$PmpG_{303-311}$, a protective vaccine epitope that persists in *Chlamydia muridarum* immune mice†

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Abstract

Urogenital *Chlamydia* serovars replicating in reproductive epithelium pose a unique challenge to host immunity and vaccine development. Previous studies have shown that CD4 T cells are necessary and sufficient to clear primary *Chlamydia muridarum* genital tract infections in the mouse model, making a protective CD4 T cell response a logical endpoint for vaccine development. Our previous work utilized proteomics to identify potential subunit vaccine candidates. Immunoprecipitation of MHC class II molecules from *Chlamydia*-infected dendritic cells, and elution of their resident peptides, identified eight candidate *Chlamydia* proteins for subunit vaccines. Subsequent vaccination studies in mice identified PmpG-1 as the most promising of the eight candidate *Chlamydia* proteins. To further that work we derived a $PmpG_{303-311}$-specific CD4 T cell clone designated PmpG1.1 from an immune C57BL/6 mouse. PmpG1.1 is a multifunctional Th1 clone stably polarized to secrete IFN-γ & TNF-α. We used this T cell clone to investigate presentation of the $PmpG_{303-311}$ epitope by infected epithelial cells. The $PmpG_{303-311}$ epitope was presented by infected epithelial cells 15-18 hours post infection. Unlike epitopes recognized by other *Chlamydia*-specific CD4 T cell clones, $PmpG_{303-311}$ persisted *in vivo*. PmpG1.1 was activated by unmanipulated irradiated splenocytes from immune mice without addition of exogenous *Chlamydia* antigen. Activation of PmpG1.1 by unmanipulated immune splenocytes was strong 6 months post-infection, long after resolution of the primary genital tract infection. Even more interesting, activation of PmpG1.1 by unpulsed immune splenocytes was greater at 6 months than 3 weeks post infection. Enhanced presentation of $PmpG_{303-311}$ epitope after clearance of active infection demonstrates a "consolidation" of a protective immune response. Understanding the antigen presenting cell populations responsible for presenting $PmpG_{303-311}$ at early (3 weeks) and late (6 months) post infection will likely provide important insights into stable protective immunity against *Chlamydia* infections.

Introduction

Public health measures to control *Chlamydia trachomatis* genital tract infections, combining case identification with partner tracing and treatment programs, have had some success in decreasing the incidence of pelvic inflammatory disease (PID) but not the incidence and prevalence of sexually transmitted infections. Development of a protective vaccine for prevention of *C. trachomatis* urogenital tract infections will be challenging as antibody has no discernable role in clearing primary infections; therefore the critical components of a *Chlamydia* vaccine will likely be its T cell epitopes. A wealth of data from the *C. muridarum* mouse model for *Chlamydia* genital tract infections suggests the critical T cell epitopes will be presented by MHC class II (human HLA-DP,-DQ, DR) molecules to *Chlamydia*-specific CD4 T cells. To date there are no examples of a protective subunit vaccine based on T cell immunity, or against an intracellular bacterial pathogen.

To facilitate rational vaccine development, we have focused on identifying *Chlamydia* peptides loaded onto MHC class II molecules using the *C. muridarum* mouse model. *Chlamydia* peptides loaded on to MHC class II molecules have the potential to be recognized by T cell receptors on *Chlamydia*-specific T cells and, by definition, are derived from *Chlamydia* proteins whose biology within infected cells makes them susceptible to host cell antigen processing and presentation machinery. Identification of *Chlamydia* proteins processed and presented by infected cells is critical for rational vaccine development as a large fraction of *Chlamydia* proteins are likely sequestered away from processing and presentation machinery by exclusive residence in the inclusion body. (? 3% unpublished data). We and others have shown that protective immunity against *C. muridarum* genital tract infections can be induced by adoptive transfer of antigen-pulsed dendritic cells, therefore our initial efforts have focused on that cell type. We previously identified a panel of CD4 and CD8 T cell epitopes by immunoprecipitation of MHC class II and class I molecules from infected C57BL/6 derived dendritic

cells, eluting the resident peptides, and identifying those peptides using Moldi-Tof Mass Spec. We found that of the identified epitope-source proteins, immunization with a PmpG-1 fusion protein, the source protein for the I-A^b-presented epitope $PmpG_{303-311}$, provided the greatest protection against *C. muridarum* infectious challenge in the genital tract.

In this study we advance that research by investigating the characteristics of the vaccine-protective T cell epitope $PmpG_{303-311}$. To that end we derived a CD4 T cell clone specific for $PmpG_{303-311}$ from an immune mouse that had previously cleared a genital tract infection. The resulting CD4 T cell clone designated PmpG1.1 was a useful tool for investigating presentation of the $PmpG_{303-311}$ epitope *in vitro* and *in vivo*. We present interesting results of those studies here.

Materials and Methods

Mice

Female C57BL/6 female mice were purchased from Harlan Laboratories (Indianapolis, IN). All mice were housed in Indiana University Purdue University-Indianapolis (IUPUI) specific-pathogen-free (SPF) facilities. The IUPUI Institutional Animal Care and Utilization Committee approved all experimental protocols.

