An adapted novel flow cytometry methodology to delineate types of cell death in airway epithelial cells

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ABSTRACT

Current methodologies to measure apoptotic and necrotic cell death using flow cytometry do not adequately differentiate between the two. Here, we describe a flow cytometry methodology adapted to airway epithelial cells (AEC) to sufficiently differentiate apoptotic and necrotic AEC. Specifically, cell lines and primary AEC (n = 12) were permeabilized or infected with rhinovirus 1b (RV1b) over 48 h. Cell death was then measured via annexin V/propidium iodide (A5/PI) or annexin V/TO-PRO-3 staining using a novel flow cytometry and gating methodology adapted to AEC. We show that A5/PI staining could not sufficiently differentiate between types of cell death following RV1b infection of primary AEC. However, A5/TP3 staining was able to distinguish six cell death populations (viable, necrotic, debris, A5** apoptotic, apoptotic bodies) after permeabilization or infection with RV1b, with phenotypic differences were observed in apoptotic populations. Collectively, using a staining and gating strategy never adapted to AEC, A5/TP3 could accurately differentiate and quantify viable, necrotic, and apoptotic AEC following RV1b infection.

Keywords: airway epithelium, cell death, rhinovirus, flow cytometry

INTRODUCTION

Cystic fibrosis (CF) lung disease is characterized by neutrophilic airway inflammation associated with progressive airway remodeling which begins early in life, even in absence of bacterial infection [1]. In infants with CF, human rhinovirus (RV) is the most commonly detected virus [2] and while children with and without CF have similar rates of viral infection, the effects and clinical outcomes of these infections are more severe in those with CF [3]. The primary target of RV infection remains the airway epithelium (AEC), and although it is capable of infecting other cell types including monocytes and macrophages, preferentially replicates in the airway epithelium [4]. Replication of RV has been shown to be significantly increased in CF AEC when compared to non-CF AEC [5]. Of significance, is the observation that AEC from children with CF elicit a damped apoptotic response following infection compared to non-CF controls, even when normalized to viral load and overall epithelial cell death [5]. It has also been recently observed that the rhinovirus 3C protease disrupts the apoptotic response to RV infection through cleavage of receptor-interacting serine/threonine-protein kinase 1 (RIPK1), resulting in a necrosis-like phenotype of cell death [6].

Studies utilizing βENaC-overexpressing mice with CF-like lung disease have suggested a link between mucus obstruction, necrosis of AEC, and sterile neutrophilic inflammation driven by the release of interleukin-1 alpha (IL-1α) from necrotic cells activating the interleukin-1 receptor (IL-1R) signaling pathway [7]. These findings suggest that triggering IL-1R signaling via IL-1α may be an important mechanism in early CF inflammation. Previous work from AREST CF has established that IL-1α is associated with structural lung disease specifically in the absence of bacterial infection [8], suggesting a role for IL-1α released from necrotic cells in the inflammatory cascade observed in the early...
with CF (mean age 2.9 ± 1.8 years old; Table S1) were used where children with CF carried at least one Pit508 del allele, and 83% were homozygous. Furthermore, six healthy children without CF (mean age 3.8 ± 1.9 years old; Table S1) were recruited when attending hospital for elective non-respiratory related surgery.

**Cell culture**

Primary AEC samples were attained by brushing of the tracheal mucosa of children with a single-sheathed nylon bronchial cytology brush (BC 25105, Olympus, Australia) as previously described [5]. After collection, primary AEC cultures were established as conditionally reprogrammed cultures as previously described [19]. Also included in the study was a healthy non-CF AEC line 16HBE14o− and equivalent CF AEC line CFBE41o—which were cultured as previously described [5].

**Rhinovirus infection**

Human rhinovirus 1b (RV1b) was propagated as previously described [20]. To simulate an acute RV infection in vitro, primary AEC were exposed to ~2.95 × 10⁴ TCID₅₀/ml which equated to a multiplicity of infection (MOI) 3. To ensure responses were due to actively replicating virus, control AEC were exposed to UV-inactivated RV1b at MOI 3. After 48 h incubation, cells from all treatment conditions were collected for analysis via flow cytometry. Viral load of RV1b to confirm infection was measured after 24 h incubation via quantitative polymerase chain reaction (qPCR) as previously described [21] (Fig. S1).

