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Title:

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

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Abbreviations: Polymeric immunoglobulin receptor (pIgR), secretory IgA (SIgA), major outer membrane protein (MOMP), inclusion membrane protein A (IncA), chlamydial protease-like activity factor (CPAF), inclusion forming unit (IFU), elementary body (EB), reticulate body (RB).

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24 **Abstract:**

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

40

41 **Introduction:**

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

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

65 The role for IgA in chlamydial infections is controversial. Naive IgA-/- female mice show no significant
66 difference to wild type mice in their ability to resolve primary or secondary *C. muridarum* infections
67 [12]. However, the concentration of IgA in the human endocervix inversely correlates with *C.*
68 *trachomatis* burden [13], and males secrete significantly more SIgA in penile secretions during *C.*
69 *trachomatis* infection indicating SIgA may play an important role in human infection and transmission

70 [14]. Passive immunization of mice with monoclonal anti-MOMP IgA can also significantly reduce the
71 magnitude of an infection in female mice [15-16]. Similarly, protection against tissue burden conferred
72 following immunization of male mice with MOMP was dependent on secretion of IgA [11]. Thus, the
73 protective role of IgA depends on the titer, which can be greatly enhanced with immunization and the
74 accessibility of the target antigen.

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

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

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

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

108

109 **Methods:**

110 ***Ethics:***

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

113 ***Mice:***

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

118 ***Cell Lines***

119 *C. muridarum* (Weiss; ATCC VR-123) was propagated in McCoy-B fibroblasts (ATCC CRL-1696) and
120 purified as previously described [23]. DMEM high glucose (Invitrogen; Melbourne, VIC, Aus) was
121 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 µg/mL gentamycin sulphate
122 (Invitrogen), 100 µg/mL streptomycin sulphate (Sigma Aldrich; Castle Hill, NSW, Aus), and 2 mM L-
123 glutamine (Invitrogen) was used to grow cells unless otherwise stated. The human endometrial
124 epithelial cell line HEC-1A (ATCC: HTB-112) and human colonic epithelium C2Bbe1 (ATCC: CRL-
125 2102) cells were purchased from the ATCC. C2Bbe1 cells had 10 µg/mL of human transferrin
126 (Invitrogen) added to the growth medium. Human endometrial epithelial cells ECC-1 (ATCC: CRL-
127 2923) were a gift from Charles Wira (Dartmouth Medical College, Lebanon, USA). Madin-Darby
128 canine kindey epithelial cell subclone MDCK I (ATCC: CCL-34) were a gift from Russell Simmons
129 (Queensland Health Scientific Services, Brisbane Australia), and subclone MDCK II cells (ATCC:
130 CRL-2936) were a gift from Finn-Erik Johansen (University of Oslo, Oslo, Norway). Human bronchial
131 epithelial cells BEAS-2B (ATCC: CRL-9609) were a gift from Phillip Hansboro (University of
132 Newcastle, Newcastle, Australia) and were grown in RPMI 1640 (Invitrogen) supplemented as for
133 DMEM. The GK1.5 (ATCC: TIB-207) hybridoma was a gift from Graham Le Gros (Malaghan Institute
134 of Medical Research, Wellington, New Zealand) and was maintained in supplemented RPMI 1640.
135 Vero E6 (ATCC: CRL-1586) green African monkey kidney epithelial cells were a gift from John
136 Aaskov (Queensland University of Technology, Brisbane, Australia) and were grown in supplemented

