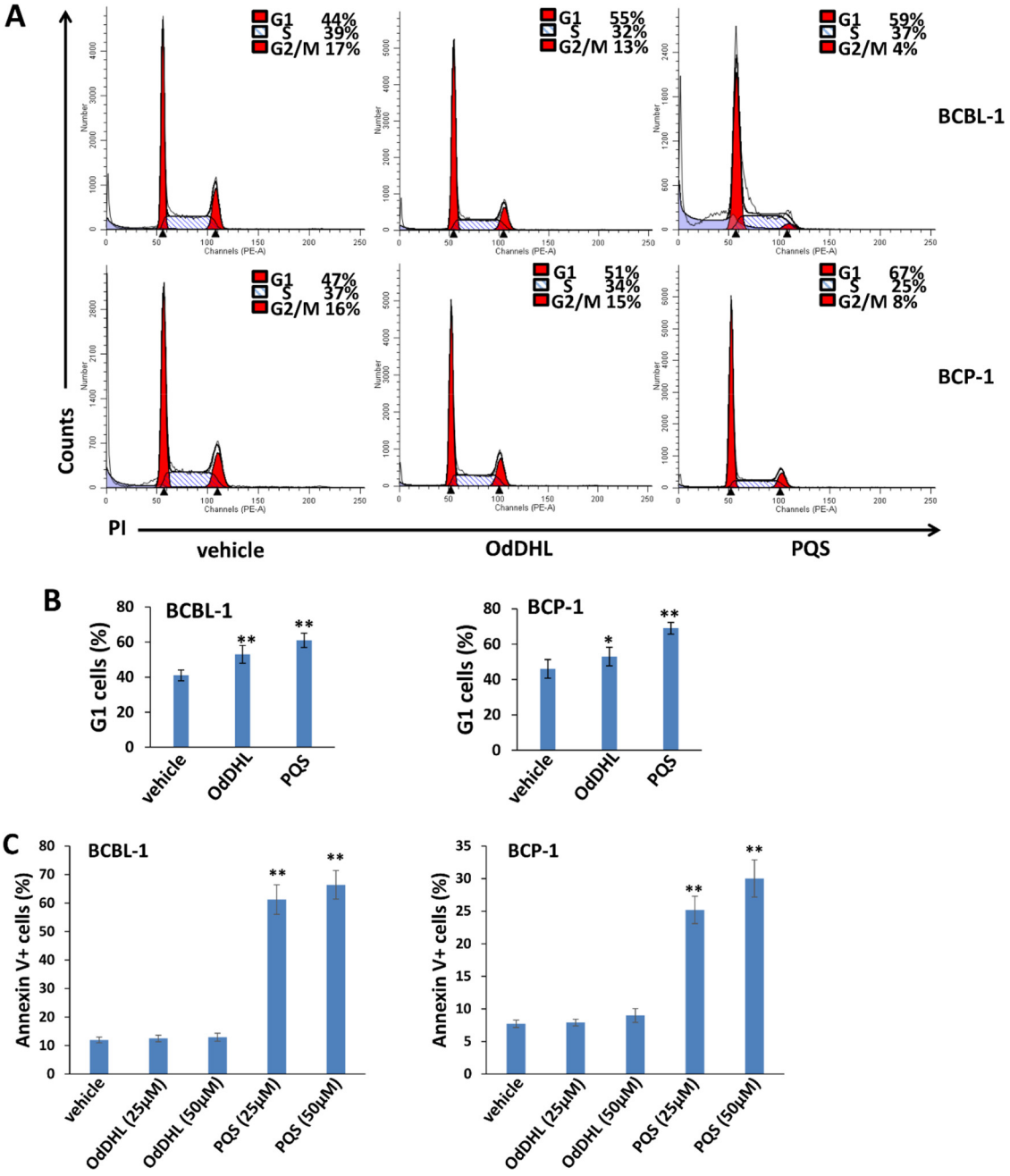

FIG 4 OdDHL or PQS regulates PEL cell cycle and apoptosis. (A and B) BCBL-1 and BCP-1 cells were incubated with 25 μM OdDHL or PQS for 48 h and then stained by propidium iodide (PI) and analyzed by flow cytometry. The solid blue area in panel A represents cell debris, which was excluded from analysis, and the G_1, S, and G_2/M subpopulations were calculated only for diploid cells. (C) Cell apoptosis was measured by using flow cytometry, as described in Materials and Methods. Error bars represent the SD from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