Cells and bacteria

C57epi.1 is a cloned oviduct epithelial cell line derived from a C57BL/6 mouse (H-2^b) (1). C57epi.1 cells are grown at 37°C in a 5% CO_2 humidified incubator in epithelial-cell media (1:1 Dulbecco's modified Eagle medium:F12K (Sigma; St. Louis, MO)), supplemented with 10% characterized fetal

bovine serum (HyClone; Logan, UT), 2 mM L-alanyl-L-glutamine (Glutamax I; Gibco/Invitrogen), 5 µg of bovine insulin/ml, and 12.5 ηg/ml of recombinant human FGF-7 (keratinocyte growth factor; Sigma).

Mycoplasma-free *Chlamydia muridarum (Nigg)*, previously known as *C. trachomatis* strain MoPn, was grown in McCoy cells (ATCC). The titers of mycoplasma-free *C. muridarum* stocks were determined on McCoy cells with centrifugation as previously described (2). UV-inactivated *C. muridarum* stocks were made by diluting concentrated stocks in PBS, then exposing roughly 3-4 ml of diluted stock in a sterile petri dish to 1200 Joules/cm^2 twice in a UV-Crosslinking Cabinet (Spectralinker, Spectronics Corporation, Westbury, NY). No viable *C. muridarum* inclusions were detectable in inoculated McCoy cell monolayers after U.V. inactivation.

Infection of mice

C57BL/6 mice were treated with 2.5 mg of depo-progesterone (Depo-Provera; Pfizer, NY, NY) injected subdermally one week prior to infection. Vaginal infections were accomplished with $5x10^4$ IFU of *C. muridarum* in 10 µl of SPG buffer. Mice were swabbed 7 days later to confirm infection. Vaginal swab IFU were recovered in SPG buffer and quantified using McCoy cell monolayers as previously described (3).

Media, epithelial cell lines, and CD4 T cell clones

Chlamydia-specific CD4 T cell clones uvm-2, uvmo-3, uvmo-4, and spl4-10 were grown in RPMI 1640 (Sigma) supplemented with 10% characterized fetal bovine serum (HyClone), 2 mM L-alanyl-L-glutamine (Glutamax I; Gibco/Invitrogen), 25 µg/ml gentamicin (Sigma), $5x10^{-5}$M 2-mercaptoethanol (Sigma), referred to as RPMI CM, and secondary MLC supernatant (2° MLC SN) with recombinant cytokines.

Chlamydia-specific CD4 T cell clone PmpG1.1 recognizing $PmpG_{303-311}$ was derived from an immune C57BL/6 ($H-2^b$) female mouse that cleared a primary *C. muridarum* genital tract infection without development of hydrosalpinx. Immune splenocytes were plated at $4x10^6$ cells per well, in tissue culture treated 24 well plates, in 1.5 ml RPMI CM containing murine recombinant IL-1α (2 ηg/ml), IL-6 (2 ηg/ml), IL-7 (3 ηg/ml), IL-15 (4 ηg/ml), human recombinant IL-2 (100 units/ml), 20% secondary mixed lymphocyte culture (MLC) supernatant; and 1.5 μg of recombinant PmpG-1 (amino acids 25-500 with N-terminal His-tag), 1.5 ug of recombinant GAP (N-terminal His-tag), or 1.5 ug of recombinant Clp-P1 (N-terminal GST tag) (4). The resulting polyclonal T cell populations were purified with ficoll-hypaque (Histopaque 1083; Sigma) and restimulated under the same conditions using $5x10^6$ irradiated immune splenocytes as antigen presenting cells. The *PmpG-1* stimulated polyclonal population was cloned by limiting dilution using irradiated immune splenocytes pulsed with UV-inactivated *C. muridarum* as specific antigen. Of the five clones from that limiting dilution, one was specific for $PmpG_{303-311,}$ designated PmpG1.1, and kept for further study.

For routine passage, $1x10^5$ CD4 clone cells were plated in 24 well tissue culture-treated wells containing 1.5 ml of RPMI CM/15% MLC supernatant supplemented with cytokines as previously described (1). PmpG1.1, uvmo-2, and uvmo-3 were passed using $5x10^6$ γ-irradiated naïve C57BL/6 splenocytes (1200 rads) that had been pre-pulsed at 37°C with 2.5 IFU equivalents of UV-inactivated *C. muridarum* per splenocyte for 20 minutes. Uvmo-4 and spl4-10 were passed under the same conditions using irradiated immune C57BL/6 splenocytes instead of naïve splenocytes. T cell clones were passed every 6-8 days under these conditions.

Epithelial cell Infections

C57epi.1 cells were plated in 48-well tissue culture plates and infected with 3 inclusion-forming-units (IFU) of *C. muridarum* per cell in 0.3 ml of culture medium. The 48-well plates were centrifuged at 1200 RPM (300 g) in a table-top centrifuge for 30 minutes then incubated at 37°C in a 5% CO_2 humidified incubator. Mock-infected wells received an equivalent volume of sucrose-phosphate-glutamic acid buffer (SPG buffer) lacking *C. muridarum*.

Flow cytometry

Cells were stained for 20 minutes on ice in RPMI CM 10% FBS with: PE-coupled GK1.5 (CD4) or PE-coupled 53-6.7 (CD8α). Cells were fixed with 1% paraformaldehyde after staining and analyzed using a FACS Calibur cytometer (BD Biosciences, San Diego, CA).

