An outbreak of legionnaires' disease at a flower show: clinical findings and studies on host defense mechanisms

Lettinga, K.D.

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CHAPTER 1

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1. Community-acquired pneumonia

Community-acquired pneumonia (CAP) is a major cause of morbidity and mortality all over the world. The incidence of CAP requiring hospitalization is 258 per 100,000 per year and increases with age to 962 per 100,000 for those 65 years of age or older [1]. The introduction of antibiotic agents dramatically reduced mortality from pneumococcal pneumonia, but the mortality rate from CAP has shown little improvement during the past 3 decades, remaining between 2 and 30% among hospitalized patients with an average of ~14% [1,2]. Mortality is estimated to be < 1% for non-hospitalized patients [2]. CAP can be caused by a variety of bacterial and viral pathogens, but the etiology is established in only 50-70% of cases. The etiology of CAP in North America over the past 20 years is listed in table 1 [3] and these numbers are supported by multiple studies [4,5].

Table 1. Etiology of CAP in North America

Etiology	Cause of CAP (in %)
Streptococcus pneumoniae	20-60
Haemophilus influenzae	3-10
Staphylococcus aureus	3-5
Gram-negative bacteria	3-10
Aspiration	6-10
Miscellaneous	3-5
Legionella spp.	2-8
Mycoplasma pneumoniae	1-6
Clamydia pneumoniae	4-6
Viruses	2-15

Data from [3].

2. Epidemiology of Legionella pneumophila

In 1976 an outbreak of pneumonia occurred among attendees of a convention for American Legion's in Philadelphia. The causative agent was identified and called *Legionella pneumophila* and the illness Legionnaires' disease (LD) [6,7]. *L. pneumophila* is a gram-negative, aerobic and facultative intracellular bacillus. Although 48 species of Legionellae have been described [8], *L. pneumophila* is responsible for about 90% of infections caused by members of the Legionellaceae family [9,10]. *L. pneumophila* serogroup 1 (70-80%), 4 and 6

account for most of the strains implicated in human infection [11,12]. In total 19 Legionella species are documented as human pathogens on the basis of their isolation from clinical material [13]. The clinical syndromes produced by members of the Legionellaceae family are designated legionellosis: LD is pneumonia, and Pontiac fever is an acute febrile, self-limiting illness without pneumonia that has been serologically linked to *L. pneumophila* or other Legionella species [13].

LD is usually community acquired (75%) and accounts for 2-13 % of all CAP cases worldwide. This range of LD incidence among CAP patients can be explained by geographical site and severity of pneumonia of the population that is studied [4,12,14]. In addition, the use of diagnostic tools may differ between countries. Hospital acquired LD (25% of cases) is often more severe, with fatality rates reaching 50% [15], and 37% of these cases are linked to outbreaks (reported to the Centers for Disease Control and Prevention) [10]. Overall, outbreaks of LD account for 10-20% of LD cases, so the majority of cases occurs sporadic. Travel is an underappreciated factor in the acquisition of LD in the community. Characteristics of typical travel-related LD make detection very difficult: a low attack rate, a long incubation period, dispersal of persons away from the source and inadequate surveillance. The sources of outbreaks and sporadic cases are similar.

Legionella bacteria are ubiquitous in both natural and engineered water supplies and a variety of equipment that disperse water has been implicated as the source of infection, including potable water. The mode of transmission is by inhalation of aerosols or micro-aspiration of contaminated water [16]. No person-to-person transmission has ever been observed, indicating that *L. pneumophila* is not adapted to the human host. In the absence of transmision to a new human host, mutations that promote survival and replication only in the human lung will not persit in the species' genome [17]. However, in water systems *L. pneumophila* can grow in a wide range of hosts: >13 species of amoebae and 2 species of ciliated protozoa [17]. It is important to note that protozoa frequently contaminate water supplies, especially heated reservoirs. The life cycle of *L. pneumophila* in amoebae strongly resembles that observed in (alveolar) macrophages. The similar cell biology suggests that the virulence of *L. pneumophila* for alveolar macrophages is a consequence of its evolution as a parasite of amoebae. Moreover, replication in amoebae not only increases the bacterial numbers but also increases virulence and resistance to anti-microbial agents. For example, mice inoculated with a mixture of bacteria and amoebae develop more severe disease than those infected with either *L. pneumophila* or *Hartemanella vermiformis* [18].

