MINIREVIEW

Innate immunity and vaccines in chlamydial infection with special emphasis on Chlamydia pneumoniae

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Abstract
Chlamydial infections are prevalent worldwide. Immunological events related to both innate and adaptive immunity during chlamydial infection can aid in recovery from the disease, but they can also cause harmful effects (immunopathology). The host genetic factors (variation in innate immunity and adaptive response-related genes) can predispose individuals to infection and its sequelae as well as determine the effects of intervention. No effective vaccine is available for human use. Modern technologies and data obtained using different ‘omics’ techniques (genomics, proteomics, transcriptomics and immunomics) might help in designing novel, more efficient vaccines, hopefully also against chlamydial infections.

Introduction
Chlamydial infection is accompanied by inflammation as the host response to infection. The inflammatory response is meant to be protective and aims at clearing the infection. However, as the inflammatory response may not only be followed by development of immunity (or at least short-lived protection) but also by immunopathology, it has been called a double-edged sword. The molecular and cellular mechanisms underlying inflammation are of major interest, as the harmful effects of inflammation can be seen in many infectious diseases and in autoimmune diseases. The available antichlamydial treatments are used to prevent transmis-

C. trachomatis seroprevalence rate (Lyytikäinen et al., 2008) and decreased ectopic pregnancy as well as tubal infertility rates (Brunham et al., 2006). Interestingly, already in the 1950s, tetracycline treatment was reported to delay the development of seroresponse in Chlamydia psittaci infection (Eddie & Meyer, 1956). Both immunity and lack of immunity in chlamydial infections can be beneficial as well as detrimental, and perhaps this has been one of the obstacles in the development of human chlamydial vaccine.

Exposure to bacteria belonging to the genus Chlamydia does not always result in clinical symptoms, and we are just beginning to understand how host genetic factors (e.g. human leukocyte antigen haplotypes and polymorphisms in genes responsible for the inflammatory response) affect the susceptibility and modify the severity of infection: a number of genetic polymorphisms associated with an increased or a decreased risk of sequelae following C. trachomatis infection have been identified (Mozzato-Chamay et al., 2000; Natividad et al., 2005; den Hartog et al., 2006; Öhman et al., 2006). We and others have shown that Chlamydia pneumoniae infection varies in different inbred mouse strains (Rottenberg et al., 1999; Vuola et al., 2000; Huang et al., 2002). More precisely, the p47 GTPases,
regulators of immunity to intracellular pathogens, have also been shown to regulate innate immunity and inflammation during murine chlamydial infection (Nelson et al., 2005; Bernstein-Hanley et al., 2006; Miyairi et al., 2007). In addition, using a different approach, Min-Oo et al. (2007) found that the mouse major histocompatibility complex (MHC) region in chromosome 17 was associated with the clearance of *C. pneumoniae* infection. Human hosts are, however, genetically complex and heterogeneous and are constantly being exposed to a number of pathogens. Data obtained using experimental infection models are not directly applicable to humans, and translations should always be performed with caution (Roshick et al., 2006). Consequently, much less is currently known about human susceptibility to *C. pneumoniae* infection. Rupp et al. (2004) reported that a CD14 promoter polymorphism was more frequent among patients with coronary artery disease and with *C. pneumoniae* DNA in peripheral blood mononuclear cells than among matched *C. pneumoniae*-negative subjects. Also, an association of mannose-binding lectin (MBL) 2 gene polymorphism and *C. pneumoniae* seroconversion was reported recently (Rantalä et al., 2008). More studies focusing on genetic heterogeneity and susceptibility of the host to infection and pathology are needed, as genetic factors can determine host response to infection and may also influence the response to therapy and vaccination (Bissonnette & Bergeron, 2006; Poland et al., 2007). Several excellent reviews on host response to *Chlamydia* spp. infection have been published just recently (Balsara & Starnbach, 2006; Darville, 2006; Rank, 2006; McClarty et al., 2007; Joyee & Yang, 2008; Roan & Starnbach, 2008). Here, the main focus will be on innate immunity and vaccines against *C. pneumoniae* infection.

