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Research paper

$\gamma\delta$ T-lymphocyte cytotoxic activity against *Mycobacterium bovis* analyzed by flow cytometry

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Abstract

Gamma Delta ($\gamma\delta$) T lymphocytes contain the unique capability of responding to pathogens in both an innate and acquired immune response. Previously, $\gamma\delta$ lymphocytes have been reported to respond to *Mycobacteria tuberculosis* determined by proliferation and IFN- γ production. Unlike alpha beta ($\alpha\beta$) lymphocytes, $\gamma\delta$ lymphocytes constitutively express a natural killer receptor providing $\gamma\delta$ lymphocytes the capability for innate cytolytic functions. A new cytolytic assay by flow cytometry was reported capable of determining natural killer activity using K562 cells as targets without the need for radioactive materials. The objectives of this study were to first apply the flow cytometer-based assay to assess $\gamma\delta$ lymphocytes natural killer activity following animal vaccination with *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG). Secondly, to optimize the flow cytometer assay in order to detect antigen specific cytolytic activity to mycobacterium and to compare the cytolytic activity of $\gamma\delta$ lymphocytes to CD-8 lymphocytes. $\gamma\delta$ lymphocytes increased in NK activity ($P=0.012$) following animal vaccination with *M. bovis* BCG. Both innate ($P=0.02$) and acquired antigen-specific cytolytic activity ($P=0.04$) increased following incubation with *M. bovis*-infected monocytes. In conclusion, flow cytometric-based assay is a sensitive and reliable tool to determine cytolytic activity of $\gamma\delta$ T-lymphocytes against mycobacterium.

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Keywords: $\gamma\delta$ lymphocytes; Flow cytometer; Natural killer cytolytic activity; *Mycobacterium tuberculosis*; *Mycobacterium bovis*

Abbreviations: $\gamma\delta$, Gamma delta; $\alpha\beta$, Alpha beta; IFN- γ , IFN-gamma; NK, Natural killer; BCG, Bacillus Calmette–Guérin.

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1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is a major cause of morbidity and mortality worldwide infecting approximately one-third of the world's population (Boom, 1999) attributing to 3 million deaths a year (Boom, 1999; Carson and Sutcliffe, 1999). There are several mechanisms involved in immunity against *M. tuberculosis* including cytokines released by sensitized T-lymphocytes activating tuberculocidal and/or tuberculostatic alveolar macrophages leading to the development of granulomas (Li et al., 1996). It has been demonstrated that CD4⁺ T-lymphocytes play a role in the protection against *M. tuberculosis*, as evidenced by the severe clinical manifestations of tuberculosis in persons with human immunodeficiency virus infection and defective CD4⁺ T-lymphocytes function (Havlir and Barnes, 1999). CD8⁺ T-lymphocytes contribute to immunity against *M. tuberculosis* (Sousa et al., 2000) primarily through cytotoxicity of infected targets. In humans infected with *M. tuberculosis*, CD8⁺ T-lymphocytes recognize mycobacterial antigens lysing *M. tuberculosis* infected monocyte-derived macrophages and alveolar macrophages (Lalvani et al., 1998; Geluk et al., 2000), and directly kill *M. tuberculosis* by secreting the antimicrobial peptide granulysin.

Although the vital function of $\alpha\beta$ lymphocytes remain unquestioned (Ladel et al., 1995), there has been increasing evidence that the unique immunological functions of $\gamma\delta$ lymphocytes contribute to immunity against tuberculosis (Boom, 1999). $\gamma\delta$ T lymphocyte subset respond to a wide variety of phosphorylated metabolites released by bacteria, parasites, and eukaryotic cells with damaged membranes (Bukowski et al., 1994; Poccia et al., 1999) secreting Th1 cytokines and exhibiting natural killer cytolytic functions (Paliard et al., 1989; Ohmen et al., 1991; Tsukaguchi et al., 1995; Boom, 1999).

Natural killer cytotoxicity (NK) is an important innate process accomplished by nonspecifically lysing infected targets, or through the use of the Fc γ II (CD16) receptor allowing the NK cells to recognize IgG bound to antigens on the target cell surface (Lee-MacAry et al., 2001). The assessment of NK cytolytic activity has been accomplished using assays detecting lactate dehydrogenase release or the most common method the release of radioactive chromium-51 (⁵¹Cr)

from lysed target cells (Lee-MacAry et al., 2001). However, these techniques have several drawbacks such as high spontaneous leaking giving a high background, short half-life, high cost, and the potential health risk due to radioactive material (Slezak and Horan, 1989).

