

# Rapid and Biologically Safe Procedures for the Evaluation of Antigen-Specific T Cell Response to Microbial Pathogens That May Be Used in the BSL-3 and BSL-4 Environment

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## Abstract

*T cell response precedes serological response, and is more feasible than pathogen detection by molecular tools. Thus, monitoring T cell response to class-A pathogens may allow for a timely treatment and a correct handling of patients containment issues, after exposure to potentially lethal agents, such as biological threat, or emerging pathogens. In this paper, we describe a procedure for the evaluation of T cell response to microbial pathogens, including class-A pathogens in BSL-3 and BSL-4 equipped laboratories.*

## Introduction

The class A pathogen list includes the most dangerous agents potentially used as bioweapons (Centers for Disease Control and Prevention [CDC], 2006b). The timely handling of possibly contaminated samples without previous knowledge about the infecting agent should be performed in BSL-3 and BSL-4 laboratories equipped with adequate facilities (CDC, 2006d). Current laboratory tests for class A pathogens include: serologic, molecular, and culture-based methods (CDC, 2006c; CDC, 2006a; Fasanella et al., 2003; Mahanty et al., 1999). PCR-based detection of nucleic acids is a good method to detect the presence of pathogens in samples, but the results are not always reliable, and the procedure requires specially equipped laboratories and specific technical expertise. Both laboratory organization and technical know-how are necessary to ensure reliable results and false-positive contamination problems (Kwok & Higuchi, 1989; Lo & Chan, 2006). Specific antibody detection by an Enzyme-Linked Immunosorbent Assay (ELISA), or immunofluorescence assay is more easily applied, and allows for the identification of both infected and exposed persons; however, a detectable antibody response may take weeks to develop. A T cell response to an infectious agent, in contrast, occurs very quickly, thus allowing for earlier detection of a pathogen-specific immune response based on the presence of circulating effector T cells (Gioia et al., 2005). With this method, it could be possible to identify exposed and infected persons in a more timely manner, allowing for early treatment and correct handling of patients containment issues following expo-

sure to potentially lethal agents, such as biological agents or emerging pathogens. Moreover, a quantitative and qualitative analysis of CD4 and CD8 T cell-mediated responses may help to understand the role of cell-mediated immunity against class A pathogens. A T cell array flow cytometry technique, based on exposure of peripheral blood mononuclear cells (PBMC) to antigens, was recently evaluated by Gioia that allowed for the characterization of an antigen-specific T cell profile, based on the detection of intracellular interferon (IFN)- $\gamma$  production (Gioia et al., 2005; Poccia et al., 2003). Unfortunately, this technique is not easily applied within the BSL-3 and BSL-4 environment. To overcome the issue of working with high-risk pathogens, an effective inactivation measure was developed to allow samples to be handled in a BSL-2 laboratory. Paraformaldehyde (PFA) has been widely used for the chemical inactivation of high threat pathogens as it is known to not alter the antigenic, scatter and fluorescence properties of the cells (Lal et al., 1988). The recommended procedures for the PFA inactivation of class A pathogens are shown in Table 1 (CDC 2006d; CDC 2006a; Flick et al., 2003; Mahanty et al., 1999; Stroher et al., 2001). These procedures can be subdivided into two main general protocols: a protocol using a < 30 minutes of PFA inactivation (short exposure), and a procedure using overnight PFA inactivation (long exposure). More specifically, an easy and safe whole blood (WhBl) flow cytometry protocol designed according to BSL-3 and BSL-4 recommendations (WhBl-BSL-3/4 assay) has been designed. This protocol can be applied to class A pathogen-containing samples to allow for the detection of T cell response by phenotypic and functional characterization of pathogen-specific T cells. This WhBl-BSL-3/4 assay was validated in comparison with standard flow cytometry assay on whole blood (WhBl assay) and on purified peripheral blood mononuclear cells (PBMC).