**Innate immunity**

The ability of the innate immune system to quickly recognize and respond to an invading pathogen is essential for controlling the infection. For this purpose, host cells express receptors that recognize evolutionarily conserved structures that are expressed by various pathogens, but are absent from host cells (the so-called pathogen-associated molecular patterns, PAMPs) (Akira et al., 2006). In the case of *C. pneumoniae* infection, this recognition primarily takes place in the respiratory tract that comprises a large surface between the host and the environment. The respiratory epithelium acts as a mechanical barrier: the bronchial epithelial cells have tight junctions, the cells are covered by mucus, the cells secrete antimicrobial substances and cilia clear the airways from microorganisms mechanically. The epithelium also detects invading pathogens with the help of pathogen-sensing pattern recognition receptors, PRRs (Bals & Hiemstra, 2004; Hippenstiel et al., 2006; Mayer & Dalpke, 2007). PRRs are also present in macrophages and dendritic cells (Akira et al., 2006). The PRRs include the Toll-like receptor (TLR) family present at the cell surface or within phagosomes (Takeda & Akira, 2005), and the cytosolic nucleotide-binding and oligomerization domain (NOD)-like receptor family (NLR) (Carneiro et al., 2007). Recognition of PAMPs by PRRs results in intracellular signaling cascades, and induces the production of inflammatory cytokines, upregulation of costimulatory molecules and activation of the antimicrobial defense. PAMPs potentially present in *Chlamydia* spp. or during the chlamydial developmental cycle include lipopolysaccharide (endotoxin) and peptidoglycan (Chopra et al., 1998), and perhaps also lipoprotein and bacterial nucleic acid.

It has been suggested that infections can already be sensed by epithelial cells, which change their activation status and transiently release professional immune cells from inhibition (Mayer & Dalpke, 2007). The inhibition is achieved by soluble factors (including transforming growth factor-β) and adaptive regulatory T cells. When pathogens appear, the epithelium-derived factors, including interleukin (IL)-1 and IL-6, can activate professional antigen-presenting cells. Interestingly, both IL-1 and IL-6 are stimulated upon *C. pneumoniae* infection (Kaukoranta-Tolvanen et al., 1998). Also, secretion of proinflammatory cytokines by epithelial cells has been observed in response to *Chlamydia* infection (Rasmussen et al., 1997), which suggests that epithelial cells play a role in chlamydial pathogenesis. However, to avoid uncontrolled permanent activation in the mucosal surface, which is prone to have contact with a variety of microorganisms, the activation process has to be strictly controlled.

**TLR**

In mammalian cells, the TLRs are the best-characterized innate immunity receptors. TLRs sense a variety of microbial ligands at the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) or within endosomes (TLR3, TLR7, TLR8 and TLR9) (Kawai & Akira, 2005). Binding of the ligands to TLRs leads to recruitment of adaptor proteins [most often myeloid differentiation factor (MyD) 88, and also TRIF, TRAM and TIRAP], and they in turn recruit a signaling complex composed of IRAKs and TRAF6 (Kawai & Akira, 2007). Eventually, this leads to activation of NF-κB and activation protein-1, induction of inflammatory cytokine expression, including TNF-α, IL-1β and IL-6 (Misch & Hawn, 2008), and enables interaction with adaptive responses.

