Title:

Evaluation of Intra and Extraepitheilal Secretory IgA (SIgA) in Chlamydial Infections

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Abstract:

IgA is an important mucosal antibody that can neutralize mucosal pathogens by either preventing attachment to epithelia (immune exclusion) or alternatively inhibit intraepithelial replication following transcytosis by the polymeric immunoglobulin receptor (pIgR). *Chlamydia trachomatis* is a major human pathogen that initially targets the endocervical or urethral epithelium in women and men, respectively. As both tissues contain abundant SIgA we assessed the protection afforded by IgA targeting different chlamydial antigens expressed during the extra and intraepithelial stages of infection. We developed an *in vitro* model utilizing polarizing cells expressing the murine pIgR together with antigen-specific mouse IgA, and an *in vivo* model utilizing pIgR-/- mice. SIgA targeting the extraepithelial chlamydial antigen, the major outer membrane protein (MOMP), significantly reduced infection *in vitro* by 24 % and *in vivo* by 44 %. Conversely, pIgR-mediated delivery of IgA targeting the intraepithelial inclusion membrane protein A (IncA) bound to the inclusion but did not reduce infection *in vitro* or *in vivo*. Similarly, intraepithelial IgA targeting the secreted protease *Chlamydia* protease-like activity factor (CPAF) also failed to reduce infection. Together, these data suggest the importance of pIgR-mediated delivery of IgA targeting extra but not intraepithelial chlamydial antigens for protection against a genital tract infection.

Introduction:

Urogenital chlamydial infections globally affect an estimated 106 million people annually [1]. Infection can cause tissue inflammation, scarring, decreased fertility and can lead to infertility. Infections are often asymptomatic (40-60% of males and 70-90% of females) facilitating continued spread throughout the community [2]. In addition to the high incidence of subclinical infections in males, risk of sexual transmission is also greatest from infected male to uninfected female, occurring in approximately 40% of encounters [3]. Whilst antibiotic intervention is widely accepted to eliminate infection, it can arrest the development of adaptive immunity limiting the appropriate responses to subsequent infections [4]. For these reasons, it is widely accepted that there is a requirement for a chlamydial vaccine [5-7].

Chlamydial vaccine research is focused primarily on protecting against the chlamydial burden and immunopathology associated with infections in females, and has identified a crucial role for CD4+ T cells secreting IFNγ and TNFα [7]. There is considerably less research devoted to developing a male vaccine [6, 8], despite males arguably being the reservoir of infection and susceptible to infertility[8]. Whilst a vaccine eliciting IFNγ and TNFα secretion in response to infection may prove efficacious in females, a similar response may be immunopathological in males [8]. The presence of *Chlamydia*-specific CD4+ T cells in male mice is associated with greater clearance of infection [9], yet CD4+ T cells secreting large amounts of IFNγ and TNFα are also associated with breakdown of immune privilege in the testes leading to infertility [10]. This suggests that a vaccine aimed at eliciting a cell-mediated response to defend against infection could facilitate the development of male infertility. Antibodies however play a non-essential but supportive role during a natural chlamydial infection [7] and considerably improve protection against infection following vaccination [11]. Thus, antibodies may be a safer alternative to potentially damaging CD4+ T cell responses in the context of a male vaccine.

The role for IgA in chlamydial infections is controversial. Naive IgA-/- female mice show no significant difference to wild type mice in their ability to resolve primary or secondary *C. muridarum* infections [12]. However, the concentration of IgA in the human endocervix inversely correlates with *C. trachomatis* burden [13], and males secrete significantly more SIgA in penile secretions during *C. trachomatis* infection indicating SIgA may play an important role in human infection and transmission

[14]. Passive immunization of mice with monoclonal anti-MOMP IgA can also significantly reduce the magnitude of an infection in female mice [15-16]. Similarly, protection against tissue burden conferred following immunization of male mice with MOMP was dependent on secretion of IgA [11]. Thus, the protective role of IgA depends on the titer, which can be greatly enhanced with immunization and the accessibility of the target antigen.

The pIgR is an integral membrane protein responsible for mucosal transport of dimeric IgA produced locally by plasma cells in the lamina propria. The pIgR is basolaterally expressed on epithelial cells where it binds dimeric IgA around the joining chain, internalizes and traffics it to the apical surface (i.e. the lumen) where pIgR is proteolytically cleaved releasing secretory component covalently bound to IgA, termed SIgA. SIgA is the dominant immunoglobulin at most mucosal surfaces and plays important roles in immune tolerance, mucosal homeostasis, commensal symbiosis, and immunity. In addition to epithelial trafficking of IgA to the mucosal lumen, pIgR transcytosis of IgA can also bind and neutralize already internalized viruses [17-19].

