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Brefeldin A, but not monensin, enables flow cytometric detection of interleukin-4 within peripheral T cells responding to *ex vivo* stimulation with *Chlamydia trachomatis*

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Abstract

Intracellular cytokine staining (ICS) assay optimization should include selection of suitable cytokine secretion inhibitors. Here, peripheral blood mononuclear cells (PBMC) from women with proven history of *C. trachomatis* genital tract infection were used to compare the ability of brefeldin A (BFA) and monensin (MN) to concurrently trap interferon- γ (IFN- γ), tumor necrosis factor (TNF), interleukin (IL)-4, and IL-17 within T cells responding to *ex vivo* stimulation with chlamydial antigen. While flow cytometric analyses showed similar intracellular levels of TNF, IFN- γ , and IL-17 among T cells treated with BFA or both BFA and MN, markedly more IL-4 was found inside T cells treated with BFA compared to those that received MN or BFA and MN. The latter findings oppose current ICS recommendations informing that ICS results are unaffected by concomitant use of BFA and MN, and also suggests that MN may be an unsuitable cytokine secretion inhibitor for ICS assays designed to measure intracellular IL-4 accumulation.

Keywords

Brefeldin A; *Chlamydia trachomatis*; Intracellular cytokine staining; Flow cytometry; Monensin; T cell effector function

1. Introduction

Chlamydia trachomatis is the most common sexually transmitted bacterium (Carey and Beagley, 2010), and infection of the female genital tract can cause pelvic inflammatory disease (Scholes et al., 1996). Prophylactic vaccination is the strategy most likely to halt transmission (Gray et al., 2009), but development of a safe and effective *C. trachomatis* vaccine requires improved understanding of the pathogen-specific responses that confer protection and elicit immunopathological tissue damage (Brunham and Rey-Ladino, 2005). While production of interleukin-13 (IL-13) by peripheral blood mononuclear cells (PBMC) stimulated *ex vivo* with chlamydial antigen correlated with resistance to genital tract re-infection (Cohen et al., 2005), more severe ocular trachomatous scarring was linked to detection of IL-4 transcript in PBMC responding to chlamydial antigen (Holland et al., 1996). Such results indicate that *Chlamydia*-specific Type 2 immunity may contribute to both host defense and immunopathological tissue damage, and imply that intracellular cytokine staining (ICS) assays aimed to detect biologically relevant *C. trachomatis*-specific

T cell effector functions should optimize detection of IL-4, the signature cytokine of the Type 2 response. As an essential ICS assay optimization step is the selection of a cytokine secretion inhibitor (Lamoreaux et al., 2006), herein we evaluated the effects of monensin (MN) and brefeldin A (BFA) on detection of tumor necrosis factor (TNF), interferon- γ (IFN- γ), IL-4, and IL-17 in T cells stimulated with chlamydial antigen. Specifically, we assessed differences in T cell viability and in the capacity of BFA, MN, or MN and BFA (BFA/MN) to simultaneously trap these 4 cytokines in T cells responding to *ex vivo* stimulation with inactivated *C. trachomatis* elementary bodies (EB).

2. Materials and methods

2.1. Study participants

After obtaining informed consent, peripheral blood was collected from 11 women with history of a nucleic acid amplification test (NAAT)-confirmed *C. trachomatis* endo-cervical infection that had been treated with a course of anti-chlamydial antimicrobial therapy (the University of Pittsburgh's Institutional Review Board approved this study design).

2.2. Antigen and PBMC preparation

PBMC were isolated using density gradient centrifugation (Lymphocyte Separation Medium, Mediatech), placed in cryopreservation solution (90% fetal bovine serum (FBS) (Atlanta Biologicals) and 10% dimethyl sulfoxide (Sigma-Aldrich)), and stored in liquid nitrogen. As described elsewhere, EB from *C. trachomatis* serovar D were prepared and stored in SPG buffer at -80°C [Cohen et al., 2000]. EB were inactivated prior to use by gamma irradiation (inactivation confirmed by an absence of inclusion forming units when EB were inoculated onto HeLa cell monolayers). For ICS assays, PBMC were thawed, washed, and re-suspended in RPMI-1640 (Cellgro) containing 10% FBS, 1 mM sodium pyruvate 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (termed complete media) (all Cellgro). Cell counts were obtained using the Countess® automated cell counter (Invitrogen), and viable cell recovery was routinely between 85 and 95%.