During *Chlamydia* spp. infection, NF-κB is activated and several proinflammatory cytokines are produced in various cell types, suggesting signaling through PRRs (Kaukoranta-Tolvanen et al., 1996; Rasmussen et al., 1997; Dechend et al., 1999; Kol et al., 1999; Prebeck et al., 2001; Gencay et al., 2009).
different Chlamydia species and a range of host cells have been used in the experiments. Despite the methodological differences, the studies have produced quite concordant data, and can be summarized as follows (Table 1). Although Chlamydiae are gram-negative bacteria and have lipopolysaccharide, signaling through TLR2 is often observed. Live chlamydial elementary bodies (EB) can initiate TLR2-mediated signaling in mice (Rodríguez et al., 2006), and human lung tissue (Droemans et al., 2007), as well as in murine and human cells (Prebeck et al., 2001; Netea et al., 2002, 2004; Yang et al., 2005; Yaraei et al., 2005a, b). Bacterial lipopolysaccharide is a classic ligand for TLR4 (Akira et al., 2006). The lipid A structures, however, may vary considerably and all gram-negative bacteria do not synthesize lipopolysaccharide with hexaacyl lipid A (Akira et al., 2006; Munford, 2008). Chlamydial lipopolysaccharide contains nonhexaacyl lipid A structures that might not effectively activate host defenses via TLR4, which could allow growth of bacteria and may even promote persistence. Indeed, the LPS of C. trachomatis has been shown to signal via TLR2 (Erridge et al., 2004). On the other hand, chlamydial heat shock protein (Hsp)60, in turn, has been suggested to act as a ligand both for TLR4 and for TLR2 (Bulut et al., 2002; Costa et al., 2002), but signal through TLR4 (Maguire et al., 2005). Hsp60, an abundant cytoplasmic chaperone molecule, is also surface exposed on some bacteria (Paju et al., 2000), is produced upon interferon (IFN)-γ treatment during Chlamydia pneumoniae infection (Molestina et al., 2002) and could thus be accessible to TLRs. Quite recently, C. pneumoniae proteins Cpn 0809, Cpn 0980 and OMP2 were shown to activate mouse macrophages through TLR2 and TLR4 (Jiang et al., 2008). MyD88, an adapter molecule associated with both TLR2 and TLR4, played an important role in initiating an early effective response against Chlamydia pneumoniae and the bacteria (Rodríguez et al., 2007). In the MyD88-deficient mice, severe inflammation with elevated inflammatory cytokine levels developed later, obviously due to the defective initial clearance of the bacteria (Naiki et al., 2005). In human ectocervical cells, MyD88 was required for IL-8 secretion, and both TLR2 and MyD88 were localized to C. trachomatis inclusions, suggesting active intracellular signaling through TLR2 (O’Connell et al., 2006). Also, the role of other downstream signaling molecules has been studied. IRAK4, a part of several TLR-signaling pathways, has been shown to control the growth of Chlamydia pneumoniae in mouse bone-marrow-derived macrophages through IFN-α and IFN-γ secretion, whereas TRAF6 regulated NF-κB activation (Trumstedt et al., 2007). TRIF (TIR domain containing adaptor-inducing IFN-β), an adaptor recruited as a consequence of recognition of double-stranded (ds) RNA by TLR3, was suggested to be responsible for IFN-β production by Chlamydia muridarum-infected oviduct epithelial cells (Derbigny et al., 2007). However, as dsRNA is not likely to occur in Chlamydia spp., signaling is likely to occur via another TLR (or yet unidentified TRIF-dependent PPR). Recently, another chlamydial component was shown to induce TLR-mediated activation: a chlamydial lipopeptide exposed on chlamydial EB, macrophage infectivity potentiator, mediated proinflammatory response in human macrophages through TLR2/TLR1/TLR6 (Bas et al., 2008), suggesting that besides lipopolysaccharide and Hsp60, other components of the bacteria are potential ligands for TLRs.

Pathogenic microorganisms have already evolved sophisticated strategies to subvert host defenses, and signaling through TLRs is not an exception. Some virulent bacteria can interfere directly with TLR function by secreting proteins that inhibit the signaling and lead to impaired host defense (GirI et al., 2008). Whether this could take place during chlamydial infection remains to be studied. TLRs seem to play an important role in inflammatory diseases that have not traditionally been linked to infection, including atherosclerosis and asthma, and variation in TLR genes (single nucleotide polymorphisms, SNPs) can confer increased risk of certain infections (Misch & Hawn, 2008). The association of Chlamydia pneumoniae infection with these inflammatory conditions (asthma, atherosclerosis, Alzheimer’s disease, multiple sclerosis and even rheumatoid arthritis and cancer) has been suggested and this area remains an important field of research. Analysis of polymorphisms in genes encoding PPR signaling can elucidate the pathogenesis of these conditions and potentially identify novel, more selective targets for therapy.