Chlamydia spp. are obligate intracellular bacteria with a biphasic lifecycle consisting of an infectious extracellular metabolically inert elementary body (EB), and an intracellular metabolically active and replicating reticulate body (RB) phase. The chlamydial EB is highly resistant to physical and environmental disruption, due primarily to highly cross-liked and disulfide-bonded membrane proteins, principally the major outer membrane protein (MOMP) [20]. Following attachment and endocytosis of the EB by the host cell, chlamydiae escape the normal endocytic pathway and differentiate within a parasitophorous vacuole, termed the inclusion. The inclusion allows the pathogen to replicate and absorb nutrients without being subjected to/attacked by innate intracellular defenses such as lysosomal fusion. Some chlamydial inclusion membrane proteins, including the inclusion membrane protein A (IncA), face the host cytoplasm and directly interact/interfere with host vesicle fusion [21]. Within the inclusion, replicating RBs also produce proteases, such as chlamydial protease activity factor (CPAF), some of which are secreted into the host cell cytoplasm and may interfere with host cell processes [22].

Chlamydia spp. express a variety of IgA-accessible epitopes. Therefore, we addressed the potential of SIgA to prevent attachment to and infection of host cells by targeting extra-epithelial chlamydial antigens presented on the EB and the ability of SIgA raised against intraepithelial chlamydial antigens

expressed during the RB phase to internalize and neutralize an already established infection. To address these questions we chose three widely studied antigens representing the EB (e.g. MOMP), inclusion membrane (e.g. IncA), and secreted chlamydial proteases (e.g. CPAF) groups. To determine the role of pIgR and antigen-specific IgA in against intra and extraepitheilal chlamydial antigens, we developed and utilized an *in vitro* Transwell® model, and confirmed the results *in vivo* using pIgR-deficient mice. We demonstrate that pIgR-mediated delivery of IgA targeting extraepithelial (MOMP), but not intracellular (IncA, CPAF), can significantly reduce chlamydial infection. These findings confirm the important role of pIgR and SIgA in chlamydial infections, and have implications for subunit chlamydial vaccines.

Methods:

Ethics:

All experiments were performed with approval from the university animal ethics committee (UAEC) of the Queensland University of Technology (QUT), (UAEC #0800000824).

Mice:

Adolescent (>6 weeks) male C57BL/6 mice were purchased from the Animal Resource Centre (Perth, Australia) and C57BL/6 pIgR -/- mice were provided by Odilia Wijburg (University of Melbourne, Melbourne, Australia). Mice were fed *ad libitum* with procedures performed under physical containment level 2 (PC2) conditions following NHMRC guidelines.

Cell Lines

C. muridarum (Weiss; ATCC VR-123) was propagated in McCoy-B fibroblasts (ATCC CRL-1696) and purified as previously described [23]. DMEM high glucose (Invitrogen; Melbourne, VIC, Aus) was supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 µg/mL gentamycin sulphate (Invitrogen), 100 µg/mL streptomycin sulphate (Sigma Aldrich; Castle Hill, NSW, Aus), and 2 mM L-glutamine (Invitrogen) was used to grow cells unless otherwise stated. The human endometrial epithelial cell line HEC-1A (ATCC: HTB-112) and human colonic epithelium C2Bbe1 (ATCC: CRL-2102) cells were purchased from the ATCC. C2Bbe1 cells had 10 µg/mL of human transferrin (Invitrogen) added to the growth medium. Human endometrial epithelial cells ECC-1 (ATCC: CRL-2923) were a gift from Charles Wira (Dartmouth Medical College, Lebanon, USA). Madin-Darby canine kindey epithelial cell subclone MDCK I (ATCC: CCL-34) were a gift from Russell Simmons (Queensland Health Scientific Services, Brisbane Australia), and subclone MDCK II cells (ATCC: CRL-2936) were a gift from Finn-Erik Johansen (University of Oslo, Oslo, Norway). Human bronchial epithelial cells BEAS-2B (ATCC: CRL-9609) were a gift from Phillip Hansboro (University of Newcastle, Newcastle, Australia) and were grown in RPMI 1640 (Invitrogen) supplemented as for DMEM. The GK1.5 (ATCC: TIB-207) hybridoma was a gift from Graham Le Gros (Malaghan Institute of Medical Research, Wellington, New Zealand) and was maintained in supplemented RPMI 1640. Vero E6 (ATCC: CRL-1586) green African monkey kidney epithelial cells were a gift from John Aaskov (Queensland University of Technology, Brisbane, Australia) and were grown in supplemented

RPMI 1640. All cells were grown in a humidified incubator at 37^{o}C and 5% CO_2. Cells were periodically determined as *Mycoplasma* spp. free by PCR.