**Flow cytometry**

A flow cytometry methodology to measure cell death and disassembly [14] was adapted for use with AEC. Briefly, AEC were detached from culture surfaces via gentle trypsinization using 0.25% (v/v) trypsin (Lonza, Basel, Switzerland). Cell supernatant was centrifuged at 1000 g for 3 min at 4°C and pelleted cells with trypsinized cells. Combined cells were then pelleted by centrifugation at 500 g for 7 min at 4°C and resuspended at a concentration of 10⁶ cells/ml in annexin binding buffer (Thermo Fisher Scientific, Waltham, MA, USA). Cells used as positive controls for dead cells were fixed and permeabilized by incubation with 3.7% paraformaldehyde for 10 min. Tubes containing 100 μl of cell suspension were stained for 15 min with 100 μl of annexin V/AlexaFluor488 (Thermo Fisher Scientific, Waltham, MA, USA) (1:40 v/v) and propidium iodide (1 μg/ml final concentration) (Thermo Fisher Scientific, Waltham, MA, USA) in annexin binding buffer or 100 μl of annexin V/AlexaFluor488 (1:40 v/v) and TO-PRO-3 (10 μM final concentration) (Thermo Fisher Scientific, Waltham, MA, USA) in annexin binding buffer. Flow cytometry was performed on a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Acquisition gate was set to record 2 × 10⁴ events total for each sample. Analysis was performed using FlowJo software v10.4 (FlowJo LLC, Ashland, OR, USA).

**Gating strategies**

A5/PI data was analyzed by excluding doublets, and sorting events into A5⁻/PI⁺, A5⁺/PI⁻, A5⁻/PI⁻, and A5⁻/PI⁺ quadrants. A5/TP3 data was analyzed using a novel flow cytometry analysis adapted in this study for epithelial cells that uses a seven step gating strategy to separate events into six populations: viable, necrotic, A5⁻ apoptotic, A5⁺ apoptotic, apoptotic bodies and cellular debris (Fig. 1) [14]. Positive staining was determined via single staining of fixed AEC using both A5 and TP3 and...
gating determined accordingly. Cut-offs used for forward scatter (FSC) and side scatter (SSC) during analysis to delineate an intact cell were $5 \times 10^4$, with everything above termed positive (+) and everything below negative (−). Expected levels of A5 and TP3 staining for each population are outlined in Table 1. A5/TP3 data were analyzed via the following pipeline: first, data was separated into TP3+ events and other events (Fig. 1A). The TP3+ events were sorted by FSC into necrotic events (FSC−) (Fig. 1B). The other events were then separated by SSC and A5 into A5−/SSC− and other events (Fig. 1C) The A5−/SSC− events were then sorted into viable (FSC+TP3+) and A5+ apoptotic events (TP3+)(Fig. 1D). The other events were then separated into A5+ and A5 (Fig. 1E), with the A5− then sorted by FSC into cellular debris (FSC−)(Fig. 1F). The A5+ was then sorted by FSC into A5+ apoptotic events (FSC+) and apoptotic bodies (FSC−)(Fig. 1G).

Figure 1. A5/TP3 gating and data analysis for differentiation of a sample into six populations. A. Data was sorted into TP3+ events and other events. B. The A5+/TP3+ events were sorted by FSC into necrotic events (FSC−). C. The A5−/TP3+ events were then separated by SSC and A5 into A5−/SSC− and other events. D. The A5−/SSC− events were then sorted into viable (FSC+/TP3+) and A5− apoptotic events (TP3+). E. The “other events” were then separated into A5+ and A5−, with the A5− then sorted by FSC into cellular debris (FSC−)(F). G. The A5− events were then sorted by FSC into A5− apoptotic events (FSC−) and apoptotic bodies (FSC−).

Statistics
Statistical analyses were conducted using GraphPad Prism v7.04 (GraphPad Software, La Jolla, CA, USA). Comparisons between paired data were performed using paired t-tests presented as mean ± standard deviation, and Wilcoxon matched pairs signed rank tests presented as median (interquartile range) where appropriate. Comparisons between unpaired data were performed using unpaired t-tests with Welch’s correction presented as mean ± standard deviation, and Mann-Whitney tests presented as median (interquartile range) where appropriate. A two tailed $P$ value $< 0.05$ was considered statistically significant.

RESULTS

A5/PI staining cannot sufficiently differentiate between cell populations using flow cytometry
To differentiate between types of cell death using A5/PI staining, primary AEC were infected with RV1b for 48 h and measured via flow cytometry. RV infection moderately but not significantly reduced viable events (A5+/PI−)(Fig. 2A), had no effect on necrotic events (A5−/PI−) (Fig. 2B) nor any effect on apoptotic events (A5+/PI+) (Fig. 2C). Furthermore, although secondary necrotic events were elevated (A5+/PI), this was not considered significant (Fig. 2D). At baseline, 21.6% ± 9.7% of total events were identified as undergoing secondary necrosis which only slightly increased following fixation (39.2% ± 15.7%). However, the minimal apoptotic events observed at baseline (0.74% ± 0.43%) was observed not to change following fixation (1.77% ± 1.53%). Additionally, A5/PI staining is unable to detect the presence of apoptotic bodies formed during cell death.