ELISA determination of IFN-γ

Relative interferon-gamma (IFN-γ) levels and IL-2 levels were determined by ELISA using monoclonal antibodies XMG1.2, 1A12 and 5H4 according to the manufacturer's protocol (Pierce-Endogen; Rockford, IL). Recombinant murine IFN-γ (R&D Systems; Minneapolis, MN) was used as the standard.

T cell Proliferation Assays

Epithelial cell targets were treated with 50 µg/ml of mitomycin C in PBS for 20 minutes at 37° C, washed twice with versene, then dislodged with enzyme free cell dissociation buffer, filtered through a 40 µM nylon filter, and counted. $5x10^4$ T cells with $5x10^4$ epithelial cells were co-cultured in 200 µl of RPMI CM. At 36 hours the experiments were pulsed with 0.5 µCi of ^3H thymidine per well for 12 hours. Assays were harvested on glass fiber filters and counted using a Packard Matrix 9600 direct beta counter.

Statistical Analysis

Summary figures for each experimental investigation are presented as 'pooled' means and with their associated standard error of the mean. Figure legends indicate the number of independent experiments pooled to generate each figure. Analysis of variance (ANOVA) models with one or two fixed effect factors, including the two-way interaction if significant, considered the time of the experiment or the run as a random block effect to account for the correlation of observations measured within the same experimental investigation. Each fixed factor or group effect was tested and the group means were compared. The Tukey-Kramer adjustment method for multiple comparisons was used to control the type I error. Statistical Analyses were performed using the statistical software packages SAS version 9.1 (SAS Institute, Cary, NC). Student's two-tailed t test was used for figures 2 and 3; presented as means and associated standard deviations.

Results

Derivation of a PmpG$_{303-311}$-specific CD4 T cell clone

We previously identified *C. muridarum* peptides bound to MHC class II molecules of infected dendritic cells, and tested whether vaccination with their epitope-source proteins could protect mice from *C. muridarum* genital tract infections. Our most protective epitope-source protein in that study was *PmpG-1,* and its epitope PmpG$_{303-311}$. Studying presentation of T cell epitopes by antigen presenting cells is experimentally difficult. One approach is to derive monoclonal antibodies specific for peptide epitopes complexed with MHC/HLA molecules that can be used to quantify epitope levels on the cell surfaces by flow cytometry (5-7). Alternatively, epitope-specific T cell clones can be used as a tool to study presentation of epitope-MHC complexes on the cell surface at or above the threshold level for T cell

activation. To study presentation of the $PmpG_{303-311}$ CD4 T cell epitope *in vitro* and *in vivo* we derived a $PmpG_{303-311}$ specific CD4 T cell clone.

Initially immune splenocytes from C57BL/6 mice that previously self-cleared primary *C. muridarum* genital tract infections were stimulated and restimulated with $PmpG_{303-311}$ peptide *in vitro*. Polyclonal T cell populations generated by this approach did not show specificity for the $PmpG_{303-311}$ peptide, even though freshly isolated immune splenocytes had strong and specific IFN-γ responses to $PmpG_{303-311}$ (*data not shown*). An alternate strategy was adopted. Immune splenocytes from a C57BL/6 mouse that self-cleared a primary C. *muridarum* genital tract infection were stimulated *ex vivo* with recombinant *PmpG-1* amino acids 25-500 (N-terminal his-tag) in primary culture, and restimulated 7 days later under the same culture conditions using irradiated immune splenocytes as the APC. Seven days after the secondary stimulation the polyclonal population was tested for $PmpG_{303-311}$ specificity (Figure 1a), and limiting diluted on irradiated immune splenocytes pulsed with UV-inactivated *C. muridarum* to generate T cell clones. Five clones resulting from the limiting dilution of the *PmpG-1* polyclonal population were screened for specificity; one clone designated PmpG1.1 was specific for recombinant *PmpG-1* aa25-500, and recognized the *$PmpG_{303-311}$* eptitope previously identified in proteomic studies (Figure 1b). PmpG1.1 is a $CD4^{+}CD8^{neg}$ T cell clone (Figure 1c).

We tested whether PmpG1.1 recognized infected epithelial cells, and whether recognition was replication dependent. C57epi.1 epithelial cells were pretreated with 10 ηg/ml IFN-γ for 12 h, then mock-infected and infected with *C. muridarum* in the absence or presence of 10 μg/ml tetracycline (stops bacterial protein synthesis & epitope generation). C57epi.1 cells were harvested 18 h post infection, then co-cultured with PmpG1.1 for 24 h in the presence of 10 μg/ml tetracycline (all wells). Culture supernatants were collected after 24 h, and IFN-γ levels determined by ELISA (Figure 2a).

PmpG1.1 recognized infected epithelial cells, and recognition of infected epithelial cells required bacterial replication.