Transwell Culture

Epithelial cells were seeded onto 0.4 µm pore Transwell® inserts (BD Falcon; North Ryde, NSW, Aus) at 10^5 cells/6.5 mm insert (24 well format). Media in the apical (200 µL) and basolateral (600 µL) chambers was changed every second day. Transepithelial electrical resistance (TEER) was monitored daily with an EVOM electrode (Millipore; Kilsyth, VIC, Aus). TEERs were determined with the formula; TEERs (ohms.cm^2) = (resistance of test (Ω) – resistance of blank insert (Ω)) x surface area of insert (cm^2). Expression of zona occludens 1 (ZO-1) was determined following 5 days of growth on transwells, followed by 24 h of *C. muridarum* infection. Cells were fixed with 100% MeOH, blocked with 5% FCS in PBST, and then stained with primary antibodies sheep-anti MOMP and rabbit-anti ZO-1 (N-terminal)(Invitrogen) for 1 h at room temperature. Inserts were then washed with PBS and then incubated with secondary antibodies donkey-anti sheep IgG-Alexa Fluor 488 (Invitrogen), and goat-anti rabbit IgG-Alexa Fluor 647 (Invitrogen) for 1 h at room temperature. Cells were then incubated with DAPI for 20 minutes and mounted with Prolong Gold (Invitrogen) overnight. Cells were then imaged using an SP5 confocal microscope (Leica)

Quantitative Real Time PCR

BEAS-2B, C2Bbe1, ECC-1 and HEC-1A cells were grown in culture, lysed and mRNA extracted with Trizol (Invitrogen), and cDNA synthesized with First Strand cDNA Synthesis Kit (Invitrogen) as per the manufacturer's instructions. Exon-spanning primers were designed from human mRNA sequences and Primer 3 software. Primers for the amplification of human pIgR (200 bp, forward: 5'-TGGCGGTCTTCCCAGCCATC -3'; reverse: 5'-GCTGGAGACGTAGCCCTCCGT-3') and GAPDH (69 bp, forward: 5'-CCACCCATG GCAAATTCC-3', reverse: 5'-TGGGATTTCCATTGATGACAA-3') were synthesized by Sigma Aldrich. Transcription of pIgR and GAPDH was quantified by PCR performed using a rotorgene thermocycler (Qiagen, Doncaster, VIC, Australia) with the conditions; 35 cycles of denaturing at 95^{o}C for 20 seconds, annealing at 60^{o}C for 20 seconds, and amplification at 72^{o}C for 20 seconds.

Recombinant Protein Production

Recombinant *C. muridarum* MOMP was a generous gift from Harlan Caldwell (Rocky Mountain Labs, Hamilton, MT, USA) and was expressed and purified as previously described [23]. Lyophilized control antigen OVA was purchased (Sigma Aldrich) and resuspended in PBS. Full-length recombinant *C. muridarum* IncA (NP_296774) and CPAF (NP_296627) were produced by amplifying full length coding sequences with primers; IncA (845bp: For with BamHI 5'-CGGGATCCATGACATCACCTACTCTAG -3', Rev with EcoRI 5'-CCGGAATTCTTAGGCGGAAGAATCAG -3'), and CPAF (1806bp: For with BamHI 5'-CGGGATCCATGAAAATGAATAGGATTTTGCTACTGC -3', Rev with KpnI 5'-CCGGTACCTTAAAAACTTCCATCCTCTGAGAGAATAATTACAC -3'). Hot start PCR was performed with conditions of 95^{o}C for 2 min, addition of *Pfu* polymerase (Promega), then 35 cycles of 95^{o}C for 1 min, 60^{o}C for 1 min, and 74^{o}C for 5 min. Amplicons were purified using Purelink PCR purification columns (Invitrogen) and restriction digested with BamHI/EcoRI (IncA) or BamHI/KpnI (CPAF) for 1 h at 37^{o}C. Digested amplicons were ligated using T4 DNA Ligase (Promega; Alexandria, NSW, Aus) into the N' terminal his-tag vector pRSET-A (Invitrogen) previously restriction digested with corresponding restriction enzymes. Vectors were transformed into BL21 (DE3) pLysS *E. coli* (Invitrogen), grown to $O.D._{600nm}$= 0.4 in LB broth, and then induced with 0.5 µM IPTG for 3 h at 30^{o}C. *E.coli* was lysed and His-tagged protein purified using Talon affinity resin (Clontech; Clayton, VIC, Aus) as per the manufacturers' instructions. Proteins were eluted with 150 mM imidazole (Sigma Aldrich), dialysed into PBS and stored at -80^{o}C.

Immunization Schedule of Mice to Obtain Antigen-Specific IgA

Mice were immunized intranasally with 20 µg of antigen and 0.5 µg of cholera toxin on days 0, 7, 14 and 25. Mice were euthanized by overdose of sodium pentobarbitone on day 35 and blood collected via cardiac puncture.

Purification of Murine Total IgA from Sera

Sera from immunized groups (n =10) were pooled and poorly solubilizing proteins precipitated by slow addition of half the serum volume of saturated ammonium sulfate, bringing the final volume of ammonium sulfate to approximately 30%. The serum was incubated at 4^{o}C on a rotating wheel for 6 h. Weakly soluble proteins were precipitated by centrifugation at 4,000 x *g* for 30 min at 4^{o}C. The supernatant was collected and half the initial volume of saturated ammonium sulfate was added to