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

139 ***Transwell Culture***

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

153 ***Quantitative Real Time PCR***

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

164 ***Recombinant Protein Production***

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

184 ***Immunization Schedule of Mice to Obtain Antigen-Specific IgA***

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

188 ***Purification of Murine Total IgA from Sera***

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

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

211 ***Transfection and Evaluation of mpIgR Transfectants***

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

223 ***Male Immunization and Challenge***

224 Mice were housed for 1 week prior to the initial immunization and immunized on days 0, 14 and 25 via
225 the intranasal (IN) route. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg),
226 placed on their backs, and received IN immunizations, in a total volume of 5 µL, 2.5 µL per nare. On
227 days 0, 7 and 14, 20 µg of antigen and 0.5 µg of cholera toxin (CT) was administered IN, and on day
228 25 mice received a boost of 50 µg of antigen, 1 µg of cholera toxin and 10 µg of CpG (5'-TCC ATG
229 ACG TTC CTG ACG TT-3')(Sigma Aldrich). Mice were depleted of CD4+ cells by IP injection of 200
230 µg of GK1.5 two days prior to penile challenge, and received continuous depletion with IP injection of
231 100 µg of GK1.5 every week until euthanasia. Mice were infected with 10^6 IFUs of *C. muridarum*
232 (Weiss) in 5 µL via the penile urethra to ensure 100% of mice were infected, as previously described
233 [25]. At sacrifice, cardiac blood was taken for ELISA, which was performed as previously described
234 [23]. Caudal and lumbar lymph nodes and spleens were taken for flow cytometry as previously
235 described [26]. The testes, bladder and penis were collected and homogenized in sucrose phosphate
236 glutamate (219 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamine) on the lowest speed
237 (5000 x rpm) with a 220V generator probe (OMNI International, Kennesaw, USA) for 10 s, and stored
238 at -80°C until IFUs were determined by culture on McCoy cells for 24 h and quantified by fluorescence
239 microscopy as described elsewhere [26].

240 ***Statistical Analysis***

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

246

247 **Results:**

248 ***Purification of Antigen-Specific IgA***

249 The ability of IgA targeting extra- and intra-cellular chlamydial antigens to prevent infection or to
250 neutralize an established infection, respectively, was determined by targeting three chlamydial
251 proteins potentially accessible to IgA binding (Figure 1A). To obtain antigens from the extra- and
252 intracellular stages of the chlamydial life cycle, recombinant MOMP, IncA and CPAF were produced
253 and purified (Figure 1B-C). Purified CPAF contained the full length protein, as well as the cleaved
254 6xHis tagged N' terminal of CPAF, consistent with recombinant CPAF's ability to be self-cleaved [27].
255 Both IncA and CPAF contained 6xHis tags confirmed by western blot; and MOMP, IncA and CPAF
256 were all recognized by sera from *C. muridarum*-infected female mice (not shown). To obtain a high
257 yield of purified IgA we immunized pIgR-/- mice as IgA pools in their plasma [28]. Because the
258 proteins Jacalin or staphylococcal superantigen-like protein 7 (SSL7) bind human IgA but not mouse
259 IgA,[29] we used Affiland® mouse IgA purification resin to purify polyclonal mouse IgA from the sera
260 of immunized mice. To ensure minimal IgG was co-purified, serum was first depleted of IgG with
261 Protein G resin. IgG-depleted sera was bound to Affiland® resin and eluted and samples from various
262 stages of purification were evaluated by western blot for IgA (Figure 1D). IgA was found to be bound
263 to washed resin and in eluted fractions, with no detectable IgM or IgG in the elutions as determined by
264 sandwich ELISA (not shown). To confirm IgA was dimeric and not monomeric, non-reduced and non-
265 denatured IgA and IgG were run on PAGE (Figure 1E). To confirm that purified IgA was able to bind
266 its corresponding antigens, IgA was used to probe chlamydial antigen separated by western blot
267 (Figure 1F). Together, these data demonstrate the Affiland® mouse IgA purification resin can be used
268 to purify polyclonal antigen-specific IgA from immunized pIgR-/- mouse sera and that purified IgA
269 retains antigen-binding specificity.