2.3. Stimulation assay and flow cytometric analysis

PBMC were labeled with CellTrace™ Violet cell proliferation dye (Invitrogen). Labeled cells (10^6 cells/ml) were re-suspended in complete media, and 200 μl aliquots put into individual wells of 96-well plates. Cells were incubated with inactivated EB (2×10^5 inclusion-forming units/ml), PHA (5 $\mu\text{g}/\text{ml}$) (Sigma-Aldrich), or complete media (as shown in Fig. 1A, the latter 2 conditions used as positive and negative controls for cellular proliferation, respectively) for 96 h at 37°C in a 5% CO_2 atmosphere. For the last 7 h of incubation, manufacturer recommended amounts of BFA, MN, or BFA/MN (all BD Bioscience) were added where indicated. Cells were stained with LIVE/DEAD® Fixable Aqua Dead Cell stain (Invitrogen), and then stained with V500-conjugated anti-CD14 (M5E2) (BD) and anti-CD19 (HIB19) (BD), PerCP-Cy5.5-conjugated anti-CD4 (RPA-T4) (eBioscience), and Pacific Orange™-conjugated anti-CD20 (HI47) (Invitrogen). After fixation and permeabilization with Cytofix/Cytoperm™ (BD), cells were stained with FITC-conjugated anti-IL-17A (eBio64DEC17), PE-conjugated anti-IL-4 (8D4-8), APC-conjugated anti-IFN- γ (4 S.B3), PE-Cy7-conjugated anti-TNF (MAb11), and APC-eFluor® 780-conjugated anti-CD3e (UCHT1) (all eBioscience) diluted in Perm/Wash buffer (BD). Cells were collected on a LSRII cytometer (BD), and evaluated using FACSDiva (BD) and FlowJo (Tree Star) software. Isotype controls were included to define the gates that measured intracellular cytokines production by live CD4^+ T cells.

2.4. Statistical considerations

Statistical analyses were performed using Prism® 5 software (GraphPad). Between-group differences (BFA alone; MN alone; BFA/MN) in T cell viability and intracellular cytokine expression were examined using repeated measures one-way ANOVA and Tukey's post-hoc test (statistically significant results were defined as *P* values <0.05).

3. Results

3.1. T cell viability was unaffected by choice of protein secretion inhibitor

At the end of the 96 h incubation period, differential effects of BFA, MN, and BFA/MN on T cell viability were compared using flow cytometry analyses that measured T cell incorporation of LIVE/DEAD® Fixable Aqua Dead Cell stain. At concentrations of these agents used in our investigation, no discernable between-group differences in T cell viability were detected (Fig. 1).

3.2. Compared to BFA, use of MN or BFA/MN sharply decreased levels of IL-4 trapped in T cells stimulated by *C. trachomatis*

We next evaluated effects of BFA, MN, and BFA/MN on simultaneous accumulation of TNF, IFN- γ , IL-4, and IL-17 inside CD4⁺ T cells stimulated with inactivated EB. Compared to BFA or BFA/MN, MN produced reduced levels of IFN- γ and TNF (representative flow cytometric analyses and between-group results displayed in Figs. 2 and 3, respectively). Compared to BFA, BFA/MN produced similar levels of TNF and small, but statistically significant, increases in IFN- γ . Conversely, intracellular IL-17 accumulation was equally low among T cells responding to stimulation with chlamydial antigen in all treatment groups (Figs. 2 and 3). Most interestingly, compared to BFA alone, use of MN (either alone or in combination with BFA) dramatically decreased levels of IL-4 that accumulated in CD3 ϵ ⁺CD4⁺ cells (Figs. 2 and 3). Though these technical studies were completed to ensure optimization of our newly developed *C. trachomatis* ICS assay protocol, the ability of BFA to trap large amounts of IL-4 inside T cells specifically responding to chlamydial antigen is intriguing and will require further study to define the biological relevance of T cell effector function that is polarized toward Type 2 immunity by prior exposure to this obligate intracellular bacterial pathogen.