peptide synthase gene cluster, *ambBCDE*, which has been shown to contribute to the virulence of *P. aeruginosa* in different animal host models. However, since the purified IQS molecule of *P. aeruginosa* is currently not available, we did not involve it in this study.

We notice that the results obtained with conditioned medium from the *P. aeruginosa* wt and QS mutants were not fully expected on the basis of the data that we obtained with pure QS molecules. One of the major reasons is the complex interconnection among these different QS systems. *las* governs the expression of both the *pqs*

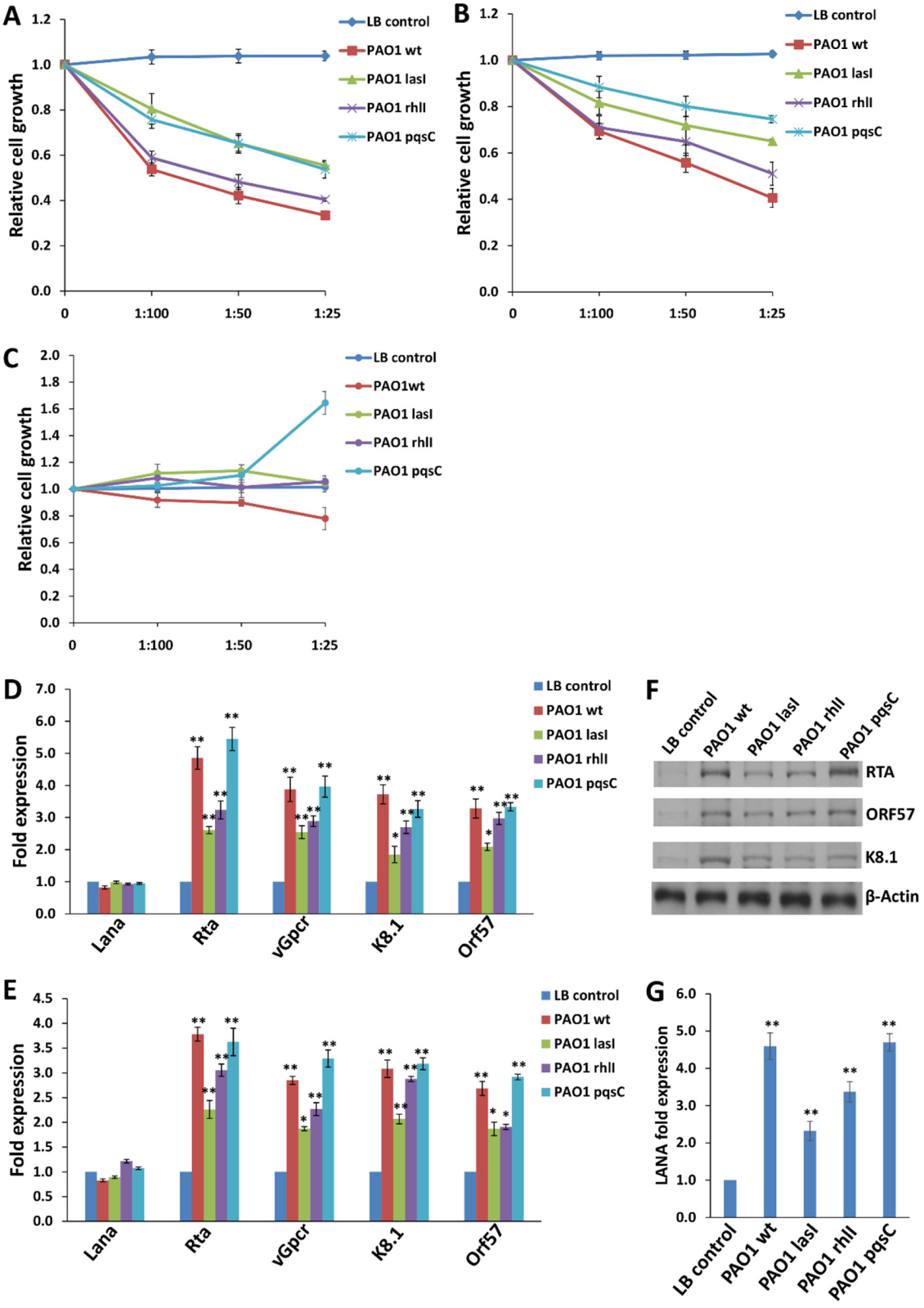

FIG 5 The conditioned medium from the *P. aeruginosa* PAO1 wild type and QS mutants regulates PEL cell growth and viral gene expression. (A to C) BCBL-1 (A), BCP-1 (B), or BL-41 (C) cells were incubated with filtered conditioned medium from overnight *P. aeruginosa* PAO1 wt or QS mutant (*lasI*, *rhlI*, *pqsC*) cultures (diluted 1:100, 1:50, and 1:25) for 48 h. The cell proliferation status was examined using WST-1 cell proliferation assays (Roche). (D and E) BCBL-1 (D) and BCP-1 (E) cells were incubated with filtered conditioned medium from overnight *P. aeruginosa* PAO1 wt or QS mutants cultures (diluted 1:25) for 48 h, and then qRT-PCR was used to quantify viral transcripts representing either latent or lytic genes. Data were normalized to those for vehicle-treated cells, and

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and *rhl* systems; on the other hand, the *rhl* system is under the control of both *las* and *pqs* (21, 22). Even the recently identified IQS has also been found to be tightly controlled by *lasRI* under rich medium conditions (20). Besides the QS signaling molecules mentioned above, the conditioned medium of *P. aeruginosa* cultures may contain a variety of QS-controlled virulence factors, such as pyocyanin and rhamnolipids (23, 24), although we found that conditioned medium from the *P. aeruginosa* PAO1 *rhlA* mutant (25) (deficient in