Flow cytometric assays have been developed to overcome some of these difficulties analyzing the differences in light scatter properties to identify targets from effector cells (Vitale et al., 1989); however, there were problems with discrimination between the two cell populations (Ellis, 1993). Radosevic et al. (1990) first reported NK cytotoxicity by staining the target cells with a green fluorescent dye in combination with the DNA intercalating dye, propidium iodide. Following this technique, use of membrane dye PKH-26 was optimized (Hatam et al., 1994; Lowdell et al., 1997), to use in combination with the viability probe TO-PRO-3 iodide (Van Hooijdonk et al., 1994; O'Brien et al., 1995).

Lee-MacAry et al. (2001) compared a flow cytometry-based assay to ⁵¹Cr release assay demonstrating greater than a 95% correlation. The strength of the flow cytometry-based assay eliminated the need for radioactive materials and allowed for the analyzing cytolytic activity at a single-cell level. The novel flow cytometric-based assay has been used to demonstrate innate activity against various target cell lines including K562, YAC Tumor cell lines, and T2 cells (Kuzushima et al., 1999; Gogoy-Ramirez et al., 2000; Fischer et al., 2002). In this study, natural killer activity following Bacillus Calmette–Guérin (BCG) vaccination is evaluated using a novel flow cytometer-based assay. Through optimization of the flow cytometer-based assay, adaptive cytolytic response to $\gamma\delta$ lymphocytes is characterized and compared to the CD-8⁺ lymphocyte.

2. Material and methods

2.1. Animal immunization

Three-week-old crossbred male pigs were allocated into nonvaccinated and BCG-vaccinated groups. Pigs were housed at the University of Minnesota isolation unit and cared for in accordance with the University of

Minnesota Institutional Animal Care and Use Committee regulations. BCG was injected at 6×10^6 CFU/ml intradermally in the left deltoid muscle.

2.2. Isolation of peripheral mononuclear cell (PBMC)

Isolation of T-lymphocytes from blood was accomplished as previously described (Bautista et al., 1999). Briefly, blood from the jugular vein of pigs was collected in sterile heparinized vacutainer tubes. Blood was layered on lymphocyte separation media at a ratio of 1:3 and centrifuged at $2000 \times g$ for 30 min at room temperature. Blood mononuclear cells enriched layer was removed and washed three times, resuspended in sterile PBS containing 1% FBS.

2.3. Monocyte isolation

Isolated PBMCs were incubated for 2 h at 37°C on 3% gelatin-coated cell culture plates. Following incubation, supernatant was removed; plates were washed three times with $1 \times \text{PBS}$ containing 0.2 mM EDTA solution was added to plates then incubated at 4°C for 20 min. Supernatant containing monocytes were washed two times and incubated overnight in 10% FBS in RPMI 1640 media at 37°C .

2.4. $\gamma\delta$ and CD-8 T lymphocyte subset purification

Purification of $\gamma\delta$ and CD-8 lymphocytes was accomplished using a previously described positive isolation method (Davis et al., 1994). Monoclonal anti-swine $\gamma\delta$ antibodies, cell line PGBL22A (VMRD, Pullman, WA) was added to the purified lymphocytes at a concentration of $1 \mu\text{g}/10^6$ $\gamma\delta$ target cells and incubated for 30 min on ice. Goat anti-Mouse IgG-coated Superparamagnetic Particles (Polysciences Warrington, PA) were calculated for a bead to cell ratio of 1:5 using the formula: (total cell number) \times (percent (%) of target cells) \times (bead to cell ratio of 5)/beads concentration. Following antibody incubation, cells were washed and resuspended in 40 ml of cold $1 \times \text{PBS}$. Magnetic beads were added to cells and incubated for 30 min on ice, inverting the samples every 10 min making sure that cells and beads remain in suspension. Following incubation, samples were placed on a magnetic separator for 5 to 10 min, drawing the beads that were bound to the $\gamma\delta$

cells out of suspension. Supernatant was removed and the beads and the bound cells were washed with $1 \times \text{PBS}$, containing 1% FBS, to remove residual nontarget cells. $\gamma\delta$ cells bound to the beads were resuspended in 1800 μl of $1 \times$ activation solution (10 mM cysteine, 50 mM NaH_2PO_4 , 1 mM EDTA) and 200 μl of papain for a final concentration of $1 \mu\text{g}/\text{ml}$ and incubated in a 37°C , 5% CO_2 incubator for 1 h, allowing enzymatic cleavage of the antibody, removing the cells from the beads. The beads were removed using the FlexiMag Separator and technique described above. The supernatant containing purified $\gamma\delta$ cells was then removed and resuspended in 10% RPMI media and incubated at 37°C , 5% CO_2 overnight. The purified cells were incubated overnight then analyzed by flow cytometry using anti- $\gamma\delta$ monoclonal antibody verifying $>90\%$ purity.

