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Zika virus-like particles (VLPs): Stable cell lines and continuous perfusion processes as a new potential vaccine manufacturing platform

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ABSTRACT

Zika virus (ZIKV) was first detected in Brazil in 2015 and then rapidly spread to more than 80 countries in Africa, Asia and the Americas. ZIKV infection was correlated with severe congenital malformations in newborns from infected mothers, as well as with Guillain-Barré syndrome in adults. Although the number of infected people has declined in the affected countries lately, the development of a vaccine for ZIKV is of great importance to avoid the future resurgence of the virus in endemic areas or the future spread to currently non-endemic regions. Among many different platforms currently under study, virus-like particles (VLPs) are a promising alternative for the development of vaccines, since tridimensional particles mimicking the virus – but lacking its genome – can be produced and present the antigen in a repetitive way, potentially eliciting robust immune responses. In this work, we demonstrated the generation of stably transfected HEK293 cells constitutively expressing Zika VLPs. Small-scale shake flask studies using a stable cell pool enriched by Fluorescence-Activated Cell Sorting (FACS) showed that daily medium exchange (intermittent perfusion) significantly enhances viable cell density and VLP production (~4-fold) over batch cultures. Continuous perfusion in a controlled bioreactor coupled to an ATF-2 cell retention device resulted in maximum VLP titers similar to those obtained under small-scale intermittent perfusion. Our results show that the use of cell lines constitutively expressing Zika VLPs, cultured in stirred-tank perfusion bioreactors, represents a promising system for the production of a VLP-based Zika vaccine candidate.

1. Introduction

Zika virus belongs to the *Flaviviridae* family, genus *Flavivirus*. It was first isolated from *Rhesus* monkeys in Uganda, Africa, in the 1940's [1]. A first human outbreak was reported in 2007 in Yap Island, Micronesia, although some human infections had been reported before [2,3]. In 2013, a larger outbreak occurred in French Polynesia and evidence was gathered that ZIKV could cause Guillain-Barré syndrome in infected adults [4,5]. In 2015, a much larger outbreak started in Brazil and it was proven that Zika virus infection in pregnant women can lead to serious congenital malformations [6,7]. Thus, due to the serious impacts that ZIKV infection could potentially generate, the WHO (World Health Organization) declared it in February 2016 as a Public Health Emergency of International Concern [8]. Nowadays, there is evidence of transmission of vector-borne ZIKV in 84 countries, territories or subnational areas, and more than 50 ZIKV vaccine candidates are being studied [9–11].

Virus-like particles are three-dimensional structures, constituted by recombinant structural proteins of the virus, lacking the viral genome and displaying the antigen in a repetitive pattern that triggers immunogenicity [12–14]. Two different works have shown that immunization of mice with different Zika VLPs constructs efficiently elicited the formation of neutralizing antibodies in mice, indicating the potential of Zika VLPs as a vaccine candidate. In these works, VLP constructs were constituted by different sets of structural proteins (containing or not the capsid protein), and the particles were obtained by transient expression in HEK293 cells [15,16].

The structural proteins prM (premembrane) and E (envelope) were chosen in this work to form the Zika VLPs, in the absence of the capsid, since it had been reported for flaviviruses that these proteins are enough for the self-assembly of the VLPs *in vitro* [17] and that epitopes recognized by protective neutralizing antibodies are mainly within the E protein of flaviviruses [18].

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The possibility of developing stable cell lines producing VLPs enables the development of a platform that could potentially lead to an affordable vaccine, overcoming the high costs of DNA and transfection reagents related to large-scale transient transfection. Moreover, stable expression allows the use of continuous perfusion processes, which can be operated at high cell densities and at steady state, providing a stable and optimal environment for the cells and, thus, allowing improvements in process productivity, reducing costs and ensuring high product quality [19–21].

2. Materials and methods

2.1. Cell line development and culture conditions

Based on the genome sequence of a ZIKV strain isolated in Brazil (strain BeH819966, GenBank #KU365779), different prM-E gene constructs, flanked by different signal peptides (SP) and containing wild-type, mutated or chimeric sequence variants of the E (envelope) protein were designed (Fig. 1), codon-optimized and synthetized at Genscript (USA). These constructs were cloned into the pCIneo vector (Promega, USA) and transfected into the suspension-adapted HEK293SF-3F6 cell line (NRC, Canada). Transfection was performed in 24-deep well plates (Porvair Sciences, UK, or Thomson Instrument Company, USA) at a density of 1×10^6 cells/mL, using 0.5 μg/mL of plasmid and 1.0 μL/mL of the transfection reagent TransIT®-2020 (Mirus Bio, USA), in HEK TF medium (Xell AG, Germany). Approximately 48 h post-transfection, samples were withdrawn, cells were diluted in fresh HEK TF medium to the final concentration of 1×10^6 cells/mL, and 100 μg/mL of the selection antibiotic G418 sulfate (Gibco, USA) was added to promote selection of cells stably expressing the heterologous DNA. Cell pools were diluted twice a week during 6 weeks under the presence of G418 sulfate, and supernatant samples were taken periodically to confirm VLP production by immunoblot assay. During the whole process of cell line development and maintenance, cells were cultivated in HEK TF medium in shake flasks (Corning, USA), spin tubes with vented caps (TPP AG, Switzerland) or 24-deep well plates, shaken at 180 rpm (25 or 50 mm orbit size). Flasks were kept in a humidified incubator at 37 °C and 5% CO_2. Cell count was performed using a Vi-CELL XR cell counter (Beckman Coulter, USA) or a Neubauer chamber, both based on the trypan blue exclusion method. When necessary, glucose and lactate concentrations were measured using a YSI 2700 Select Biochemistry Analyzer (YSI Inc., USA).