A5/TP3 staining allows differentiation between six cell populations following fixation
Since using the traditional A5+/PI− staining resulted in a large proportion of double positive staining that was further compounded by highly variable staining between samples, another flow cytometry methodology was then trialed using AEC lines. Utilizing a submerged monolayer model that ensures a single population of basal airway epithelial cells we exposed them to a typical fixative to determine cell death responses. Results generated showed that staining with A5/TP3 allowed differentiation into six different populations, namely: viable events, necrotic events, debris, A5+ apoptotic events, A5− apoptotic events, and apoptotic bodies. When quantified, fixed AEC had reduced viable events (67.69% ± 13.4% vs. 4.81% ± 10.1%; $P < 0.05$), increased necrotic events (12.69% ± 7.15% vs. 28.73% ± 16.9%; $P < 0.05$), de-
creased debris (4.11% ± 4.09% vs. 1.40 ± 1.24%; *P < 0.05), increased A5+ apoptotic events (9.81% ± 5.41% vs. 56.35% ± 26.7%; *P < 0.05) and had no effect on A5- apoptotic events or apoptotic bodies (Fig. 2E).

To determine the overall responses to fixation, cell line responses were grouped into “viable”, “necrotic” and “apoptotic” events for analysis. Fixation of cell lines significantly reduced viable events (67.69% ± 13.43% vs. 4.81% ± 10.13%; *P < 0.05) (Fig. 2F), significantly increased necrotic events (12.69% ± 7.15% vs. 28.73% ± 16.94%; *P < 0.05) (Fig. 2G), and significantly increased apoptotic events (15.03 ± 8.01% vs. 64.53% ± 21.60%; *P < 0.05) (Fig. 2H).

Figure 2. A5/PI cannot differentiate types of cell death in primary AEC but A5/TP3 is effective to differentiate viable, apoptotic, and necrotic AEC lines. Primary AEC (*n = 4) were infected with RV1b for 48 h, collected and stained with A5/PI. RV1b infection decreased viable events (A), had no effect on necrotic events (B), had no effect on apoptotic events (C), and increased secondary necrotic events (D). AEC lines (*n = 7) were collected, fixed with paraformaldehyde, and analyzed via flow cytometry. After fixation with paraformaldehyde, viable events were reduced compared to control, necrotic events were increased compared to control, debris was reduced compared to control, A5+ apoptotic events were increased compared to control, and no changes in apoptotic bodies or A5- apoptotic events were observed compared to controls (E). When grouped into “viable”, “necrotic”, and “apoptotic” events, viable events were reduced compared to control (F), necrotic events were increased compared to control (G), and apoptotic events were increased compared to control (H). * *P < 0.05.
A5/TP3 staining allowed measurement of viable, necrotic, and apoptotic events in primary AEC after fixation and RV infection

As differences were observed between control and fixed cell lines, primary AEC were then fixed, stained, and measured using flow cytometry to confirm initial results utilizing immortalized cell lines were translatable to primary cells. Fixation of primary AEC and staining with A5/TP3 resulted in similar staining to cell lines, with reduced viable events (79.53% ± 4.17% vs. 8.23% ± 1.87%) (Fig. 3A), increased necrotic events (6.66% ± 3.52% vs. 56.02% ± 13.0%) (Fig. 3B), and increased apoptotic events (9.90% ± 4.14% vs. 31.31% ± 11.24%) (Fig. 3C). To confirm A5/TP3 staining via flow cytometry could sufficiently detect differences after an infectious stimulus with clinical relevance, primary AEC were exposed to RV1b over 48 h. Viable events were significantly reduced compared to control following infection with RV1b for 24 h (61.35% ± 14.1% vs. 42.59% ± 12.9%; P < 0.001) and 48 h (58.07% ± 12.9% vs. 22.18% ± 13.6%; P < 0.001) (Fig. 3D). Additionally, viable events were significantly lower at control at 48 h compared to 24 h (P < 0.05), and lower following RV1b infection for 48 h compared to 24 h (P < 0.05). There were also increased necrotic events compared to control following infection with RV1b for both 24 h (8.560% ± 1.97% vs. 12.20 ± 4.09%, P < 0.01) and 48 h (10.12 ± 4.24% vs. 18.84% ± 6.42%, P < 0.001) (Fig. 3E). Additionally, necrotic events were significantly higher after 48 h of RV1b infection when compared to 24 h (P < 0.001). RV1b infection for 24 h also significantly increased debris (2.359% (1.62–2.53) vs. 6.725% (4.55–8.26); P < 0.001) (Fig. 3F) but had no effect on levels of A5− apoptotic events (P > 0.05) (Fig. 3G). However, RV1b infection significantly reduced A5− apoptotic events when compared to control after 24 h (3.58% ± 1.20% vs. 2.68% ± 1.08%; P < 0.001) and 48 h (4.33% ± 1.80% vs. 2.93% ± 1.45%; P < 0.05) (Fig. 3H). Finally, apoptotic bodies were significantly increased when compared to control following RV1b infection for 24 h (9.92% (4.92–24.49) vs. 17.30% (9.84–28.49); P < 0.05) and 48 h (15.84% (7.05–29.81) vs. 39.28% (27.29–60.08); P < 0.01) (Fig. 3I). Thus, we report the ability to measure and differentiate events into viable, necrotic, and apoptotic populations and determine changes in cell viability and death in primary AEC following injurious stimuli like RV infection.