3. Microbiology

Legionellaceae are gram-negative, small coccobacilli measuring up to 0.5 μm by 1-3 μm with polar or lateral flagella up to 8 μm in length and 14-20 ηm in diameter. The organism is nutritionally fastidious and does not grow on standard bacteriological media. Charcoal yeast extract buffered to pH 6.9 (BCYE) is the primary medium used for isolation. L-Cysteine is a critical ingredient in culture and keto-acids and ferric ions stimulate growth. *L. pneumophila* can be visualized by Gram stain with some difficulty. Legionella stains poorly when examined on smears of infected secretions or in tissue, but they stain more readily after growth on culture plates.

Antigens of *L. pneumophila* that have been studied extensively are the lipopolysaccharides (LPS), responsible for endotoxic properties of the bacterium and also the determinant of the serogroup specificity. LPS of *L. pneumophila* has a unique structure compared to LPS of other gram-negative bacteria and this may explain the observed low endotoxicity. There are 15 antigenically distinct serogroups of *L. pneumophila* [8]. The major outer membrane protein of Legionella is involved in binding of the bacteria to complement receptor 1 and 3 on mononuclear phagocytic cells [8]. *L. pneumophila* produces several extracellular products: a hemolysin, several proteinases with collagenase activity and a heat-stable polypeptide cytotoxin (Hsp60). The latter toxin is involved in reducing superoxide generation in PMN, thus reducing bacterial killing. A number of virulence factors involved in intracellular growth and programmed cell death have been the focus of recent studies. The macrophage infectivity protiator (Mip)-gene is involved in the process of establishing efficient infection in macrophages and protozoa [17]. In macrophages, the Dot/Icm type IV secretion machinery (Dot, defective for organelle trafficking; Icm, intracellular multiplication) of *L. pneumophila* enables the bacterial phagosome to evade maturation along the endosomal-lysosomal degradation pathway [19]. Upon termination of intracellular replication the bacteria undergo phenotypic modulations resulting in Dot/Icm dependent pore-formation-mediated cytotoxicity [20] and apoptosis of the host cell via activation of caspase 3 [21,22].

4. Clinical features

LD originally was believed to cause the typical clinical syndrome that fits into the group of diseases called "atypical pneumonia". However, several prospective comparative

studies have determined that on the basis of clinical findings, non-specific laboratory findings or chest roentgenographic results it is not possible to distinguish between LD and common causes of pneumonia [4,23,24]. LD encompasses a broad spectrum of illnesses, ranging from a mild cough and slight fever to widespread pulmonary infiltrates and multi-organ failure. Early in the disease most patients experience nonspecific flu-like symptoms such as fever, myalgia, anorexia and headache. Cough with sputum production is reported in 25 to 75 % of the patients and differences with other causes of pneumonia are not convincing [25]. The frequency of diarrhea in patients with LD ranged from 0-25%, which was not different from other causes of pneumonia. Change in mental status is the most common neurological abnormality. Fever is virtually always present and often higher than 40 °C. Hyponatremia and elevated levels of serum transaminase enzymes are the two most common types of non-specific laboratory abnormalities that clinicians associate with LD. However, in several prospective studies differences in laboratory values from other causes of pneumonia could not be demonstrated [25]. Chest radiographic findings in LD are also non-specific and all types of roentgenographic patterns are seen. Progression of infiltrates despite appropriate therapy is often observed. The extent of radiographic infiltration does not correlate with severity of clinical manifestations or with ultimate outcome [26]. The outbreak in the Netherlands once more demonstrated that patients with LD are not easily recognized by physicians (this thesis). On the other hand, physicians are more likely to prescribe erythromycin to patients with LD than to patients with "typical" bacterial pneumonia [27]. This suggests that there may be clues on admission which alert the physician to suspect LD, for example, severity of pneumonia or a history of traveling. For a subset of patients with LD findings are classic, but use of these classic findings as a sole index for suspicion inevitably results in missed diagnoses.

Cigarette smoking, chronic obstructive pulmonary disease (COPD) and immunosuppression have consistently been implicated as risk factors for acquisition of LD [10,15,28]. Surgery and organ transplantation are major risk factors for acquisition of nosocomial Legionella infection [29]. All these conditions are predictors for fatal outcome [10,23,30] although results depend extensively on the population studied.