Non-Toll-like innate immune proteins

The TLRs work in synergy with the cytosolic NLRs (which sense bacteria), RIG-1 (retinoic acid-inducible gene 1)-like receptors (RLRs) (which sense viruses) and C-type lectin receptors (CLRs) (which sense fungi) (Ishii et al., 2008). The TLRs and CLRs activate the transcription factor NF-κB and also MAP kinases, which are required for the expression of many immune and inflammatory genes, most notably cytokines and chemokines (Pálsson-McDermott & O’Neill, 2007). NOD1 and NOD2 respond to muropeptides derived from bacterial peptidoglycan (McDonald et al., 2005). The chlamydial genome encodes enzymes required for peptidoglycan synthesis (Kalman et al., 1999; Stephens et al., 1998), and the bacteria are somewhat sensitive to penicillin and β-lactam antibiotics (Chopra et al., 1998), but peptidoglycan 


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Table 1. Role of the PRRs and their adaptor molecules in the recognition of Chlamydiae or chlamydial components

<table>
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<th>Receptor/adapter protein</th>
<th>Chlamydial species/ chlamydial component</th>
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<td>Foam cell formation in RAW 264.7 cells</td>
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<td><em>Sonicated C. pneumoniae</em></td>
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<td><em>C. pneumoniae</em> Hsp60</td>
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<td><em>C. trachomatis</em> LPS</td>
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<td>MyD88</td>
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<td><em>C. trachomatis</em></td>
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<td>TRIF</td>
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<td>Nod1</td>
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LPS, lipopolysaccharide; TNF, tumor necrosis factor; LDL, low-density lipoprotein; PBMC, peripheral blood mononuclear cells. PMN, polymorphonuclear neutrophils; MBP, mannose-binding protein.
as such has not been detected in the bacteria themselves. A few studies suggest that a motif needed for NOD recognition is present during Chlamydia spp. infection. In C. pneumoniae-infected HEK293 cells, NOD1- and NOD2-mediated NF-kB activation was demonstrated (Opitz et al., 2005). The results obtained using overexpression of dominant-negative NOD1 or silencing of NOD1 by RNA interference also suggest that NOD1 recognition plays a role in NF-kB activation and secretion of proinflammatory cytokines during in vitro infection with C. trachomatis (and C. muridarum), although during vaginal infection, NOD1 deficiency in mice did not influence the course of the infection, cytokine secretion or pathology (Welter-Stahl et al., 2006). It remains to be shown how and which chlamydial structures recognized by NLRs are able to enter the host cell cytoplasm and establish contact with intracellular receptors. Certain NLRs (e.g. Nalp3, IPAF and NAIP) are important components of the so-called inflammasomes that contain caspase-1, which cleaves pro-IL-1b and pro-IL-18 into their mature forms (Akira et al., 2006; Ishii et al., 2008). Similar to polymorphisms in TLRs, those in NLR genes are associated with susceptibility to inflammatory conditions (Carneiro et al., 2007; Tattoli et al., 2007; Franchi et al., 2008). Genetic variations in TLR and/or NOD, more specifically multiple SNPs in several genes (carrier traits), were suggested to be associated with persistent infection and tubal pathology after C. trachomatis infection (den Hartog et al., 2006).

The majority of CLRs mediate endocytosis and/or phagocytosis, play a role in antigen presentation and keep endogenous glycoprotein levels constant (Pålsson-McDermott & O’Neill., 2007). A subset of CLRs, for example, Dectin-1, respond to various PAMPs. CLRs can also act as phagocytic receptors, and the mannose receptor has been implicated in the entry of C. trachomatis into host cells (Kuo et al., 2002). PAMP recognition by CLRs is also potentially connected to the induction of innate response genes (Robinson et al., 2006). MBL variant alleles are associated with accelerated development of severe atherosclerosis (Madsen et al., 1998), and C. pneumoniae infection may promote development of severe coronary artery disease in predisposed individuals (Rugonfalvi-Kiss et al., 2002). Also, MBL polymorphism could be associated with tubal damage following C. trachomatis infection (Sziller et al., 2007).