Figure 3. A5/TP3 staining in primary AEC results in measurable changes in viable, necrotic, and apoptotic events via flow cytometry. Primary AEC were collected, fixed with paraformaldehyde (n = 4) or infected with RV1b (n = 12) over 48 h, stained with A5/TP3, and analyzed via flow cytometry. After fixation with paraformaldehyde, viable events were reduced (A), necrotic events were increased (B), and apoptotic events were increased (C). RV1b infection: decreased viable after infection for 24 h and 48 h, with lower viable events at 48 h compared to 24 h in both control and RV1b (D), increased necrotic events after RV1b infection for 24 h and 48 h, with increased necrotic events at 48 h compared to 24 h (E), significantly increased debris following RV1b infection for 24 h (F) no change in A5− apoptotic events after RV1b infection over 48 h (G), significantly increased apoptotic bodies following RV1b infection for 24 and 48 h, with higher apoptotic bodies after 48 h of infection compared to 24 h (H), and significantly decreased A5− apoptotic events following RV1b infection for 24 h and 48 h (I). *P < 0.05.
A5/TP3 staining allows the observation in phenotypical differences in apoptotic cell disassembly

Following the optimization of the seven-step gating protocol for AEC, and the observation that RV infection of AEC resulted in increased apoptotic cell disassembly, we then determined phenotypical differences between non-CF and CF AEC infected with RV1b over 48 h. Infection with RV1b had no statistically significant effect on A5+ apoptotic events after 24 h (Fig. 4A) or 48 h (Fig. 4B). However, RV1b infection significantly decreased A5− apoptotic events when compared to control after 24 h in non-CF (4.18% ± 1.10% vs. 3.31% ± 1.11%; P < 0.01) and CF AEC (2.98% ± 1.06% vs. 2.05% ± 0.62%; P < 0.05), with A5− apoptotic events following RV infection significantly lower in CF when compared to non-CF AEC (P < 0.05) (Fig. 4C). After 48 h of infection, there was a significant decrease in A5− apoptotic events in CF AEC (3.45% ± 1.67% vs. 2.11% ± 1.22%; P < 0.01) but not non-CF AEC (Fig. 4D). Additionally, after 48 h of infection A5− apoptotic events were significantly lower in CF AEC when compared to non-CF AEC (2.11% ± 1.22% vs. 3.74% ± 1.25%; P < 0.05) (Fig. 4D). Finally, infection with RV1b for 24 h significantly increased apoptotic bodies in non-CF (15.23% (5.01–22.77) vs. 20.86% (13.99–31.15); P < 0.05) but not CF AEC (Fig. 4E). After 48 h, significantly increased apoptotic bodies were observed in both non-CF (21.20% (11.29–28.67) vs. 45.63% (36.77–67.80); P < 0.05) and CF AEC (7.35% (5.69–31.92) vs. 28.31% (22.71–50.66); P < 0.05) (Fig. 4F).