A previous study showed that *in vivo* protection by CD4 T cell clones in adoptive transfer correlated with a clones ability to control *C. muridarum* replication in epithelial cells (8). We tested whether PmpG1.1 could control *C. muridarum* replication in C57epi.1 epithelial cells. C57epi.1 epithelial cell monolayers in 48 well plates were pretreated with 10 ηg/ml IFN-γ for 12 h; then infected with *C. muridarum* (moi 3). Four hours later the inocula were removed, cell monolayers washed then cultured in T cell medium with or without PmpG1.1 T cells (150K) at an effector to target ratio of ~0.75: 1. 28 h later (32 post infection) the cell monolayers were harvested by adding SPG buffer and scraping. Recovered IFU per well were enumerated on McCoy monolayers (Figure 2b). PmpG1.1 terminates *C. muridarum* replication in epithelial cells with an efficiency comparable to other potent previously described *Chlamydia*-specific CD4 T cell clones (9).

Cytokine profile of PmpG1.1

We have shown that multifunctional Th1 T cells secreting both IFN-γ and TNF-α correlate strongly with protective genital tract immunity in murine vaccine studies with either recombinant *Chlamydia* antigens or *Chlamydia*-pulsed dendritic cells (10, 11). Accordingly we analyzed the cytokine profile of PmpG1.1, and investigated whether that profile was influenced by culture conditions *ex vivo*. Over a 6 month period, PmpG1.1 T cells were passed in parallel using the standard protocol (multiple cytokines plus secondary MLC supernatant; see materials & methods), and the same conditions except limiting exogenous cytokines to the IL-2 and IL-7 components of the polykine growth media. PmpG1.1 T cells grown in parallel under these two different conditions for 6 months were activated by immobilized anti-

CD3 antibodies at the end of their usual culture cycle. 20 h post activation culture supernatants were collected and analyzed for IL-2, IFN-γ, TNF-α, IL-6, IL-10, IL-17 and IL-21 (Figure 3). Activated PmpG1.1 produced significant levels of IFN-γ and TNF-α. The low levels of IL-6 & IL-17 were close to and IL-10 levels overlapped 0 ρg/ml within 95% confidence intervals. IL-17 production was not induced by addition of combination of TGF-β, IL-23 and IL-6 to the culture medium (*data not shown*). No IL-2 or IL-21 was detectable in the culture supernatants. There was an approximately 2-fold difference in TNF-α production between the two culture conditions, but no meaningful difference in the overall cytokine profile. The lack of a significant difference between PmpG1.1's cytokine profile over 6 months of culture in dramatically different cytokine milieus suggests that the diverse intracellular cytokine patterns seen in activated immune T cells from vaccinated and infected animals reflect stable T cell cytokine differentiation patterns. PmpG1.1 has the multifunctional IFN-γ & TNF-α cytokine profile associated with protective immunity in *C. muridarum* vaccination studies. We attempted to test PmpG1.1's protective capability by adoptive transfer into *Rag1* knockout mice followed by *C. muridarum* infectious challenge. Those studies were inconclusive as PmpG1.1 T cells were not found (zero $CD4^+$ cells) in the spleen, lymph nodes or genital tracts of adoptively transferred *Rag1* ko mice, even with pre-transfer natural killer cell depletion using monoclonal antibody NK1.1 (*data not shown*).

Timing of the presentation of the PmpG303-311 epitope on infected epithelial cells

Derivation of a $PmpG_{303-311}$-specific CD4 T cell clone provided an opportunity to investigate the mechanism and timing of infected epithelial cell presentation of a protective *Chlamydia* epitope. C57epi.1 epithelial cell monolayers in 96 well plates, untreated or pretreated with IFN-γ, were infected on a staggered interval over 12 h to generate mock-infected (0 h), and infected monolayers 9, 12, 15, 18,

and 21 h post infection. At endpoint the inocula were removed, monolayers washed and then co-cultured with PmpG1.1 T cells in the presence of tetracycline, an antibiotic that halts bacterial protein synthesis and therefore epitope generation. Culture supernatants were harvested at 48 h, and levels of IFN-γ determined by ELISA (Figure 4). The $PmpG_{303-311}$ epitope reaches cell surface in levels sufficient to activate PmpG1.1 T cells between 15 and 18 h post infection. IFN-γ pretreatment of epithelial cells did not affect the timing of recognition or the magnitude of PmpG1.1 activation as measured by IFN-γ production.

$PmpG_{303-311}$ epitope persists in vivo after clearance of a primary genital tract infection

During characterization of PmpG1.1 a "high background" was noted when irradiated immune splenocytes were used as antigen presenting cells. Irradiated immune splenocyte APC were included in the PmpG1.1 derivation strategy because they are more efficient antigen presenting cells, both lowering the level of antigen required and magnitude of the T cell response (12-14). On reflection, the only likely difference between immune and naïve splenocytes is exposure of immune splenocytes to *Chlamydia* antigens during the primary infection. During the first 10 days of a primary genital tract infection in wild type C57BL/6 mice there is low level dissemination of *C. muridarum* as demonstrated by recoverable IFU from lung and spleen (15, 16). To formally investigate the irradiated immune APC phenomenon, C57BL/6 mice in two experimental groups were vaginally infected with *C. muridarum* ~ 6 months apart, and used as the source of immune splenocyte APC at days 21/22 and days 185/186 post infection. Unmanipulated immune splenocytes were compared to naïve splenocyte APC controls for their ability to activate PmpG1.1 and 4 other previously characterized *Chlamydia*-specific CD4 T cell clones, uvmo-2, -3, uvmo-1, & spl4-10 (1, 9). The antigen specificity of the non-PmpG1.1 *Chlamydia*-