2.2. Analysis of VLP production

VLP production was evaluated by immunoblots, based on the application of 3-μL samples of centrifuged supernatant (200*g*, 5 min, room temperature) onto a nitrocellulose membrane (Vita Scientific, USA). The monoclonal antibody 4G2 (1:8000) (MAB10216, Merck, USA), which recognizes the conserved fusion loop of the E protein of flaviviruses, or anti-Zika prME mouse serum (1:50), was used as primary antibody, followed by incubation with horseradish peroxidase (HRP)-conjugated donkey anti-mouse antibody (1:10,000) (A16011, Invitrogen, USA). Antibody binding to the E protein of the VLPs was visualized via chemiluminescence with ECL™ Prime detection reagent (GE Healthcare, USA). Dengue envelope protein (DEN-026, Prospec, Israel) or an *in house* Zika VLP standard (300 μg/mL stock concentrate) was used as positive control. Image analysis for densitometry determination was performed using ImageJ software (National Institutes of Health, USA). To each blot analyzing duplicates of unknown samples, different concentrations of the positive control were added in duplicate, allowing determination of VLP concentration in terms of E protein.

2.3. Enrichment of stable cell pools producing Zika VLPs

Lecc3 and lecc9 cell pools were enriched 21 and 6 weeks post-transfection (wpt), respectively. In order to enrich cell pools for high-producer subpopulations, approximately 200×10^6 viable cells were stained with 4G2 antibody biotinylated *in house* using EZ-Link™ Sulfo-NHS-Biotin kit (Thermo Scientific, USA) and streptavidin-PE (Invitrogen, USA). After incubation with biotinylated 4G2 for 20 min on ice bath, cells were washed three times with phosphate-buffered saline (PBS) containing 2% (m/v) of bovine serum albumin (BSA) (Sigma-Aldrich, USA). Cells were then stained with streptavidin-PE for 15 min on ice bath. Stained cells showing higher fluorescence signal were collected by FACS

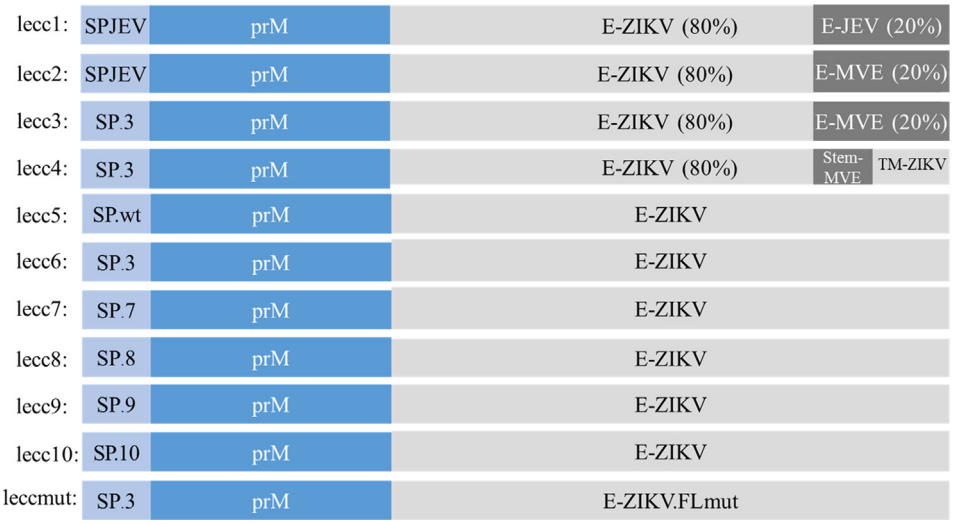

Fig. 1. ZIKV constructs based on the structural proteins prM and E. Signal peptides (SP) from Japanese encephalitis virus (JEV) and other peptides designed *in house* were evaluated. E protein sequences were either chimeric, where 20% of the C-terminal end of ZIKV E protein was substituted by 20% of the C-terminal end of E of JEV or Murray Valley encephalitis virus (MVE) sequences (lecc1-3) or based on the wild-type sequence of ZIKV strain BeH819966 (lecc5-10). E protein of construct leccmut was also designed according to ZIKV strain BeH819966, except for substitutions in amino acids 106 (Gly-Asp) and 107 (Leu-Asp), present in the fusion loop. Construct lecc4 is also chimeric, considering that the aminoacids of the stem part of the E protein of Zika were substituted by the MVE amino acids sequence.

(Fluorescence-Activated Cell Sorting) and transferred to 24-deep well plates to a final concentration of 0.5×10^6 cells/mL in HEK TF medium. Penicillin/streptomycin was added to the medium during the sorting procedure and for 2 passages thereafter to prevent microbial cell growth. Non-stained cells were used as negative control in the FACS runs, and non-sorted cells were used in immunoblots for comparison with the enriched (sorted) cell pools.