Two years after the first described outbreak of LD in Philadelphia [6], 58% of the patients were still not fully recovered [31]. Information on the follow-up of pulmonary abnormalities in survivors of LD is only limited available. Radiological abnormalities were shown to persist for at least several months after recovery from the acute phase of LD [32]. In lung biopsies taken shortly after LD there were structural changes and fibrosis of lung

parenchyma [33]. Despite considerable progress in medical and epidemiological management of outbreaks of LD, little is known about the overall health impact of such an event on survivors. An important measure of patient well-being is the health-related quality of life (HRQL) [34,35] which can be assessed by symptom-based questionnaires or The Medical Outcomes Study Short Form 36-item Health Survey (SF-36) questionnaire. Using a symptom-based questionnaire, the recovery of patients with low-risk pneumonia to pre-morbid health status requires more than 90 days [34,35]. In patients who survived ICU admission for lung injury, an impaired quality of life compared with matched population controls was demonstrated even after one to two years follow-up [36,37]. For patients recovered from LD, follow-up quality of life data are described in this thesis.

5. Diagnostic tools

LD can be diagnosed with the use of specialized laboratory tests and general use of these tests may prevent under-diagnosis, underreporting and delay of adequate therapy. Most of the data are applicable to *Legionella pneumophila,* since sensitivity or specificity estimates for non-pneumophila species are not known.

Culture of Legionella: Culture diagnosis remains the gold standard for diagnosis of Legionnaires' disease. Based upon serologically positive patients (fourfold rise in titer), sensitivity is near 60% and the specificity is near 100% [8,38]. Sensitivity is limited by several factors. First, laboratories experienced in the isolation of Legionellae are more likely to recover the organism than inexperienced laboratories [8]. Second, hospital laboratories commonly reject sputum samples containing many squamous cells or few polymorphonuclear leukocytes. However, many patients with LD produce non-purulent sputum in which Legionella is present. Finally, bacteria survive poorly in respiratory secretions and immediate culture is critical, a factor that hampers adequate cultures in clinical practice. Legionella can be cultured from blood, lung tissue, lung biopsy material, respiratory secretions (sputum, bronchoalveolar lavage fluid) and stool. Occasionally, Legionella has been cultured from extrapulmonary sites such as bone marrow, prostetic heart valves and sternal wounds. Culture has the disadvantage of delay, because a positive result is not available until after at least 3 days of incubation and antibiotic treatment should be initiated before test results are known. In addition, certain commercially available culture media are selective for *L. pneumophila* but may inhibit growth of other Legionella species [13].

Direct Fluorescence Antibody staining (DFA): The sensitivity of DFA testing for Legionella in respiratory secretions has varied from 25 to 75%, and the specificity is >95%. Estimates of sensitivity of DFA staining are highly dependent on the golden standard used. Not surprisingly, sensitivity is higher when a positive culture is used as the golden standard compared to serology. The sensitivity and specificity for species other than *L. pneumophila* is not exactly known [13]. Immunofluorescent microscopy is technically demanding and should only be performed by experienced laboratory personnel. There are several species-specific polyvalent and monoclonal reagents available with different specificity and sensitivity, and cross reactions have been reported [8,25,39]. In conclusion, DFA staining is not useful for routine clinical practice.

Serological diagnosis: Seroconversion is a diagnostic tool with a high sensitivity and a high (serogroup dependent) specificity. The indirect fluorescent antibody test (IFAT), developed by the Centers for Disease Control, Atlanta (CDC), is the most widely used test and detects immunoglobulin (Ig) G, IgM and IgA antibodies to *L. pneumophila* with a sensitivity of ± 80%, using 99% cut-off values [8]. ELISA assays (detecting *L. pneumophila* serogroup 1-6; IgM and IgG antibodies) have the advantage of being simple and rapid and several studies showed a high overall correlation between IFAT and ELISA testing. The micro-agglutination test (MAT) detects *L. pneumophila* serogroup 1-15 IgM antibodies with a specificity of > 99% [40]. Harrison et al [41] found a sensitivity of 63% for a fourfold rise in titer to ≥ 1:128 using positive culture or positive DFA staining in respiratory secretions as the golden standard. However, serology assays are not used in clinical practice on a large scale. The time required for seroconversion is variable and can be more than 10 weeks [8,42]. In one study, 41% of 42 patients with culture-proven *L. pneumophila* pneumonia did not seroconvert within 4-6 weeks [43]. A single high titer ≥ 1:256 can be used in an outbreak situation, but a single high titer in solitary cases should be carefully considered [43].

