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Endosulfatases SULF1 and SULF2 limit *Chlamydia muridarum* infection

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Summary

The first step in attachment of *Chlamydia* to host cells is thought to involve reversible binding to host heparan sulfate proteoglycans (HSPGs), polymers of variably sulfated repeating disaccharide units coupled to diverse protein backbones. However, the key determinants of HSPG structure that are involved in *Chlamydia* binding are incompletely defined. A previous genome-wide Drosophila RNAi screen suggested that the level of HSPG 6-O sulfation rather than the identity of the proteoglycan backbone maybe a critical determinant for binding (Elwell *et al*., 2008). Here, we tested in mammalian cells whether SULF1 or SULF2, human endosulfatases which remove 6-O sulfates from HSPGs, modulate *Chlamydia* infection. Ectopic expression of SULF1 or SULF2 in HeLa cells, which decreases cell surface HSPG sulfation, diminished *C. muridarum* binding and decreased vacuole formation. ShRNA depletion of endogenous SULF2 in a cell line that primarily expresses SULF2 augmented binding and increased vacuole formation. *C. muridarum* infection of diverse cell lines resulted in downregulation of *SULF2* mRNA. In a murine model of acute pneumonia, mice genetically deficient in both endosulfatases or in SULF2 alone demonstrated increased susceptibility to *C. muridarum* lung infection. Collectively, these studies demonstrate that the level of HSPG 6-O sulfation is a critical determinant of *C. muridarum* infection *in vivo* and that 6-O endosulfatases are previously unappreciated modulators of microbial pathogenesis.

Introduction

Chlamydiae are obligate intracellular bacteria that are associated with a wide spectrum of diseases in humans. *C. trachomatis* is the most common bacterial cause of sexually transmitted diseases and non-congenital infertility in Western countries and the leading cause of acquired blindness in developing countries (Mandell *et al*., 2010). *C. pneumoniae* is an important cause of upper and lower respiratory tract infections and has been associated with a variety of chronic diseases including atherosclerosis (Campbell *et al*., 2003). The capacity of *Chlamydia* to cause sexually transmitted, ocular, and respiratory tract infections

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and the extraordinary prevalence and array of these infections make them public concerns of primary importance.

All *Chlamydia* species share a dimorphic life cycle in which they alternate between an extracellular, spore-like form, the elementary body (EB), and an intracellular, metabolically active but non-infectious form, the reticulate body (RB) (Moulder, 1991). EBs are rapidly internalized by most cultured cells, even nonprofessional phagocytes such as epithelial cells, suggesting that the receptor(s) is widespread and/or that there are multiple receptors (Dautry-Varsat *et al.*, 2005). The initial step in infection, binding to host cells, is a two-step process involving reversible binding to host heparan sulfate proteoglycans (HSPGs) followed by higher affinity irreversible binding (Carabeo *et al.*, 2001, Wuppermann *et al.*, 2001, Su *et al.*, 1996). Subsequent entry occurs through receptor-mediated endocytosis in clathrin-coated pits and/or pinocytosis in non-clathrin-coated pits (Hybiske *et al.*, 2007, Dautry-Varsat *et al.*, 2005).

The involvement of HSPGs in the initial interaction of *Chlamydia* species, including *C. trachomatis*, *C. muridarum*, and *C. pneumoniae* (Wuppermann *et al.*, 2001, Su *et al.*, 1996), with host cells is intriguing. HSPGs regulate a diverse array of biological processes, including the interaction of numerous growth factors and morphogens with their receptors (Esko *et al.*, 2001). HSPGs are extraordinarily heterogeneous structures that are composed of a linear array of repeating dissacharide units that are variably sulfated and that are covalently linked to various core proteins. HSPGs can be sulfated at four different sites (N-, 3-O, and 6-O of glucosamine and 2-O of uronic acid) during biosynthesis at the Golgi, giving rise to almost limitless structural diversity. 6-O-sulfation is subject to regulation, both through the action of 6-O-sulfotransferases during biosynthesis in the Golgi and by post-synthetic editing of 6-O-sulfation by dedicated HSPG-specific endosulfatases at the cell surface.

Two extracellular HSPG-specific 6-O-endosulfatases, SULF1 and SULF2, have been characterized in humans, mice, quail and other species (Morimoto-Tomita *et al.*, 2002, Dhoot *et al.*, 2001). These neutral pH endosulfatases are secreted and act on cell surface or on extracellular HSPGs to remove 6-O sulfates from internal glucosamine residues present in highly sulfated subdomains of HSPGs. Studies in cell lines and in transgenic mice reveal important functions of these endosulfatases in development and in neoplasia. Expression of the Sulfs in different tissues or cell lines is variable and suggests in part overlapping functions; some tissues, such as lungs, express both SULF1 and SULF2, while others express SULF1 or SULF2 predominantly (Lum *et al.*, 2007). Their functional redundancy in development has been revealed by detailed studies of transgenic mice. While individual SULF1- or SULF2- deficient mice exhibit a relatively mild phenotype affecting general growth and lung development, the double knockout mice exhibit severe developmental defects and early post-natal lethality (Holst *et al.*, 2007, Ai *et al.*, 2007, Lamanna *et al.*, 2006, Lum *et al.*, 2007, Lamanna *et al.*, 2007, Ratzka *et al.*, 2008). Many tumor or tumor-derived cells lines demonstrate enhanced expression of SULF2, and depletion of SULF2 in several cancer cell lines decreases cellular growth and cell migration (Nawroth *et al.*, 2007, Lai *et al.*, 2008b, Lemjabbar-Alaoui *et al.*, 2010, Phillips *et al.*, 2012) suggesting that SULF2 could be a novel therapeutic target. SULF1, on the other hand, has been suggested to be a tumor suppressor (Khurana *et al.*, 2011, Lai *et al.*, 2008a). The biological activity of the Sulfs relates to their ability to modulate growth factor/morphogen signaling pathways, including Wnts, Bone Morphogenic Proteins, glial cell-derived neurotrophic factor, transforming growth factor-β, FGF2, and PDGF-BB (Dhoot *et al.*, 2001, Li *et al.*, 2005, Lai *et al.*, 2003, Otsuki *et al.*, 2010, Yue *et al.*, 2008, Lai *et al.*, 2008b).