2.4. Kinetic studies in shake flasks

Batch and pseudoperfusion (PP) studies were performed to assess cell behavior in spin tubes, using 15 mL as initial working volume. Cells were inoculated at 0.5×10^6 cells/mL in HEK TF medium and samples were taken daily to evaluate cell growth, viability, glucose consumption, lactate concentration and also to evaluate VLP production. All experiments were performed in biological duplicates. In the case of pseudoperfusion, two different feeding strategies were applied, both starting feeding on day 4, when the volume of the spin tubes was adjusted to 10 mL. From day 4 on, cell suspension was centrifuged daily (200*g*, 5 min, room temperature) to promote cell retention, and then medium was exchanged. In the first pseudoperfusion strategy, a stepwise feeding was performed based on cell demand for glucose: 0.5 vvd (volume of fresh medium per volume of culture per day) when glucose concentration upon sampling was higher than 2 g/L; 0.75 vvd if between 1 and 2 g/L; and 1 vvd when lower than 1 g/L. The second pseudoperfusion strategy was based on a constant medium exchange rate of 1 vvd from day 4 on.

2.5. Perfusion cultivation in stirred-tank bioreactor

A perfusion cultivation was carried out in a stirred-tank bioreactor (ez-Control, Applikon Biotechnology, The Netherlands) fitted with a pitched-blade impeller and using an alternating tangential filtration system (XCell™ ATF-2, Repligen, USA) as cell retention device. The microfiltration polyethersulfone hollow fiber module for ATF-2 system had pore size of 0.2 μm, 1.0 mm of lumen and 0.13 m^2 of surface area (Repligen, USA). The lab-scale bioreactor was inoculated with 0.5×10^6 cells/mL and cultivation was performed at 37 °C, pH 7.1 and a dissolved oxygen (dO_2) setpoint of 40% of air saturation. Automatic control of dO_2 was performed by injection of air in the beginning of the run until cell concentration reached $2–3 \times 10^6$ cells/mL, and then injecting pure oxygen through a drilled hole sparger, both at a maximum rate of 0.25 L/min. Stirring speed was maintained between 200 and 300 rpm, and working volume was 0.9–1.1 L.

Perfusion started on day 3 with a medium exchange rate of 0.35 vvd and was gradually increased up to 1.5 vvd, in order to keep glucose concentration at approximately 1 g/L during the steady state. Controlled removal of cells (cell bleed) was initiated on day 7 to maintain cell concentration at approximately $25–30 \times 10^6$ cells/mL.

3. Results and discussion

3.1. Design of different gene constructs and development of stable cell lines

With the final aim of obtaining stable cell lines constitutively expressing and secreting Zika VLPs, different gene constructs containing the sequences of the structural proteins premembrane (prM) and envelope (E) were designed (Fig. 1). The chimeric versions of E protein evaluated in constructs 1–4 were based on previous reports showing that replacement of the stem domain (54 amino acids in E) or both the stem and transmembrane domains (99 amino acids in the C-terminal end of E) of the E protein of flaviviruses by the analogous sequence of the Japanese encephalitis virus (JEV) led to more efficient secretion of flavivirus VLPs [25,26]. Additionally, the use of the JEV signal peptide had shown to increase secretion of prM-E constructs of flaviviruses [25,26], so we decided to compare the JEV and ZIKV wild-type signal peptides to different signal peptides designed in our lab (named SP3-SP10). Furthermore, in one of the constructs (lecc-mut), two mutations (G106R and L107D) were included in the fusion loop region of the E protein according to previous reports related to other flaviviruses [27,28].

Mammalian cell lines known for good recombinant protein expression and/or virus production (CHO-K1, HEK293, Vero and BHK-21) were transfected with the different constructs, but the highest transient expression level was observed in HEK293 cells, so only data for this cell line are shown. HEK293 cells are well established as a robust bioprocess platform for the production of viruses and viral vectors [21], and have also been used to produce VLPs of HIV, rabies and influenza in continuous perfusion mode, coupling the bioreactor to cell retention devices such as acoustic filters and spin filters [22–24]. As an additional point regarding HEK293 cells, since Zika VLPs are enveloped and the cell membrane of the host cell line is used during assembly and budding of the VLPs, the use of a human cell line might be an advantage for this purpose.

Initially, four different constructs (lecc1-lecc4) bearing chimeric E sequences were used to evaluate VLP production by transient expression in the suspension-adapted HEK293-3F6 cell line. Only the supernatants were analyzed, because we were aiming at secreted VLP production, which can be continuously harvested in the setting of a continuous perfusion process. In this first part of the study, it was possible to observe the expression of secreted VLPs related to the constructs lecc1-lecc3 at 48 h post-transfection (hpt). The JEV-chimera showed stronger signal than the MVE-chimera (lecc1 × lecc2 constructs). However, the use of a novel signal peptide designed *in house* was able to improve secretion of the Murray Valley encephalitis virus (MVE) chimera (lecc2 × lecc3 chimera). The construct lecc4, which had the E sequence altered only in the stem region, and not in both the stem and transmembrane domains as lecc1-lecc3, was not detected in the supernatant (Fig. 2A).