The specificity of serological tests for *L. pneumophila* is ~95-99%. False-positive result have been reported [39,44]. However, cross-reactions occur especially with non-*L. pneumophila* spp.

Urine Antigen detection: The urinary antigen tests combine reasonable sensitivity and high specificity with rapid results. Approximately 80% of patients with *L. pneumophila* serogroup 1 infection excrete Legionella antigen in their urine [45]. Therefore, detecting these antigens permits early diagnosis and initiation of appropriate therapy. Although there is some cross reactivity with *L. pneumophila* serogroups 4, 5 and 10, the capture antibody used in the

majority of urinary antigen tests is considered to be specific for *L. pneumophila* serogroup 1 (the antigen detected is a component of the LPS portion of the cell wall and is heat stable). Therefore, even though most cases of LD are caused by *L. pneumophila* serogroup 1, total dependence on this assay may miss as many as 30-40 % of cases. Currently, there are three commercially available ELISA urinary antigen tests (Binax EIA, Bartels EIA and Biotest EIA). Two tests intend to detect *L. pneumophila* serogroup 1 and the Biotest EIA is intended to detect antigens of other serogroups and species in addition to *L. pneumophila* serogroup 1 [46]. However, the Binax EIA and Bartels EIA are capable to detect antigens from non-*L. pneumophila* serogroup 1, although with a lower sensitivity [47]. The reported sensitivity ranges from 50-90%, depending on the golden standard used , the patient characteristics, the serogroup with which the patient is infected, the timing of urine collection and whether the urine is concentrated or not [39,43,48,49]. A new immunochromatographic test (Binax Now) intends to detect *L. pneumophila* serogroup 1, is simple to perform (like a home pregnancy test) and results can be obtained in 15 min. This test showed 98% overall agreement with the Binax EIA assay [50]. Concerns have been raised about the sensitivity of urinary antigen testing early in the course of the disease [51]. In a study with patients with nosocomial *L. pneumophila* serogroup 1 infection, 5 of 7 patients with a negative urine antigen test during the first 5 days of illness had a subsequent positive test. Because these patients had nosocomial infection, they may have been tested earlier in the disease than patients with community-acquired infection. False-negative urinary tests in early disease were not found by others [45] (Dr. E. Yzerman, pers. communication). Specificity for all tests is ~98-100% and cross reactivity with other micro-organisms is rare [52,53].

In general, considering the sensitivity of urinary antigen testing and the limitation of serogroup and species specificity, Legionella as the cause of pneumonia can not be excluded when the test is negative.

PCR: Amplification of DNA by polymerase chain reaction is a very sensitive tool, able to detect low quantities of Legionella DNA in respiratory secretions, serum and urine. PCR represents one of the few diagnostic tests with the potential to detect infections caused by any of the known species of Legionella, although PCR lacks the ability to discriminate between Legionella species [46,54].

Urine screening with a commercially available PCR kit (Enviro-Amp kit) within the first 2 days of illness showed a sensitivity of 75% and a specificity of 90% [39]. However, the sensitivity is so high that positive results may occur due to low level Legionella

contamination of drinking water [39,55]. Validation of PCR assays will be difficult, because deficiencies in existing techniques will make it difficult to rule out false-positive results.

6. Therapy

Since the first described outbreak of LD, erythromycin has been the drug of choice [6]. Increased mortality associated with delay of starting adequate therapy for LD has been reported [56-58]. In patients suspected of having LD adequate therapy should therefore be started as soon as possible. It is unlikely that a comparative clinical trial testing newer drugs could be completed, since such a study would require 300-900 patients in each group for adequate statistical power and thus would take years to perform [59]. Therefore, assessment of the efficacy and potential utility of antimicrobials against Legionella spp. is based on 3 methods: first, *in vitro* extracellular susceptibility testing can be used to screen for active agents. However, the MIC can be influenced by inactivation of drugs by the test media and also high extracellular susceptibility can not be extrapolated to *in vivo* activity. For example, β-lactams are highly active *in vitro* but do not penetrate the intracellular compartment. Second, *L. pneumophila* has been cultured *in vitro* in a number of cell lines to assess the intracellular activity of antimicrobials. The third method are studies with guinea pigs that develop severe pneumonia when infected with *L. pneumophila.* Clinical efficacy in humans corresponds to efficacy in this animal model. *In vitro* data (MIC data and intracellular culture methods) and guinea pig models suggested that newer macrolides and many fluoroquinolone agents have superior activity against Legionella spp. compared to erythromycin. Human studies are limited to case reports and small retrospective studies.