rhamnolipid production) still effectively inhibited PEL cell growth (data not shown). Therefore, the construction of double- or even triple-knockout QS mutants in future studies will be helpful to answer these questions. Of course, we cannot exclude the possibility that some QS-independent factors, such as the type III secretion system of *P. aeruginosa* (26), may also affect PEL cell growth and/or viral gene expression.

Although both the QS molecules (e.g., PQS) and the conditioned medium of *P. aeruginosa* cultures displayed inhibitory effects on PEL cell growth, we think that coinfection by *P. aeruginosa* should not be enough to eliminate all the tumor cells in patients (due to their highly aggressive progression). On the other hand, these QS molecules and/or conditioned medium from *P. aeruginosa* laboratory strains and clinical isolates displays a strong ability to induce viral lytic gene expression and the release of viral infectious particles from PEL cells, which may greatly facilitate virus dissemination, the establishment of new infection, and, finally, the promotion of tumor development. We are now working on determining the underlying mechanisms by which *P. aeruginosa* or its QS molecules induce viral lytic gene expression. One of the possible mechanisms is the repression of some KSHV microRNAs, which have been shown to maintain viral latency in infected cells through either direct targeting of the viral lytic reactivation activator Rta or indirect mechanisms targeting some host factors (27–29). Since our methods used in this study represent an indirect evaluation of virion release, there are several alternative interpretations for the increased LANA expression in HUVEC exposed to QS molecules or conditioned medium-induced supernatant; for example, QS molecules enhance KSHV attachment to HUVEC or relieve heterochromatin formation on incoming virions to enhance LANA transcription. Therefore, we will design respective experiments to test these alternative interpretations in future studies.

