Title:

Divergent outcomes following transcytosis of IgG targeting intracellular and extracellular chlamydial antigens

Short Title:

IgG neutralization/enhancement in chlamydial infection

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Abstract:

Antibodies can play a protective but non-essential role in natural chlamydial infections dependent on antigen specificity and antibody isotype. IgG is the dominant antibody in both male and female reproductive tract mucosal secretions, and is bi-directionally trafficked across epithelia by the neonatal Fc receptor (FcRn). Using physiologically relevant pH-polarized epididymal epithelia grown on Transwells®, IgG specifically targeting an extracellular chlamydial antigen; the Major Outer Membrane Protein (MOMP), enhanced uptake and translocation of infection at pH 6-6.5 but not at neutral pH. This was dependent on FcRn expression. Conversely, FcRn-mediated transport of IgG targeting the intracellular chlamydial inclusion membrane protein A (IncA), induced aberrant inclusion morphology, recruited autophagic proteins independent of lysosomes, and significantly reduced infection. Challenge of female mice with MOMP-specific IgG-opsonized *C. muridarum* delayed infection clearance but exacerbated oviduct occlusion. In male mice, MOMP-IgG elicited by immunization afforded no protection against testicular chlamydial infection, whereas; the transcytosis of IncA-IgG significantly reduced testicular chlamydial burden. Together these data show that the protective and pathological effects of IgG are dependent on FcRn-mediated transport as well as the specificity of IgG for intracellular or extracellular antigens.

Keywords

IgG, Chlamydia, FcRn, vaccine

Introduction:

Chlamydia trachomatis genital infections affect an estimated 90 million people annually [1]. Infections are often asymptomatic (30-50% males, 70-90% females [2]), leading to undiagnosed and untreated epidemics. In females, *C. trachomatis* commonly infects the endocervix, leading to an ascending infection that can cause pelvic inflammatory disease (PID) in an estimated 30% of patients, with 10-20% of PID patients progressing to tubal infertility [3]. Similarly in males, chlamydial infection of the penile urethra can ascend to colonize the prostate, epididymes and testes leading to inflammation, pathology and potentially infertility [4]. CD4+ T helper (Th) 1 cells secreting interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) are known to be crucial in clearance of chlamydial infection [5], yet this phenotype of T cells are known to breakdown immune privilege in the testes, leading to autoimmunity against sperm resulting in infertility [6]. Thus, the conventional approach to vaccine development in females (inducing potent Th1 responses) may promote infertility in males, making antibodies an attractive alternative. As a widely used model of human *C. trachomatis* infections, vaginal or penile infection of mice with *C. muridarum*, leads to an ascending infection and upper reproductive tract sequelae [7] that closely mimics the human immunopathology [8].

C. trachomatis is an obligate intracellular bacterium with a biphasic life cycle consisting of an extracellular elementary body (EB) phase, and an intracellular replicative reticulate body (RB) phase. The EB is resistant to environmental and physical disruptions despite having no detectable peptidoglycan, but is stabilized with highly cross-linked disulfide-bonded proteins in the outer membrane (primarily MOMP) [9]. Following attachment and infection of the host cell, the EB differentiates into a RB within a non-fusogenic parasitophorous vacuole termed an inclusion, which is made up of at least 22 inclusion membrane proteins (e.g. IncA) [10]. Within the inclusion, RBs acquire host nutrients and replicate whilst also secreting proteases (e.g. CPAF) into the host cell cytosol. After 72 hours of infection most RBs have differentiated back into the EB phase and are then released from the cell by extrusion or lysis allowing further infection. In the context of a vaccine, temporal expression of chlamydial antigens across the spectrum of the life cycle offer the potential the prevent host cell attachment, and arrest intracellular replication.

Antibodies are arguably the first line of defense against infection and are responsible for the sterilizing immunity elicited by the most successful vaccines. However, the role of antibodies in urogenital chlamydial infections remains controversial. Whilst IgG and Fc gamma receptors (FcγR) appear to play a pivotal role in acquired immunity against *Chlamydia* [11-13], EB opsonization by IgG has also been shown to enhance chlamydial uptake and infection of cells with mouse IgG_{2b} and mouse $Fc\gamma R_{II}$ [14], and also mouse IgG_3 and human $Fc\gamma R_{III}$ [15]. Thus there appears to be contrasting roles for IgG targeting extracellular EB antigens (specifically MOMP) *in vitro* and *in vivo*. More importantly, the trafficking of these potential enhancing/neutralizing antibodies has received minimal research interest.

The neonatal Fc receptor (FcRn) is ubiquitously expressed throughout the mucosal tissues of mammals on antigen presenting cells (APCs), syncytiotrophoblasts, endothelial and epithelial cells [16]. FcRn is a major histocompatibility complex class I (MHC-I)-like IgG transporter that requires

dimerization with beta-2 microglobulin (β2m) to function [17-18]. FcRn reversibly binds IgG between domains CH2-CH3 under acidic conditions (pH 5-6.5) and releases at neutral pH (pH 7.4) [16]. Therefore, FcRn facilitates bidirectional IgG delivery in and out of the lumen of the male and female reproductive tracts in a pH-dependent manner [19-20]. FcRn can also increase the half-life of bound ligands (IgG and albumin) *in vivo* [18], translocate IgG-bound antigen through epithelial cells for delivery to antigen-presenting cells [21], and intraepithelial IgG can also bind and eliminate internalized virus [22]. As FcRn binds IgG at acidic pH and is expressed by reproductive tract epithelia (vagina, uterus, epididymes, prostate) all of which have an acidic luminal pH, IgG specific for chlamydial extracellular antigens may increase infectivity by enhancing the uptake of IgG-opsonized *Chlamydia*. Conversely, FcRn bound IgG specific for chlamydial intracellular antigens could potentially target internalized *Chlamydia* for degradation. In this study we sought to determine the role of FcRn and IgG targeting intracellular and extracellular chlamydial antigens on infection outcomes at an acidic pH similar to that of both the male and female reproductive tracts.

