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Design of chemical and irradiation techniques for safe, complete, efficient and downstream research-compatible inactivation of SARS-CoV-2

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Introduction

We are currently undertaking a research study determining actual SARS-CoV-2 peptide targets presented by HLA of infected human lung/respiratory tract cells with the intent of guiding the cytotoxic T cell response to such peptide-HLA targets. Importantly, our analysis pipeline detects actual peptide targets, not those that are just *predicted* from sequence and host HLA display. Further, through a multi-institution collaboration, we have access to physiologically appropriate Type 2 alveolar epithelial cells induced from human stem cells (iAEC2; Kotton Laboratory, Boston University Medical Center [BUMC]) and have established collaboration with the Saeed Laboratory at the National Emerging Infectious Disease Laboratory (NEIDL/BUMC) to infect such iAEC2 with SARS-CoV-2, already an established practice at the NEIDL. Using minimal numbers of cells (10^6 or less), we have shown we can determine using mass spectrometry and *in house*-designed software the rich display of peptides presented by HLA, whether from normal cells, transformed or tumor cells, or virally-infected cells (1-3).

Given that our mass spectrometer (MS) machine, specifically calibrated for analysis of HLA-presented peptides, is housed in a BSL2 facility, while the infections with SARS-CoV-2 are undertaken in a BSL3 laboratory, we have undertaken a study with the NEIDL laboratory to establish a reliable, efficient technique for SARS-CoV-2 inactivation at the NEIDL for safe transfer to the MS facility in the Immunobiology Laboratory at the Dana-Farber Cancer Institute on the 5th floor of the Jimmy Fund Building. Our MS facility includes AB SCIEX TripleTOF 6600 that has been fitted for use with a low flow (nL/min) plot column fabricated *in house* for the Poisson detection liquid chromatography data independent acquisition (DIA) MS experiments that offer the attomole detection sensitivity required here.

Methodology

Once appropriate target cells for the viral immune peptidome have been isolated, our current protocol requires detergent lysis of those cells, releasing clusters of membrane HLA presenting peptides derived from intracellular proteolysis into micelles in suspension. The micelles are enriched by immunoprecipitation using an anti-monomorphic human HLA-specific monoclonal antibody (W6/32) bound to beads followed by extensive washing of the beads to remove as much non-HLA-associated material as possible. This complete procedure is non-denaturing and the peptides remain bound to the HLA. The peptides are then eluted from the antibody/beads and prepared for MS analysis. There are two time points in this procedure where SARS-CoV-2 inactivation may be performed without compromising the integrity of the assay pipeline.

The first possible inactivation point is immediately after the cells have been disassociated and collected. Either in up to 100 μL medium in a 1.5 mL Eppendorf low protein-binding microfuge tube or else as a centrifuged cell pellet in a similar tube, the cells may be exposed for up to 30 s to high-intensity UV-C light (4, 5). Note that in reference 5, where complete inactivation of SARS-CoV-2 was observed, UV illumination was delivered at 2 cm distance by a low-pressure mercury lamp delivering 1.082 mW/cm^2 over 130 s. Our system is directly comparable to this but uses a much more manageable LED UV-C source delivering ~12 mW/cm^2 at the same distance, i.e. not only comparable but with the capacity to exceed the performance of the mercury lamp.

In the test assays here, for ease of transfer of the viral material to subsequent viable cells for plaque assays, only the cells in medium option will be examined for this submission.

Notwithstanding, although the UV-C inactivation is our preferred path, inactivation results of the actual samples to be delivered to us are not yet complete so we are requesting consideration only of the detergent protocol as described in the following paragraph.

The second possible inactivation point is during the detergent lysis itself, extending out our established lysis protocol from 15-20 min to 3 h. Dilutions of the detergent lysate will then be added to cultures of viable cells for plaque assays.

Experimental set-up

Special materials required:

Cell lysis buffer: Triton X-100, 1.2%; octyl-β-D-glucopyranoside, 1.77%; Tris-HCl, 20 mM; EDTA, 1 mM; NaCl; 100 mM; cOmplete protease inhibitor cocktail, ×1 (Roche).

High-Intensity UV-C light (SteriLED; PM-UVC-PO3), kindly provided by Principal Lighting Group, San Angelo TX, providing ~18 mW/cm^2 irradiation at 1 cm, ~12 mW/cm^2 at 2 cm.