Despite their emerging important roles in growth, development, and neoplasia, the role of HSPG sulfation in microbial pathogenesis is largely underexplored. We have recently shown that *Chlamydia* co-opts the FGF2 signaling pathway in an HSPG-dependent manner to enhance infection (Kim *et al.*, 2011). FGF2 binds directly to EBs, where it may function as a bridging molecule to facilitate binding of EBs with HSPGs to activate FGFR. Since FGF2-FGFR interactions are stabilized by HSPGs and are affected by HSPG sulfation (Rusnati *et al.*, 1994, Lundin *et al.*, 2000), these results suggest that Sulfs may modulate *Chlamydia* binding. A study using chemically modified synthetic heparin molecules has shown that 6-O sulfation on the heparin is critical for *Chlamydia* attachment to mammalian cells (Yabushita *et al.*, 2002). In a genome-wide Drosophila RNAi screen designed to identify host genes required for *Chlamydia* infection, we identified 3 genes involved in heparan sulfate biosynthesis and postsynthetic editing (Elwell *et al.*, 2008): (i) Sulf1, whose depletion *increased* infection; (ii) 2-sulfotransferase, an enzyme required to add sulfate to the 2-O position of uronic acid in HS, which must occur before the addition of the sulfate at the 6-O position, and whose depletion *decreased* infection; and (iii) 6-sulfotransferase, an enzyme required to add sulfate to the 6-O position of glucosamine with HS after the 2-O sulfation step, and whose depletion *decreased* infection. These results suggested that sulfation is critical for *Chlamydia* infection. We further utilized the Drosophila S2 cell system to systematically deplete each of the 4 different core proteoglycans by RNAi (perlecan, syndecan, and 2 glypicans) and found that depletion of each of the known classes of proteoglycans partially decreases vacuole formation (unpublished data). These results suggested that 6-O-sulfation, rather than the identity of core proteoglycans, is an important determinant of *Chlamydia* binding. We were particularly interested in the Sulfs, as these enzymes have not previously been shown to modulation pathogenesis.

In this study, we tested the hypothesis that the Sulfs regulate *Chlamydia* infection in vitro and in vivo, using *C. muridarim* (also known as Mouse Pneumonitis), a murine strain that is closely related to *C. trachomatis* and that has served as a useful model for genital tract and pulmonary disease (Ramsey *et al.*, 2009). Overexpression of SULF1 or SULF2 decreased *Chlamydia* binding and vacuole formation, whereas depletion of SULF2 enhanced infection. In a mouse model of acute *pneumonia*, mice deficient in SULF2 or in both SULF1 and SULF2 exhibited enhanced susceptibility to *Chlamydia* infection. Collectively, these studies suggest that HSPG 6-O sulfation is a critical determinant of *Chlamydia* infection in *in vivo* and implicate the HSPG-specific endosulfatases as novel modulators of pathogenesis.

Results

Ectopic expression of SULF1 or SULF2 decreases *Chlamydia* binding and vacuole formation

We first examined endogenous expression of *SULF1* and *SULF2* mRNA and protein in HeLa cells. qRT-PCR analysis revealed that *SULF1* and *SULF2* mRNA expression was low (relative to *GAPDH* mRNA) but detectable in HeLa cells, with steady state levels of *SULF2* mRNA approximately ~3 fold greater than those of *SULF1* (Fig S1). Endogenous SULF2 protein was too low to detect by immunoblot analysis (data not shown), and SULF1 proteins levels were not evaluated due to lack of commercially available antibodies. The low expression of Sulfs in HeLa cells makes this cell line useful for testing the effect of overexpression of Sulfs on *Chlamydia* infection.

Transient transfection of Myc-tagged human SULF1 or SULF2 in HeLa cells resulted in similarly robust expression as assayed by immunoblot analysis with Myc antibodies (Fig 1A). Immunofluorescence (IF) microscopy of the Sulf-transfected cells with anti-Myc antibodies revealed intracellular expression as well as cell surface associated and extracellular protein, (Fig S2, white arrows) as SULF1 and SULF2 are known to be secreted

onto the cell surface and extracellularly (Morimoto-Tomita *et al.*, 2002, Tang *et al.*, 2009) (Fig S2, see bottom panels). We compared *C. muridarum* binding and vacuole formation in HeLa cells transiently transfected with Myc-SULF1, Myc-SULF2, or vector control. For binding studies, we quantified the number of cell-surface associated EBs per cell at 1 hr post infection. For vacuole formation, we quantified the average number of vacuoles per cell at 20–24 hpi. We observed a 60–70% decrease in *Chlamydia* binding (Fig 1B) and, similarly, a 40% decrease in vacuole formation (Fig 1C and Fig S2) compared to vector-transfected cells. Under conditions that reduced vacuole formation, there was no decrease in vacuole size and no alteration in vacuole morphology, although there were fewer vacuoles per cell (Fig S2).

To confirm that the catalytic activity of Sulfs was required for binding and vacuole formation, we ectopically expressed a catalytically inactive version of SULF1, in which the critical cysteines are changed to alanine (IHS1) (Tang *et al.*, 2009). When Myc-IHS1 was overexpressed at levels similar to Myc-SULF1 or Myc-SULF2, there was no decrease in *Chlamydia* binding or vacuole formation compared to vector-transfected cells (Figs 1B, 1C, and S2). Together, these results suggest that increased expression of either SULF1 or SULF2 can negatively regulate *Chlamydia* infection in a manner dependent on their enzymatic activity.