Results:

Characterization and Silencing of FcRn in mECap18 Cells.

The mECap18 cells seeded on Transwell® inserts were found to have low TEERs, but were able to prevent passive flux of 4 kDa FITC dextran by 95% after 5 days and 98% by day 7 (Figure 1B). To confirm epithelial tight junction formation, cells grown on Transwells® for 5 days were probed for ZO-1 expression (Figure 1C). After 5 days, mECap18 cells had visible ZO-1 protein expression at cell-cell barriers. Untreated mECap18 cells were found to constitutively transcribe both FcRn (4.5×10^{-2} relative copies to β2m) and $Fc\gamma R_{II}$ (4.4×10^{-4} relative copies to β2m) with no detectable $Fc\gamma R_{I}$ or $Fc\gamma R_{III}$ mRNA by qRT-PCR (not shown). Protein expression of $Fc\gamma R_{II}$ and FcRn was confirmed by western blot (Figure 1D). Following transfection with shRNA targeting FcRn mRNA, mECap18 cells were found to down regulate FcRn mRNA transcription by PCR (Figure 1E), and FcRn protein expression by western blot (Figure 1D). Silencing of FcRn expression caused a significant 72% decrease in IgG transcytosis ($P < 0.05$) (Figure 1F).

FcRn-mediated Uptake and Translocation of IgG-Opsonized Chlamydia

To determine if polyclonal anti-MOMP IgG neutralized or enhanced infection under polarizing conditions, mECap18 cells (+/- shRNA FcRn) were grown on Transwell® inserts for 5 days, and then apically infected with EBs opsonized with increasing concentrations of MOMP-IgG. The pH of the apical medium was adjusted to 6.5 to replicate *in vivo* epididymal lumen conditions [23]. Non-silenced cells were also treated with wortmannin to chemically inhibit $Fc\gamma R_{II}$/FcRn-mediated enhancement of infection [14,24]. There was an IgG dose-dependent enhancement of infection of MOMP-IgG opsonized EBs (MOMP-IgG:EBs) ($P < 0.01$) (Figure 2). EBs treated with 1 μg/mL of polyclonal MOMP IgG demonstrated a 50% enhancement of infection ($P < 0.001$), and EBs opsonized with 100 μg/mL enhanced infection by 74% ($P < 0.01$). This enhancement of infection was abrogated in shRNA-transfected or wortmannin-treated mECap18 cells. The purified MOMP-IgG contained antigen-specific IgG of all subclasses with the majority of the EB-binding activity seen in IgG1 and IgG2b subclasses (Supplementary Figure 1).

Translocation of IgG-Opsonized Chlamydial EBs

Having shown that MOMP-IgG enhanced infection of pH-polarized epithelial cells, and transcytosis of IgG:OVA immune complexes (IC) across epithelia has previously been demonstrated [21], we sought to determine if epithelial cells could traffic IgG:EB IC from the apical to the basolateral chamber where they could infect other cells. As chlamydial EBs are approximately 0.31-0.44 μm in size [25], we seeded mECap18 cells onto 3.0 μm inserts. Infection of mECap18 cells grown on the insert membrane was enhanced by IgG-opsonization of EBs by 77% at pH 6, increasing to 167% ($P < 0.01$) when $Fc\gamma R_{II}$ was blocked with 2.4G2 (Figure 3A). When observing basolateral infection, a significant increase in infection (106%, $P < 0.01$) was observed only in basolateral wells with apical inserts infected at pH 6, but not blocked with 2.4G2. Similar results were observed when seeding murine macrophage cells (RAW 264.7) in the basolateral chamber (not shown). Taken together, these data suggest IgG (IgG_1

or IgG_{2b}) targeting extracellularly-exposed domains of the chlamydial EB can enhance binding and uptake of infectious particles by $Fc\gamma R_{II}$ and FcRn. When both receptors are chemically inhibited with wortmannin or FcRn expression alone was silenced the enhancement of infection was abrogated, yet when $Fc\gamma R_{II}$ was blocked the translocation of opsonized EBs was significantly reduced suggesting $Fc\gamma R_{II}$ may load FcRn with IgG:IC and direct it for basolateral trafficking..