Day 1: Seed A549 human lung cell line cells into three 10 cm dishes @ 3 million cells per dish

Day 2: Infect cells in two dishes with SARS-CoV-2 @ MOI of 3; leave cells in the third dish uninfected. Seed Vero E6 cells into 24-well plates for a plaque assay

Day 3: Harvest culture medium and store on ice; then harvest cells on ice as follows:
Dish 1 (Infected): Remove media, wash once with cold 1X PBS, add 1 mL of cold 1X PBS and scrape the cells. Collect in a 1.5 mL low protein-binding Eppendorf microfuge tube and let the tube sit on ice for 3 h.
Dish 2 (Infected): Remove media, wash once with cold 1X PBS, add 1 mL of cold lysis buffer and scrape the cells. Collect lysate in a 1.5 mL low

protein-binding Eppendorf microfuge tube and let the tube sit on ice for 3 h.
Dish 3 (Uninfected): Remove media, wash once with cold 1X PBS, add 1 ml of cold lysis buffer and scrape the cells. Collect lysate in a 1.5 mL low protein-binding Eppendorf microfuge tube and place on ice for 3 h.
Dish 1 will provide a culture supernatant containing intact SARS-CoV-2 virus and a disaggregated cell preparation infected with SARS-CoV-2 in PBS (Positive control)

Dish 2 will provide a culture supernatant containing intact SARS-CoV-2 virus and a disaggregated, lysed cell preparation infected with SARS-CoV-2 exposed to detergent for 3 h (Test condition).

Dish 3 will provide a culture supernatant free of virus and a disaggregated cell preparation also free of virus but lysed in detergent for 3 h (Negative control).

The detergent lysates are ready to use after dilution as described below. One further condition is added at this point to test the direct effect of the detergent upon cell viability. In this instance, the lysis buffer itself is simply diluted in 10-fold dilutions without prior incubation on cells ("mock" Triton condition).

The conditioned culture media collected from infected and uninfected cells is then exposed on ice (100 µL *in* Eppendorf microfuge tubes) to the UV-C source for 5 s, 10 s or 30 s prior to preparation of 10-fold dilutions for the plaque assay.

The plaque assay is set up as follows:

Prepare 10-fold serial dilutions of the cell lysates or culture supernatants in OptiMEM (10^{-1}-10^{-4})
Remove media from the 24-well plates containing the Vero E6 cells
Add 100 µl of virus dilutions (culture supernatant or cell lysate) per well
Incubate at 37ºC for 1h with occasional swirling/rocking
Add the overlay

Day 6: Process the plates for fixation/staining and count plaques.

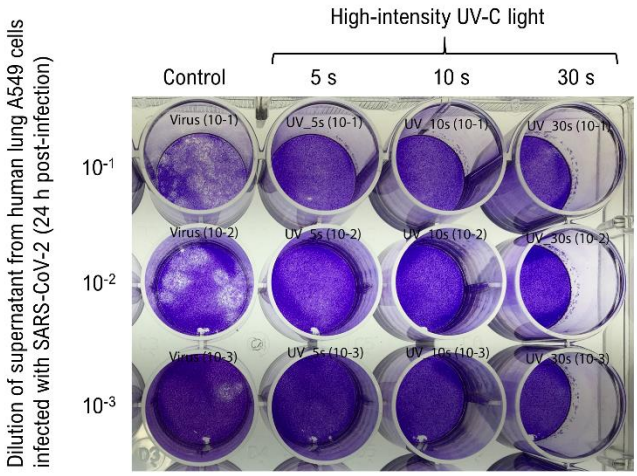
Results

Note: In every instance utilizing detergent lysates, irrespective of source, addition at 10-1 dilution was not compatible with cell growth and no Vero E6 cells survived to assay. Accordingly, the 10^{-1} condition is not presented.

Culture supernatant SARS-CoV-2 inactivation by UV-C irradiation:
Control culture supernatant from Dishes 1, 2 above shows a significant coverage of plaques over the dish surface at 10^{-1} with decreasing but detectable plaques down to

10^{-3} dilution (Fig. 1). The same supernatants, after exposure to 5 s, 10 s or 30 s, reveal not a single viral plaque at any virus titer dilution.

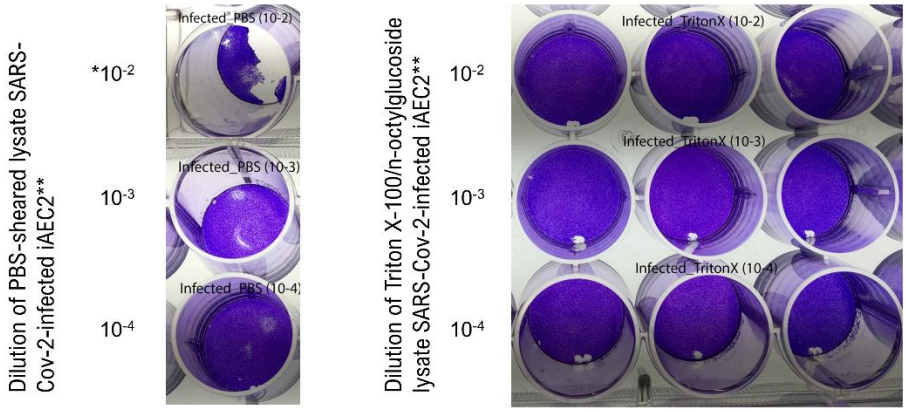
Figure 1. Complete inactivation of SARS-CoV-2 by exposure to high-intensity UV-C light for 5s – 30 s