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

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

276 polarize in a Transwell® model, determined their susceptibility to infection once polarized, and
277 transfected them with a plasmid constitutively expressing mpIgR. The TEER of the cell lines in
278 Transwells was determined over one week and human HEC1A, ECC-1 and C2Bbe1 cells, as well as
279 canine MDCK I cells were found to strongly polarize (Figure 2B). However, upon polarization only
280 C2Bbe1 cells remained susceptible to chlamydial infection (Figure 2C), consistent with other findings
281 [30]. Expression of human pIgR mRNA was also determined by qRT-PCR in BEAS-2B, C2Bbe1,
282 ECC-1 and HEC-1A cell lines with BEAS-2B and ECC-1 lacking expression, C2Bbe1 with a small
283 amount (1.5×10^{-6} relative copies), and HEC-1A with the largest amount (9×10^{-4} relative copies)
284 (Figure 2D). As C2Bbe1 cells strongly polarized, remained susceptible to infection, and had low
285 expression of human pIgR, these were selected for transfection with mpIgR and used in subsequent
286 experiments.

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

292 To determine if monolayer integrity was affected by chlamydial infection, electrical resistance, passive
293 flux and tight junction protein expression was investigated. Following 24 hrs of infection TEERs were
294 non-significantly reduced (P = 0.15) in infected cells when compared to uninfected cells (Figure 2F).
295 Interestingly, passive transport of FITC-dextran (4 kDa) was slightly but significantly reduced (P =
296 0.03) in *C. muridarum* infected C2Bbe1 cells (0.3 ± 0.4 μM/hr) compared to mock treated C2Bbe1
297 cells (1.25 ± 0.3 μM/hr) (not shown). Additionally, tight junction protein zona occludens 1 (ZO-1)
298 expression was observable following *C. muridarum* infection (Figure 2G). The ability of polarized
299 C2Bbe1 cells to transcytose mouse IgA was also significantly greater following mpIgR transfection
300 compared to untransfected WT C2Bbe1 cells ($P < 0.05$) (Figure 2H). Taken together, these data
301 confirm that C2Bbe1 cells can be infected when polarized, remain polarized when infected, and
302 following transfection with murine pIgR bind and traffic murine IgA from the basolateral to the luminal
303 compartment.

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

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

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

324 Unlike *C. muridarum* infection of female C57BL/6 mice which is generally resolved within 3-5 weeks
325 [32], infected male C57BL/6 mice continued to have a viable infection in the testes, bladder and penis
326 for at least 7 weeks (Figure 4A). To access the ability of vaccination to reduce infection, 3 weeks post-
327 infection was chosen as this was found experimentally to be the point where infection plateaued in
328 mice. To determine if the *in vitro* results could be replicated *in vivo*, we initially attempted IP and
329 systemic retro-orbital passive immunization of 0.2 mg of biotinylated purified IgA, and quantified the
330 amount of IgA delivered to the bladder (in urine) and prostate (prostatic fluid). Passive immunization
331 of mice supplied minimal concentrations of biotinylated IgA to the reproductive tract and this rapidly
332 declined to the limit of detection by ELISA (<10 ng/mL) within 48 hours (Supplementary Figure 1A-B).
333 To overcome the limitations observed from passive immunization, we followed the same immunization

334 schedule utilized to produce polyclonal IgA against MOMP and IncA for *in vitro* experiments, but
335 depleted mice of CD4+ T cells prior to and throughout the infection to isolate the effects of antibodies
336 (Figure 4B). Previous studies have also identified that live respiratory infection can provide some
337 degree of protection against a genital challenge [33]; therefore, we included this group to determine if
338 protection was mediated by SIgA/CD4+ T cells. There was no significant change in cachexia between
339 WT and pIgR-/- mice following respiratory challenge suggesting a limited role for IgA in resolution of
340 respiratory infection (Supplementary Figure 2A). Following 3 weeks of infection or immunization and
341 23 days of CD4+ depletion, immunized and intranasally infected mice developed a robust Ag-specific
342 IgA (Figure 4C) and IgG responses (Supplementary Figure 2B) and responses were equivalent
343 between both WT and pIgR-/- mice. All groups receiving αCD4 treatment showed >95% depletion of
344 CD3+CD4+ T cells in the spleen and draining lymph nodes (Supplementary Figure 2C-D).