FcRn Internalized IgG can bind and Neutralize Intracellular Chlamydia

Following infection of polarized mECap18 cells, polyclonal IgG from mice immunized with IncA, IncMem, CPAF or OVA was added to both apical and basolateral media and IFU determined after 24 h (Figure 4A). IncA-IgG provided significant partial protection (25 %; $P < 0.05$), in an FcRn-dependent manner ($P < 0.05$). IgG targeting inclusion membrane lysates (IncMem) or secreted protease CPAF failed to provide significant protection against infection compared to OVA-IgG controls. When observing chlamydial inclusions by microscopy, both IncA/IncMem IgG treated cells exhibited aberrant inclusion formation (Figure 4B), consistent with microinjection of polyclonal anti-IncA IgG into cells [26]. We also confirmed that *C. muridarum* begins expressing detectable IncA at 7-8h post infection using the mouse IncA IgG and anti mouse-IgG-AlexaFluor568 (not shown). Only internalized FcRn and inclusion-specific IgG was found to co-localize at the inclusion membrane (Figure 4C). Interestingly however, infection caused FcRn to be internalized and accumulate at the inclusion regardless of the addition of IgG. Further investigation found that accumulation occurred between 6-8 h post infection (Supplementary Figure 2A), was *Chlamydia*-mediated and required an intact microtubule network (Supplementary Figure 2B). This accumulation was also observed in human epithelial cells infected with *C. trachomatis* serovars D and L2 (Supplementary Figure 2C), or *C. pneumoniae* AR39 (not shown). FcRn-mediated IncA-IgG neutralization was found to be associated with accumulation of sequestosomal protein p62 (Figure 4D), and independent of lysosomal activation (Figure 4E).

Infection of Female mice with IgG-Opsonized EBs Delays Recovery from Infection and Enhances Pathology

To determine if enhancement or translocation of IgG-opsonized EBs occurred *in vivo*, female mice were infected with MOMP IgG:EBs, or OVA-IgG treated EBs. Normal infection of WT and β2m -/- mice saw a peak of infection at day 6, which had resolved by day 24 (Figure 5A). There were no significant differences in vaginal shedding in any groups, until late in the infection (day 18) when initial MOMP-IgG opsonization of EBs extended the duration of infection (83% vs WT OVA-IgG infected; $P < 0.05$) (Figure 5B). Interestingly, WT mice infected with MOMP-IgG:EBs had an 80% increase in hydrosalpinx incidence ($P < 0.05$) and severity ($P < 0.01$) (Figure 5C) despite not expressing FcRn [20], suggesting FcRn-translocation may exaserbate downstream immunopathological responses. WT mice infected with MOMP-IgG:EBs also and a two-fold increase in intra-stromal lymphocytes in the uterine horns ($P < 0.001$)(Figure 5D) compared to WT uninfected or OVA-IgG/EB infected mice. There were no significant differences in shedding or pathological outcomes in either group of β2m -/-

mice, suggesting β2m and its heterodimers (FcRn, MHC-I, CD1) play a minimal role in reducing infectious burden, but an important role in protection from pathology.

Transcytosis of IgG targeting intracellular, but not extracellular chlamydial antigens enhances protection in male mice

To determine the role of transcytosed IgG targeting extracellular and intracellular chlamydial antigens *in vivo*, WT and β2m -/- male C57BL/6 mice were utilized. Initally, intravenous and intraperitoneal passive immunization of purified IgG was trialled but only transient concentrations (peak 0.1 µg/mL 6h post immunization) reached the reproductive tract (not shown). To overcome this, we immunized male mice as done in the *in vitro* experiments as this schedule has previously been shown to produce reproductive tract IgG [27], but also depleted mice of CD4+ T cells to maximize the influence humoral responses on infection. CD4+ T cells were depleted both prior to challenge to eliminate immunization-acquired CD4+ T cells, and continuously throughout infection to prevent T cell-dependent activation of infection-specific B cells.

Mice were intrasnasally immunized with MOMP, IncA or OVA, depleted of CD4+ T cells and urogenitally challenged with *C. muridarum* (Figure 6A). The inability of β2m -/- mice to transport IgG into tissues was validated by western blot of testes lysates (Figure 6B). To remove the protective role of CD4+ T cells in immunity, mice were continuously depleted of CD4+ T cells following immunization, and throughout urogenital infection with all GK1.5-treated groups having >90% reductions in CD4+ T cells at sacrifice (Figure 6C). All mice had serum antigen-specific IgA (Supplementary Figure 3) and IgG (Figure 6D) responses as determined by ELISA, however all β2m -/- had a 1-log reduction in Ag-IgG ($P < 0.05$) likely due to decreased IgG half-life [16,28]. Following 3 weeks of infection, WT mice immunized with either MOMP or IncA had reductions of testicular chlamydial burden of 13% ($P < 0.01$) and 33% ($P < 0.01$) respectively when compared to CD4-depleted OVA controls (Figure 6E). Immunization of β2m -/- mice with MOMP also provided a significant reduction in burden (38%; $P < 0.05$) but this was not significantly different from MOMP-immunized WT mice ($P > 0.05$). Interestingly, protection afforded from IncA immunization of WT mice was completely absent in β2m -/- ($P < 0.001$). As naïve testicular infection of C57BL/6 male mice lasts at least 2 months (Supplementary Figure 4), and live respiratory infections are cleared within 2 weeks and have been shown to provide protection against genital challenge in females [29], some mice were also intranasally infected with *C. muridarum* prior to CD4-depletion and urogenital challenge. Recovery from respiratory infection as determined by cachexia was found to be supported by β2m expression (60% increase in recovery time of β2m -/- mice), but non-essential as all strains had recovered by day 15 (Supplementary Figure 5). Mice that had received a respiratory infection prior to challenge had no significant protection in the reproductive tract in the absence of CD4+ T cells; however there was a 30% reduction in burden when comparing IN-infected to OVA-immunized β2m -/- mice. OVA-immunized, mock CD4-depleted (naïve response) showed no significant differences to OVA-immunized CD4-depleted mice (CD4-defiencent naïve response) in either strain.