SULF2 limits *Chlamydia* infection

We next tested whether depletion of endogenous SULF1 or SULF2 would enhance *Chlamydia* infection, as the resultant increased cell surface HSPG 6-O sulfation would be predicted to increase binding and vacuole formation. To avoid issues caused by functional redundancy of the Sulfs, we utilized HaCaT cells, a human keratinocyte cell line which expresses high levels of *SULF2* mRNA but barely detectable levels of *SULF1* mRNA (Fig S3A). HaCaT cells were transduced with a lentivirus containing either one of two specific human *SULF2* shRNAs or a scrambled control shRNA. Puromycin-selected HaCaT cells were examined for depletion of *SULF2* mRNA by qRT-PCR (Fig. 2A). Transduction of two different *SULF2* shRNAs resulted in at least a 5-fold reduction in *SULF2* mRNA levels (Fig 2A). Consistent with previous reports (Lamanna *et al.*, 2006), *SULF1* expression was increased by 2~3 fold upon depletion of *SULF2* but the total *SULF1* mRNA levels were still negligible compared to *SULF2* (Fig S3B). To verify that transduction of *SULF2* shRNA decreased SULF2 protein levels, we immunoprecipitated secreted SULF2 using heparin sepharose beads followed by immunoblotting with an antibody to SULF2 (Fig 2B, upper panel). As quantified by densitometry, depletion of *SULF2* by either shRNA decreased secreted SULF2 levels by over 5-fold (Fig 2B, lower panel).

The puromycin selected shRNA-transduced cells were examined for *Chlamydia* binding and vacuole formation. Depletion of SULF2 by either of the two SULF2-directed shRNAs resulted in a 2 to 3 fold increase in *Chlamydia* binding (Fig 2C, $p<0.05$) and 30–40% increase in vacuole formation (Fig 2D, $p<0.05$). While the effect of SULF2 protein depletion on *Chlamydia* infection was modest compared to the efficiency of depletion, the fact that it was observed with two different shRNAs makes off-target effects unlikely. Together, our results indicate that endogenous endosulfatase activity limits *Chlamydia* infection

Chlamydia infection decreases SULF2 expression

In previous work, we have established that FGF2 enhances *Chlamydia* binding, while *Chlamydia* infection stimulates *FGF2* transcription and enhances production and release of FGF2, thus establishing a positive feedback loop that increases secondary infection (Kim *et al.*, 2011). We therefore tested the notion that *Chlamydia* infection might regulate *SULF* expression. We assayed three different cell lines that primarily express SULF2: HeLa cells,

HaCaT cells, and H292 cells, a human lung mucoepidermoid cell line (Lemjabbar-Alaoui *et al.*, 2010). Each cell line was infected with *Chlamydia,* and mRNA levels were examined by RT-PCR at 24 hrs post-infection (hpi). Although we could not detect significant changes in *SULF1* mRNA expression at this time point, *SULF2* mRNA expression was decreased by 70% in HeLa cells, by 40% HaCaT cells, and by 60% in H292 cells (Fig 3). Together, these results indicate that *Chlamydia* infection results in decreased *SULF2* expression in multiple cell lines. Decreased SULF2 protein, particularly in cells that express little SULF1, might cause local increases cell associated levels of 6-O sulfation and thus could lead to increased susceptibility to *Chlamydia* infection.

SULF1 and SULF2 limit *Chlamydia* infection *in vivo*

Our results thus far suggest that overexpression of SULF1 or SULF2 decreases *Chlamydia* binding while depletion of SULF2 (in the absence of significant SULF1 expression) enhances *Chlamydia* infection. We next tested whether Sulf activity would modulate *Chlamydia* infection in vivo. For these experiments, we assessed the susceptibility to infection of wild type compared to SULF knockout mice, utilizing a well-described murine model of acute pneumonia in which mice are intranasally infected with *C. muridarum*. This mouse model faithfully recapitulates important aspects of *C. trachomatis* and *C. pneumoniae* pneumonia (Rank, 2006, He *et al.*, 2011, Coalson *et al.*, 1987, Murthy *et al.*, 2004).

We confirmed that *Chlamydia* replicates in the lung upon intranasal inoculation and determined the optimal duration of infection for our experiments. Whole lungs were harvested at 5 and 48 hpi, frozen, thawed, and homogenized to release infectious progeny. HeLa cells were infected with these lysates, and progeny quantified by vacuole formation from this second round of infection. While no infectious progeny could be isolated at 5 hpi, EB production was robust at 48 hpi (Supplemental Fig 4A), indicating that replication had occurred in the lungs. Consistent with this finding, lung inflammation, as assessed by H&E staining, was clearly evident at 48 hpi (Supplemental Fig 4B).