Macrolides and azalides: Erythromycin is widely used, but it is less active *in vitro* and in animal models than azithromycin, clarithromycin and roxithromycin. Azithromycin is either bactericidal or irreversibly inhibitory against the intracellular bacterium, whereas erythromycin is only bacteriostatic. Single-dose azithromycin treatment of guinea pigs is much more effective than multiple-dose erythromycin therapy and is also more active than clarithromycin [60]. When azithromycin is used in treating community-acquired Legionella, cure has been observed in 8 out of 8 patients with a total dose of 1.5 g given over 3-5 days [61]. Newer macrolides have less side effects than erythromycin [62]. On the basis of the available data, azithromycin appears to be the best macrolide to treat LD, followed by clarithromycine.

Quinolones: Fluoroquinolones probably have the best activity against *L. pneumophila* in experimental models with MIC values ≤ 0.01 μg/ml [63-65]. Many quinolones kill rather than just inhibit the intracellular bacterium and they readily concentrate within phagocytes. Animal studies have confirmed that the fluoroquinolones are more effective than erythromycin [59]. Levofloxacin is more active than ofloxacin and ciprofloxacin, and it can administered orally or IV. Failures have occurred in patients receiving low-dose ciprofloxacin or ofloxacin [59,63] and high dosages (800-1000 mg/d) need to be administered for better effectiveness. The newer quinolones, e.g. moxifloxacin, levofloxacin and sparfloxacin, seem better choices than older quinolones (ciprofloxacin and ofloxacin), but clinical comparisons are lacking.

Other antimicrobials: Rifampicin is very active against extracellular and intracellular Legionella spp. In the clinical setting monotherapy is not recommended since rifampicin resistance has been thought to emerge quickly. However, rifampicin resistance did not occur in guinea pigs with Legionella pneumonia who were treated with rifampicin alone [66]. Rifampicin has been reserved for adjunctive therapy, although there are no good clinical data demonstrating the superiority of combination therapy with rifampicin. Synergism of erythromycin and rifampicin has been reported in the guinea pig model [63].

Trimethoprim-sulfamethoxazole, tetracyclines and chloramphenicol have been tested in vitro, in animal models and in the clinical setting, but they are less active than macrolides, quinolones and rifampicin [59].

Newer drugs like ketolides are currently investigated [67]. They are active against extracellular and intracellular Legionella, but clinical data are awaited.

Choice of therapy: There are a number of antimicrobial agents that are active *in vitro* and *in vivo*, concentrate intracellularly and achieve high concentrations in lung tissue. The fluoroquinolones, especially levofoxacin, have excellent activity against *L. pneumophila* but azithromycin is a good alternative. Quinolones should be used in preference to erythromycine for the treatment of patients with severe (nosocomial or community-acquired) LD and immunocompromised patients. Azithromycine can be used in patients with mild community acquired LD. Combination therapy of quinolones and azithromycine may be considered in severely ill patients who do not improve on initial therapy with a quinolone, but data supporting an additional effect are lacking.

7. Pathophysiology of LD

Phagocytosis of *L. pneumophila*

Once Legionella enters the upper respiratory tract, clearance is effected by cilia and respiratory epithelial cells. This probably explains the increased risk of LD in smokers and in persons with COPD or alcoholism, in whom mucociliary clearance is impaired.

Alveolar macrophages (AM) phagocytose *L. pneumophila* by an unusual process termed "coiling phagocytosis" [68]. When Legionella bacteria are opsonized with complement C3, phagocytosis is enhanced by binding to complement receptor (CR) 1 and 3 on human monocytes [17,69]. CR1 and CR3 recognize fragments of C3; CR1 is the primary receptor for C3b and CR3 is the primary receptor for iC3b. Phagocytosis is serum dependent and C3 binds selectively to the major outer membrane protein of *L. pneumophila* [70]. Others, have identified LPS as the surface molecule that primarily activates the classical pathway [71]. However, complement levels in the human lung are normally low and therefore it is likely that in early infection *L. pneumophila* attaches to phagocytes by another mechanism. Indeed, in the absence of complement or antibodies, the microorganism still binds to phagocytic mononuclear cells (PMC) that express Fcγ-receptors and CR1 and CR3 receptors. Furthermore, bacterial growth after complement-independent attachment (pre-incubation with monoclonal antibodies against CR1 and CR3) has been observed in guinea pig alveolar macrophages and different cell lines [72]. Thus the mechanism of binding is not yet elucidated and does not appear to influence the intracellular fate of Legionella bacteria [73].