Discussion:

Antibody-mediated enhancement of chlamydial infections was first reported with HeLa 229 cells where a murine IgG3 monoclonal antibody enhanced uptake of EBs, facilitated by human $Fc\gamma R_{III}$ [14-15]. IgG2b-opsonization of EBs also enhanced infection of Chinese hamster ovary (CHO) cells transfected with mouse $Fc\gamma R_{II}$ [14]. However, neither HeLa nor CHO cells express human FcRn [20,30], and would have poor affinity for mouse IgG even if present [20,31-32]. Thus, we developed a model to investigate FcRn-mediated enhancement in mouse epithelial cells expressing $Fc\gamma R_{II}$ and FcRn. We report that in addition to $Fc\gamma R_{II}$-mediated enhancement, IgG-opsonized chlamydiae can utilize FcRn to both gain entry in apical epithelia negating the neutralizing effects of IgG, but also transcytose across apically acidified epithelial cells where they can go onto infect pH-neutral basolateral cells (epithelial, fibroblasts, APCs). These data also suggest that epithelial $Fc\gamma R_{II}$ binding of IgG:IC may be priming FcRn to translocate IgG:IC as opposed to internalizing and in an effort to increase IgG halflife. This cooperation of FcγRs and FcRn has been shown to be important in neutrophil phagocytosis and cross presentation in DCs [33-34]. Interestingly in female mice, enhancement of infection was not observed in terms of bacterial shedding in the vagina, but upper reproductive tract pathology was exacerbated in the uterine horns and oviducts simply by initially opsonizing EBs with MOMP-IgG prior to inoculation. There was a two-fold increase of lymphocyte infiltrate in WT mice inoculated with MOMP-IgG:EBs compared to all other groups suggesting functional FcRn may have translocated opsonized-EBs and exacerbated the immune response. Prior inoculation of WT mice with MOMP-IgG:EBs caused a significant increase in oviduct occlusion compared to OVA-IgG controls, but both β2m-/- groups had immunopathology demonstrating the vulnerability of β2m-/- mice to oviduct pathology, and makes enhancement of pathology due to MOMP-IgG:EBs difficult to interpret in this model. Regardless, the oviduct pathology was surprising as this tissue does not express FcRn [20], suggesting a downsteam response of FcRn-translocation may be responsible for enhanced immunopathology. We are currently investigating whether the enhanced pathology in these mice may have been due to CD8-CD11b+ dendritic cell uptake of IgG:IC and FcRn-mediated cross presentation to CD8+ T cells [34], which are implicated in aggravated pathological sequelae during chlamydial infection of female mice [35]. Due to volume and physiological limitations in the male mouse model, we immunized and depleted of CD4+ T cells to remove the protective effect previously published [36]. In male mice immunized with MOMP and depleted of CD4+ T cells, we saw a small 13% reduction in testicular burden, which was not dependent on the presence of IgG in the testes as there was no significant difference in the absence of functional FcRn and IgG. Together, this suggests a limited protective role for IgG targeting membrane proteins on the EB and that other mechanisms are providing protection in this environment, likely secretory IgA [27].

Our *in vitro* Transwell® data suggests translocation of IgG-opsonized EBs to the sub-epithelia may be driving deleterious immunopathology *in vivo.* We recently reported similar findings showing enhanced pathology following infection with EBs pre-incubated with vaginal lavages from MOMP-immunized mice [37]. MOMP is a multipass transmembrane protein that is a potent immunogenic antigen which induces robust cell-mediated and humoral responses during normal infections of both females and

246 males, and is the most widely studied vaccine candidate [38]. IgG specificity to specific domains of
247 MOMP is also clinically used in *C. trachomatis* infections to differentiate serotypes. Thus, the potential
248 for MOMP-IgG mediated enhancement of infection is not limited to vaccination, but may also enhance
249 infection/pathology following repeat infections, or in a primary infection contracted from a MOMP-IgG
250 seropositive sexual partner. In support of increased risk of transmission, human males have been
251 shown to have up to 24 fold more IgG in urethral secretions when infected with *C. trachomatis* [39]. To
252 expand on previous studies indentifying antibodies associated with upper reproductive tract pathology
253 [40], it would be interesting to determine if IgG coating of EBs is a predictor for the outcome of
254 pathological sequelae in females.

255 To determine the role IgG/FcRn *in vivo*, we used WT and β2m -/- mice which lack functional FcRn [17-
256 18]. Whilst the β2m-/- mice obviously have a deficit in all MHC class I-like proteins that may impact
257 infection outcomes, the use of appropriate controls (control antigen, with or without CD4-depletion)
258 should ameliorate concerns.

259 Whilst IgG and FcRn were found to enhance infection when targeting the extracellular antigen MOMP,
260 we report that IgG targeting intracellular chlamydial proteins such as IncA was able to target the
261 inclusion during FcRn-mediated transcytosis. We found that FcRn is recruited independent of IgG to
262 the inclusion membrane along microtubules, between 6-8 hrs post infection and remains there for the
263 rest of the infection. This is likely due to the recruitment of RabGTPases by *Chlamydia* spp. to the
264 inclusion membrane [41]. Whilst the chlamydial inclusion is traditionally considered non-fusogenic with
265 the endocytic pathway [42], it obtains nutrients including sphingomyelin via fusion with exocytic
266 trafficking vesicles, primarily using the host cell Rab4+ and Rab11+ GTPase pathways [41,43-44].
267 Interestingly, both Rab4+ and Rab11+ are crucial in cellular trafficking of FcRn which may suggest
268 why FcRn was detectable at the inclusion membrane [45].This *Chlamydia*-mediated accumulation of
269 FcRn at the inclusion membrane may reduce the ability of IgG to transcytose and bind intracellular
270 antigens later in the infection cycle.

