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Cancer remains a devastating condition that a ects human health and quality of life

[1–4]. Immune compromised patients tend to be more susceptible to developing malignancy, including Kaposi's sarcoma (KS), primary e usion lymphoma (PEL), and multicentric Castleman's disease[5,6]. Such conditions are tightly linked with Kaposi's sarcoma-associated herpesvirus (KSHV, also known as Human Herpesvirus-8 (HHV-8)). KSHV, a gamma-2 herpesvirus, is an oncogenic virus with a double-stranded deoxyribonucleic acid (DNA) genome[6–9]. KSHV infection is primarily latent, including in tumor cells[6,10]. During latent infection, the virus persists as a multiple copy, extrachromosomal episome[6]. e latency-associated nuclear antigen (LANA) is one of several genes expressed

ension) two-dimensional (2-D) cultures. 2-D cultures lack many features of the native As a result, many *in vivo* physiologic properties that may be crucial to de ning a cell's n, such as signaling through certain pathways (*e.g.*,

Various 3-D culturing techniques (*e.g.*, hanging drop, micro uidic systems, bioprinting, assembly, spinner asks, and rotary system) have been successfully used to generate 3-D tumor models[19–33]. For example, hanging drop approach has been increasingly used to generate 3-D models due its simplicity; however, it is still challenging to use this method to provide long-term cultures. e rotary system and the spinner asks are suitable for long-term cultures; however, they are unable to generate consistently sized 3-D constructs and require special equipment[34]. Further, bioprinting and assembly are fabrication techniques that may require a subsequent culturing system (*i.e.*, bioreactors) to grow and mature cells[19,35]. While micro uidic systems have shown promise in 3-D culture, high uid ow induced-shear stress can a ect cell physiology[22]. A detailed description of advantages and disadvantages of each technique is shown in Supplementary Information (SI) Table S1. Although such techniques have been successfully applied for tissue engineering and regenerative medicine applications (*e.g.*, generation of 3-D models of stem cells[36] and hepatocytes[37,38]), only a few were utilized to culture virus-infected tumor cells[18]. In one report, a 3-D *in vitro* model for KSHV infection was developed using spheroids embedded in clotted- brin gel[15].

and 300 μm microwells at the same location. us, based on this simulation data and the size distribution results (Fig. 1b), the 450 μm microwell platform was chosen to be the basis of our subsequent experiments.

To evaluate the feasibility of growing virus-infected cells in 3-D, KSHV-infected BJAB and uninfected (control) BJAB cells were seeded into the microwells and cultured both with and without puromycin selection (Figs 1c and 3). e cells were seeded at a low-density ($\leq$100 cells/microwell) to allow for direct observation and monitoring of proliferation during the experiment. Although precise control over the number of seeded cells per microwell is a challenge, it was clear that increasing the cell concentration when seeding the microwells resulted in increased numbers of cells settling into microwells[45]. e cell occupancy in a microwell array has been previously investigated both theoretically and experimentally[46,47]. In our microwell system, as the microwell size is much larger than the cell size, the cells already present in a given microwell will not a ect addition of new cells to that same microwell. e possibility (f) of a microwell containing n cells can be described by the equation of Poisson distribution as below,

$$f(n;\lambda) = \frac{\lambda^n e^{-\lambda}}{n!} \tag{3}$$

where λ is the average number of cells per microwell area on the glass slide. As λ increases the Poisson distribution approaches the Gaussian form. e peak or maximum of the Poisson distribution corresponds to the mean (λ). us, the average cell number in microwells can be modulated by the cell seeding density on the glass slide.

Once cells settle inside the microwells, they do not displace during exchange of cell culture media. e cells can be harvested without compromising their viability[48] by tilting the platform at 45°.5(o)12.6(n).9(t)6()8(e n3p -8(y [(c

grow in aggregates determined by the size and shapes of the constructed microwells, and with cell-to-cell contact occurring in three dimensions.

Subsequent to cell seeding, a series of bright eld photomicrographs were obtained of each microwell for 15 days. e results demonstrated that there was no signi cant di erence in the proliferation rate between KSHV-infected BJAB cells cultured with or without puromycin selection (Figs 3a and 4a). is result is expected since the recombinant KSHV expresses the puromycin resistance gene. Proliferating cells grew efficiently until they lled the entire microwell (at around day 15), and subsequently began to spill out from the microwells (SI Figure 3). is cell outgrowth occurs due to the non-adhesive properties of PEG, and results in some non-ho12.4(n)o Tc -0.8rs cell mTc -0.8-8.7(de)4.3(l)13.(s a)9.2( )6.3(er d)-2.8(a)18.3(y 15.)4.3(e ini)12.3(t)-4.7(i)-2. summarizes the volumTc -0es of the 3-D models that were ge Tc -0erated rsing di erent sizes of microwells. e size of the