2.5. Natural killer assay

A K562 cell line was used to assess $\gamma\delta$ lymphocytes NK activity (Rodriguez-Carreno et al., 2002). K562 cells were membrane stained as a target using PKH 64 (Sigma, St. Louis, MO; Lee-MacAry et al., 2001). Briefly, cells were resuspended in 2 ml of diluents C reagent provided in the kit and stained at a final dilution of 1:2000 PKH stain for 5 min, washed and resuspended in 2 ml of FBS to stop the reaction, washed and adjusted to a concentration of 1.0×10^4 cells/100 μl in sterile FACS tubes. Purified porcine $\gamma\delta$ lymphocytes were added to target cells at an E/T ratio of 50:1 and placed in a 37°C , 5% CO_2 incubator for 4 h. Following incubation, cells were brought up to 500 μl with $1 \times \text{PBS}$, 50 μl of a 1:2000 dilution of ToPro-3 stain (Molecular Probes, Eugene, OR), a stain that will distinguish live from dead cells, was added and each sample was placed on ice for 15 min then analyzed by flow cytometry.

2.6. Antigen-driven cytotoxicity

Purified monocytes were washed and incubated for 3 h with BCG at a MOI 5. Following incubation, monocytes were washed three times and incubated for 24 h. Infected autologous monocytes were membrane stained with PKH stain (as described above) and adjusted to 1.5×10^4 cells/100 μl in sterile FACS tubes. Purified $\gamma\delta$ or CD-8 lymphocytes at a concentration of

4.9×10^5 were added to target cells and placed in a 37 °C, 5% CO₂ incubator for 4 h. Following incubation, cells were brought up to 500 µl with 1×PBS, 50 µl of a 1:2000 dilution of live/dead ToPro-3 stain was added and each sample was placed on ice for 15 min then analyzed by flow cytometry.

2.7. Data analysis and statistical analysis

Results were analyzed by CellquestPro, figures were derived by WinMDI software. Data was analyzed using a dot plot separated into four quadrants. Percentages of cells killed by cytotoxic activity were calculated by (upper right)/(upper and lower right)×100 then subtracting the percentage of spontaneous death from target cells only control tube. Statistically significant differences were assessed using unpaired Student's *t*-test for analysis between two groups. Differences between data were consid-

ered statistically significant at $p < 0.05$. All tests were performed with commercially available software for personal computer (Statista 6.0, StatSoft).

3. Results

3.1. Optimization of flow cytometry cytolytic assay

The use of flow cytometry to detect cytolytic activity in response to pathogens is a relatively new and novel method. The most widely used method for detection of cytolytic activity detection has been ⁵¹Cr release assay. Flow cytometer cytolytic-based assay have been compared to ⁵¹Cr release assays determining greater than 95% correlation using various cell lines (Lee-MacAry et al., 2001). By applying the novel flow cytometric-based assay using PKH membrane and ToPro-3 live/dead stain, it was possible to