Figure 4. A5/TP3 staining in primary AEC results in measurable phenotypic differences in apoptotic populations. Non-CF (n = 6) and CF (n = 6) primary AEC were infected with RV1b over 48 h, stained with A5/TP3, and analysed via flow cytometry. RV1b infection: had no significant effect on A5+ apoptotic events after 24 h (A) and 48 h (B), significant decreased A5− apoptotic events after 24 h in both non-CF and CF AEC, with significantly lower events in CF (C), significantly decreased A5− apoptotic events in CF AEC after 48 h, with significantly lower events in CF compared to non-CF AEC (D), significantly increased apoptotic bodies in non-CF AEC after 24 h (E), and significantly increased apoptotic bodies in non-CF and CF AEC following 48 h of RV1b infection (F). *P < 0.05.
**DISCUSSION**

This study investigated the effectiveness of the gold standard A5/PI staining to assess viability, apoptotic, and necrotic cell death and compared the results to a novel flow cytometry analysis pipeline adapted to AEC, utilizing A5/TP3 staining combined with a seven-step gating strategy. While staining with A5/PI was able to differentiate apoptotic and necrotic cells within a sample, the lack of clarity about the double-positive population represents was of concern. There is no consensus whether the double-positive population represents apoptotic cells with permeabilized membranes or necrotic samples with exposed phosphatidylserine which then bind A5 [9]. Furthermore, recent data suggests that necrotic cells stain A5+/PI- initially before becoming A5-/PI+ [13]. As a result, a novel flow cytometry method was adapted to AEC and assessed for its ability to differentiate viable, apoptotic, and necrotic events with more certainty [14]. The study by Jiang et al. (2016) utilized T cells, monocytes, squamous cells, endothelial cells, and fibroblasts but was successfully adapted to AEC in this manuscript. Furthermore, although A5 with PI or other membrane-exclusion dyes could detect primary necrosis, debris and apoptotic bodies were unable to be detected using the outlined method [14].

A large amount of apoptotic bodies were detectable after chemical induction of necrosis in a number of cell types [14]. The generation of apoptotic bodies was also observed in this study when using primary AEC following RV infection, with phenotypical differences observed between non-CF and CF AEC. As part of the homeostatic process in the airway, effective phagocytosis and removal of apoptotic cells termed efferocytosis [22] is vital to maintain the "immunologically silent" status commonly prescribed to apoptotic cells [23]. In the airway, this is performed by professional phagocytes such as macrophages and neutrophils [24], or via engulfment by non-professional phagocytes including AEC [25]. Since defective apoptotic responses have been observed in AEC and neutrophils in CF [5,26], reported accumulation of apoptotic cells in the CF airway may be suggestive evidence of defective efferocytosis [27]. Impaired phagocytic capacity in a number of cell types has been reported in CF [22,28]. Failure to successfully clear apoptotic cells can result in an immune response since apoptotic cells undergo secondary necrosis [29] and apoptotic cell disassembly. Interleukin-1 released by non-CF AEC during RV1b infection is associated with apoptosis [30], suggesting signaling may result from the immunological response to secondary necrosis and apoptotic breakdown of AEC. As apoptotic bodies can stimulate neutrophilic inflammation via IL-1α [17], it is important to elucidate the contribution of early apoptotic events during cell disassembly.

We were able to adapt the novel flow cytometry methodology utilizing A5/TP3 staining in conjunction with a seven-step gating analysis to differentiate viable, necrotic, and apoptotic events to an airway epithelial cell population after stimulation. Using this adapted flow and gating methodology, which can capture particles resulting from cells breaking down due to secondary apoptosis in a sample, we can have greater confidence in data generated when differentiating between viable, necrotic, and apoptotic cells. Utilizing AEC lines, comparable staining to the study by Jiang et al., (2016) was detected via flow cytometry [14]. After infection of AEC with RV confirmed via PCR, we were also able to observe decreased viable events, increased necrotic events, and increased apoptotic events in primary AEC. From the experiments conducted in this study, future work investigating cell death should be conducted utilizing A5/TP3 staining for differentiation between viable, necrotic, and apoptotic events via flow cytometry rather than the traditional A5/PI staining. After successfully adapting this methodology to primary AEC, there is now a robust approach to test whether there are phenotypic differences between the response of non-CF and CF AEC following RV infection and if the resultant inflammation is IL-1R driven. In the future, this methodology can be used successfully and with ease to investigate whether RV infection drives necrosis in the CF airway, and how it is linked to early life inflammation driven by IL-1R signaling observed in vivo. More broadly, this method can easily be adapted to other chronic airway diseases where mucus obstruction and cell death of airway epithelial cells is a typical observation including diseases such as asthma, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis.

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**References**


Supplementary information

Table S1. Demographics of the study population.

Figure S1. Infection of primary AEC with RV1b results in increased viral titer after 24 h.

Supplementary information of this article can be found online at http://www.jbmethods.org/jbm/rt/suppFiles/336.

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