Expression of triggering receptors expressed on myeloid cells (TREM-1; Klesney-Tait et al., 2006), another non-TLR that can increase inflammation, can be modified by C. trachomatis infection (Cooper et al., 2008). In conclusion, the recognition of host-invading pathogens is essential in the host response to infections. As there are a plethora of sensors (TLR and non-TLR proteins), host cells obviously attempt to integrate signals from multiple pathogen-sensing receptors to mount an appropriate response to infection (Klesney-Tait et al., 2006; Sansonetti, 2006).

### Chlamydia pneumoniae vaccine

In general vaccinology, conventionally killed or live-attenuated organisms and subunit vaccines have been used and attempts to develop new vaccines have largely been based on the hit-and-miss strategy. Later approaches include the use of expression libraries and other technologies relying on the genomic sequence of the microorganisms. The availability of complete microbial genome sequences and advancements in high-throughput technologies for molecular profiling (the ‘omics’ approaches: genomics, transcriptomics, proteomics and immunomics) have the potential to dramatically change vaccine development strategies. To develop a successful vaccine, the correlates of protection must be known irrespective of the antigen selection strategy. For human chlamydial infections, this has not been straightforward and no vaccine is currently available for human use. The adaptive immunity is obviously essential in controlling chlamydial infection and in attempts to eliminate the bacteria upon subsequent exposure. However, the immune response may also be associated with coexistence (chronic infection) or enhanced pathology. Susceptibility to reinfections suggests that immunity developing after a natural chlamydial infection might not be protective or long lived. In addition, Chlamydiae are obviously able to persist for long periods of time within the host, and chronic infections are associated with several clinical manifestations of public health importance. Chlamydiae can actively inhibit the expression of MHC class I and class II molecules in host cells by secreting a chlamydial protease- or proteasome-like activity factor (CPAF) (Zhong et al., 2001) that is responsible for the degradation of RFX-5, a transcription factor required for expression of MHC molecules. This strategy can promote chlamydial persistence in the host cells while detection by CD4+ T cells is restrained. Antigenic variation, the ability of microorganisms to change their surface properties to avoid recognition and clearance by the adaptive immune response, has been known for many years. Whether C. pneumoniae can utilize the polymorphic outer membrane proteins (pmp; Kalman et al., 1999) in such a way and undergo phase variation (switching between pmp genes) in response to immune pressure is an intriguing thought (Pedersen et al., 2001).

Studies using immunocompetent and different knock-out mice indicate that several immune effector mechanisms play a role in the control of C. pneumoniae infection. The correlates of protection include the production of IFN-γ (Rottenberg et al., 2000; Vuola et al., 2000), development of T-cell responses (Penttilä et al., 1999; Rottenberg et al., 1999; Rothfuchs et al., 2004) and production of mucosal antibody.
A computer-based method has been used to predict C. pneumoniae (Sarén et al., 2006; Rodríguez et al., 2006). IFN-γ is a critical cytokine in protection against infection and in the reduction of pulmonary C. pneumoniae loads (Rottenberg et al., 2000). In addition, T cells are essential in immunity to C. pneumoniae. Although both CD4+ and CD8+ T cells contribute to protection, the CD8+ T cells play the predominant role (Penttilä et al., 1999; Rottenberg et al., 1999; Tvinnerem & Wizel, 2007). On the other hand, C. pneumoniae can infect phagocytic cells, especially macrophages, and use them as host cells (Godzik et al., 1995; Mannonen et al., 2004), which can enhance replication and dissemination of the infection from the respiratory tract to other locations (Moazed et al., 1998). As the prevalence of circulating IgG antibodies in the general population is quite high, antibodies do not seem to play a major role in the control of C. pneumoniae infection, although they can neutralize pathogen infectivity in vitro (Puolakkainen et al., 1995; Rodríguez et al., 2006), but mucosal antibodies could be associated with protection (Penttilä et al., 2006). Because the natural C. pneumoniae infection induces only partial protection against the repeated reinfections, sterilizing protection requires novel approaches to develop safe human vaccines that achieve the above-mentioned correlates. The key question here has been and still is how to induce protective immunity and to avoid immunopathology, and moreover, how to induce longer-lasting protection than is achieved at least in certain individuals after natural infection.