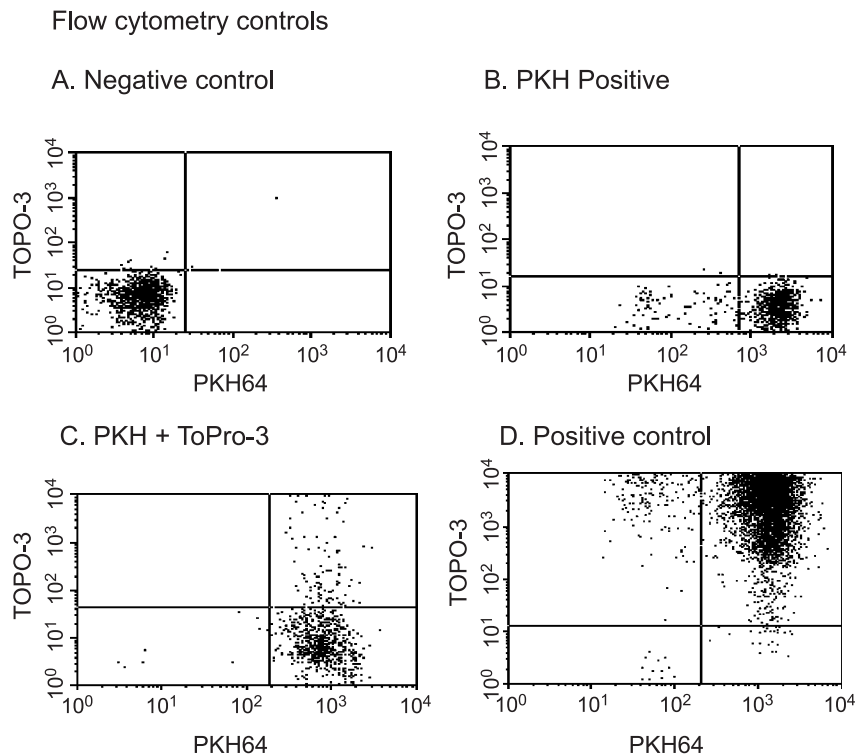


Fig. 1. Optimization of the flow cytometer for cytotoxicity. To determine if the flow cytometry is calibrated correctly, various controls were used prior to analysis. (A) Nonstained target cells used to set the gains and voltages. Target cells were set within the first decade of dot plot allowing for maximal separation from effector lymphocytes. (B) Target cells stained with PKH membrane stain verifying good separation from effector cells and FL-4 settings were correct. (C) PKH and ToPro-3 stained target used to verify nonspecific staining shifting the target population up the Y-axis. (D) Ethanol-lysed target cells used as a positive control verifying good separation between viable and killed target cell populations.

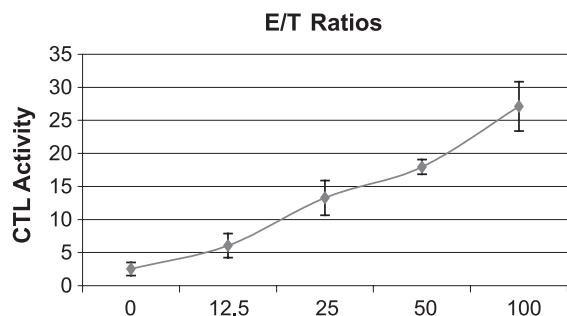


Fig. 2. *E/T* ratios. To determine the optimal *E/T* ratio, purified $\gamma\delta$ lymphocytes were added to K562 cells at *E/T* ratios of 0, 12.5:1, 25:1, 50:1, and 100:1 and incubated for 4 h. Cytotoxicity was expressed as a mean of $n=4$.

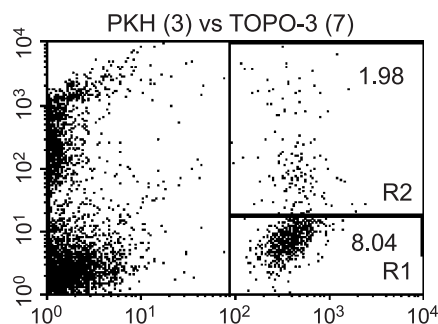
investigate the unique ability of innate and acquired antigen directed cytolytic immune response by $\gamma\delta$ lymphocytes against *Mycobacterium bovis*. ToPro-3 requires the fluorescence parameter (fl) FL-4 laser requiring calibration using the manufacturer's calibration beads (Becton Dickinson Immunocytometry System, San Jose, CA). To properly calibrate the flow cytometer, nonstained target cells were used as a negative control to set the amps and gain (Fig. 1A). Stained target cells were used to verify the separation of effector/target cells (Fig. 1B). PKH-ToPro-3 target cells were used to verify the flow cytometry was setup properly (Fig. 1C). Ethanol permeabilized PKH stained target cells stained with ToPro-3 were used to set fl-4 channel of the flow cytometer (Fig. 1D). Event acquisition was set for 10,000 events in a region encompassing the PKH/ToPro-3 positive quadrants (upper and lower right quadrants only). To determine the optimal *E/T* ratio, purified $\gamma\delta$ lymphocytes were incubated to membrane stained K562 target cells at various *E/T* ratios of 12.5:1, 25:1, 50:1, and 100:1, and incubated for 4 h. ToPro-3 was immediately added to assay tubes and analyzed by flow cytometry. A steady increase of cytolytic activity was observed with the increase of $\gamma\delta$ lymphocytes demonstrating the assay was optimized (Fig. 2). An *E/T* ratio of 50:1 was used for the subsequent assays.