Interestingly, the QS molecules of *P. aeruginosa* have been found to regulate host immune cell functions and cytokine production (30), which may modify the tumor microenvironment to accelerate tumor development. For example, during *P. aeruginosa* infection, innate immune cells can migrate toward the site of infection and remain in close proximity to the bacterial biofilms, but their functions are inhibited rather than stimulated by enhanced concentrations of QS molecules and QS-controlled bacterial traits (31, 32). In other studies, it has been reported that the expression and secretion of different pro- and anti-inflammatory cytokines in host cells are influenced by bacterial QS molecules (33–35). So, it will be interesting to explore the role of the immunoregulatory function of *P. aeruginosa* QS molecules in KSHV pathogenesis and tumorigenesis in future studies.

The current study focused only on KSHV$^+$ PEL cells, but it will be interesting to find out the impacts of *P. aeruginosa* or its QS molecules on other KSHV-related malignancies, such as KS and MCD. For example, *P. aeruginosa* is one of most common pathogens causing skin and soft tissue infections (36, 37), which may also affect KS development.

FIG 5 Legend (Continued)
β-actin was used as a loading control. (F and G) BCBL-1 cells were incubated with the filtered conditioned medium described above for 4 days, and then protein expression was measured using immunoblots. Released virions were isolated, purified from the supernatant, and used to infect fresh HUVEC. After 24 h postinfection, *Lana* transcripts were quantified using qRT-PCR. Error bars represent the SD from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