After *L. pneumophila* has entered the phagocyte, they survive in a ribosome-lined phagosome, which does not fuse with lysosomes and therefore evades the antimicrobial defenses of mononuclear cells [17]. The bacteria multiply in this phagosome and ultimately destroy the host cell and parasite newly recruited cells. Infection of macrophages with Legionella results in rapid multiplication of the bacteria. Within 24-48 hours, there is a 100-1000 fold increase in the number of bacteria in infected cultured macrophages. Therefore, intracellular replication within macrophages and, as recently shown, within alveolar epithelial cells [74] is an important hallmark of LD. The magnitude of intracellular replication in epithelial cells and the contribution to disease are not known.

Recognition of *L. pneumophila*: the role of toll-like receptors

L. pneumophila is a gram-negative bacterium that possesses a variety of antigens usual for gram-negative bacteria, such as LPS, heat-shock proteins (Hsp60), outer membrane proteins (OmpS), flagella and fimbria. These antigens are known to stimulate immune cells to produce a number of cytokines.

Toll like receptors (TLR) are thought to be an important link between pathogen detection and the induction of innate immunity [75,76]. The TLR family consists of transmembrane proteins with leucin-rich motifs in their extracellular domain and a cytoplasmic domain which is homologous to the signaling domain of the IL-1 receptor. Expression of *TLR* has been found in monocytes/macrophages, B-cells, T-cells and dendritic cells [77]. To date, at least 10 TLR's have been identified of which TLR4 is the predominant, if not the exclusive, receptor for LPS and TLR5 selectively recognizes flagellin on gram-negative and gram-positive bacteria [78]. Activation via TLR4 has been shown to induce an intracellular signaling cascade involving the MyD88, IL-1R accessory protein kinase (IRAK), TNFR-associated factor 6 (TRAF-6) and NF-κB-inducing kinase, leading to the activation of NF-κB and subsequent transcription of NF-κB controlled genes resulting in immune activation [79,80]. Signal transfer requires several other factors such as LBP, the LPS binding protein, MD-2 and the CD14 receptor on the surface of PMC [81].

It is not known whether TLR's are involved in cellular recognition of Legionella antigens. Activation of host cells by *L. pneumophila* LPS might pass off via TLR4 resulting in NF-κB activation and the production of pro-inflammatory cytokines as has been demonstrated for several other gram-negative bacteria and mycobacteria [75,76,82]. However, LPS of *L. pneumophila* has an unique structure: the O-chain is highly hydrophobic as is the outer core oligosaccharide. The lipid A moiety consists of unusually long, branched-chain fatty acids, which could be responsible for the observed low endotoxicity. Legionella LPS is a weak inducer of pro-inflammatory cytokines by PMC *in vitro* and *in vivo* compared to enterobacterial LPS [83,84]. Moreover, LPS of Legionella does not bind to CD14 or soluble CD14, which in the presence of a secreted protein MD-2 results in activation of TLR4 [84].

Studies with *TLR*4 deficient mice (C3H/HeJ) that are hyporesponsive to LPS showed that TLR4 plays an important role in host defense mechanisms against at least some gram-negative bacteria [85,86]. The role of TLR4 in host defense against *L. pneumophila* infection is not known. It might be that LPS or other Legionella cell wall components, like flagellin, are able to activate PMC via other (toll-like) receptors. Recently, it has been shown that TLR5

recognizes bacterial flagellin from both gram-positive and gram-negative bacteria, and that activation of the receptor mobilizes the nuclear factor NF-κB [78].