To provide proof of concept for the suitability of the proposed protocol, it was necessary to observe cellular responses. At present, it is impossible to find any individual whose T cells are able to respond to Class-A antigens, while normal samples could make it very easy to set up a flow cytometry protocol including the requested inactivation step. We decided to use CMV antigens to set up the protocol by using normal donors tested for CMV whose T cells were known to have the ability to respond to

**Table 1**

Established protocols for the inactivation of Class-A pathogens by paraformaldehyde (PFA).

Agent	% PFA	Time of Exposure	References
<b>Short Treatment</b>			
<i>Smallpox virus</i>	2 %	15 minutes	1, 2
<i>Brucella spp</i>	3-8 %	≤ 30 minutes	2
<i>Francisella tularensis</i>	3-8 %	≤ 30 minutes	2
<i>Yersinia pestis</i>	3-8 %	≤ 30 minutes	2
<b>Long Treatment</b>			
<i>Bacillus anthracis</i>	4-12 %	6-10 hours	2
<i>Clostridium botulinum</i>	4-12 %	6-10 hours	2
Hemorrhagic fever viruses	2-4 %	12-16 hours	2, 3, 4, 5

1—CDC, 2006b; 2—CDC, 2006c; 3—Flick, 2003; 4—Mahanty, 1999; 5—Stroher, 2001

CMV antigens. In this way it was possible to validate a general inactivation procedure and maintain T cell activity readout, fulfilling the requested protocols for safe Class-A handling that can be applied in any situation.

Peripheral venous blood was collected in heparinized Vacutainer tubes (BD Biosciences) from normal controls known for CMV status by venipuncture. Blood samples were processed within our BSL-3 facility by diluting 1:2 in culture medium (RPMI-1640 medium containing 10% v/v heat-inactivated fetal calf serum [FCS]), and incubated overnight in 5% CO<sub>2</sub> atmosphere in the presence of the stimulating antigen(s), i.e., CMV lysate (2 µg/ml, Biowhittaker, East Rutherford, New Jersey); CMV p65 protein (2 µg/ml, Biodesign International, Saco, Maine); control peptide pool (enclosing CMV, Epstein Barr Virus, Influenza Virus peptides, 1 µg/ml, kindly provided by NIH) in the presence of the co-stimulatory antibodies anti-CD28 (1 µg/ml) and anti-CD49d (1 µg/ml, BD Biosciences). As additional controls, anti-CD28 (1 µg/ml) and anti-CD49d (1 µg/ml) cultures and anti-CD3 antibody (2 ng/ml, Biosource International, Camarillo, California) cultures were used. One hour after the start of the cultures, Brefeldin A (BFA, 10 µg/ml final, Serva, Heidelberg, Germany) was added to block intracellular transport and allow cytokine accumulation in the Golgi compartment. After overnight incubation, stimulated whole blood cultures were stained for surface markers (such as CD8, BD Biosciences), treated by FACS Lysing Solution, washed, inactivated by PFA 4% (according to short or long treatment protocols), and brought out of the BSL-3 facility. Fixed samples were incubated with IFN-γ specific monoclonal antibody (IFN-γ-APC: IgG2b, clone 2573.11, BD Biosciences) according to the manufacturer's instructions in labeling buffer (PBS containing 1% BSA, 0.1% sodium azide and 0.5% saponin) for 15 minutes at room temperature, and acquired by FACS-Calibur cytometer accordingly to standard procedures. In particular, lymphocytes were identified by morphologic parameters, and

responder T cells were identified by CD3, CD8, and IFN-γ expression.