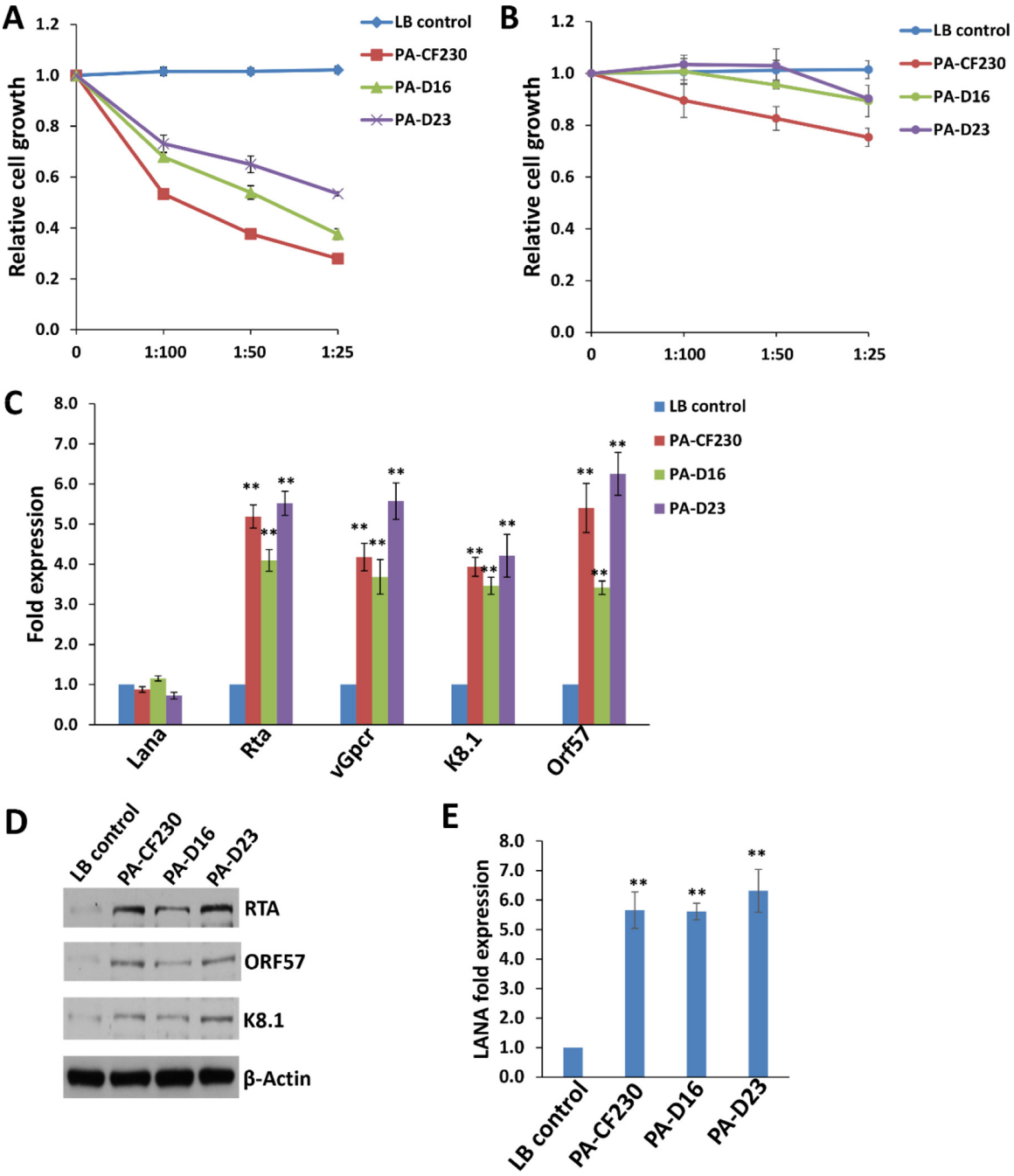

FIG 6 Conditioned medium from *P. aeruginosa* clinical isolates regulates PEL cell growth and viral gene expression. (A and B) BCBL-1 or BL-41 cells were incubated with filtered conditioned medium from overnight *P. aeruginosa* clinical isolate (PA-CF230, PA-D16, PA-D23) cultures (diluted 1:100, 1:50, and 1:25) for 48 h. The cell proliferation status was examined as described in the legend to Fig. 5. (C) BCBL-1 cells were incubated with filtered conditioned medium from overnight *P. aeruginosa* clinical isolate cultures (diluted 1:25) for 48 h, and then qRT-PCR was used to quantify the viral transcripts. (D and E) BCBL-1 cells were incubated with filtered conditioned medium from clinical isolates for 4 days, and then protein expression was measured using immunoblots. Released virions were isolated, purified from the supernatant, and used to infect fresh HUVEC. After 24 h postinfection, *Lana* transcripts were quantified using qRT-PCR. Error bars represent the SD from 3 independent experiments. **, $P < 0.01$.

MATERIALS AND METHODS

Cell culture, bacterial strains, and reagents. Cells of the KSHV$^+$ PEL cell line BCBL-1 and Burkitt's lymphoma cell line BL-41 (KSHV negative, Epstein-Barr virus negative) were kindly provided by Dean Kedes (University of Virginia) and maintained in RPMI 1640 medium (Gibco) with supplements as described previously (38). Cells of another KSHV$^+$ PEL cell line, BCP-1, were purchased from the American Type Culture Collection (ATCC) and maintained in complete RPMI 1640 medium (ATCC) supplemented

with 20% fetal bovine serum. Primary human umbilical vein endothelial cells (HUVEC) were cultured as described previously (7). All the cells were cultured at 37°C in 5% CO_2. All experiments were carried out using cells harvested at low passage numbers (<20). *P. aeruginosa* PAO1 (strain PAO0001) was obtained from the Pseudomonas Genetic Stock Center (East Carolina University School of Medicine, Greenville, NC, USA). The *lasI* and *rhlI* mutants were constructed by allelic displacement in PAO1 as described previously (39). The *pqsC* mutant was constructed via transposon insertion in PAO1 (40). *P. aeruginosa* clinical isolates were collected as described previously (18, 19). Luria-Bertani (LB; Oxoid) broth was used as the culture medium for *P. aeruginosa* growth. Purified QS molecules from *P. aeruginosa*, OdDHL, BHL, and PQS, were purchased from Sigma.