Macrophage apoptosis

Apoptosis is a highly regulated process of cell death that is found in response to infections with many organisms, including *L. pneumophila*. *L. pneumophila* is capable of inducing apoptosis in macrophages and alveolar epithelial cells within a few hours after infection [87]. Apoptosis of AM could be an effective weapon of the host to kill or restrict intracellular growth of Legionella bacteria, but it could also be beneficial to the bacterium by blocking the immune functions that are critical for host defense. In a mice model with *M. tuberculosis* AM depletion led to improved clearance and survival of *M. tuberculosis* infection [88]. This is supported by observations of an inverse relationship between apoptosis induction and virulence in *M. tuberculosis* [89]. Selective depletion of activated macrophages led to impaired resistance to *M. tuberculosis*, suggesting that host-induced apoptosis is directed against non-activated macrophages (J. Leemans et al., submitted).

Legionella serogroup 1 induces the highest amount of apoptosis [90] and it has the highest cytotoxicity to macrophages compared to other serogroups. In this way, the induction of apoptosis may serve as a pathogen-encoded virulence determinant.

Resolution of *L. pneumophila*: cytokine production

Pro-inflammatory cytokines: IFN-γ, TNF-α and IL-12

Limitation of multiplication in alveolar macrophages and monocytes is the key defense mechanism against LD. In general, mice are resistant to *L. pneumophila* infection, but the A/J mouse strain is permissive for growth of Legionella bacteria. Nevertheless, mortality is only slightly higher than in other mouse strains [91]. Therefore, this is a suitable model to study the inflammatory response during Legionella infection. A/J mice have a recessive mutation on chromosome 13, called the *lgn*1 gene. Encoded in this region is the murine homolog of the gene encoding neuronal apoptosis inhibitory protein (NAIP). These genes are also expressed in macrophages [17]. A/J mouse tissue contains less NAIP RNA and protein than does resistant wild type mouse. In addition, impaired IFN-γ production by macrophages of A/J mice has also been implicated to play a role in the susceptibility of these mice [92]. In A/J mouse, the course of infection can be divided into an early phase, during which a rapid multiplication and inflammatory response can be observed, and a second phase, after 2-3 days

post-infection, with a decrease in the pulmonary bacterial count. The early inflammatory response mainly consists of influx of monocytes/macrophages, lymphocytes and NK cells [93] and these cells represent a first and effective line of defense against infection by producing cytokines and chemokines that limit the growth of bacteria in macrophages.

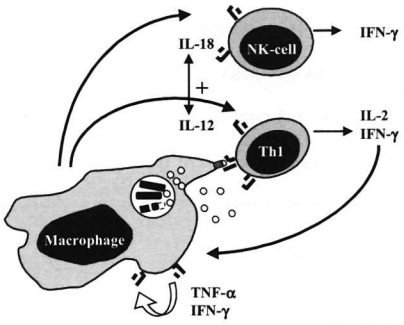

Figure 1. The immune response to *L. pneumophila*

TNF-α, mainly produced by macrophages, has been shown to enhance the bactericidal activity of macrophages and to protect against a variety of intracellular bacterial infections [94]. TNF-α is produced during replicative Legionella pneumonia and depletion of TNF-α in a rat model resulted in limited recruitment of monocytes to the lung and failure to clear Legionella bacteria from the lungs [94]. It has been shown that treatment of macrophages with r-TNF-α caused a decrease in the ability of *L. pneumophila* to replicate within these cells [95], indicating that TNF-α is crucial for resistance to Legionella infection. On the other hand, systemic high levels of TNF-α during illness are associated with adverse outcome [96].

The role of cell-mediated immunity (CMI) in *L. pneumophila* infection is known to be critical for outcome[97]. In patients with LD a relative predominance of Th1 type cytokines was found [98]. A T helper 1 (Th1) response is characterized by IFN-γ production; IFN-γ plays a critical role in activation of monocytes and alveolar macrophages in a dose and time dependent way, such that it inhibits intracellular multiplication of *L. pneumophila* (figure 2) [99-102]. IFN-γ can be produced, in low levels, by macrophages itself and the autocrine biological functions of IFN-γ on the macrophage include up-regulation of MHC class II and the activation to a non-permissive state.