Based on these results and on the fact that the use of the JEV signal peptide and JEV stem/transmembrane regions included in constructs lecc1 and lecc2 had been patented by Chang [29], construct lecc3 was chosen for further studies designed to compare chimeric to wild-type and fusion-loop mutated E sequences (lecc3 × lecc5 × leccmut), as well as different signal peptides (wild-type ZIKV SP in lecc5 to others designed in-house in lecc6-10). The results of transient expression at 48 hpt of VLPs in the supernatants are shown in Fig. 2B. Constructs lecc7-10 showed higher VLP secretion levels in comparison to constructs 3, 5 and 6. This means that among the signal peptides designed *in house*, all but one (SP3 in lecc3 and lecc6 constructs) enabled higher secreted VLP concentrations than the ZIKV wild type signal peptide in construct lecc5.

Approximately 48 hpt, G418 sulfate was added to the cultures to select cells stably expressing the VLPs. After 6 weeks under selection pressure, mock transfected cells had died and transfected cell pools had viability in the range of 84–96% and were considered stable. VLP monitoring in the supernatants of transfected cells confirmed continued VLP production in all of them (Fig. 2C). The levels of secreted VLPs varied between the different stable cell pools as observed for the transient expression samples, reflecting the performance of the different signal peptides used in the gene constructs. Also, except for the samples withdrawn 1 wpt, when cells were recovering from transfection and selection, and had very low cell density and viability, variations in detected signal over time for a given cell pool were minor.

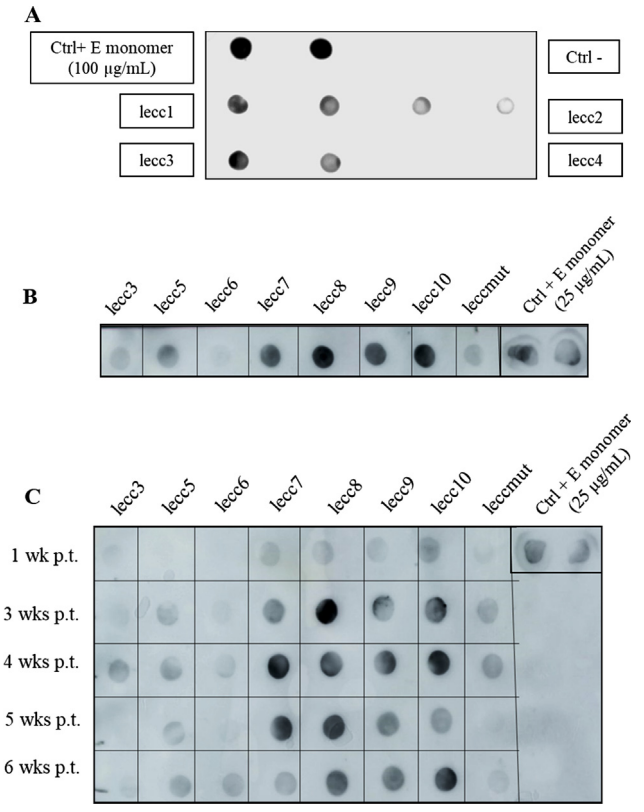

Fig. 2. Immunoblot for confirmation of VLP production by the different cell pools. Dengue E protein was used as a positive control to assure that E proteins were recognized effectively by this methodology. (A) First evaluation of VLP transient expression in HEK293-3F6 cell line using constructs lecc1-lecc4 at 48 h post-transfection (p.t.). Samples from both biological transfection replicates were evaluated. As observed, it was possible to detect expression of constructs lecc1-lecc3, but no production of lecc4 was visualized. 4G2 monoclonal antibody was used as primary antibody to detect E protein. (B) Transient expression of different VLP constructs at 48 hpt., showing higher expression level for constructs lecc7-lecc10. Anti-Zika (prME) mouse serum was used as primary antibody to detect E protein. (C) VLP expression of constructs lecc3, lecc5-lecc10 and leccmut from 1 wkt until 6 wpt. Anti-Zika (prME) mouse serum was used as primary antibody to detect E protein.

It is generally believed for flaviviruses that constitutive expression of VLPs would be toxic to the host cells, so usually transient or inducible expression methods are used to obtain flavivirus VLPs [30]. However, in our experiments cells presented high viability after recovering from transfection, indicating that constitutive expression of Zika VLPs was not deleterious to the cells and enabling the development of a continuous process for flavivirus VLP production. Among the few works found in literature using continuous perfusion cultivation of mammalian cells for the production of VLPs [22–24], none of them is related to flaviviruses, and just one of them [23] focuses on constitutive expression of the VLPs. However, differently from the present work, which uses a plasmid expression vector, their recombinant cell line was obtained by lentivirus-based transduction of HEK293 cells to express rabies VLPs. In the other two works from literature adopting perfusion processes for VLP production, either repeated transient expression [22] or inducible stable expression [24] is used. In order to develop an affordable high-cell density perfusion platform, a constitutive stable expression is expected to have lower costs and be advantageous when compared to inducible or transient expression systems.

Since the aim of this study was to produce VLPs in stirred-tank bioreactors, small-scale evaluation of the cell pools was first performed 4 wpt in batch mode. In general, cell growth was similar among the different cell pools (Fig. 3). The cell growth profile and the high cell densities obtained in this experiment confirmed that the stable constitutive expression of Zika VLPs was not deleterious to the cells.