Cell proliferation assays. Cell proliferation was measured by using the WST-1 assay (Roche) according to the manufacturer's instructions. Briefly, after the period of treatment, 10 μl/well of the WST-1 cell proliferation reagent was added into the 96-well microplate, and the plate was incubated for 3 h at 37°C in 5% CO_2. The absorbance of the samples was measured by using a microplate reader at 450 nm.

Microarray analysis. Microarray analysis was performed by and the results were analyzed at the Stanley S. Scott Cancer Center Translational Genomics Core at LSUHSC. Total RNA was isolated using a Qiagen RNeasy kit (Qiagen), and 500 ng of total RNA was used to synthesize double-stranded cDNA. Biotin-labeled RNA was generated using a TargetAmp-Nano labeling kit (Epicentre) for the Illumina Expression BeadChip system and hybridized to the HumanHT-12 (v4) Expression BeadChip system (Illumina) at 58°C for 16 h. The chip was washed, stained with streptavadin-Cy3, and scanned with Illumina BeadStation 500 and BeadScan systems. Using Illumina's GenomeStudio software, we normalized the signals using the cubic spline algorithm, which assumes that the distribution of the transcript abundance is similar in all samples. The background signal was removed using the detection *P* value algorithm to remove targets with signal intensities equal to or lower than those of irrelevant probes (which have no known targets in the human genome but which are thermodynamically similar to the relevant probes). The microarray experiments were performed twice for each group, and the average values were used for analysis. Common and unique sets of genes and enrichment analysis were performed using MetaCore software (Thompson Reuters).

Cell cycle analysis. PEL cell pellets were fixed in 70% ethanol and incubated at 4°C overnight. Cell pellets were resuspended in 0.5 ml of 0.05 mg/ml propidium iodide (PI) plus 0.2 mg/ml RNase A and incubated at 37°C for 30 min. The cell cycle distribution was analyzed on a FACSCalibur 4-color flow cytometer (BD Bioscience).

Cell apoptosis assays. Flow cytometry was used for quantitative assessment of apoptosis using a fluorescein isothiocyanate-annexin V-propidium iodide (PI) apoptosis detection kit I (BD Pharmingen).

qRT-PCR. Total RNA was isolated using an RNeasy minikit (Qiagen), and cDNA was synthesized from equivalent total RNA using a SuperScript III first-strand synthesis SuperMix kit (Invitrogen) according to the manufacturer's instructions. The primers used for amplification of the target genes are listed in Table S1 in the supplemental material. Amplification was carried out using an iCycler IQ real-time PCR detection system, and cycle threshold (C_T) values were tabulated in duplicate for each gene of interest in each experiment. No-template (water) controls were used to ensure minimal background contamination. Using the mean C_T values tabulated for each gene and paired C_T values for β-actin as a loading control, fold changes in expression for experimental groups relative to the assigned controls were calculated using automated iQ5 (v2.0) software (Bio-Rad).

Immunoblotting. Cells were lysed in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5 mM NaF, and 5 mM Na_3VO_4. Total cell lysates (30 μg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and incubated with 100 to 200 μg/ml of K8.1 (ABI), KSHV ORF57 (Santa Cruz), and RTA (Abbiotec). For loading controls, lysates were also incubated with antibodies detecting β-actin (Sigma). Immunoreactive bands were developed using an enhanced chemiluminescence reaction (Perkin-Elmer).

Statistical analysis. Significance for differences between the experimental and control groups was determined using the two-tailed Student's *t* test (Excel software, v8.0), and *P* values of <0.05 or <0.01 were considered significant or highly significant, respectively.

Accession number(s). The microarray original data have been submitted to the Gene Expression Omnibus (GEO) database (accession number GSE110076).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.00478-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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