271 Intracellular IgG was found to colocalize with FcRn, which together colocalized in the presence of
272 IncA and IncMem IgG. We also show that the resulting IgG-bound inclusion exhibited aberrant
273 morphology consistent with microinjection of anti-IncA IgG into infected cells identifying antigen-
274 specific IgG and FcRn dependent colocalization with the inclusion [26]. Thus we confirm that IncA is
275 cytoplasmic facing and can be bound by intracellular IgG, but also that normal cellular transport
276 mechanism (ie FcRn) can deliver IgG to the inclusion membrane where it can bind, block and
277 enhance sequestosomal activity. Scidmore *et al.* have also reported that inclusion membrane proteins
278 IncG, IncF and CT299 are cytoplasmic facing and can be bound by intracellular IgG, and that
279 microinjection of CT229 antibodies into infected cells inhibits chlamydial infection [26,44]. These three
280 inclusion membrane proteins are also all expressed within 2 hours of infection making them much
281 more potent targets for intracellular IgG targeting as FcRn is not internalized to the inclusion
282 membrane at this time [44,46]. If high titers of anti-CT229 IgG could be achieved by vaccination, this may
283 provide sterilizing immunity in reproductive tract tissues expressing FcRn.

Unlike FcRn-mediated clearance of internalized virus by IgG via lysosomal degradation [22], we did not observe LAMP1 aggregates, but rather observed colocalization of IgG, FcRn and the apoptosis/ubiquitin proteosome marker p62. This is unsurprising as chlamydiae actively inhibit lysosomal fusion with the inclusion membrane as part of its normal growth [42], but the addition of IncA-IgG led to an accumulation p62 at the inclusion membrane. Whether or not this was responsible for the reduction in growth requires further investigation.

Antibodies, and in particular IgG, are the principle reason many commercial vaccines are a success due to their high affinity for foreign antigens, long-lived production, and both systemic and mucosal circulation. This makes IgG an interesting target in the development of a chlamydial vaccine, particularly in a male vaccine. Here we demonstrate that FcRn plays a pivotal role on the outcome of IgG neutralization or enhancement of chlamydial infections, and more excitingly, that intracellular chlamydial antigens (inclusion membrane proteins and potentially other inclusion-secreted proteins) are viable candidates for a sub-unit chlamydial vaccine.

298 **Methods:**

299 ***Ethics Statement***

300 All experiments were performed with approval from the university animal ethics committee (UAEC) of
301 Queensland University of Technology (QUT), (UAEC #0800000824 and #0700000346).

302 ***Mice***

303 Adolescent (>6 weeks) male and female C57BL/6 WT, and β2m -/- mice were used in these studies.
304 Adolescent BALB/c male and female mice were also immunized and/or infected for production of
305 antigen-specific IgG. WT C57BL/6 and BALB/c mice were purchased from the Animal Resource
306 Centre (Perth, Australia), and breeding pairs of C57BL/6 β2m -/- mice were generously donated by
307 Prof. Mark Smyth (Peter MacCallum Cancer Centre, East Melbourne, Australia). Mice were fed *ad*
308 *libitum* with procedures performed under physical containment level 2 (PC2) conditions following
309 NHMRC guidelines.

310 ***Cell lines and culture***

311 *C. muridarum* (Weiss; ATCC VR-123) were propagated in McCoy B cells (ATCC CRL1696) and
312 purified on a discontinuous Renografin gradient as described elsewhere [9]. The hybridomas 2.4G2 and
313 GK1.5 were generous gifts from Prof. Graham Le Gros (Mallaghan Institute of Medical Research,
314 Wellington, New Zealand). The SV40-immortalized murine caput epididymal epithelial (mECap18)
315 cells were a generous gift from Dr. Petra Sipila (Turku University, Turku, Finland)[47], and were
316 maintained in DMEM, 10% heat-inactivated FCS, 100 µg/mL streptomycin sulfate, 50 µg/mL
317 gentamycin sulfate, 2 mM L-glutamine and 50 nM dihydrotestosterone as described elsewhere [47]. An
318 FcRn knockdown mECap18 clone was established by stable transfection with a short hairpin RNA
319 (shRNA) pGIPZ vector targeting FcRn mRNA (Open Biosystems # V2LMM-43474). Positively
320 transfected cells were selected for with 0.1 µg/mL puromycin. Clones were established by limiting
321 dilution. Silencing of FcRn was determined by qRT-PCR on cDNA isolated from mECap18 cells,
322 McCoy cells and splenocytes using PCR conditions of 95^{o}C for 10 min, followed by 35 cycles of 95^{o}C
323 for 20 s, 65^{o}C for 20 s, and 72^{o}C for 20 s on a Rotorgene thermocycler (Qiagen). Exon-spanning
324 primers for mouse FcRn (NM_010189) and mouse GAPDH (NM_008084.2) mRNA are listed (Table
325 1). Western blotting was performing following transfer of cell lysates onto nitrocellulose and blocking
326 with 5% skim milk PBS for 1 h. Blots were incubated with rabbit anti-mouse FcRn-cytoplasmic domain
327 (purified from the sera of rabbits immunized with the cytoplasmic domain of murine FcRn), rat anti-
328 mouse CD16/32 (2.4G2) or rabbit anti-β-actin (Abcam) for 1 h at room temp, then washed and probed
329 with corresponding secondary antibody (goat anti-rabbit IgG-HRP or goat anti-rat IgG heavy chain-
330 HRP (Southern Biotech)) for 1 h. Blots were washed with PBS, treated with ECL (Thermo Fischer),
331 and visualized with a Universal Hood II (Biorad).