bring the final concentration to 50%. The sera were incubated on a rotating wheel overnight at 4^{o}C. Ig was precipitated at 4,000 x *g* for 30 min at 4^{o}C. The supernatant was discarded, and the Ig resuspended in PBS to 10 times the initial volume of sera. Ig was pooled and depleted of IgG by passing over Protein G resin (Genscript; Piscataway, NJ, USA) and collecting the flow through. IgG-depleted ammonium sulfate fractioned antibody was further purified with Mouse IgA Purification Resin Kit (# MIKA-FF Kit; Affliland SA, Ans-Liege, Belgium) as per manufacturer's instructions. Briefly, Ig was diluted in15 mL PBS, and IgA precipitated with 35 mL of precipitation buffer (Affliand SA) for 15 min at room temperature. Protein was allowed to rest at 4^{o}C for 30 min, and then centrifuged at 4,000 x *g* for 15 min. The supernatant was collected (containing IgG, IgD IgE and other highly soluble proteins), and the precipitate (containing polymeric IgA and IgM) was resuspended in 10 mL of Binding buffer (Affiland SA). The soluble IgA/IgM in binding buffer was run over an equilibrated Mouse IgA Resin bed by gravity flow, washed with PBS, and eluted using Elution Buffer (Affiland SA). Protein containing fractions were pooled and concentrated using a 30 kDa molecular weight cut-off centrifuge filter (Millipore). To confirm purification of IgA, eluates were separated on non-reducing/non-denaturing SDS-PAGE, and western immunoblotting and was determined to be >90 % IgA with no detectable IgM or IgG by sandwich ELISA. A typical yield following purification was 3-4 mg of IgA per mL of pIgR-/- serum. Aliquoted IgA was stored at -80^{o}C until required.

Transfection and Evaluation of mpIgR Transfectants

C2Bbe1 cells were transfected with a vector encoding murine pIgR with Lipofectamine 2000 (Invitrogen) and Plus Reagent (Invitrogen) as per the manufacturer's instructions. Briefly, Lipofectamine complexed with pcDNA3.1 murine pIgR (pcDNA_mpIgR) generously donated by Finn-Erik Johansen (University of Oslo, Norway) was transfected into equilibrated C2Bbe1 cells, and positive cells selected for in DMEM supplemented with 550 µg/mL of G418 (Invitrogen). This plasmid has previously been shown to express functional mouse pIgR in MDCK cells [24]. Transfected clones were obtained by limiting dilution for monoclonal cell populations. Clones were evaluated on their ability to bind mouse IgA by incubating them with pIgR-/- sera (which pools IgA), fixing with 100% methanol, probed with goat anti-mouse IgA-HRP (Southern Biotechnology), and detecting with DAB precipitation (Thermo Fisher Scientific; Scoresby, VIC, Aus) and counter stained with Mayer's hematoxylin (Sigma Aldrich). Clone 1 of 8 was found to bind the most IgA and was used thereafter.

Male Immunization and Challenge

Mice were housed for 1 week prior to the initial immunization and immunized on days 0, 14 and 25 via the intranasal (IN) route. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), placed on their backs, and received IN immunizations, in a total volume of 5 µL, 2.5 µL per nare. On days 0, 7 and 14, 20 µg of antigen and 0.5 µg of cholera toxin (CT) was administered IN, and on day 25 mice received a boost of 50 µg of antigen, 1 µg of cholera toxin and 10 µg of CpG (5'-TCC ATG ACG TTC CTG ACG TT-3')(Sigma Aldrich). Mice were depleted of CD4+ cells by IP injection of 200 µg of GK1.5 two days prior to penile challenge, and received continuous depletion with IP injection of 100 µg of GK1.5 every week until euthanasia. Mice were infected with 10^6 IFUs of *C. muridarum* (Weiss) in 5 µL via the penile urethra to ensure 100% of mice were infected, as previously described [25]. At sacrifice, cardiac blood was taken for ELISA, which was performed as previously described [23]. Caudal and lumbar lymph nodes and spleens were taken for flow cytometry as previously described [26]. The testes, bladder and penis were collected and homogenized in sucrose phosphate glutamate (219 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamine) on the lowest speed (5000 x rpm) with a 220V generator probe (OMNI International, Kennesaw, USA) for 10 s, and stored at -80^{o}C until IFUs were determined by culture on McCoy cells for 24 h and quantified by fluorescence microscopy as described elsewhere [26].

Statistical Analysis

Statistical analysis of data was performed using Graphpad Prism version 5. Unpaired two-tailed Student's t tests and one-way ANOVA with Tukey's post hoc tests were performed where indicated. All mouse work was performed using 5 animals per group as it was determined to have >80% statistical power. Significance was determined as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Graphs with error bars represent the mean ± the standard error of the mean (S.E.M).