3.2. Natural killer activity of $\gamma\delta$ lymphocytes

$\gamma\delta$ lymphocytes express a natural killer receptor capable of being upregulated following exposure to

a pathogen (Rajan et al., 1996). In this study, we observed an increase in cytolytic activity against the K562 target cells following *M. bovis* BCG vaccination determined by ToPro-3 live/dead stain shifting from R1 to R2 (Fig. 3A and B). By gating on R2 region, a histogram showed an increase in ToPro-3 staining between control and BCG-vaccinated pigs (Fig. 4A and B). Overlaying control and BCG-vaccinated groups, an increase in cytolytic activity is observed (Fig. 4C). Prior to BCG vaccination, $\gamma\delta$ lymphocytes had 18% natural killer activity, which increased to 34.5% 10 days post-vaccination ($P=0.012$; Fig. 5).

A. Dot plot PKH vs. ToPro-3, control pigs



B. Dot plot PKH vs. ToPro-3, BCG vaccinated

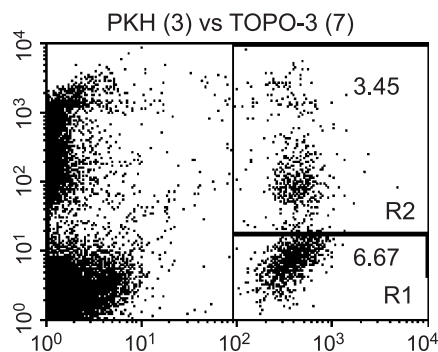
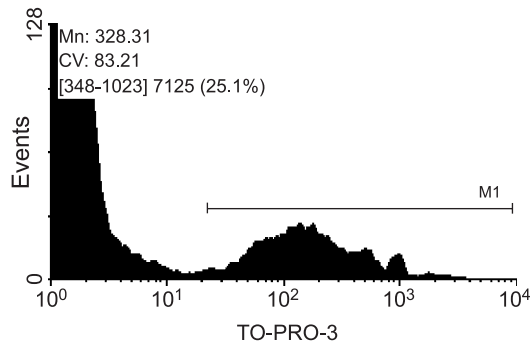
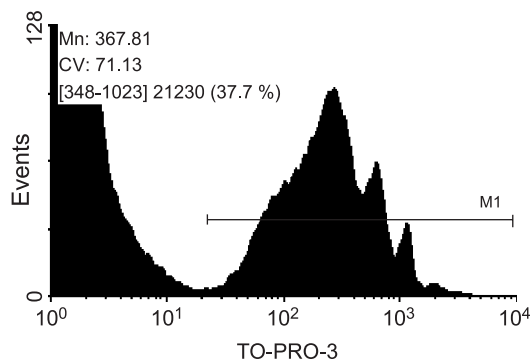


Fig. 3. Natural killer activity determined by flow cytometry. Using WinMDI flow cytometry software data was imported in a dot plot PKH (*X*-axis) vs. ToPro-3 (*Y*-axis). Cytolytic activity was determined by a shift up the *Y*-axis from R1 to R2 regions due to an increase of ToPro-3 staining dead or dyeing K562 cells following cytolytic activity. (A) Dot plot of nonvaccinated control group. (B) Dot plot of BCG-vaccinated group.

A. Histogram derived by flow cytometry software, control pigs



B. Histogram derived by flow cytometry software, BCG vaccinated group



C. Overlay of control and BCG vaccinated groups

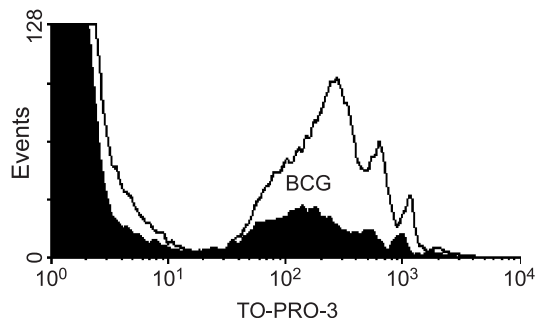


Fig. 4. Analysis of natural killer activity. Gating on region R2 (Fig. 3 dot plots) histograms was constructed demonstrating ToPro-3 staining on target cells can be measured. (A) Amount of events and intensity detected by ToPro-3 staining on target cells in the nonvaccinated control group. (B) Amount of events and intensity detected by ToPro-3 on the target cell population in BCG-vaccinated group (Schild et al.). Overlay of nonvaccinated and BCG-vaccinated groups comparing the increase of ToPro-3 staining on target cells due to cytolytic activity. Plots are representative of $n=4$ control and $n=4$ BCG-vaccinated pig groups.