332 ***Transwell® Assays***

mECap18 cells (± pGIPZ shRNA FcRn) were seeded at 10^5 cells/insert onto 6.5 mm, 0.4 µm polyester Transwell® inserts (BD Bioscience), and grown for 5 days, changing the media every second day (Figure 1A). The transepithelial electrical resistance (TEER) was measured with an EVOM electrode (Millipore). Electrical resistance was calculated from the formula: resistance of cells (ohms.cm^2) = (resistance of cells – resistance of insert) x surface area of insert (cm^2). Passive flux of FITC dextran (4 kDa) (FD4) was performed as described elsewhere [48]. Zona occludens 1 (ZO-1) was observed in mECap18 cells grown on Transwell® inserts for 5 days, fixed, blocked and probed with rabbit anti-ZO1 IgG (Invitrogen) for 1 h, and detected with goat anti-rabbit IgG-Alexa Fluor 488 (Invitrogen). DNA was stained with DAPI (Invitrogen) for 20 mins. Transcytosis of mouse IgG (polyclonal naïve purified IgG), or chicken IgY (Sigma Aldrich) was determined on day 5 post-seeding as previously described [49]. Briefly, 20 µg of polyclonal IgG or IgY was added to the apical chamber of pH-polarized mECap18 cells (apical pH 6.5, basolateral pH 7.4) and basolateral transport quantified after 2 h at 37°C by sandwich ELISA.

Production of Antigen-Specific Polyclonal IgG

Full length recombinant *C. muridarum* MOMP was a generous gift from Dr. Harlan Caldwell (Rocky Mountain Labs, Hamilton, MT, USA) and was expressed and purified as previously described [50]. 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 (Table 1) using *Pfu* polymerase (Promega) and hotstart PCR conditions of 95°C for 2 min, addition of *Pfu* polymerase, then 35 cycles of 95°C for 1 min, 60°C for 1 min, and 74°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°C. Digested amplicons were ligated using T4 DNA Ligase (Promega) 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 and induced with 0.5 µM IPTG for 3 h at 30°C. *E.coli* was lysed and His-tagged protein purified using Talon affinity resin (Clontech) as per the manufacturers' instructions. Proteins were eluted with 150 mM imidazole and dialysed into PBS and stored at -80°C. Inclusion membrane lysates (IncMem) from McCoy B cells infected for 24 h with *C. muridarum* were semi-purified by centrifugation as previously described [51]. Mice were intranasally immunized with 20 µg of antigen, 2.5 µg of cholera toxin (CT) (Sapphire Bioscience), and 10 µg of CpG-ODN (Sigma Aldrich) on day 0, 7, 14 and 25. Serum was collected on day 35 and polyclonal IgG purified using Protein G resin (Genscript) as per the manufacturer's instructions. Purified IgG was stored in PBS at -80°C until required.

In vitro *Enhancement or Neutralization of Infection*

In vitro neutralization was determined from the formula: neutralization (%) = 100 – ((IFUs per well/average IFUs of OVA controls) x 100). Enhancement of infection was determined as negative neutralization. mECap18 (+/- shRNA FcRn) were grown on Transwell® inserts for 5 days, serum starved in HBSS pH buffered to 7.4 with 30 mM HEPES (HBSS+7.4) for 1 h at 37°C. Cells were then

pH-polarized for 30 min at 37°C by incubating cells with HBSS+7.4 in the basolateral chamber, and HBSS buffered to pH 6.5 with 30 mM MES (HBSS+6.5) in the apical chamber. At this point, some non-transfected mECap18 cells were incubated for 1 h at 37°C with 150 nM wortmannin (Sigma Aldrich) to inhibit both FcRn and $Fc\gamma R_{II}$-mediated endocytosis [14,24,52]. Purified IgG at various concentrations were incubated with 5 x 10^4 IFUs of *C. muridarum* in HBSS+6.5 for 1 h at 4°C to allow opsonization. The pH-polarized cells were then apically infected for 4 hrs at 37°C. HBSS media was then removed, and replaced with growth media for 20 hrs (total 24 hrs infection) before fixation with methanol, and IFUs enumeration by fluorescence microscopy using sheep anti-Cmu MOMP sera as previously described [53] For intracellular IgG neutralizations, mECap18 cells (+/- shRNA) were grown for 5 days and then infected with 10^5 IFUs at 37°C for 1 h. Fresh media (apical pH 6.5, basolateral pH 7.4) containing 100 µg/mL purified mouse IgG (IncA, IncMem, CPAF or OVA-specific mouse IgG) was then added. After 24 hrs of infections cells were fixed with methanol and IFUs enumerated as above. For co-localization experiments, fixed cells were blocked with 5% FCS in PBS for 1h, and probed with rabbit anti-mouse IgG-AlexaFluor568 (Invitrogen), rabbit anti-mouse FcRn-CT, followed by goat anti-rabbit IgG-AlexaFluor488 (Invitrogen); or rabbit anti-LAMP1 (Abcam), or rabbit anti-SQSTM1(p62) (Abcam) and detected with goat anti-rabbit IgG-AlexaFluor568 (Invitrogen). Coverslips were then incubated in DAPI (Invitrogen) for 20 min, and mounted onto glass slides with Prolong Gold (Invitrogen) overnight. Coverslips were imaged with an SP5 confocal microscope (Leica).