Results:

Purification of Antigen-Specific IgA

The ability of IgA targeting extra- and intra-cellular chlamydial antigens to prevent infection or to neutralize an established infection, respectively, was determined by targeting three chlamydial proteins potentially accessible to IgA binding (Figure 1A). To obtain antigens from the extra- and intracellular stages of the chlamydial life cycle, recombinant MOMP, IncA and CPAF were produced and purified (Figure 1B-C). Purified CPAF contained the full length protein, as well as the cleaved 6xHis tagged N' terminal of CPAF, consistent with recombinant CPAF's ability to be self-cleaved [27]. Both IncA and CPAF contained 6xHis tags confirmed by western blot; and MOMP, IncA and CPAF were all recognized by sera from *C. muridarum*-infected female mice (not shown). To obtain a high yield of purified IgA we immunized pIgR-/- mice as IgA pools in their plasma [28]. Because the proteins Jacalin or staphylococcal superantigen-like protein 7 (SSL7) bind human IgA but not mouse IgA,[29] we used Affiland® mouse IgA purification resin to purify polyclonal mouse IgA from the sera of immunized mice. To ensure minimal IgG was co-purified, serum was first depleted of IgG with Protein G resin. IgG-depleted sera was bound to Affiland® resin and eluted and samples from various stages of purification were evaluated by western blot for IgA (Figure 1D). IgA was found to be bound to washed resin and in eluted fractions, with no detectable IgM or IgG in the elutions as determined by sandwich ELISA (not shown). To confirm IgA was dimeric and not monomeric, non-reduced and non-denatured IgA and IgG were run on PAGE (Figure 1E). To confirm that purified IgA was able to bind its corresponding antigens, IgA was used to probe chlamydial antigen separated by western blot (Figure 1F). Together, these data demonstrate the Affiland® mouse IgA purification resin can be used to purify polyclonal antigen-specific IgA from immunized pIgR-/- mouse sera and that purified IgA retains antigen-binding specificity.

Establishment of an in vitro *Model to Access the Protection Afforded from Intra- and Extra cellular IgA in Chlamydial Infections*

To determine the neutralizing potential of IgA targeting intra- and extraepithelial chlamydial antigens, a Transwell model using polarized epithelial cells expressing murine pIgR (mpIgR) was utilized (Figure 2A). As we were unable to locate a mouse cell line that constitutively expresses mpIgR and forms tight cell-cell junctions (i.e. polarizes), we screened a variety of epithelial cell lines known to

polarize in a Transwell® model, determined their susceptibility to infection once polarized, and transfected them with a plasmid constitutively expressing mpIgR. The TEER of the cell lines in Transwells was determined over one week and human HEC1A, ECC-1 and C2Bbe1 cells, as well as canine MDCK I cells were found to strongly polarize (Figure 2B). However, upon polarization only C2Bbe1 cells remained susceptible to chlamydial infection (Figure 2C), consistent with other findings [30]. Expression of human pIgR mRNA was also determined by qRT-PCR in BEAS-2B, C2Bbe1, ECC-1 and HEC-1A cell lines with BEAS-2B and ECC-1 lacking expression, C2Bbe1 with a small amount (1.5×10^{-6} relative copies), and HEC-1A with the largest amount (9×10^{-4} relative copies) (Figure 2D). As C2Bbe1 cells strongly polarized, remained susceptible to infection, and had low expression of human pIgR, these were selected for transfection with mpIgR and used in subsequent experiments.

Following transfection with pcDNA-mpIgR, antibiotic selection, and cloning, the ability of transfected C2Bbe1 clones to bind murine IgA was determined by immunocytochemistry (Figure 2E). In the presence of 1% pIgR -/- sera (high in IgA), C2Bbe1 cells bound a small amount of mIgA consistent with the weak binding of mouse IgA by hpIgR, but when transfected with mpIgR bound considerably more IgA.

To determine if monolayer integrity was affected by chlamydial infection, electrical resistance, passive flux and tight junction protein expression was investigated. Following 24 hrs of infection TEERs were non-significantly reduced ($P = 0.15$) in infected cells when compared to uninfected cells (Figure 2F). Interestingly, passive transport of FITC-dextran (4 kDa) was slightly but significantly reduced ($P = 0.03$) in *C. muridarum* infected C2Bbe1 cells (0.3 ± 0.4 μM/hr) compared to mock treated C2Bbe1 cells (1.25 ± 0.3 μM/hr) (not shown). Additionally, tight junction protein zona occludens 1 (ZO-1) expression was observable following *C. muridarum* infection (Figure 2G). The ability of polarized C2Bbe1 cells to transcytose mouse IgA was also significantly greater following mpIgR transfection compared to untransfected WT C2Bbe1 cells ($P < 0.05$) (Figure 2H). Taken together, these data confirm that C2Bbe1 cells can be infected when polarized, remain polarized when infected, and following transfection with murine pIgR bind and traffic murine IgA from the basolateral to the luminal compartment.