Supernatants of several of these constructs have been purified by chromatographic methods, and the purified VLPs analyzed by ELISA, as well as by transmission electron microscopy and dynamic light scattering, confirming the expected size and structure of the VLPs [31].

3.2. Evaluation of cell stability and FACS-aided enrichment of cell pools

In order to evaluate stability of VLP expression, supernatant samples were taken over time and evaluated by immunoblot for lecc3 (Fig. 4A) and lecc9 (Fig. 4C) cell pools. Lecc3 pools showed similar VLP production when samples from 6 wpt and 21 wpt were evaluated. Part of the cell pool had been maintained in the absence of G418 from 16 wpt on and immunoblot analysis did not indicate any differences (Fig. 4A), confirming stability of expression even after 5 weeks in the absence of selection pressure. Lecc3-expressing cells at 21 wpt (with and without G418) were additionally submitted to FACS-aided enrichment of high-producers, and expression in both sorted cell populations (with and without

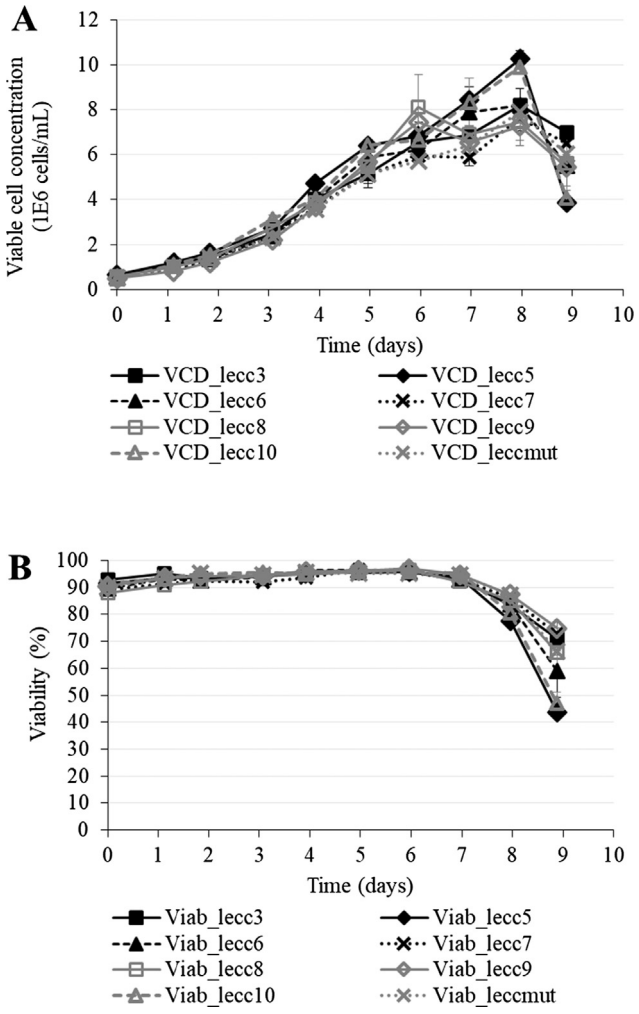

Fig. 3. Evaluation of the cell pools when cultivated in small-scale shaken flasks, performed 43 days post-transfection, in HEK TF medium. (A) Viable cell concentration (VCD) obtained for the different stable cell pools cultivated in batch mode. (B) Viability of the cell pools during the experiment was kept above 90% until day 7, when glucose became depleted.