specific CD4 T cell clones is unknown, but not $PmpG_{303-311}$ or any of the other 7 *C. muridarum* CD4 T cell epitopes we previously identified (4) (*data not shown*). The T cell clones were co-cultured with irradiated naïve & immune irradiated splenocytes for 48 h, then culture supernatants collected and IFN-γ quantified by ELISA (Figure 5a). APC presentation of $PmpG_{303-311}$ is uniquely preserved *in vivo* compared to epitopes recognized by the 4 other T cell clones and, remarkably, presentation of $PmpG_{303-311}$ was stronger 6 months post infection than it was 3 weeks post infection. These results suggest some type of consolidation of $PmpG_{303-311}$ epitope presentation by splenic APC. Three of the other four CD4 T cell clones' epitopes showed a similar, but much smaller in magnitude (~10-fold), consolidation of epitope presentation. Conversely, the *Chlamydia*-specific T cell clone spl4-10 showed no activation by either naïve or immune APC, largely ruling out a non-specific immune APC activation of T cell clones by immune APC due to unknown cytokine or accessory molecule interactions.

To get a sense of how robust persistent presentation of the $PmpG_{303-311}$ epitope was on immune splenocytes, we compared the ability of *Chlamydia*-pulsed naïve splenocytes to activate PmpG1.1 with that of unpulsed and antigen-pulsed immune irradiated splenocytes (Figure 5b). Unpulsed immune irradiated splenocytes activated PmpG1.1 to the same degree as antigen-pulsed naïve irradiated splenocytes. Presentation of $PmpG_{303-311}$ on immune irradiated splenocytes was not saturated as addition of UV-*C. muridarum* to immune irradiated splenocytes increased PmpG1.1 IFN-γ production by roughly 3-fold.

Discussion

Previous identification of $PmpG_{303-311}$ as protective *C. muridarum* CD4 T cell epitope provided an opportunity to investigate the immuno-mechanics of protective immunity. For this report we derived a

CD4 T cell clone specific for the $PmpG_{303\text{-}311}$ epitope in order to investigate *PmpG-1* antigen processing and presentation by infected epithelial cells. Understanding how protective *versus* non-protective T cell epitopes are processed and presented by infected epithelial cell targets may reveal antigen characteristics associated with protective immunity, and contribute to rational selection of *Chlamydia* proteins for inclusion in subunit vaccines.

A previous study showed that a CD4 T cell clones ability to terminate *C. muridarum* replication in epithelial cells *in vitro* correlated with its ability to protect against genital tract *C. muridarum* infections in adoptive transfer experiments (8). Consistent with those results the PmpG1.1 CD4 T cell clone, specific for the protective vaccine epitope $PmpG_{303\text{-}311}$, was able to recognize infected epithelial cells and terminate infection in them. We attempted to directly test whether PmpG1.1 could protect mice in adoptive transfer experiments. Those experiments were inconclusive because we could not find any PmpG1.1 cells in *Rag1* knockout mice after adoptive transfers. This may relate to the fact that activated PmpG1.1 produces IFN-γ & TNF-α, but not IL-2. In humans $IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}/IL\text{-}2^{neg}$ CD4 T cells are found in a $perforin^{+}/CD28^{neg}$ CD4 subset that has little or no proliferative capacity with T cell activation absent exogenous cytokines, and may undergo activation induced cell death (AICD) under those conditions (17).

The $PmpG_{303\text{-}311}$ epitope appears on the surface of infected epithelial cell in levels sufficient to activate PmpG1.1 between 15 and 18 h post infection. This is the same window of time post infection that epitopes for three other *C. muridarum*-specific CD4 T cell clones appear on the cell surface (1). Unlike the epitopes for the previously characterized CD4 T cell clones, $PmpG_{303\text{-}311}$ is completely unaffected by pretreatment of the epithelial cells with IFN-γ prior to infection; suggesting that neither alternations in levels of MHC class II expression nor IFN-γ triggered changes in antigen processing such as the transition to immunoproteosomes (18), had any significant effect on processing of *PmpG-1* in

infected epithelial cells. Of the four epitopes characterized to date, $PmpG_{303-311}$ is the only epitope whose time to presentation and level on infected epithelial cells, as measured by T cell activation, is not affected by IFN-γ pretreatment. Only one of the four *C. muridarum* CD4 T cell clones characterized to date could recognize an infected epithelial cell earlier than 15 h post infection, and that was very modest recognition 12 h post infection that required IFN-γ pretreatment of the epithelial monolayer. $PmpG_{303-311}$, a protective T cell epitope, is not visible to CD4 T cells until >15 h post infection. That time point occurs after the *C. muridarum* EB to RB transition is well underway, and supports our previous hypothesis that protective immunity is not likely based on non-specific disruption of epithelial cell physiology or viability during the "eclipse" phase, ~2 to 15 h post infection, when non-infectious RB predominant in the infected cell. Rather, protective CD4-mediated T cell immunity is more likely a direct attack on intracellular infectious EB (9).