***SIgA Specific for Extra but not Intraepithelial Chlamydial Antigens, Reduces Infection* in vitro**

Utilizing the purified antigen specific IgA and the *in vitro* Transwell cell model, we evaluated the ability of pIgR-transported SIgA to neutralize the extraepithelial chlamydial EB and intraepithelial replicating chlamydial RB. When anti-MOMP and control anti-OVA IgA was added to polarized cells basolaterally prior to addition of EBs to the apical chamber, only MOMP-IgA added to cells expressing mpIgR caused a significant reduction in apical infection (24%; $P < 0.05$) (Figure 3A). When IgA targeting the chlamydial inclusion membrane protein (IncA) or secreted protease (CPAF) was added to infected cells basolaterally, no significant protection was afforded relative to OVA-IgA controls, regardless of pIgR expression (Figure 3B). There was also no significant reduction in viability of replicating *C. muridarum* in subsequent infections (not shown). To confirm that IgA in the process of transcytosis could interact with the chlamydial inclusion, confocal microscopy was also performed (Figure 3C). IgA targeting IncA was found to co-localize with the inclusion but did not induce aberrant morphology, which has been observed from microinjection of anti-IncA IgG, or neonatal Fc receptor (FcRn) delivery of IgG [21, 31]. Addition of anti-CPAF IgA showed diffuse staining consistent with negative controls (not shown). Taken together, these data demonstrate that IgA targeting extraepithelial chlamydial antigen (MOMP) prevents infection in a pIgR-dependent manner but IgA targeting intraepithelial chlamydial antigens is unable to neutralize an established infection when targeting secreted protease CPAF or inclusion membrane protein IncA, despite colocalizing with the chlamydial inclusion.

***SIgA Specific for Extra but not Intraepithelial Chlamydial Antigens, Reduces Infection* in vivo**

Unlike *C. muridarum* infection of female C57BL/6 mice which is generally resolved within 3-5 weeks [32], infected male C57BL/6 mice continued to have a viable infection in the testes, bladder and penis for at least 7 weeks (Figure 4A). To access the ability of vaccination to reduce infection, 3 weeks post-infection was chosen as this was found experimentally to be the point where infection plateaued in mice. To determine if the *in vitro* results could be replicated *in vivo*, we initially attempted IP and systemic retro-orbital passive immunization of 0.2 mg of biotinylated purified IgA, and quantified the amount of IgA delivered to the bladder (in urine) and prostate (prostatic fluid). Passive immunization of mice supplied minimal concentrations of biotinylated IgA to the reproductive tract and this rapidly declined to the limit of detection by ELISA (<10 ng/mL) within 48 hours (Supplementary Figure 1A-B). To overcome the limitations observed from passive immunization, we followed the same immunization

schedule utilized to produce polyclonal IgA against MOMP and IncA for *in vitro* experiments, but depleted mice of CD4+ T cells prior to and throughout the infection to isolate the effects of antibodies (Figure 4B). Previous studies have also identified that live respiratory infection can provide some degree of protection against a genital challenge [33]; therefore, we included this group to determine if protection was mediated by SIgA/CD4+ T cells. There was no significant change in cachexia between WT and pIgR-/- mice following respiratory challenge suggesting a limited role for IgA in resolution of respiratory infection (Supplementary Figure 2A). Following 3 weeks of infection or immunization and 23 days of CD4+ depletion, immunized and intranasally infected mice developed a robust Ag-specific IgA (Figure 4C) and IgG responses (Supplementary Figure 2B) and responses were equivalent between both WT and pIgR-/- mice. All groups receiving αCD4 treatment showed >95% depletion of CD3+CD4+ T cells in the spleen and draining lymph nodes (Supplementary Figure 2C-D).

To determine the chlamydial burden across the urogenital tract, we measured infectious load in the testes (Figure 4D), bladder (Figure 4E), and penis (Figure 4F). MOMP-immunization of WT mice afforded a significant reduction in total chlamydial burden in the testes (73 %; $P < 0.01$), bladder (50 %; $P < 0.05$) and penis (73 %; $P < 0.01$), relative to OVA-immunized controls. Importantly, the protection provided by MOMP immunization was entirely abrogated in pIgR-/- (and hence SIgA-/-) mice. Interestingly, prior intranasal infection and CD4-depletion of WT or pIgR-/- produced no significant protection suggesting a limited role of SIgA in immunity acquired from natural infection. WT and pIgR-/- mice immunized with IncA had significant reductions of infectious burden in the testes (73 % in WT, 46% in pIgR-/-; $P < 0.01$) and bladder (50 % in WT, 41% in pIgR -/-; $P < 0.05$) when compared to control OVA-immunized mice suggesting protection observed was not dependent on SIgA. . Taken together, these findings suggest pIgR-mediated transport of IgA specific for MOMP provides significant protection in the male reproductive tract. Conversely, targeting intraepithelial antigen IncA with transcytosing IgA has little effect on reducing infectious burden.

Discussion

SIgA and the pIgR play pivotal roles in mucosal homeostasis and immunity [34]. During infectious challenge, the pIgR-mediated delivery of secretory component (non-specific innate defense) and more importantly SIgA to the mucosal lumen provides protection from tissue invasion. All *Chlamydia* spp. infect via the mucosa of either the ocular, respiratory, anorectal or urogenital tracts and thus come into direct contact with lumenal SIgA, but also potentially transcytosing dIgA during intraepithelial chlamydial replication. As pIgR is expressed in both the male and female lower reproductive tracts [35], it likely plays an important protective role in preventing initial infection, but also can prevent ascending infection to the gonads.