Translocation of Opsonized EBs

As opsonization of EBs with MOMP-IgG enhanced apical infection, we sought to determine if IgG-opsonized EBs (0.3-0.45 µm in size) could also be translocated across the monolayer to the basolateral chamber. mECap18 cells were seeded onto 3.0 µm Transwell® inserts for 5 days. On day 3, 10^5 mECap18 cells were also seeded into each basolateral chamber of 24 well Transwell® plates. On day 5, cells were serum starved and pH-polarized as described above. EBs were incubated with 100 µg IgG/10^5 EBs in HBSS+6 or HBSS+7.4 for 1 h at 4°C to allow opsonization. Prior to infection, some inserts were apically treated with 2 µg/mL 2.4G2 in HBSS+7.4 for 30 min at 4°C to allow $Fc\gamma R_{II}$ blocking. Unbound antibody was then washed away with ice-cold HBSS+7.4, and cells re-incubated in HBSS+6 for 15 min at 37°C. Inserts were then apically infected for 4 h at 37°C with IgG-treated EBs, in HBSS+7.4, HBSS+6, or HBSS+6/2.4G2 block. HBSS was then discarded, and replaced with growth media for an additional 20 h at 37°C. Following incubation, cells on the inserts and in the basolateral chambers were washed, fixed and IFUs quantified by fluorescent microscopy.

Chlamydial Infection of Mice

Male C57BL/6 WT and β2m -/- mice were intranasally immunized with MOMP, IncA, or OVA as described above. To determine if protective antibodies were produced from a previous infection, some mice were intranasally infected with 10^3 IFUs on day 0. On day 33, mice were depleted of CD4+ T cells by intraperitoneal injection of 0.2 mg GK1.5 antibody. Mice were then continuously depleted every week with 0.1 mg of GK1.5 until sacrifice. On day 35, male mice were urethrally challenged with 10^6 IFUs of *C. muridarum* via the glans penis [27,54]. Mice were infected for a further 21 days, before

409 they were euthanized on day 56. Testes were collected and homogenized in sucrose phosphate
410 glutamate (SPG) with a 220V generator probe (OMNI International, Kennesaw, USA) for 10 s, and
411 stored at -80^{o}C until IFUs were determined by culture on McCoy cells. Testes of uninfected mice were
412 also collected, homogenized, and solubilized in RIPA buffer, and Western blotted to determine
413 endogenous IgA and IgG within the tissue using goat anti-mouse IgA-HRP (alpha heavy chain)
414 (Southern Biotech) and goat anti-mouse IgG-HRP (gamma heavy chain) (Southern Biotech).

415 Seven days prior to vaginal inoculation, female WT and β2m -/- mice were subcutaneously injected
416 with 2.5 mg of depot medroxyprogesterone acetate (Pfizer) to synchronize animals in diestrus and
417 facilitate chlamydial infection [7]. Mice were then infected with MOMP-IgG opsonized, or OVA-IgG
418 treated EBs (40 µg IgG/5 x 10^3 IFUs in 10 µL SPG). Infection was monitored every 3 days by vaginal
419 swabbing until euthanasia. On day 35, mice were euthanized, hydrosalpinx formation in the oviducts
420 recorded, and tissues fixed in 100 % ethanol and prepared for histochemistry.

421 ***Statistical Analysis***

422 Statistical analysis of experiments was performed using Graphpad Prism version 5. Unpaired two-
423 tailed Student's t tests and one way ANOVA with Tukey's post hoc tests were performed where
424 indicated. All mouse work was performed using 5 animals per group which was predicted to give
425 >80% statistical power for shedding data. Significance was determined as * = $P < 0.05$, ** = $P < 0.01$,
426 *** = $P < 0.001$. Graphs with error bars represent the mean ± the standard error of the mean (S.E.M).

427

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Disclosure

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598 **Figure Legends**

599

600 **Figure 1:** ***Characterization and Silencing of FcRn in Epididymal Epithelial Cells***

601 (A) Experimental system used throughout this study. (B) Passive apical to basolateral flux of FITC
602 dextran (FD4) over 2 hrs, and TEERs over time was determined for mECap18 cells grown on 0.4 μm
603 Transwell® inserts. (C) Tight junction protein ZO-1 expression by mECap18s grown for 5 days on a
604 Transwell® inserts was determined by anti-ZO-1 antibody and observed with an SP5 confocal
605 microscope. (D) mECap18 cells were transfected with a shRNA FcRn mRNA silencing vector and
606 clones screened by Western blot using anti-FcRn, anti-CD16/32, and β-actin antibodies. (E)
607 mECap18 cells transfected with non-silencing (Mock), or FcRn silencing shRNA1 (#58263) or
608 shRNA2 (#43474) were analyzed for transcripts of FcRn and GAPDH mRNA by qRTPCR. Murine
609 fibroblasts (McCoy B) and female BALB/c splenocytes were used as positive controls. Image shows
610 agarose electrophoresis following qRT-PCR quantification. (B) Apical to basolateral transcytosis of
611 murine IgG or chicken IgY was determined by growing mECap18 (+/- shRNA2) for 5 days and adding
612 antibody apically, and measuring the amount of antibody detected in the basolateral chamber after 2
613 hrs. Statistical significance determined with Student's t test (n = 3-5 wells per condition). All results
614 representative of at least 2 individual experiments. Scale bar = 10 μm. Error bars indicative of mean ±
615 S.E.M.