The selection of immunogen, formulation of vaccine, choice of adjuvant and immunization strategy need to be carefully studied and evaluated. The strategy to identify protective vaccine antigens has moved from educated guesses to reverse vaccinology. When chlamydial genome sequences were not available, the proteins that were surface associated and/or abundant were considered the most likely vaccine antigen candidates (Penttilä et al., 2000; Svanholm et al., 2000). Despite multiplying in their own vacuole (inclusion), Chlamydiae communicate with the host cells through the type III secretion system (TTSS) (Hsia et al., 1997), and it was shown to be a likely method for delivery of several chlamydial proteins into the host cell cytoplasm (Subtil et al., 2005). Consequently, the secreted proteins or even components of the TTSS can be considered as vaccine candidates and potential CD8 T-cell antigens. Indeed, use of such proteins, such as CPAF and LcrE (CopN), are of particular interest in vaccine development (Sambri et al., 2004; Murthy et al., 2007; Tammiruusu et al., 2007).

The genome-based approach has been applied to identify putative surface-exposed proteins of C. pneumoniae (Montigiani et al., 2002) and to construct DNA vaccines (Murdin et al., 2000; Penttilä et al., 2000; Svanholm et al., 2000). Also, a computer-based method has been used to predict C. pneumoniae peptides that could bind to MHC class I molecules (Sarén et al., 2002). Later, a CD8+ T-cell heptapeptide minigene vaccine was suggested to induce protective immunity against C. pneumoniae in mice (Pinchuk et al., 2005). For discovery of novel vaccine antigens, it could be of importance to identify the genes that are upregulated during infection as they might represent protective antigens. Transcriptional profiling and proteomics-based approaches are just beginning to elucidate whether the acute chlamydial infection state is distinct from chronic infection and whether both have a characteristic genetic program (Belland et al., 2003; Mukhopadhyay et al., 2006; Ouellette et al., 2006). For practical reasons, most microarray studies are performed with the organism grown in vitro because it is technically very challenging to obtain sufficient amounts of bacterial RNA from infected human tissues. Again, the differences between cell culture systems and the human host need to be considered.

Immunome is the set of pathogen epitopes that interfere with the host immune system (de Groot, 2006). A key focus of immunomics has been the development of algorithms for the design and discovery of new vaccine antigens. The in silico prediction methods have a great capacity to accelerate epitope discovery, and several programs are available that can help to design and optimize vaccines (de Groot, 2006; Davies & Flower, 2007). For T-cell epitope prediction, many programs are available, including MHC-Bench (http://www.imtech.res.in/raghava/mhcbench/), an interface developed specifically for evaluating the various MHC-binding peptide prediction algorithms (Davies & Flower, 2007). B-cell prediction is more problematic owing to the difficulties in defining linear and discontinuous epitopes from the protein of interest. Potential novel vaccine candidates can be identified using reverse vaccinology: starting from genomic information, likely cell surface proteins and secreted proteins, or genes showing sequence and/or structural homology to known virulence factors are identified. Use of reverse vaccinology has tentatively been validated: molecules conferring protection against experimental Streptococcus pneumoniae and Neisseria meningitides infection were discovered using such an approach (Wizemann et al., 2001; Giuliani et al., 2006). Similarly, the protective capacity of the 53 putative surface-exposed proteins identified among the selected ORFs from C. pneumoniae (Montigiani et al., 2002) could be investigated. It is claimed that if reverse vaccinology can be applied in vaccine design, it can save enormous amounts of money, time and labor. Whether these methods are of any practical use remains to be seen. Eventually, the vaccine candidates have to be validated using traditional experimental approaches, including an appropriate animal model and careful immunological evaluation. To decide which of these candidates to take forward to clinical trials still remains a real challenge.

Infections due to Chlamydia spp. remain an important public health problem throughout the world. The
complexity of the organism and its life cycle has, thus far, thwarted vaccine development and elucidation of immune correlates of protection. Nevertheless, vaccines against *Chlamydia trachomatis* that reduce development of chronic conditions, and ideally also reduce transmission, were an ideal component of *Chlamydia trachomatis* control strategy. Similarly, a *Chlamydia pneumoniae* vaccine that is able to prevent or reduce development of chronic conditions associated with infection could be of public health importance.

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