Sulf2$^{-/-}$ single knockout mice and *Sulf1*$^{-/-}$*Sulf2*$^{-/-}$ double knockout C57BL/6 mice (Lum *et al.*, 2007) were derived by breeding. We were unable to generate sufficient numbers of *Sulf1*$^{-/-}$ single knockout mice and thus could not assess their susceptibility to infection. Because *Sulf1*$^{-/-}$*Sulf2*$^{-/-}$ mice demonstrated decreased body size and weight compared to wild-type mice (data not shown and (Lamanna *et al.*, 2006, Lum *et al.*, 2007, Ai *et al.*, 2007)), we utilized two different control groups: wild type littermates that were age-matched (10–12 week old mice that weighed more than the *Sulf1*$^{-/-}$*Sulf2*$^{-/-}$ mice) or that were weight-matched (younger mice that weighed the same as the *Sulf1*$^{-/-}$*Sulf2*$^{-/-}$ mice). Control littermates, *Sulf2*$^{-/-}$ mice, and *Sulf1*$^{-/-}$*Sulf2*$^{-/-}$ mice were infected intranasally with *C. muridarum* (5×10^6 IFU), lungs harvested at 48 hrs, and infectious progeny quantified. Consistent with our cell culture based results, we observed a statistically significant enhanced susceptibility to *Chlamydia* infection in the *Sulf1*$^{-/-}$*Sulf2*$^{-/-}$ mice, with a mean increase of 26-fold in IFU compared to the age-matched controls ($P < 0.001$) and a 5-fold increase in IFU compared to the weight-matched controls ($P < 0.05$). A trend towards increased susceptibility was observed in the *Sulf2*$^{-/-}$ knockout mice, with a 13-fold increase in mean production of infectious progeny compared to the age-matched controls. However, this result but did not reach statistical significance as determined by ANOVA, consistent with redundant roles of SULF1 and SULF2. We conclude that by controlling HSPG 6-O sulfation, Sulfs regulate susceptibility to *Chlamydia* infection both *in vitro* and *in vivo* and thus function as novel modulators of microbial pathogenesis.

Discussion

The variability in HPSG sulfation accounts in part for the enormous complexity and diversity in the glycan code, allowing subtle regulation of important cellular processes ranging from development to cell adhesion and growth factor signaling (Bernfield *et al.*, 1999, Lamanna *et al.*, 2007, Esko *et al.*, 2001). Pathogens can also subvert cell surface gycosaminoglycans for binding (Chen *et al.*, 2008), but the precise role of the sulfation of specific residues has only been elucidated in a few instances. For example, HSPG 3-O sulfation is critical for Herpes Simplex Virus binding (Shukla *et al.*, 1999) to host cells, while 6-O- and N-sulfation of GlcNAc of HS is crucial for Coxsackie B virus binding through secondary receptors (Zautner *et al.*, 2006). While 6-O-sulfated heparin mimics have been reported to inhibit *Chlamydia* entry (Yabushita *et al.*, 2002), we provide the first evidence that the endosulfatases SULF1 and SULF2, which control the level of 6-O-sulfation on cell surface HSPGs, are important modulators of *Chlamydia* infection. We demonstrate that overexpression of either SULF1 or SULF2, which has been shown to decrease surface HSPG 6-O sulfation (Hossain *et al.*, 2010), results in diminished *Chlamydia* binding and vacuole formation, while depletion of SULF2 in cells that predominantly express SULF2 results in increased *Chlamydia* binding and vacuole formation. Most importantly, we use genetically modified mice to show that mice deficient in SULF2 or in both SULF1 and SULF2 show increased susceptibility to *Chlamydia*-induced pneumonia after intranasal inoculation. Our study is amongst the few that extends findings from cell culture to an in vivo animal model of the pathogenesis of *Chlamydia* infections. Whether Sulfs play a role in modulating genital tract infection will be of interest to determine in the future.

There is a long history of the involvement of HSPGs in *Chlamydia* attachment. Despite some reports suggesting that *Chlamydia* might synthesize an HSPG-like molecule (Zhang *et al.*, 1992, Chen *et al.*, 1996, Chen *et al.*, 1997, Rasmussen-Lathrop *et al.*, 2000), the general consensus in the field is that *Chlamydia* co-opt host cell HSPGs for the initial low affinity reversible binding step (Dautry-Varsat *et al.*, 2005). Various glycosaminoglycans have been shown to competitively inhibit binding (Yabushita *et al.*, 2002). Studies utilizing chemically derived host cell mutants deficient in one or more biosynthetic steps have pointed to a role in host cell HSPGs (Zhang *et al.*, 1992, Rasmussen-Lathrop *et al.*, 2000). However, these studies have been limited by conflicting results (Stephens *et al.*, 2006), possibly reflecting the presence of multiple or compensatory mutations in these chemically mutagenized genomes. More recently, we have shown in a genome-wide RNAi screen performed in Drosophila S2 cells that host enzymes involved in HSPG biosynthesis were required for intracellular infection (Elwell *et al.*, 2008). While this paper was in preparation, Rosmarin et al performed a loss-of-function genetic screen in human haploid cells to identify host factors important in *Chlamydia* infection and found that inactivation of three host genes involved in HSPG biosynthesis decreased the susceptibility of these cells to *C. trachomatis* infection (Rosmarin *et al.*, 2012). Our work, in conjunction with our previous RNAi screen (Elwell *et al.*, 2008), provides strong evidence that the HSPG 6-O sulfation plays a critical role in the initial binding of *Chlamydia* to host cells. Our recent finding of a role for FGF2 in mediating HSPG-dependent binding (Kim *et al.*, 2011) suggests the intriguing possibility that 6-O sulfation may have dual roles in *Chlamydia* infection: it may contribute to initial interactions between *Chlamydia* and the host cell as well as stabilize a receptor complex, comprised of FGF2, HSPG, and FGFR (Rusnati *et al.*, 1994), to which *Chlamydia* binds and utilizes for entry.

Our studies do not rule out an additional role for HSPG 6-O-sulfation in post-binding steps during *Chlamydia* infection. For example, HSPGs have also been reported to play roles in viral entry, in addition to serving as a binding receptor or co-receptor for viruses (Chen *et*

al., 2008, Zautner *et al.*, 2006). Our finding that the modulation of 6-O sulfation affected vacuole formation in parallel with binding without affecting vacuole morphology suggests that if there is a separate role in entry, it is likely to be minimal.