Cell-resident SARS-CoV-2 inactivation by detergent lysis:

At concentrations of 10^{-2} to 10^{-4}, the addition of the Triton-X100-based lysis buffer directly to the Vero E6 cells used for the plaque assay had no effect upon the cell growth characteristics (data not presented). Addition of the positive control, where cells were scraped/sheared in PBS but not lysed with lysis buffer, resulted in viral plaques developing from 10-2 to 10-4 dilutions (Fig. 2). In contrast, upon incubation of the SARS-CoV-2-infected cells with lysis buffer, not a single viral-induced plaque was observed at any dilution.

Figure 2. Complete inactivation of SARS-CoV-2 by incubation in Triton X-100/n-octylglucoside detergent for 3 h



n = 3 for each dilution

* For the PBS control 10^{-2} condition, presence of multiple viral plaques led to instability of cell sheet during staining/washing

**iAEC2: Human alveolar epithelial Type 2 cells induced from stem cells

July 20th 2020

Conclusion.

We are requesting approval for transfer of cells and cell lysates previously-infected with SARS-CoV-2 following treatment with detergent at Stage 2 as highlighted in blue below, with the Stage 1 protocol to be considered at a later date.

Full protocol for immunoprecipitation of peptide-HLA complexes for MS analysis

All cell procedures are carried out under sterile, high efficiency particulate air (HEPA)-filtered conditions at 4°C using low-protein binding tubes (Eppendorf).

Cells ($0.5 – 1 \times 10^6$ cells) are centrifuged at 900g for 5 min, and the supernatant removed.

Stage 1 possible intervention for virus inactivation. For UV-C irradiation-based viral inactivation, the cell pellet is exposed on ice at a distance of 1-2 cm to high-intensity UV-C irradiation for 30 s following which the cells are frozen at -80°C prior to transfer in this state to DFCI where the pellet will be gently flicked to resuspend the cells and 0.4 mL lysis buffer (Triton X-100, 1.2%; octyl-β-D-glucopyranoside, 1.77%; Tris-HCl, 20 mM; EDTA, 1 mM; NaCl; 100 mM; cOmplete protease inhibitor cocktail, ×1; Roche) added. The protocol will continue from the asterisk indicated below.

Stage 2 possible intervention for virus inactivation. Alternatively, for detergent-based inactivation, the pellet will be gently flicked to resuspend the cells and 0.4 mL lysis buffer added. The cells will be incubated on ice with the lysis buffer for 3 h with gentle agitation every 20 min. At this point, the microfuge tube will be sealed with parafilm and frozen at -80°C prior to transfer in this state to DFCI.

*The lysate will be centrifuged for 10 min at 21,000 g and the pellet retained for subsequent analysis of HLA Class I by Western blotting of transfers from native Tris-glycine PAGE gels. The supernatant lysate is then centrifuged for 1 h at 105,000 g (Airfuge; Beckman-Coulter) and 4°C to pellet cellular debris. During this centrifugation, Protein G Sepharose beads (20 µL of 50% suspension in PBS; GE Healthcare) are preincubated with anti-monomorphic HLA Class I antibody (4 µg in 8 µL PBS; clone W6/32; Biolegend). Following ultracentrifugation of the lysate, a sample (25 µL) is removed for subsequent detection of HLA Class I as described above. The remaining lysate is then added to the W6/32 pre-incubated beads and rotated for 3 h at 4°C. The lysate-bead suspension is then centrifuged at 900 g for 3 min and the supernatants retained for confirmation of depletion of HLA Class I. The HLA Class I-loaded beads are then washed ×6 in wash buffer (0.5 mL/wash; octyl-β-D-glucopyranoside, 1.77%; Tris-HCl, 20 mM; EDTA, 1 mM; NaCl; 100 mM) followed by ×2 washes in salt buffer (Tris-HCl, 20 mM; EDTA, 1 mM; NaCl; 100 mM). Residual liquid is removed using fine pipette tips and the bead samples stored at -80°C until processed for MS analysis.

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