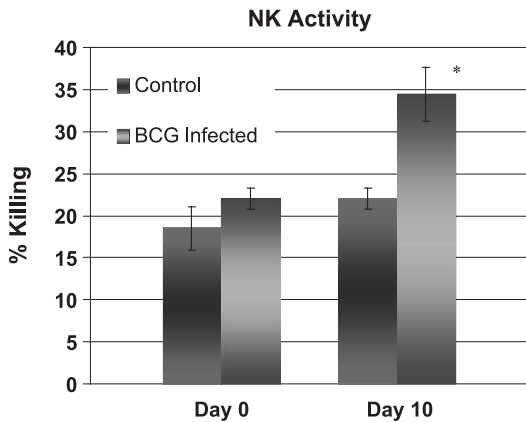


Fig. 5. Natural killer activity following BCG exposure. Following analysis of $\gamma\delta$ lymphocytes with K562 cells, data was imported in to a dot plot and cytolytic activity was calculated (as described in material and methods). *Statistically significant change determined by $p < 0.05$. Plots are representative of $n=4$ control and $n=4$ BCG-vaccinated pig groups.

3.3. Antigen-specific cytotoxic activity

$\gamma\delta$ lymphocytes encompass the ability to function in both an innate and adaptive response to presented antigens (Groh et al., 1998). $\gamma\delta$ lymphocytes innate cytolytic capability is due to the constitutively expressed natural killer receptor, which are relatively rare on $\alpha\beta$ lymphocytes (Mingari et al., 1995). To compare innate cytotoxicity between $\gamma\delta$ and $\alpha\beta$ CD-8 lymphocytes, $\gamma\delta$ and CD-8 lymphocytes were purified from non-vaccinated animals and exposed to BCG-infected autologous monocytes; noninfected autologous monocytes were used as a control. $\gamma\delta$ lymphocyte had 9% cytotoxic activity compared to 2% activity by CD-8 ($P=0.02$).

To characterize $\gamma\delta$ lymphocytes adaptive response to presented antigens, purified autologous monocytes were incubated with BCG at a MOI 5 for 3 h, washed and incubated for 24 h for antigen presentation. Following incubation, purified $\gamma\delta$ lymphocytes from BCG-vaccinated or nonvaccinated animals were adjusted to achieve a final E/T ratio of 50:1 and incubated for 4 h. $\gamma\delta$ lymphocyte incubated with noninfected monocytes were used for a control and subtracted for nonspecific spontaneous death. $\gamma\delta$ T lymphocytes displayed innate cytotoxic activity (Fig. 6A), increasing to 22% by day 14 post-vaccination

($P=0.04$) compared to nonvaccinated control group (Fig. 6A).

Previous reports have demonstrated that $\gamma\delta$ lymphocytes respond prior to $\alpha\beta$ lymphocytes in various immune responses (Ladel et al., 1995). It was important here to compare antigen-specific cytolytic activity between $\gamma\delta$ and CD-8⁺ $\alpha\beta$ lymphocytes using the flow cytometric-based assay. Autologous monocytes were incubated with BCG at a MOI 5 for 3 h, washed and incubated for 24 h for antigen presentation. Purified $\gamma\delta$ or CD-8 lymphocytes were adjusted to achieve a final E/T ratio of 50:1 and incubated for 4 h; noninfected monocytes were used for a control and for nonspecific spontaneous death. $\gamma\delta$ lymphocytes increased to 23 % by day 14 post-vaccination ($\gamma\delta$ vs. CD-8, $P=0.044$), while CD-8⁺ $\alpha\beta$ lymphocytes showed low cytolytic activity on day 14 increasing by day 21 post-vaccination (Fig. 6B). While the data demonstrated an increase in cytolytic activity by CD8 lymphocytes, there was no significant difference compared to $\gamma\delta$ lymphocytes on day 21 pi.