An incidental finding during the course of this investigation was that the $PmpG_{303-311}$ epitope persists on splenic APC cell surfaces 6 months post infection. In fact, there is greater activation of PmpG1.1 with unmanipulated/unpulsed immune irradiated splenocytes taken from mice 6 months post infection than there is from immune irradiated splenocytes from mice 3 weeks post infection. Unmanipulated/unpulsed immune splenocytes from either time point, 3 weeks or 6 months, were superior to naïve irradiated splenocytes pulsed with UV-inactivated *C. muridarum* for activation of PmpG1.1. *C. muridarum* has a low grade dissemination from the genital tract during the first 10 days of infection, including to the spleen (<100 IFU) . One would have predicted that 3 week post infection splenocytes with recent exposure to live *C. muridarum* would have been superior to 6 month post infection splenocytes for activating PmpG1.1. Significantly great activation of PmpG1.1 by the 6 month post infection splenocytes argues that either the level of $PmpG_{303-311}$ epitope increased over 6 months or, more likely, the available $PmpG_{303-311}$ on splenic APC 6 months post infection resides on a more

effective type of antigen presenting cell. Determining which antigen presenting cell subset continues to present the $PmpG_{303\text{-}311}$ epitope 6 months post infection, and whether vaccination can reproduce that state, will likely provide important insights into protective immunity against *Chlamydia* genital tract infections that may be useful for rational development of a subunit vaccine.

Acknowledgements

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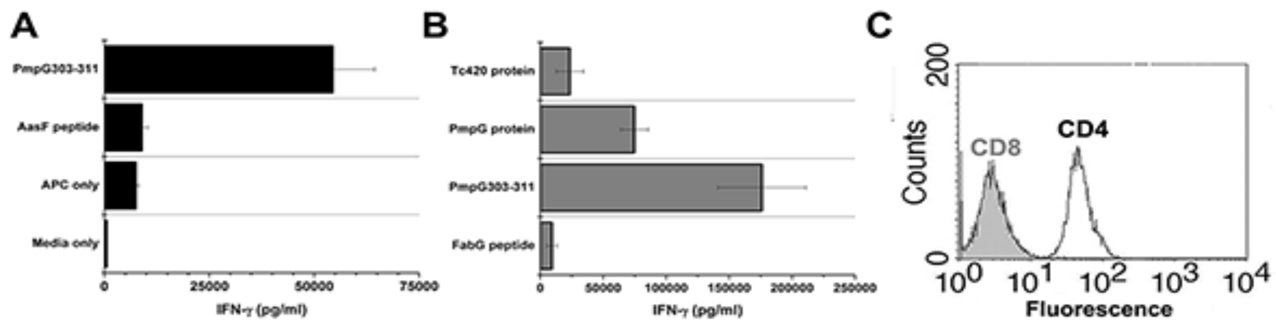

Figure 1. Derivation of $PmpG_{303-311}$ specific CD4 T cell clone PmpG1.1. A) Immune splenocytes stimulated and restimulated *in vitro* with *PmpG-1* 25-500, were tested for specificity for the $PmpG_{303-311}$ eptitope. The polyclonal population was activated with irradiated naïve splenocyte APC plus 2 μg/ml of indicated peptides or media control; IFN-γ in 48 h culture supernatants quantified by ELISA. B) Specificity of the PmpG1.1 T cell clone. The PmpG1.1 T cell clone was activated with immune irradiated splenocyte APC plus 1.5 μg/ml of indicated proteins or 2 μg/ml of indicated peptides; IFN-γ in 48 h culture supernatants quantified by ELISA. C) PmpG1.1 was stained for CD8a and CD4 and analyzed by flow cytometry.