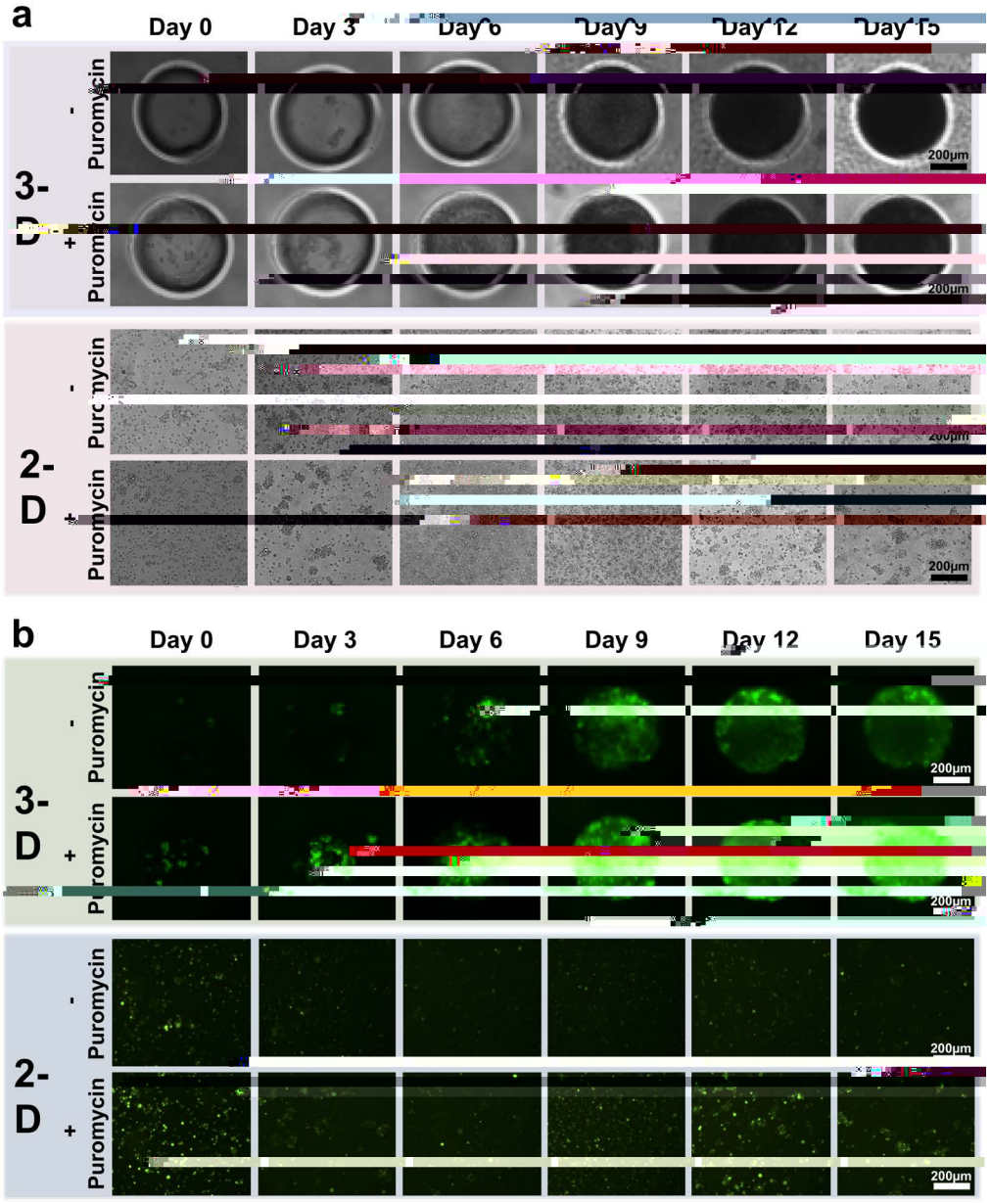

Fig e 3. KSHV i fec ed BJAB cell mai ai ed i 3-D 450 μm mic ´ ell ´ 2-D t e i´ , b´ h i h (+) a d i h´ t (−) t ´m ci elec i´ . (**a**) Bright eld photomicrographs of infected BJAB cells grown for 15 days. Images were taken at 3-day intervals, beginning with the day of seeding. Scale bars indicating 200 μm are shown. (**b**) Fluorescent photomicrographs of infected BJAB cells grown for 15 days. Expression of GFP (green) by the infected cells, which were cultured in 2-D or 3-D, indicates KSHV infection. Scale bars represent 200 μm. In the absence of puromycin selection, less uorescence was observed over time in cells in 2-D and 3-D culture due to loss of KSHV infection. is is a representative gure for the subsequent quanti cation gure (*i.e.*, Fig. 4). Scale bars indicating 200 μm are shown.