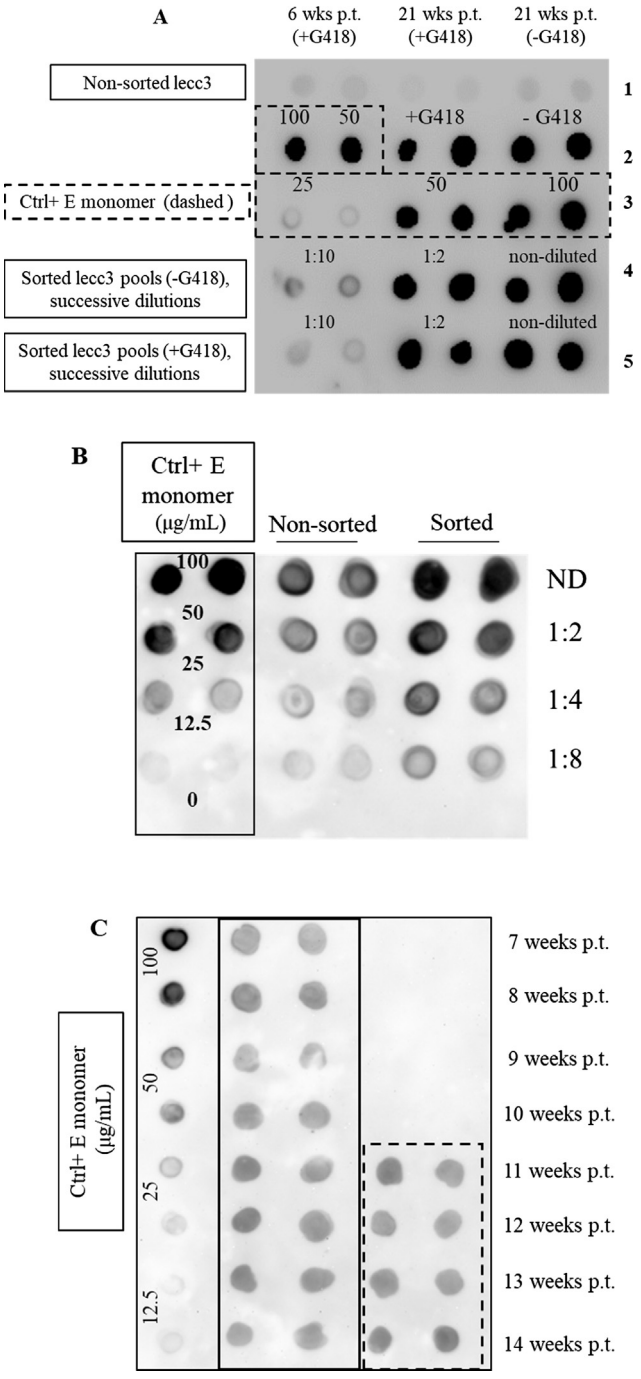

Fig. 4. Immunoblot confirming stability in production over time and the increase in VLP production by cell pools after sorting procedure. All conditions were evaluated in replicates. (A) Non-sorted lecc3 cells were kept for over 20 weeks in culture and it showed similar expression with samples collected 6 weeks post-transfection, indicating stability in VLP production over time. Part of the cell pool was maintained in the absence of selection pressure (G418) after 16 weeks p.t. (wpt) and VLP expression was comparable to samples taken 6 wpt and 21 wpt, both in the presence of G418 (line 1). Samples from sorted lecc3 cells (lines 2, 4 and 5) indicate increased production when compared to samples from non-sorted cells (line 1). Dengue E protein in different concentrations (dashed rectangle, lines 2 and 3) was used as positive control. (B) VLP expression obtained by sorted and non-sorted lecc9 cell pools. (C) Evaluation of the stability of VLP expression by lecc9 cell pool (non-sorted) in the presence of selection pressure (black rectangle). Stability was evaluated only in the presence of selection antibiotic (G418 sulfate) until week 10 post-transfection. Additionally, part of the cell pool was cultivated in the absence of G418 from week 11 post-transfection on (dashed rectangle) and production showed to be stable even with no addition of the selection antibiotic. Dilution of samples was carried out to assure sorted cells were producing more than pre-sorted cell pool. Dengue E protein in different concentrations was used as positive control.

G418) was equivalent. In the case of lecc9, VLP expression was monitored weekly from 7 wpt to 14 wpt, with no indications of expression loss. Part of the cell pool had been maintained in the absence of G418 from 11 wpt on (dashed rectangle in Fig. 4C) and immunoblot analysis did not indicate any differences.

Besides stability evaluation, selection of high-producer cells was desired. For HEK293 cells, there is a lack of an effective amplifiable expression system, such as CHO-DHFR$^-$ and CHO-GS systems. Therefore, it was important to pursue other strategies to obtain cells with increased productivity, such as the use of FACS to select high producer cells [32]. Since it has been observed in non-human primates that chimeric and wild-type versions of E contained in prM-E constructs can elicit the generation of antibodies with different neutralizing profile and different protective capacity [33], lecc3 (chimeric E protein) and lecc9 cell pools (wild-type E protein) were chosen for further studies and were used in FACS experiments for pool enrichment.

Based on the hypothesis that secreted products may be transiently found on the cell membrane [34,35], cells were stained with a fluorescent marker in order to sort for high-producer cells using preparative FACS. Upon sorting of a heterogeneous cell pool that survived selection pressure, the 0.3–0.8% of the cells presenting highest fluorescence intensity were gated. From this gate, 150,000–200,000 cells were collected into tubes and then transferred for cultivation in 24-deep well plates. Cells were expanded under orbital shaking, and supernatant samples were taken to compare VLP production before and after cell FACS enrichment. Improved production after sorting was confirmed by immunoblot for both lecc3 and lecc9 cell pools (Fig. 4A and B). Based on the immunoblot results, lecc3 cell pool was selected for further studies to develop the perfusion process.

3.3. Use of stable pools to develop continuous perfusion studies: small-scale evaluation

In order to evaluate the effect of feeding strategies on the behavior of FACS-enriched lecc3 cell pool, pseudoperfusion was used as a scale-down model for high-cell density cultivations [36]. Experiments in spin tubes were carried out testing a stepwise daily feeding strategy, based on the cell demand for glucose, and a constant feeding strategy based on 100% daily medium exchange, both with feeding starting on day 4. As observed in Fig. 5A, cell growth was substantially increased when cells were fed daily compared to batch mode. There was no significant difference in the behavior of cells fed according to the two different strategies, especially because differences in the amount of medium fed were restricted to days 4 to 7, when glucose concentration was not limiting (>1 g/L) (Fig. 5B). Therefore, the stepwise strategy based on cell demand was chosen to be applied in the subsequent perfusion bioreactor experiment.

Regarding VLP production (Fig. 5C), cell specific productivity (q_p) was similar in batch and pseudoperfusion (stepwise feeding strategy) modes. Since cells grown under both feeding strategies reached higher concentrations, maximum VLP concentration was approximately 4-fold higher in pseudoperfusion than the batch control. These results indicate that an intermittent perfusion process allows an increase in both cell concentration and VLP production. Thus, a truly continuous perfusion system could be even more advantageous and provide high volumetric productivities, contributing to an affordable production of the VLP vaccine candidate.