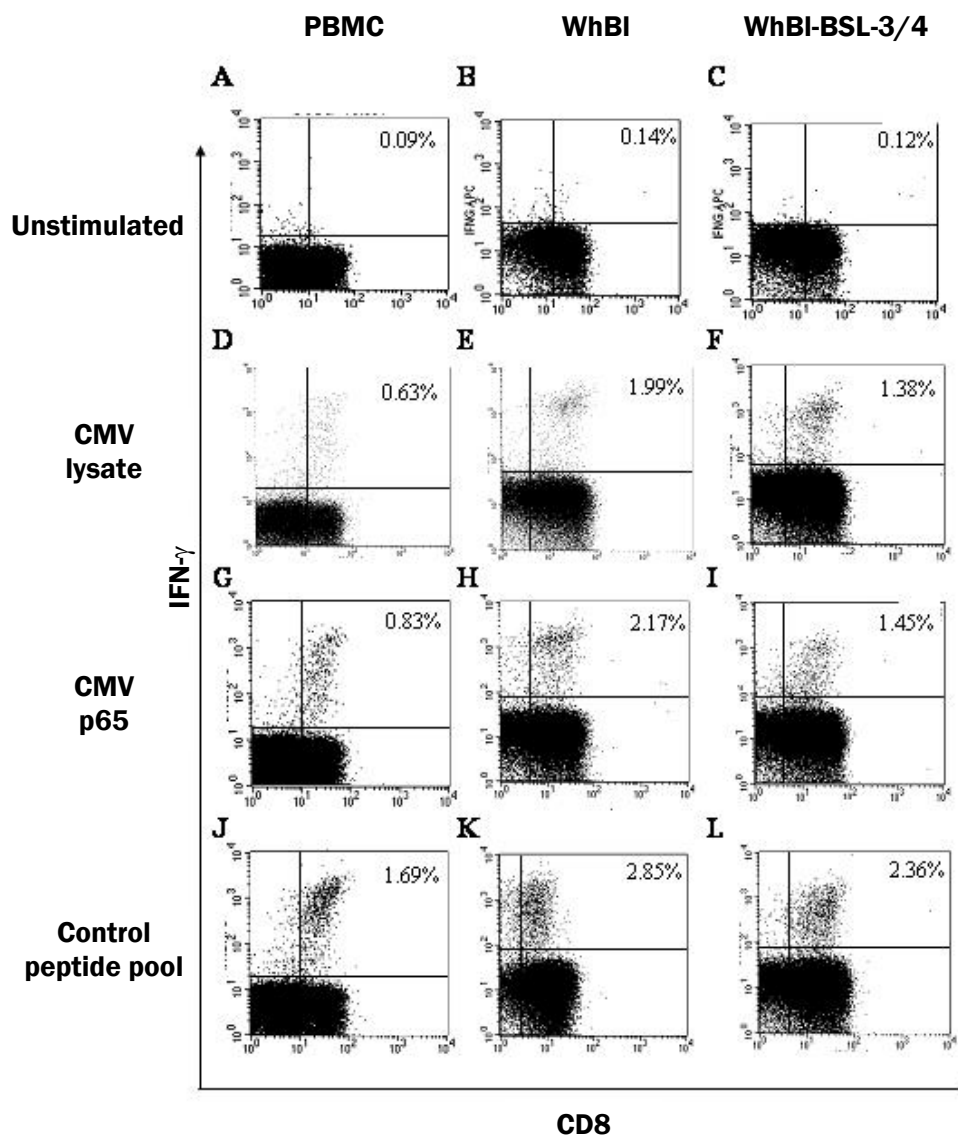
## Results

Figure 1 shows the CD8 T cell response to different Ags preparations to include CMV lysate (panels D-F), CMV p65 protein (panels G-I) and control peptides (panels J-L) in a healthy subject previously characterized to be a CMV responder. The panels show the IFN-γ production (vertical axis) by CD8 T cells (horizontal axis) obtained by culturing responder's PBMC with stimuli as described on the left column. The assay was performed by the WhBl-BSL-3/4 procedure (long inactivation protocol, right column) and compared to the standard WhBl (center column) and PBMC (left column) assays. Anti-CD3 and anti-CD28 plus anti-CD49d antibodies were used as controls (data not shown). The numbers in the upper-right quadrant represent the frequency of IFN-γ producing CD8 T cells in the various experimental conditions. The evaluation of CMV-specific IFN-γ producing CD8 T cells by the WhBl-BSL-3/4 procedure (Figure 1, panels F, I, L) was at least equivalent to those obtained by the standard procedures (PBMC: panels D, G, J; WhBl: panels E, H, K) for both whole CMV lysate, p65 and control peptides. Similar results were obtained in multiple experiments and are representative of at least three tests. The short-term (one hour) PFA treatment also gave similar results (data not shown). Interestingly, in both the standard WhBl and WhBl-BSL-3/4 procedures, slightly higher frequencies of antigen-specific CD8-T cells were detected, as compared to the PBMC assay, possibly reflecting a more efficient stimulation due to more physiological conditions.

The CD8 T cell response detection allows for identification of infected/exposed/immunized persons weeks before the occurrence of detectable antibody response (Gioia et al., 2005; Poccia et al., 2003), but it is not appli-

### Figure 1

**Antigen-specific T cell response.** After *in vitro* stimulation with cytomegalovirus (CMV) antigen preparations (CMV lysate and p65) or with a positive control peptide pool (enclosing CMV, Epstein Barr Virus, Influenza Virus peptides), the frequency of IFN- $\gamma$ -producing CD8 T cells was analyzed by flow cytometry. These experiments were performed on peripheral blood mononuclear cells (PBMC) (Panels A, D, G, J) and on whole blood (WhBI) both using standard (WhBI: Panels B, E, H, K), and BSL-3/4 (WhBI-BSL-3/4, Panels C, F, I, L) protocols.



cable for samples suspected to be infected with class A pathogens that must be handled in BSL-3 or BSL-4 facilities. We therefore standardized a flow cytometry procedure that by an inactivation step, performed according to recognized biosafety procedures, may be applied to CD8 T cell response to pathogen-related antigen preparation (such as viral lysates, proteins and peptides and, possibly, live viruses) without affecting its sensitivity. As T cell response to infectious agents occurs very early (days or weeks before the development of a serological response) this pro-