To address the potential for antigen-specific IgA to interact with intra- and extra-epithelial chlamydiae, we established a method to purify dIgA from immunized pIgR-/- mice, and apply it to polarized epithelia in the presence or absence of murine pIgR. Apically delivered MOMP-SIgA afforded 24 % neutralization of 10^5 IFUs, and protection was dependent of transcytosis by pIgR. The rate of transcytosis in this model was calculated in mpIgR transfectants to be approximately 1 μg/cm^2/day (equivalent to 0.35 μg/well or 1.75 μg/mL of polyclonal total IgA transcytosed into the apical compartment). Additionally, the concentration is lower than total polymeric IgA levels observed in rodent vaginal washes (5.29 ± 5.81 μg/mL) [36], and much lower than human vaginal fluid (21 - 118 μg/mL), uterine cervical fluid (3 - 330 μg/mL) and ejaculate (11 - 23 μg/mL) [37]. This suggests that improving anti-MOMP IgA production in the reproductive tract via mucosal immunizations is an attractive target for future vaccine development.

In MOMP-immunized mice the expression of pIgR, and thus transport of SIgA, significantly reduced chlamydial burden in the testes, bladder and penis. In the absence of pIgR (and CD4+ T cells) there was no protection, revealing a limited role for other antigen-specific effectors in the male genital tract (eg. IgG, CD8+). In fact, we have previously shown that the presence and transcytosis of anti-MOMP IgG provides no protection in the context of infectious burden or pathology [31, 38]. This inability of MOMP-IgG to neutralize EBs outside of defined *in vitro* conditions is due to Fc gamma receptor or FcRn-mediated uptake of IgG-opsonized EBs, and subsequent EB escape from lysosomal degradation [39]. Interestingly, despite minimal pIgR expression in the upper reproductive tract [35], we also observed a significant reduction in chlamydial infection in the testes of MOMP-immunized WT

388 mice, but not pIgR-/- mice suggesting SIgA is important in preventing ascending infection. Conversely
389 in mice previously infected, there was no significant pIgR/SIgA mediated protection on secondary
390 challenge, consistent with the knockout of IgA in previous studies [12]. Interestingly, in control groups
391 (OVA immunized ± CD4-depletion) there was no significant protection in any tissues screened
392 revealing the limited ability of CD4+ T cells to control infection in naïve males within the first three
393 weeks. A limitation of this *in vivo* model is that pIgR also transports pentameric IgM, however, the
394 concentration of IgM in mucosal secretions is 10-100 fold lower than of IgA or IgG [37]. Together, this
395 suggests that vaccines that induce SIgA targeting extraepithelial chlamydial antigens may significantly
396 reduce infection in males and may also reduce the transmission of infection to females.

397 Intra-epithelial IgA has been shown to neutralize internalized HIV and influenza viruses and the
398 intracellular niche *Chlamydia* establishes during infections may also be vulnerable to trafficking IgA.
399 We demonstrate that pIgR-dependent transcytosis of anti-IncA IgA is able to colocalize with the
400 chlamydial inclusion yet does not significantly reduce infection *in vitro*. We demonstrated that IncA-
401 immunization conferred protection *in vivo*; however, this was not dependent on SIgA as there were
402 negligible differences between infectious burden in WT and pIgR-/- mice. The *in vivo* reduction in
403 burden afforded from IncA-immunization was likely due to intraepithelial IgG transported by FcRn,
404 which we have previously demonstrated *in vitro* and *in vivo* [31]. Unlike intraepithelial IgA which can
405 neutralize pathogens through recycling endosomes [18], intraepithelial IgG bound to antigen can
406 mediate lysosomal degradation [40], as well as the recruitment of sequestomes providing a
407 neutralizing mechanism beyond steric blocking [31]. Vaccines targeting other cytoplasmic-facing Incs
408 (e.g. CT813 or CT229) may produce more promising results as they are expressed earlier during the
409 infectious cycle and may have the potential to arrest chlamydial escape from the endocytic pathway.
410 Intraepithelial IgA targeting other chlamydial antigens within the inclusion (proteins associated with
411 the replicating RBs) is unlikely to neutralize as SIgA is a large heterodimeric protein (405 kDa) and
412 the permeability of the inclusion membrane excludes molecules larger than 0.5 kDa [41]. IgA targeting
413 the secreted protease CPAF provided no protection, consistent with other findings [42]. Targeting
414 other inclusion-secreted chlamydial proteases; e.g. high temperature requirement A (HtrA) or tail-
415 specific protease (Tsp), with trafficking IgA may also provide little protection as these proteases are
416 also secreted into the host cytoplasm but are unlikely to interact with the microtubule network
417 unilaterally trafficking IgA. Recently, we have demonstrated that FcRn-mediated (bidirectional)

418 trafficking of IgG targeting CPAF also fails to neutralize infection and together with these data
419 demonstrate that neither intraepithelial CPAF-IgA or IgG is likely to play a significant role in reducing
420 infectious burden.