3.4. Perfusion run using XCell-ATF2 as cell retention device

A proof-of-concept perfusion cultivation was performed in a stirred-tank bioreactor coupled to an ATF-2 system, which promotes cell retention by alternating tangential filtration (ATF),

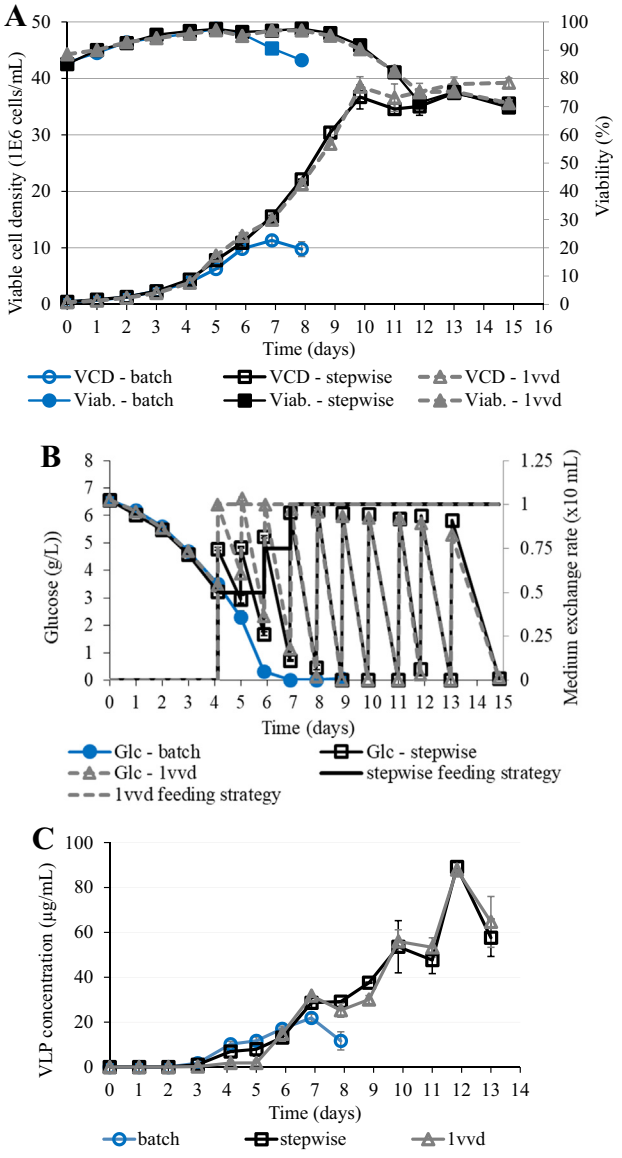

Fig. 5. Production of VLPs by lecc3 cell pool (sorted) in spin tubes cultivated under batch (blue circles) and pseudoperfusion modes (black squares - stepwise feeding; grey triangles - constant 1vvd feeding). (A) Viable cell density (VCD – open symbols) and viability (filled symbols) during the cultivation of cells. (B) Glucose concentration (Glc) and daily medium exchange rate during the cultivation. Feeding strategies started on day 4 and samples were taken before and after feed regimen was performed. (C) VLP concentration referred to Dengue E protein obtained when cells were grown in batch (blue circles) or pseudoperfusion modes (black squares - stepwise feeding; grey triangles - constant 1vvd feeding). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

allowing complete retention of the cells. Although no reports in literature have been found regarding the use of ATF for VLPs, an ATF-based perfusion system has been reported by Nikolay et al. [37] as efficient for the production of native Zika virus from infected BHK-21 cells.

The ATF system was started on day 2, one day before the beginning of perfusion itself and no negative effects on cell behavior were observed, indicating that the shear stress levels in the ATF cartridge are not deleterious and might even help to decrease HEK293 cell aggregation. However, although Zika VLPs are approximately 50 nm in size and the nominal pore size of the ATF-2 cartridge used was 0.22 µm, we observed unexpected retention of the VLPs inside the bioreactor, resulting in poor VLP transmission to the harvest, especially from day 15 of cultivation on (Fig. 6C).

Regarding cell growth, due to the high oxygen demand of HEK293 cells and in order to avoid oxygen limitations, cell bleed was started on day 7, when viable cell concentration reached approximately 25×10^6 cells/mL (Fig. 6A). A steady state at 25–30×10^6 cells/mL was kept from that moment on, and perfusion rate was recalculated daily according to cell demand, in order to maintain glucose level around 1 g/L (Fig. 6B). The stepwise feeding strategy was successful, since it decreased medium usage in the beginning of perfusion, as compared with a constant feeding strategy of 1 vvd, as previously shown in the small-scale pseudoperfusion studies.