4. Discussion

The objectives of this study were to use a newly developed flow cytometry-based assay to determine the innate and adaptive antigen driven cytolytic response by $\gamma\delta$ lymphocytes following *Mycobacterium* vaccination. Traditionally, effector cell-mediated cytotoxicity has been determined by lysing target cells pulsed with various compounds containing radioactive isotopes such as the ⁵¹Chromium (⁵¹Cr) assay, ⁷⁵Selenium (⁷⁵Se), or Tritium (³H) (Cerottini and Brunner, 1974). These assays contain many unfavorable characteristics such as radioactive materials, short half-life, high cost, potential health risk, and spontaneous release from target cells altering results (Slezak and Horan, 1989). Several non-radioactive indirect assays have been developed such as Europium (Eu³⁺; Patel and Boyd, 1995), bisbenzamide dye (Toka et al., 1996) Calcein-AM (acetoxymethyl ester of calcein) (Lichtenfelsa et al., 1994). However, indirect measurement of the supernatant frequently fails to offer sufficient accuracy.

Vitale et al. (1989) described a flow cytometric assays for the detection of NK cytolytic activity through light scatter properties to identify targets from

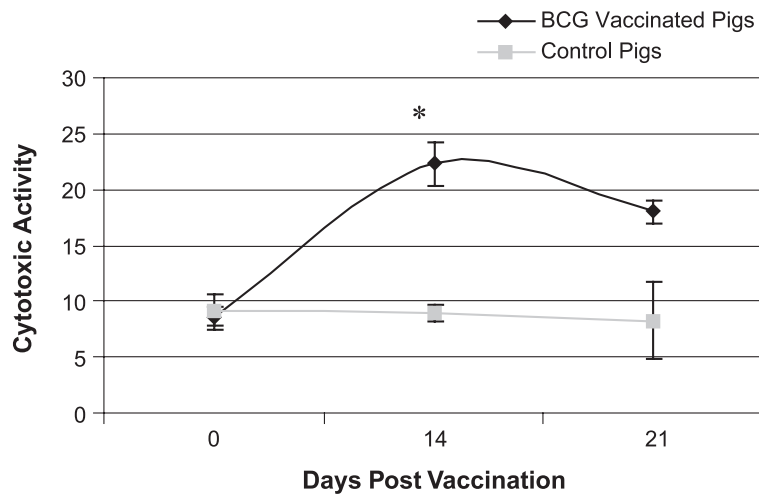
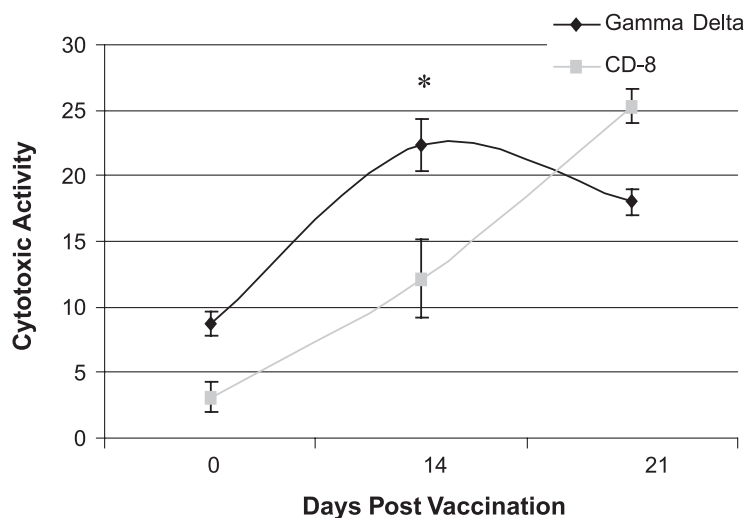
A. Antigen specific cytotoxic activity by $\gamma\delta$ lymphocytesB. $\gamma\delta$ vs CD-8 antigen specific cytotoxicity

Fig. 6. $\gamma\delta$ lymphocytes cytolytic activity following incubation with BCG infected monocytes. $\gamma\delta$ lymphocytes were purified and incubated with BCG-infected autologous monocytes. Data was imported in to a dot plot and cytolytic activity was calculated (as described in material and methods). (A) BCG-vaccinated pig group was compared to nonvaccinated control group demonstrating cytolytic activity due to mycobacterial exposure. (B) Antigen-specific cytolytic activity of $\gamma\delta$ and CD-8 lymphocytes. Purified $\gamma\delta$ or CD-8 lymphocytes were incubated with autologous BCG infected monocytes. Data was imported in to a dot plot and cytolytic activity was calculated (as described in Material and methods) then plotted to comparing the two lymphocyte subsets. *Statistically significant change determined by $p < 0.05$.