The regulation of *SULF1* and *SULF2* transcription is incompletely understood. TGF-β upregulates *SULF1* expression while P53 increases *SULF2* expression (Chau *et al.*, 2009, Yue *et al.*, 2008). The mechanism and pathways by which *Chlamydia* modulate *SULF2* expression remain to be explored. In cell types that predominantly express SULF2, we found that *SULF2* expression was consistently reduced 40–70% upon *Chlamydia* infection. The work presented here suggests the intriguing possibility that *Chlamydia*-induced down regulation of Sulf transcription could function in a feedforward loop that enhances secondary infection and increases susceptibility to *Chlamydia* infection. Through the predicted subsequent decrease in SULF2 production and secretion, cells in the local environment could have higher levels of surface 6-O sulfation. They may thus be more susceptible to secondary infection with *Chlamydia* and/or with other pathogens, including including Human Immunodeficiency virus, Herpes Simplex Virus, Human Papilloma virus, Syphilis, and *Neisseria gonorrhea*, which are common co-infecting pathogens (Mandell *et al.*, 2010). Together with our recent findings that FGF2 expression and secretion is also enhanced upon *Chlamydia* infection (Kim *et al.*, 2011), it is clear that this organism may utilize multiple mechanisms to facilitate local spread of infection as well as to enhance susceptibility to other sexually transmitted diseases.

In summary, our studies reveal a novel role for 6-O endosulfatases in modulating *Chlamydia* infection. Furthermore, these studies underscore the potential for using therapies directed against HSPGs as multivalent antimicrobials (Tiwari *et al.*, 2012). It will be of interest to determine whether modulating HSPG sulfation is a common theme in microbial pathogenesis. If so, combination therapy involving antagonists of the Sulfs, in conjunction with HSPG mimetics, may be a potent and broad strategy to prevent infection with multiple STDs.

Materials and Methods

Reagents

8G1, a SULF2 monoclonal mAb, was developed by immunizing a SULF2 null mouse (*Sulf-1*$^{-/-}$*Sulf2*$^{+/+}$) with human SULF2 protein as previously described (Lemjabbar-Alaoui *et al.*, 2010). Other antibodies were obtained from the following sources: goat anti-*Chlamydia* MOMP was purchased from Fitzgerald; mouse anti-GAPDH from Chemicon; HRP-rabbit anti-goat IgG from Zymed; goat anti-mouse IgG HRP from Amersham Biosciences; all fluorescently labeled secondary antibodies and phalloidin from Molecular Probes; Effectene, QIAshredder, RNeasy kit, RNase-free DNase, and cDNA synthesis kit from Qiagen; SYBR GreenER qPCR SuperMix from Invitrogen; Heparin sepharose beads from GE health care; Puromycin from Millipore.

Cell culture and *Chlamydia* propagation

HeLa 229, L929, and H292 cells were obtained from ATCC and passaged as previously described (van Ooij *et al.*, 1997, Lemjabbar-Alaoui *et al.*, 2010). HaCaT cells were obtained from Rosen lab (UCSF) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. *C. muridarim* was propagated in L929 cells grown in suspension culture and purified by discontinuous density gradient centrifugation with Renografin (Mallinckrodt, Inc) as previously described (Caldwell *et al.*, 1981).

Immunofluorecent quantitation of *C. trachomatis* binding and vacuole formation

Cells were grown on glass coverslips in 24-well plates in DMEM containing 10% FBS overnight, and were infected with *C. trachomatis* for 1 hr at a multiplicity of infection (MOI) of 2–10 for binding studies and an MOI of 0.5–1 for vacuole formation studies. For quantitation of binding, non-adherent bacteria were removed by washing three times with PBS, and the infected cells were fixed with 4% PFA for 30 min. For quantitation of vacuole formation, PBS-washed cells were incubated in fresh DMEM containing 10% FBS for 20–24 hr. The cells were fixed and were permeabilized with 0.2% Triton X-100 for 15 min, followed by blocking in 2% FBS/1% Fish Skin Gelatin in PBS for 30 min. Bound EBs or vacuoles were visualized by staining with anti-*Chlamydia* MOMP antibody followed by staining with Alexa-488 conjugated secondary antibody. The host cell was visualized by staining the actin cytoskeleton with phalloidin-Alexa 594. Immunofluorescence images were acquired under identical exposure time for each set of experiments on a Nikon TE2000 perfect focus microscope using Nikon Elements. Metamorph (Molecular devices) or Adobe Photoshop CS4 was used to quantify nuclei, EBs, or vacuoles.

For binding studies, the number of bound EBs per cell was quantified and then normalized to the average number of bound EBs in untreated samples. For vacuole formation studies, the number of vacuoles and the number of nuclei per field was quantified to yield an average number of vacuoles per cell (vacuole formation). We chose this metric rather than measuring the fraction of cells infected because multiple vacuoles per cell are often observed at high multiplicities of *C. muridarum* infection. We then normalized to untreated samples (relative vacuole formation). Under the conditions of our experiments, increased binding efficiency (ie number of EBs bound per cell) is also reflected in increased vacuole formation (ie number of vacuoles/cell).

For all experiments utilizing image analysis, a minimum of 10 fields was analyzed per treatment. Data were compiled from at least 3 independent experiments unless otherwise specified. Data is presented as relative EB binding (normalized to untreated samples) or relative vacuoles formation (number of vacuoles/cell normalized to untreated samples).

Transfection studies

HeLa cells were seeded on 12 mm glass coverslips in 6 well plates and transfected with Effectene according to the manufacturer's instructions. 48 hrs after transfection, cells were infected with *Chlamydia* at 37 °C. Transfected protein levels were determined by immunoblotting as previously described (Kim *et al.*, 2011).