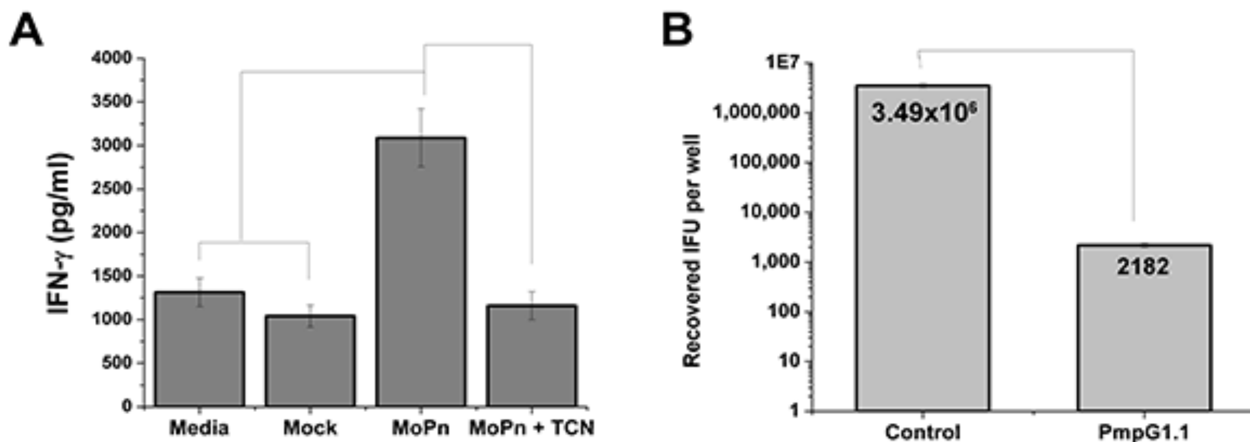

Figure 2. PmpG1.1 recognition of infected epithelial cells, and termination of replication in them. A) PmpG1.1 cells were co-cultured 1: 1 with C57epi.1 cells that were mock-infected or 18 h post infection (moi 5), without and with tetracycline co-treatment; IFN-γ in 24 h culture supernatants quantified by ELISA. B) C57epi.1 monolayers, pretreated 12 h with 10 ηg/ml IFN-γ, were infected with *C. muridarum* (moi 3). Four hours later monolayers were washed, and then co-cultured with PmpG1.1 T cells at an effector to target ratio of 0.75: 1. Wells were harvested 32 h post infection and recovered IFU enumerated on McCoy monolayers. Panels A & B represent single experiments done in quadruplicate.

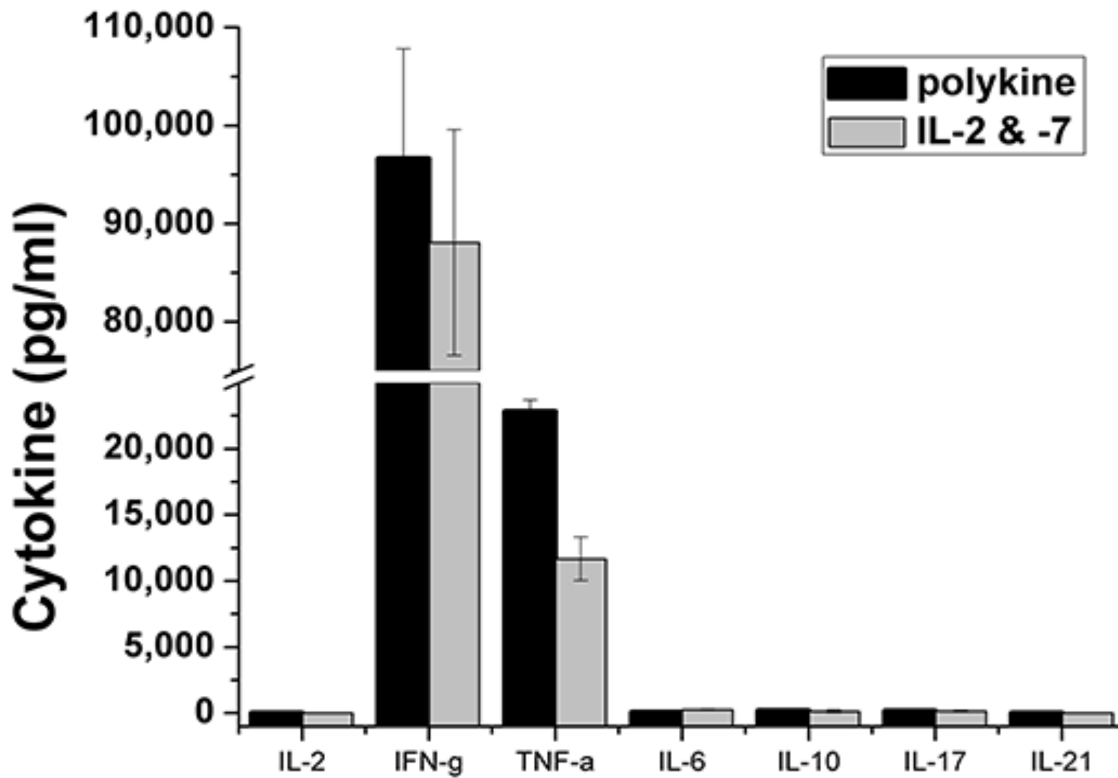

Figure 3. Cytokine profile of PmpG1.1. PmpG1.1 T cells were activated by immobilized anti-CD3 antibody 145-2c11 in media containing 1 ηg/ml IL-7. 20 h later culture supernatants were collected and analyzed for indicated cytokines by ELISA. Aggregate data from six independent experiments determining IFN-γ levels; with two independent experiments for each of the other cytokines.