4. Conclusions

As quality of the T cell response influences outcomes to many infectious diseases, ICS assays that simultaneously evaluate multiple T cell effector functions can be used to better delineate disease pathogenesis and guide vaccine development (Seder et al., 2008). BFA and MN, the 2 agents evaluated in this investigation, are the protein transport inhibitors most frequently used in ICS assays to detect cytokine secretion. However, these reagents possess different modes of action, and therefore, do not always provide identical results. For example, when incubation periods exceed 18 h, MN is more toxic than BFA (Dinter and Berger, 1998). Of greater practical importance for ICS assays that more typically treat cells with protein transport inhibitors 12 h or less, MN traps TNF less efficiently inside antigen-stimulated cells. As a result, ICS assays require use of BFA to optimally determine TNF secretion levels (O'Neil-Anderson and Lawrence, 2002). In analogous results, we report that MN or BFA/MN abrogates intracellular IL-4 accumulation compared to the levels that were detected using BFA alone. The mechanism responsible for this novel observation awaits elucidation, but we postulate it may be the result of the increased susceptibility of IL-4 to degradation during Golgi stress, as MN was recently shown to promote Golgi stress responses (Oku et al., 2011). In summary, our results are contrary to current guidelines recommending that the concomitant use of MN and BFA is unlikely to alter ICS results

(Lamoreaux et al., 2006), and serve to highlight the need for procedural studies, including selection of a cytokine secretion inhibitor, that ensure the optimization of experimental ICS procedures and the standardization of ICS assay protocols.

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Fig. 1.

BFA and MN have comparable effects on T cell viability. PBMC were isolated from non-pregnant women with history of NAAT-confirmed genital tract *C. trachomatis* infection and stimulated 96 h with inactivated *C. trachomatis* EB. During the last 7 h, cultures were treated with BFA, MN, or BFA/MN as indicated, and then stained with a live/dead cell marker, followed by staining as indicated in Materials and methods. A) Contour plots depict the gating strategy used to define CD4⁺ T cells that proliferated in response to stimulation with chlamydial antigen. Plots show sequentially (left to right) the gating hierarchy used to interrogate for live non-B cell non-monocyte cells (Live/Dead^{low}CD14⁻CD19⁻CD20⁻), singlets, CD3e⁺CD4⁺ cells, and finally to define proliferating cells as events that diluted the cell proliferation dye. B) Representative contour plots show percentages of Live/Dead^{low}CD3e⁺ cells (i.e., live T cells) treated with BFA, MN, or BFA/MN that responded to chlamydial antigen. C) Graph depicts percentages of live T cells stratified by treatment group (n=11 samples per group). For B and C, Live/Dead staining was performed, with no addition of any fluorescently labeled antibodies in the same channel.

Fig. 2.

MN alters intracellular cytokine levels detected within CD4⁺ T cells that responded to chlamydial antigen. PBMC cultures were stimulated 48, 72, and 96 h with inactivated *C. trachomatis* EB. During the last 7 h, cultures were treated with BFA, MN, or BFA/MN as indicated, and processed for flow cytometric analyses as described in the Materials and methods. A) PBMC stimulated for 48, 72, and 96 h, and treated with BFA during the last 7 h of the respective incubation times are shown. Representative contour plots depict increased accumulation of IFN- γ -producing CD4⁺ T cells among proliferating cells with longer stimulation periods. B) PBMC stimulated for 96 h were treated during the last 7 h of incubation with BFA, MN, or BFA/MN as indicated, and then processed for flow cytometric analyses. Representative contour plots show gates used to identify proliferating CD4⁺ T cells that produced cytokines in cultures treated with BFA, MN, or BFA/MN. Numbers indicate percentages within each gate (data shown are from one representative experiment of three independent experiments performed).

Fig. 3.

MN severely decreases levels of IL-4 detected within T cells stimulated by chlamydial antigen. Graph depicts the integrated median fluorescence intensity (iMFI) levels of IFN- γ , IL-4, TNF and IL-17 in CD4⁺ T cells that proliferated in response to inactivated EB. iMFI was calculated as (percentage of positive cells) $\times$ (MFI for each indicated cytokine), and treatment groups were compared using repeated measures one-way ANOVA and Tukey's test for multiple comparisons (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$). Each symbol represent individual samples (n=11 samples per treatment group), and horizontal bars indicate mean values. Data shown are pooled from three independent experiments.