Immunoblot analysis

Cells were lysed for 15 min on ice in Lysis Buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50mM NaF, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 0.1 mM okadaic acid, and Complete protease inhibitors (Roche Diagnostics)). Cell lysates were collected, centrifuged at 20,800 g for 15 min to remove cell debris, and the supernatant was boiled in NuPage 4X LDS sample buffer (Invitrogen) with 100 mM DTT for 10 min. Proteins in the supernatant were separated on 10% NuPAGE Novex Bis-Tris gels (Invitrogen) and transferred to 0.45 mm Trans-blot nitrocellulose membranes using iBlot (Invitrogen). Membranes were rinsed in water, followed by blocking with 3% milk (Upstate) in Tris-buffered saline (TBS) for 1 hr. Each membrane was incubated with the indicated antibody in 3% milk in TBS with 0.02% Tween-20 (TBST) overnight at 4 °C, followed by an incubation with the appropriate HRP-conjugated antibodies for 1 hr. HRP-conjugated antibodies were detected by ECL (Amersham Biosciences) according to the manufacturer's protocol. For quantification, band intensity

was analyzed using ImageJ analysis software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,).

Isolation of RNA and DNA, cDNA synthesis, and Real Time PCR

RNA was isolated using the QIAshredder and the RNeasy kit according to the manufacturer's instructions (Quiagen). RNA was treated with RNase-free DNase according to the manufacturer's instruction. RNA concentrations were measured using Nanodrop Spectrophotometer (Thermo-scientific). One μg of RNA was reverse transcribed using the cDNA synthesis (Qiagen) kit in a 20 μL reaction. Quantitative PCR (qPCR) was performed with 2 μL of the cDNA preparation using SYBR GreenER qPCR SuperMix (Invitrogen) in a 25 μL reaction using DNA Engine Opticon-2 Real-Time PCR Detection System in the Opticon-2 Real-Time Cycler (BioRad). Primers for human *SULF1* were 5′-cgtgctatgaaggaagatgga3′ forward and 5′-tgcccagttcgtttcagt-3′ reverse, for human sulf2 5′-cgtgctatgaaggaagatgga3′ forward and 5′-tgcccagttcgtttcagt-3′ reverse, and for human *gapdh* were 5′-CTTCTCTGATGAGGCCCAAG-3′ forward and 5′-GCAGCAAACTGGAAAGGAAG-3′ reverse. qPCR included initial denaturation at 94 °C for 10 min, followed by 35 cycles of 94 °C for 10 s, 53 °C for 15 s, 72 °C for 20 s, 72 °C for 1 s and then 72 °C for 10 min followed by a dissociation curve every 0.5 °C from 55 °C to 95 °C. In a dissociation curve, a single peak was confirmed in each of the amplified sequences. For the quantification of *SULF1* or *SULF2* expression relative to *gapdh* in different samples, the threshold cycle (Ct) values of targets were expressed as 2-ΔΔ Ct (fold) as described previously (Winer *et al.*, 1999). Each sample was additionally amplified without reverse transcription reaction to confirm the absence of contaminating DNA in the RNA sample.

Lentiviral shRNA depletion

pLKO-puro vectors containing human *SULF2* specific shRNAs (*SULF2*A: *ccgggggcgaaagtcattggaatttctcgagaaattccaatgactttcgccctttttg, SULF2B: ccggcatcaatgagactcacaatttctcgagaaattgtgagtctcattgatgtttttga*) or scrambled shRNA were purchased from Sigma. Human embryonic kidney 293 FT cells with transfected with Virapower lentiviral packaging mix (Invitrogen) according to manufacturer's instructions. The next day, transfection complexes were removed and cells were allowed to produce virus for 48 hrs. Media containing virus were collected and used to directly transduce HaCaT cells. The cells were allowed to recover for 24 h and subject to puromycin selection (5 μg/ml). Puromycin resistant cells were harvested, RNA extracted, and qRT-PCTR performed to assess the depletion of *SULF2* mRNA.

To assess efficiency of SULF2 protein depletion, culture media was collected from Lentiviral shRNA-transduced HACaT cells grown in 10 cm-dishes for 48 hr. Following centrifugation for 10 min at 240 × g, the clarified supernatants were incubated overnight with Heparin coated Sepharose beads (GE HealthCare) at 4 °C. The beads were washed, and bound proteins were eluted by boiling in NuPage 4X LDS sample buffer (Invitrogen) containing 100mM dithiothreitol followed by immunoblotting with anti-Sulf2 antibodies.

Animal experiments

$Sulf1^{+/+}Sulf2^{+/+}$, $Sulf\text{-}1^{-/-}Sulf2^{+/+}$, and $Sulf1^{+/+}Sulf2^{-/-}$ C57BL/6 mice (Ratzka *et al.*, 2008) were bred to generate $Sulf1^{+/-}Sulf2^{+/-}$ and $Sulf1^{+/-}Sulf2^{-/-}$ heterozygoes. The heterozygotes were then bred to generate $Sulf1^{+/+}Sulf2^{-/-}$ and $Sulf1^{-/-}Sulf2^{-/-}$. double knockout mice. Genotyping was performed by PCR of tail vein DNA. Mice were fed a chow diet and water ad libitum throughout the study. Mice were anesthesized by isofurane inhalation and infected intranasally with 50 ul of PBS containing 5×10^6 IFU of *C. muridarum*. Mice were euthanized by CO2 inhalation and cervical dislocation at 48 hours

post infection. Lungs were dissected intact and immediately placed on ice prior to freezing at −80 °C. All animal protocols were approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

Quantitation of lung bacterial burden

Lungs were thawed in SPG buffer to make a 10% (wt/vol) solution and then pulverised through 70 μm cell strainers using the rubber plunger from a sterile plastic syringe. Tissue homogenates were centrifuged at 500 ×g for 10 min at 4 °C to remove coarse tissue debris. 10-fold dilutions of the clarified supernatants were then added for 1 hr to 24 hr HeLa cell monolayers grown on coverslips. Unbound EBs were removed by washing 3x with media followed by incubation at 37 C in 5% CO2 for 24 hours. Vacuoles/cell were quantified by staining with a MOMP-specific monoclonal antibody conjugated to fluorescein isothiocyanate (1:3 dilution, Fitzgerald). Vacuole quantitation was normalized to age-matched wild type mice

Statistical analysis

Data are expressed as mean ± SE. Data were analyzed by ANOVA, followed by the Bonferroni multiple comparisons. All statistical calculations were performed using InStat software and GraphPad Prism 4.0. Differences were considered statistically significant at $p < 0.05$, compared with control.