421 Taken together, we demonstrate the pIgR-mediated delivery of SIgA targeting extraepithelial
422 chlamydial antigens significantly reduces infectious burden *in vitro* and *in vivo* whereas IgA targeting
423 prominent intraepithelial chlamydial antigens provides no significant protection *in vitro* or *in vivo*. We
424 confirm that in addition to IgG, transcytosing IgA can also interact with the inclusion revealing the
425 potential to target chlamydial proteins necessary for growth, viability, nutrient acquisition, or escape
426 from host endosomal degradation or antigen processing/presenting pathways. In the context of a
427 male vaccine, SIgA targeting EB surface-exposed proteins are attractive vaccine candidates to
428 reduce infectious burden throughout the reproductive tract, and will likely also reduce the transmission
429 dose to sexual partners. The converse protection afforded from extraepithelial IgA but not IgG, and
430 intraepithelial IgG but not IgA may explain why antibodies have such contradictory roles in many
431 vaccine studies. These data reveal that SIgA targeting surface-exposed EB antigens is indeed
432 important in protective chlamydial immunity, but also that intraepithelial binding of prominent
433 chlamydial antigens IncA and CPAF by trafficking IgA provides no protection.

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Figure Legends:

Figure 1: Purification of Antigen-Specific Dimeric Mouse IgA

(A) Potential chlamydial antigen targets for intra and extra epithelial IgA. SDS-PAGE gels of purified recombinant *C. muridarum* antigens MOMP, IncA (B), and CPAF (C). (D) Serum from MOMP/IncA/CPAF or OVA-immunized mice was pooled (n =10), depleted of IgG and purified with Affiland® Mouse IgA Purification Resin. Samples were separated on SDS-PAGE, blocked and probed with anti-mouse IgA (alpha chain) HRP conjugated antibodies. (E) Non-reducing/non-denaturing SDS-PAGE of IgA and IgG elutions. (F) Protein antigens were separated by SDS-PAGE, and western blotted with corresponding purified IgA. Bound IgA was detected with anti-mouse IgA (alpha heavy chain)-HRP IgG.

Figure 2: A model to Evaluate Efficacy of Intra and Extraepithelial IgA against chlamydial infection

Schematic showing *in vitro* model used to access intra and extraepithelial neutralization. (B) MDCK I-II, HEC-1A, ECC-1, C2Bbe1, Vero E6 and BEAS-2b cells were grown on Transwell® inserts and the TEERs recorded. (C) Susceptibility of cell lines to apical infection following 5 days of polarization on Transwell® inserts. (D) Quantitative expression of human pIgR mRNA in BEAS2b, ECC-1, C2Bbe1, and HEC-1A cells was determined by qRT-PCR. (E) C2Bbe1 cells (+/- mpIgR) were fixed and incubated with pIgR-/- mouse sera, and bound IgA was detected with goat-anti mouse IgA-HRP antibody. (F-G) C2Bbe1 cells were grown on Transwell® inserts for 5 days then apically infected with *C. muridarum* for 24 h. (F) TEER of C2Bbe1 cells following 24h of infection. (G) Confocal microscopy demonstrating tight junction (ZO-1) expression in mock and *C. muridarum*-infected C2Bbe1 cells. (H) C2Bbe1 cells (+/- mpIgR) were grown on Transwell® inserts for 5 days and then purified mouse IgA was basolaterally loaded. Apical samples were taken and quantified by sandwich ELISA at 1, 3, 6 and 24 h post inoculation. Errors bars represent mean +/- S.E.M (n=3-4). Scale = 25 μm. ND = none detected.

Figure 3: The pIgR mediates delivery of neutralizing IgA to Extra but not Intraepithelial chlamydial antigens

C2Bbe1 cells (+/- mpIgR) were seeded on Transwell® inserts for 5 days. 100 µg of purified IgA was loaded basolaterally and allowed to transport for 24h. Cells were then apically infected with 10^5 IFUs of *C. muridarum* for 24h. Inclusion forming units were quantified by fluorescence microscopy. (A) Neutralization of chlamydial infection in polarized epithelia loaded basolaterally with polyclonal IgA from mice immunized with MOMP or OVA. (B) Neutralization of chlamydial infection in polarized epithelia loaded basolaterally with polyclonal IgA from mice immunized with IncA, CPAF or OVA. (C) Confocal microscopy of OVA and IncA-IgA treated cells staining for DNA (DAPI), *Chlamydia* (anti-MOMP), and mouse IgA (IgA). Results representative of 3 individual experiments (n = 4 inserts per group). Error bars showing mean +/- S.E.M. Scale = 10 µm.

Figure 4: SIgA targeting Extra but not Intraepithelial chlamydial antigen reduces burden in the MRT

(A) Chlamydial burden in the male mouse testes, bladder and penis over 7 weeks was quantified by cell culture (n = 5 per time point). (B) Schematic representing immunization schedule, CD4 depletion and urogenital chlamydial challenge. (C) Antigen-specific serum IgA titers in WT and pIgR-/- mice following immunization were determined by ELISA with corresponding immunized antigen (IN Cmu mouse sera was screened using UV-inactivated EBs). Following 3 weeks of infection, chlamydial burden in the testes (D), bladder (E), and penis (F) of immunized mice was quantified by cell culture. Statistics determined by one way ANOVA. Error bars represent mean +/- S.E.M.

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Conflict of Interest:

The authors have no conflict of interest to declare.