cedure may be used for the early detection of signs of exposure to a class A pathogen. Therefore, the availability of an easy-to-perform and safe assay that is able to reveal the presence of a T cell based pathogen-specific immune response may be a very helpful tool that can be used, in parallel with the detection of nucleic acids of the etiological agent, to reliably identify in the early stages class A pathogen-exposed and/or infected persons. Moreover, this procedure allows one to monitor the cell-mediated immunity in patients infected by class A pathogens.

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## Development and Validation of a Pilot Scale Enhanced Biosafety Level Two Containment for Performance Evaluation of Produce Disinfection Technologies

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### Abstract

The development and validation of the Biosafety Level 2 (BSL-2) enhanced containment system located at the produce pilot plant facility of the U.S. Department of Agriculture—Eastern Regional Research Center is presented here. This multi-purpose containment is used to enclose pilot and pilot scale washing and sanitizing equipment for fruits and vegetables, or other decontamination equipment where aerosol generation is likely, and complete protection is required for researchers. This containment is operated under a negative pressure with all exhausted air (approximately eight containment air exchanges per hour) being passed in parallel through two hydrophobic HEPA filters. During operation, personnel are

excluded from the containment, materials are introduced into and removed from the containment via pass-throughs, and equipment is operated via computer control and glove ports.

At the completion of any series of processing trials, vegetative bacterial cells remaining within the containment, and in the processing water, are inactivated by raising the internal temperature of the containment, and all contents, including equipment, processing water and waste water to sufficiently inactivate all bacterial cells, except spore formers, using industrial steam at atmospheric pressure. We have demonstrated the feasibility and safety of conducting multiple trials using pathogenic bacteria with pilot scale processing equipment within the containment, and then inactivating surviving vegetative bacterial cells with the steam-in-place proc-