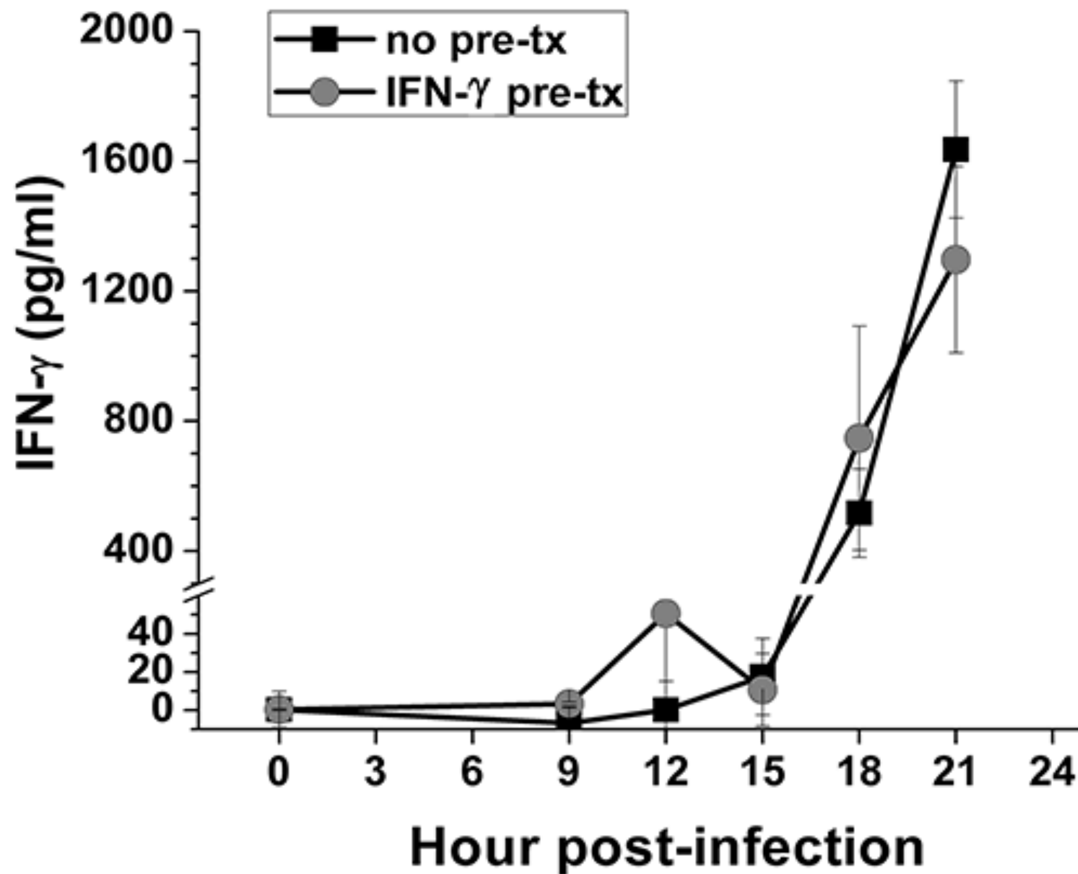

Figure 4. Processing and presentation of the $PmpG_{303-311}$ epitope by infected epithelial cells. C57epi.1 monolayers in 96 well plates were infected with *C. muridarum* (moi 3) over a staggered interval to generate mock (0 h), 9, 12, 15, 18, and 21 h infected monolayers at endpoint. Infected monolayers were then co-cultured with PmpG1.1 T cells in the presence of 10 µg/ml tetracycline; IFN-γ in 48 h culture supernatants was quantified by ELISA. Aggregate data from two independent experiments;

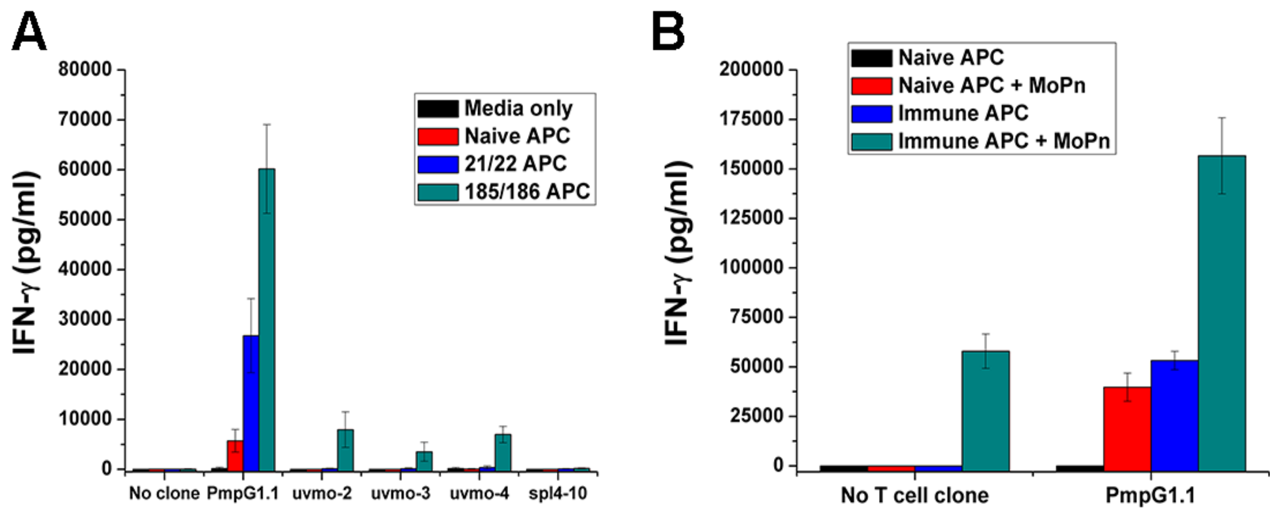

Figure 5. Presentation of the $PmpG_{303-311}$ epitope persists *in vivo* >6 months post infection. A) T cell clone cells were cultured in media (control), or co-cultured with naïve and immune irradiated splenocytes *without* exogenous antigen. Culture supernatants were collected at 48 h and IFN-γ quantified by ELISA. Aggregate data from two independent experiments. B) PmpG1.1 cells were co-cultured with naïve or immune irradiated splenocytes in the absence and presence of UV-inactivated *C. muridarum* (MoPn). Culture supernatants were collected at 48 h and IFN-γ quantified by ELISA. Data presented are from one independent experiment done as triplicates.