Gene ID

Human *SULF1* (GC08P070428) and *SULF2* (GC20M046285)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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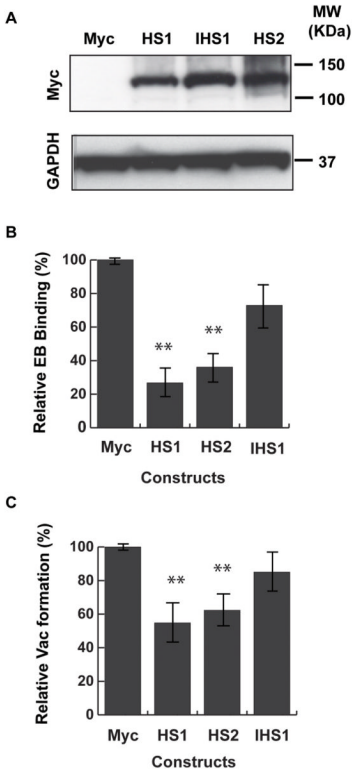

Fig. 1. Overexpression of SULF1 or SULF2 in HeLa cells decreases *Chlamydia* binding and vacuole formation

HeLa cells were transiently transfected with the vector expressing Myc-tag alone (Myc) or Myc-tagged SULF1 (HS1), Myc-tagged SULF2 (HS2), or a catalytically inactive version of Myc-tagged SULF1 (IHS1). (A) Levels of Myc-tagged transfected protein were determined by immunoblotting with an antibody to Myc. GAPDH serves as a loading control. (B) For quantitation of EB binding by immunofluorescence microscopy, HeLa cells were transfected for 48 hrs and then infected with *C. muridarum* for 1 hr. The number of EBs bound per cell was normalized to vector-transfected controls. For vector-transfected controls, the average number of bound EBs/cell was 10.5. Shown is the mean EB binding (± SEM) relative to vector-transfected controls for at least 5 independent experiments. At least 150 cells were counted in each experiment (C) HeLa cells were transfected for 48 hrs and then infected with *C. muridarum* for 24 hrs. The average number of vacuoles per cell was normalized to vector-transfected controls. For vector-transfected controls, the average number of vacuoles per cell was 0.54. Shown is the mean (± SEM) number of vacuoles per cells normalized to vector-transfected controls for at least 3 experiments. At least 500 cells were counted in each experiment **$p < 0.01$ ANOVA.