effector cells. This assay was further enhanced by staining the target cells with the green fluorescent dye (Radosevic et al., 1990) in combination with the DNA viability probe TO-PRO-3 iodide (Van Hooijdonk et al., 1994; O'Brien et al., 1995). In this study, we

attempted to use PKH-26 membrane stain as reported by Hatam et al., 1994 and Lowdell et al., 1997. Our laboratory determined that PKH-64 gave a tighter population when analyzed by flow cytometer important for the separation of target and effector cells.

In this study, the first component was to use the flow cytometer-based assay to characterize $\gamma\delta$ lymphocytes NK functions using the K562 cell line as target cells. Following the determination of NK activity, our second goal was to optimize the flow cytometer assay to determine antigen directed cytolytic functions. To achieve the first goal of NK activity, $\gamma\delta$ lymphocytes were incubated with K562 cell line at various E/T ratios to determine the ratio to be used through out this study. An E/T ratio of 50:1 was chosen because it demonstrated cytolytic activity. To optimize the assay for antigen specific responses, monocytes were purified and stained with various concentrations of PKH-64 membrane stain to determine whether there are cytotoxic effects on the cells due to staining. Moreover, various dilutions of ToPro-3 live/dead stain were tested for nonspecific staining. It was determined that 1:2000 PKH stain was optimal for both K562 and monocytes for separation from effector cells and 50 μ l of a 1:2000 dilution of ToPro-3 live/dead stain was used in 500 μ l of cells.

Using the novel flow cytometer-based assay, it was determined that $\gamma\delta$ lymphocytes function in an innate natural killer cytolytic activity prior to mycobacterium vaccination. Following *M. bovis* BCG vaccination, an increase in NK cytolytic activity was observed in $\gamma\delta$ lymphocytes. The findings in this study are all in agreement with Battistini et al. (1997) and Paliard et al. (1989) who reported that $\gamma\delta$ lymphocytes express functional natural killer receptors demonstrating natural killer activity. Although virtually all $\gamma\delta$ lymphocytes carry natural killer receptors, natural killer receptor expression on $\alpha\beta$ lymphocytes is relatively rare (Fuertes et al., 1999; Jason et al., 2000; Steele et al., 2000). To test potential innate cytolytic activity differences between $\alpha\beta$ and $\gamma\delta$ T-lymphocyte subsets, purified naïve $\gamma\delta$ and $\alpha\beta$ lymphocyte subsets were compared for innate cytolytic activity against mycobacterially exposed autologous monocytes. This comparison verified that $\gamma\delta$, not $\alpha\beta$ CD-8⁺ lymphocyte subset showed evidence of innate antigen-specific cytolytic activity. Innate cytolytic activity may occur because $\gamma\delta$ lymphocyte, not $\alpha\beta$ lymphocytes respond through the CD-1 molecule, a MHC-like surface molecule that processes and presents nonpeptide antigens to T lymphocytes, including mycobacterial lipids (Beckman et al., 1994).

The ability of $\gamma\delta$ lymphocytes to recognize antigens through the CD-1 molecule may be responsible for the bridge between innate and adaptive immune responses demonstrated by $\gamma\delta$ lymphocytes (Spada et al., 2000). To test $\gamma\delta$ lymphocytes antigen-specific cytolytic potential against mycobacterium, on day 14 post-vaccination, autologous monocytes were infected with *M. bovis* and incubated with purified $\gamma\delta$ lymphocytes from nonvaccinated or vaccinated animal groups. $\gamma\delta$ lymphocytes from vaccinated pigs increased in antigen-specific cytolytic activity compared to non-vaccinated pigs following exposure to mycobacteria. Moreover, $\gamma\delta$ lymphocytes responded prior to $\alpha\beta$ cytotoxic CD-8⁺ lymphocytes.

In conclusion, using the flow cytometer-based cytolytic assay, it was demonstrated that $\gamma\delta$ lymphocytes respond to the K562 cell line in an innate cytolytic response, which increased following vaccination. Moreover, $\gamma\delta$ lymphocytes responded in both an innate and adaptive cytolytic responses against mycobacterium-infected autologous monocytes as a target cells.

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