~2–3 fold more LANA dots present in infected cells in 3-D compared to 2-D culture (Fig. 5a), indicating a higher KSHV genome copy number since each LANA dot corresponds to a viral genome. Consistent with this nding, both in the presence or absence of puromycin, the 3-D cultures had much brighter uorescence compared to the 2-D cultures (Figs 3 and 5). We also investigated lytic reactivation at 15 days by detecting the KSHV lytic protein product, ORF59 (Fig. 5b). Reactivation from BJAB-KSHV cells was very ine cient, and even a er induction with 12-O-tetradecanoyl phorbol-13-acetate (TPA) and sodium butyrate fewer than 1% of cells expressed ORF59 (data not shown). However, we observed that ~3 fold more cells expressed ORF59 in 3-D versus 2-D culture in the presence of puromycin as there were ~15 ORF59 expressing cells in 3-D compared to ~5 ORF59 expressing cells in 2-D (with similar numbers of total cells plated per slide). In the absence of puromycin, fewer cells underwent reactivation in 3-D, perhaps related to lower episome copy number, but there still appeared to be more ORF59

expressing cells compared to 2-D culture without puromycin. ese ndings of increased episome copy number and increased lytic reactivation are similar to previous observations made by Cheng *et al.* when KSHV infected primary endothelial cells were grown in 3-D a er embedding spheroids in brin gel[15]. erefore, it is likely that autocrine and paracrine signaling in the 3-D microenvironment contributes to enhanced infection with higher viral genome copy per cell and a higher rate of lytic reactivation, perhaps more similar to conditions present *in vivo*[49,50].

In this study, we report the ability to culture human KSHV-infected BJAB cells in 3-D using a microwell format. e use of microwell-based culture for the purpose of growing virus-associated tumor cells in 3-D has not been well studied or applied. One challenge of this concept is the di culty to extend the culture for a longer period ($>$3 weeks) of time without the detachment of the PEG hydrogel from the TMSPMA coated slides as a result of the weakening of the attachment between the PEG microwells and the glass. However, stability of the PEG attachment can be enhanced by etching the glass surface with a mixture of sulfuric acid and hydrogen peroxide (piranha etch)[51] as well as treating the surface with oxygen plasma to form an oxygen-rich layer[52,53]. Furthermore, the present method has the potential to allow further investigation of the recovered KSHV-infected BJAB cells a er long term culture, such as for ow cytometry or polymerase chain reaction. Morever, this approach would provide the basis for future work to further investigate the biology of KSHV infected cells grown in 3-D, including potential e ects on cytokine production. In addition, investigation of other infected cell types, such as endothelial cells, would be a logical next step for the applications of this approach. is innovative platform can also be usetive platfotf2

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Here, we demonstrate the ability to grow KSHV-infected human BJAB cells in 3-D cultures. e cells were cultured in PEG-based 3-D microwell array system for up to 15 days. Compared with culture in 2-D, cells in 3-D had more nuclear LANA dots, indicating a higher KSHV genome copy number, and cells in 3-D also underwent lytic reactivation at a higher rate. e 3-D microwell array system has the potential to improve cell culture strategies and allow for better-controlled studies for drug discovery and experimental biology.

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&ÁÂÒÉÃÁÔÏÎ ÏÆ0$-3-ÏÌÄ8 Poly(dimethylsiloxane) (PDMS) molds were fabricated by thoroughly mixing (10:1) of elastomer and curing agent (SYLGARD 184 Silicone Elastomer Kit; Sigma). e mixture was poured on a silicon master patterned with SU-8 photoresist, degassed in a vacuum chamber for 30 minutes, and then allowed to cure at 70 °C for 2 hours. e silicon master (positive structure) has circular depressions, which were replicated into PDMS (negative structure) for the following fabrication steps. ree di erent silicon masters were used to prepare di erent sizes of PDMS molds with cylindrical protrusions of: 150 μm (diameter)9

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49. Hamilton, S. K. *et al.* Development of 3D hydrogel culture systems with on-demand cell separation. *Biotechnology journal* **8,** 485–495 (2013).
50. Bogdanowicz, D. R. & Lu, H. H. Studying cell-cell communication in co-culture. *Biotechnology journal* **8,** 395–396 (2013).
51. Yoshimoto, K., Ichino, M. & Nagasaki, Y. Inverted pattern formation of cell microarrays on poly (ethylene glycol)(PEG) gel patterned surface and construction of hepatocyte spheroids on unmodi ed PEG gel microdomains. *Lab Chip* **9,** 1286–1289 (2009).
52.