345 To determine the chlamydial burden across the urogenital tract, we measured infectious load in the
346 testes (Figure 4D), bladder (Figure 4E), and penis (Figure 4F). MOMP-immunization of WT mice
347 afforded a significant reduction in total chlamydial burden in the testes (73 %; $P < 0.01$), bladder (50
348 %; $P < 0.05$) and penis (73 %; $P < 0.01$), relative to OVA-immunized controls. Importantly, the
349 protection provided by MOMP immunization was entirely abrogated in pIgR-/- (and hence SIgA-/-)
350 mice. Interestingly, prior intranasal infection and CD4-depletion of WT or pIgR-/- produced no
351 significant protection suggesting a limited role of SIgA in immunity acquired from natural infection. WT
352 and pIgR-/- mice immunized with IncA had significant reductions of infectious burden in the testes (73
353 % in WT, 46% in pIgR-/-; $P < 0.01$) and bladder (50 % in WT, 41% in pIgR -/-; $P < 0.05$) when
354 compared to control OVA-immunized mice suggesting protection observed was not dependent on
355 SIgA. . Taken together, these findings suggest pIgR-mediated transport of IgA specific for MOMP
356 provides significant protection in the male reproductive tract. Conversely, targeting intraepithelial
357 antigen IncA with transcytosing IgA has little effect on reducing infectious burden.

358

359 **Discussion**

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

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

379 In MOMP-immunized mice the expression of pIgR, and thus transport of SIgA, significantly reduced
380 chlamydial burden in the testes, bladder and penis. In the absence of pIgR (and CD4+ T cells) there
381 was no protection, revealing a limited role for other antigen-specific effectors in the male genital tract
382 (eg. IgG, CD8+). In fact, we have previously shown that the presence and transcytosis of anti-MOMP
383 IgG provides no protection in the context of infectious burden or pathology [31, 38]. This inability of
384 MOMP-IgG to neutralize EBs outside of defined *in vitro* conditions is due to Fc gamma receptor or
385 FcRn-mediated uptake of IgG-opsonized EBs, and subsequent EB escape from lysosomal
386 degradation [39]. Interestingly, despite minimal pIgR expression in the upper reproductive tract [35],
387 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.

434

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558 **Figure Legends:**

559 **Figure 1: Purification of Antigen-Specific Dimeric Mouse IgA**

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

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

570 Schematic showing *in vitro* model used to access intra and extraepithelial neutralization. (B) MDCK I-
571 II, HEC-1A, ECC-1, C2Bbe1, Vero E6 and BEAS-2b cells were grown on Transwell® inserts and the
572 TEERs recorded. (C) Susceptibility of cell lines to apical infection following 5 days of polarization on
573 Transwell® inserts. (D) Quantitative expression of human pIgR mRNA in BEAS2b, ECC-1, C2Bbe1,
574 and HEC-1A cells was determined by qRT-PCR. (E) C2Bbe1 cells (+/- mpIgR) were fixed and
575 incubated with pIgR-/- mouse sera, and bound IgA was detected with goat-anti mouse IgA-HRP
576 antibody. (F-G) C2Bbe1 cells were grown on Transwell® inserts for 5 days then apically infected with
577 *C. muridarum* for 24 h. (F) TEER of C2Bbe1 cells following 24h of infection. (G) Confocal microscopy
578 demonstrating tight junction (ZO-1) expression in mock and *C. muridarum*-infected C2Bbe1 cells. (H)
579 C2Bbe1 cells (+/- mpIgR) were grown on Transwell® inserts for 5 days and then purified mouse IgA
580 was basolaterally loaded. Apical samples were taken and quantified by sandwich ELISA at 1, 3, 6 and
581 24 h post inoculation. Errors bars represent mean +/- S.E.M (n=3-4). Scale = 25 µm. ND = none
582 detected.

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

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

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

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

603

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609 **Conflict of Interest:**

610 The authors have no conflict of interest to declare.

611