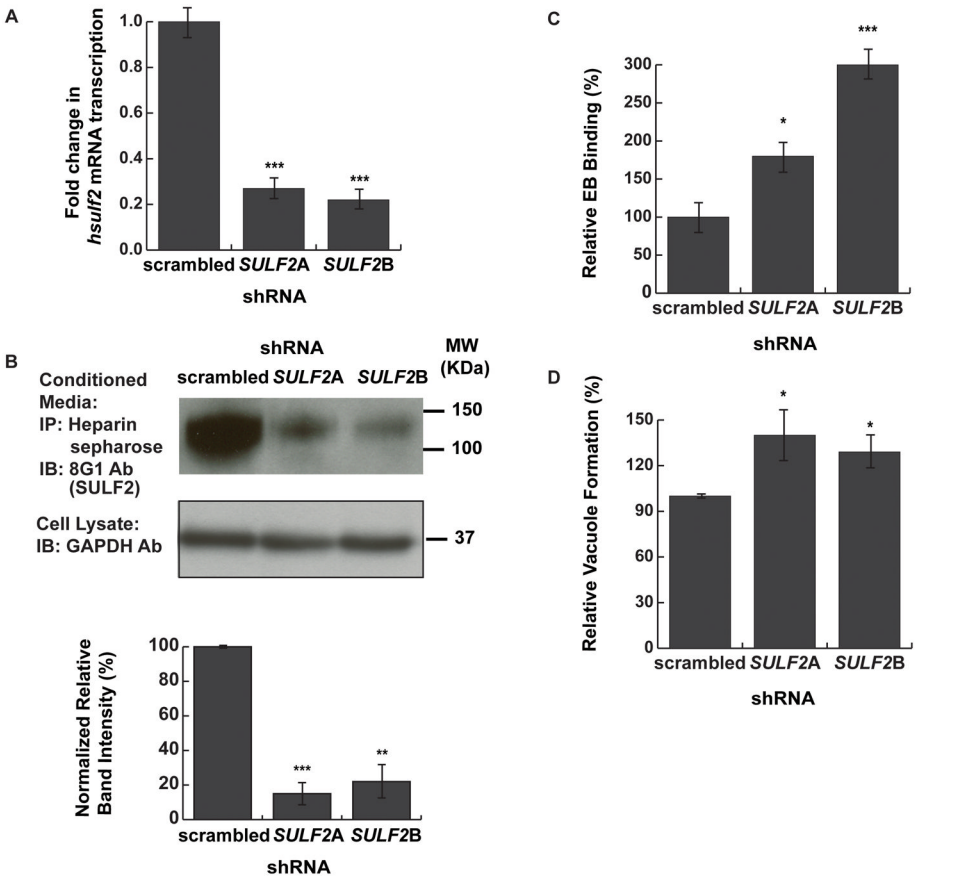

Fig 2. Depletion of SULF2 increased *Chlamydia* binding

HaCaT cells were transduced with lentivirus expressing scrambled shRNA or two different shRNAs targeting *SULF2* (*SULF2*A and *SULF2*B) and selected for puromycin resistance. (A) Total mRNA was isolated from shRNA-transduced cells, and *SULF2* levels were quantified by qRT-PCR. mRNA expression was compared to *GAPDH* and then further normalized to scrambled shRNA-transduced cells. (B) Secreted SULF2 from conditioned media from the puromycin-selected shRNA transduced cells was affinity purified by binding to heparin-sepharose beads and immunoblotted with a monoclonal antibody to SULF2 (8G1). As a control for cell number, lysates of the transduced cells were immunoblotted with GAPDH. The intensity of SULF2 bands was quantitated and compared to GAPDH by densitometry using ImageJ and then further normalized to scrambled shRNA-treated cells. The lower panel shows the average from 3 blots each for scrambled or *SULF2* shRNA-depleted cells. (C) EB binding was quantified from Puromycin-selected shRNA-transduced HaCaT cells by immunofluorescence microscopy. The number of EBs bound per cell was normalized to scrambled shRNA-transduced controls. For scrambled shRNA-transduced controls, the average number of bound EBs/cell was 2.4. Shown is the mean EB binding (± SEM) relative to scrambled shRNA transduced controls from 3 independent experiments. At least 150 cells were counted in each experiment. (D) Vacuole formation was quantified from Puromycin-selected shRNA-transduced HaCaT cells by immunofluorescence microscopy. The number of vacuoles per cell was normalized to scrambled shRNA-transduced controls. For scrambled shRNA-transduced controls, the average number of vacuoles per cell was 0.29. Shown is the mean number of vacuoles/cell (± SEM) relative to scrambled shRNA transduced controls from 3 independent experiments. At least 500 cells were counted in each experiment. * $p<0.05$, *** $p<0.001$ ANOVA

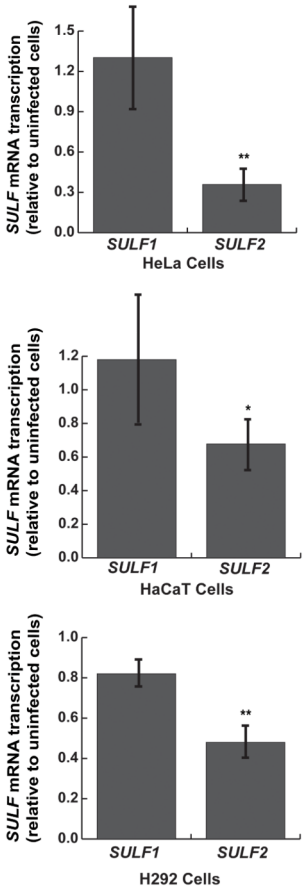

Fig 3. Chlamydia infection decreases SULF2 expression

HeLa, HaCaT, and H292 cells were infected with *C. muridarum*. At 24 hpi, total RNA was isolated and *SULF1* and *SULF2* mRNA levels were quantified by qRT-PCR. *SULF1* or *SULF2* mRNA was normalized to *GAPDH* mRNA and compared to uninfected cells. * $p<0.05$, ** $p<0.01$ ANOVA

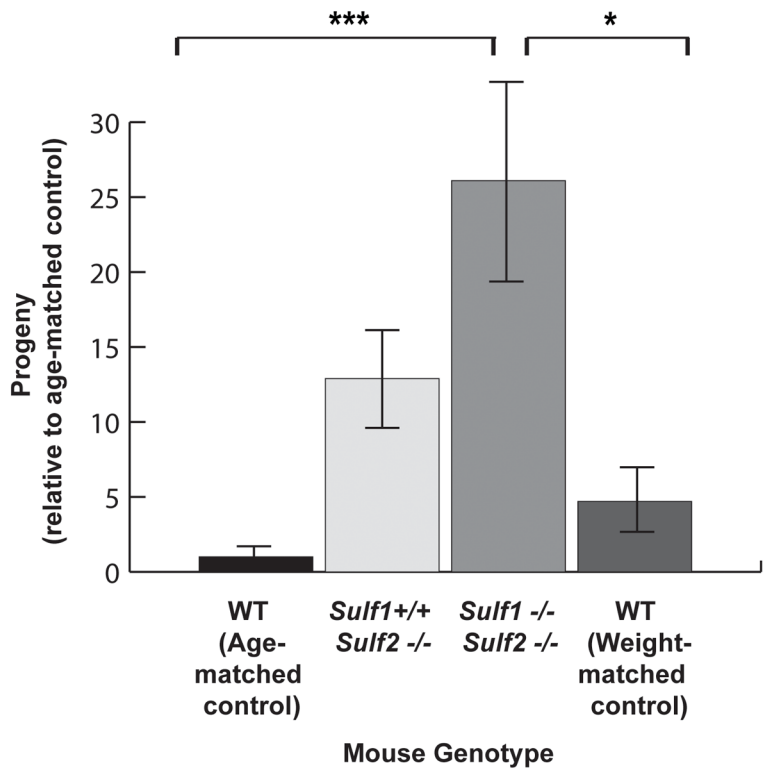

Fig. 4. Sulf-deficient mice are more susceptible to lung infection by *Chlamydia*

Wild type (age or weight matched), $Sulf1^{+/+}Sulf2^{-/-}$, and $Sulf1^{-/-}Sulf2^{-/-}$ mice were intranasally infected with 5×10^6 IFU of *C. muridarum*. At 48 hpi, lungs were harvested from mice and infectious progeny determined by plating serial dilutions onto HeLa cells followed by quantitation of vacuoles/cell at 24 hpi. Data was normalized to age-matched mice and were pooled from 4 experiments. Data were expressed as means ± SEM; ***$p<0.001$ compared to age-matched control; *$p<0.05$ compared to weight control. Data were analyzed by one-way ANOVA with post hoc Bonferroni correction.