616 **Figure 2:** ***FcRn-mediates Enhancement of Infection via Uptake of IgG-Opsonized EBs***

617 mECap18 cells (mock or FcRn shRNA) were grown on 0.4μm Transwell® inserts for 5 days and then
618 apically infected with media at pH 6.5 containing EBs pre-incubated with varying concentrations of
619 polyclonal MOMP-IgG. Enhancement of infection was determined by comparison with wells infected
620 with EBs pre-incubated with 100 μg/mL polyclonal OVA-IgG. IFUs were enumerated by fluorescence
621 microscopy. Groups run in triplicate insert/wells per group. Results are representative of 5 individual
622 experiments. Statistics determined with Student's t test. Error bars indicate mean ± SEM.
623

624 **Figure 3:** ***FcRn Transcytosis of IgG Opsonized EBs Facilitates Translocation of Infection***

625 mECap18 cells were grown on 3 μm Transwell® inserts for 5 days and infected with EBs pre-
626 incubated with polyclonal MOMP or OVA IgG. Cells were apically infected under neutral pH (7.4), or
627 acidic pH (6) with or without CD32-blocking antibody (2.4G2). (A) IFUs quantified in cells in the apical
628 epithelia. (B) IFUs quantified in the basolateral epithelia following translocation. IFUs were determined
629 by fluorescence microscopy. Groups run in triplicate insert/wells per group. Results representative of
630 3 repeat experiments. Statistics determined with Student's t test. Error bars indicate mean ± SEM.

631 **Figure 4:** ***FcRn internalized IgG binds chlamydial antigens reducing growth and mediating***
632 ***sequestasomal activation***

(A) Epididymal cells were grown on Transwells® for 5 days, apically infected with *C. muridarum* for 3-4 hours, and replaced with fresh apical media (pH 6.5) and basolateral media (pH 7.4) containing polyclonal IgG (100 µg/mL) purified from IncA, IncMem, CPAF or OVA immunized mice for a further 20 hours. Neutralization was determined relative to the mean OVA-IgG controls. (B-E) mECap18 cells grown on coverslips overnight, and infected for 24 hours with media containing various antigen-specific IgG fixed and stained for fluorescence microscopy. (B) Aberrant inclusion formation, (C) colocalization of IgG and FcRn with chlamydia inclusion, (D) sequestosome (p62) and (E) lysosomal (LAMP1) localization. Results representative of 2-3 individual experiments. White arrows identify chlamydial inclusions. Statistics determined with Student's t test. Error bars indicate mean of triplicate wells ± SEM.

Figure 5: ***Infection with MOMP-IgG-Opsonized EBs Attenuates Infection Clearance, and Enhances pathology in the murine FRT***

Wild type and β2m -/- female mice were vaginally inoculated with 5 x 10^3 IFUs pre-incubated with MOMP IgG or OVA IgG. (A) Kinetics of vaginal shedding following challenge. (B) Cervicovaginal swab burden at late in infection (day 18). (C) Oviduct occlusion and dilation (hydrosalpinx) after 35 days of chlamydial challenge. Asterisks represent significant difference in total oviducts with hydrosalpinx (D). H&E staining of uterine horns following 35 days of infection. Images are representative of 5 separate mice. Arrows identify lymphocyte infiltration. Number of lymphocytes per 10^3 cells was determined by counting H&E sections. Scale bars = 50 µm. Statistics determined with one way ANOVA with Tukey's post hoc test.

Figure 6: ***Protection Provided from Antigen-Specific IgG Targeting Intracellular Chlamydial Antigen, but not Extracellular Chlamydial Antigen is Dependent on Functional FcRn***

(A) Immunization and challenge schedule. (B) Homogenates of testes from naïve WT and β2m -/- mice were Western blotted for IgA, IgG and β–actin. (C) Representative FACS blot of CD4+ T cells in the spleen at euthanasia. (D) Serum antigen-specific IgG endpoint titers of WT and β2m -/- mice immunized with corresponding antigen at euthanasia were determined by direct ELISA. (E) Viable chlamydial burden in the testes of immunized WT and β2m -/- mice after 3 weeks of infection was determined by cell culture and quantified by fluorescent microscopy. OVA-immunized, non-depleted control mice (OVA+). Statistics determined with Student's t test (D) and one-way ANOVA with Tukey's post hoc (E) (n = 5). Asterisks above graph indicate group is significantly different from OVA-immunized CD4-depleted group of the same strain. Dotted lines identify mean of OVA-immunized CD4-depleted WT and β2m -/- mice. Error bars indicate mean ± SEM.

Supplementary Figures

Supplementary information is available at Immunology and Cell Biology's website.