VLP concentration inside the bioreactor showed a peak on day 12 (9 days after perfusion start and 5 days after cell bleeding start) and then decreased progressively until the end of the run. Since this proof-of-concept perfusion run was inoculated with cells at a

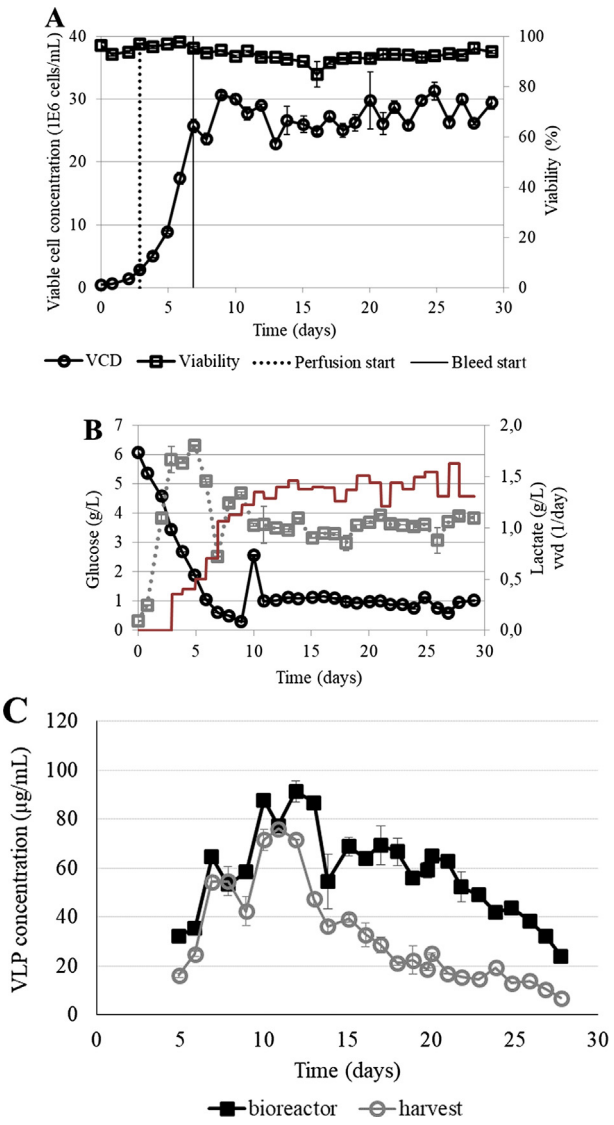

Fig. 6. Production of VLPs by lecc3 cell pool (sorted) in perfusion mode. (A) Viable cell density (VCD – circles) and viability (squares) during the cultivation of cells. (B) Glucose and lactate concentrations and perfusion rate medium exchange during the perfusion run, given as volume of fresh medium per culture volume per day (vvd). (C) VLP concentration in the bioreactor (squares) and in the harvest (circles) referred to E protein of the Zika VLP (internal standard). Results confirm retention of the product inside the bioreactor due to the use of XCell™ ATF-2 system.

high passage number (beyond the 21 wpt that had been tested for stability), further studies are needed comparing cells at low and high *in vitro* cell ages. Maximum VLP concentration in the continuous bioreactor harvest was approximately 80 μg/L on day 11, but improved results are expected to be achieved using a cell retention device that does not retain the product and cells that are within the passage number for which stability has been demonstrated.

In this study, a perfusion rate of 1.5 vvd was applied, in order to ensure a reasonable glucose concentration in the bioreactor and to guarantee a robust continuous operation of the bioreactor for one month. This perfusion rate corresponds to a cell-specific perfusion rate (CSPR) of approximately 50–60 pL/cell/day, which is about 3 times higher than CSPRs considered optimal [20]. Further studies aiming at the optimization of feeding strategy and of perfusion medium composition can certainly lead to a perfusion process with less medium usage, contributing to further reduction of costs. Despite this perspective of further improvement, we believe that the current proof-of-concept process would be cost-effective when compared to traditional vaccine platforms based on batch production of whole viruses, especially considering that VLPs can be produced in lower biosafety level plants, and that perfusion systems generally provide higher volumetric productivities and require reduced bioreactor sizes, less plant footprint and lower investment costs when compared to batch processes [19,20,21,37]. Further studies employing more sophisticated VLP quantification assays, cells at lower passage number and a cell retention device that doesn't cause product retention are ongoing and will allow an exact calculation of cell-specific productivities and volumetric productivities achieved for Zika VLP production in continuous perfusion mode.

4. Conclusions

VLPs are a promising vaccine platform for flavivirus vaccines due to safety and repetitive presentation of the antigen. Furthermore, VLPs represent the possibility of incorporating mutations to have customized molecular features, which is very interesting in the case of flaviviruses, in order to decrease cross-reactivity and the risk of antibody-dependent enhancement (ADE) of infection, which affect diagnostics and vaccine development.

Our present results confirm that perfusion technology using stably transfected cells constitutively expressing Zika VLPs is highly promising as a vaccine manufacturing platform and brings prospects for the development of large-scale, cost-effective technologies for production of vaccines for ZIKV. Moreover, the strategy developed in this work can be easily applied for the production of other Zika VLP constructs incorporating customized molecular features, as well as for the production of VLPs of other flaviviruses circulating in the Americas that we have been expressing at our laboratory, such as Yellow Fever, Dengue 1–4, Saint-Louis encephalitis, Ilheus, and Cacipacore viruses.

Declaration of Competing Interest

The authors declare no conflict of interest.

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All authors attest they meet the ICMJE criteria for authorship.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.05.064.

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