IL-12 is a pivotal denominator of the balance between Th1 and Th2 lymphocyte subsets, as it drives naive T cells into a Th1 direction [103]. IL-12 is mainly produced by monocytes and polymorphonuclear cells (PMN) [97]. Mice depleted of IL-12 have significantly more Legionella bacteria in their lungs compared to control mice and a significant decrease in intrapulmonary TNF-α activity within the first 24 hours of infection [97,104]. In addition, pre-treatment of mice with anti-IL-12 and anti- IL-18 antibodies resulted in a 97% decrease of IFN-γ levels at 72 hours after infection and more bacteria were recovered from the lungs [105]. *In vitro* viable *L. pneumophila* selectively suppress IL-12 production by macrophages of A/J mice at the level of both mRNA and protein secretion by an MCP (monocyte chemotactic protein)-1 independent mechanism [106]. This may be one of the mechanisms which Legionella bacteria use to escape from innate immune responses.

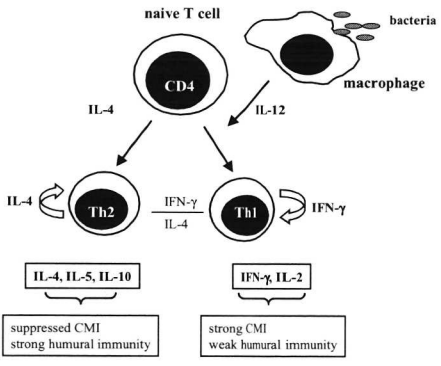

Figure 2. Model of induction of Th1 and Th2 cells. Naive T cells differentiate into Th1 or Th2 cells depending on the cytokine mileu provided by antigen oresenting cells. IL-12 promotes TH1 cells and IL-4 promotes TH2 cells. IFN-γ and IL-4 can act as autocrine growth factors and can inhibit the opposite Th subset. Th1 cells mediate the elimination of Legionella bacteria by inducing a strong CMI.

Despite the activation of macrophages by IFN-γ and TNF-α, they do not kill the intracellular bacteria, even in the presence of specific antibodies [100]. Instead, activated macrophages only restrict replication. IFN-γ activated macrophages inhibit intracellular multiplication by limiting the availability of intracellular iron [107]. This is the result of down regulation of transferrin receptor expression on monocytes and a decrease of the concentration of intracellular ferritin [108,109]. On the other hand, iron also plays a major role in phagocytic cell interactions with pathogens serving as a catalyst in the generation of toxic oxygen metabolites (respiratory burst) used in anti-microbial defense. Ferric iron reacts with hydrogen peroxide to produce hydroxyl radical (Fenton reaction) and *in vitro* this is one of the

most active oxidative metabolites against Legionella. Since activated phagocytic cells do not kill *L. pneumophila*, the respiratory burst may not play a significant role in host defense against *L. pneumophila.*

Activation of macrophages by IFN-γ also induces synthesis of nitric oxide (NO) from L-arginine [110]. NO release leads to cell damage but also to the death of many pathogens. Endogenous NO seems to play a role in IFN-γ mediated resolution of *L. pneumophila* infection in a susceptible host [111], but IFN-γ mediated inhibition in a resistant murine strain is NO-independent [102].

Anti-inflammatory cytokines: IL-10 and IL-4

The role of IL-10 and IL-4 in *L. pneumophila* infection is rather complicated. IL-10 suppresses TNF-α and IFN-γ production and macrophage activation *in vitro* [112]. IL-10 is produced by alveolar macrophages, monocytes and Th2 lymphocytes. In a BALB/c mouse model, IL-10 could not be detected in *ex vivo* splenocyte cultures after an intravenous challenge with *L. pneumophila* [113]. In A/J mice with a pulmonary Legionella infection IL-10 and IL-4 were not induced [97,113]. On the other hand, IL-4 deficient mice were found to be more susceptible (higher mortality) to Legionella infection than wild type Balb/c mice and splenocyte cultures of wild type mice demonstrated an early transient production of IL-4 (3-5 hours post-infection) [113]. IL-4 deficient mice also produce more TNF-α, IL-1β and IL-6, suggesting that IL-4 attenuates the mobilization of acute-phase cytokines during the early immune response [113]. Thus, IL-4 and IL-10 may regulate acute-phase cytokines, especially TNF-α.

Chemokines

Chemokines are a family of small chemotactic proteins that play an important role in migration to the site of infection and activation of inflammatory cells [114]. They are divided into several families based on their structural differences. The two major families are the CC and CXC chemokine families: the latter can be further distinguished by the presence or absence of an amino-acid sequence, glutamine acid-leucine-arginine (the ERL-motif). IFN-γ - inducible protein IP-10 is a non ERL-CXC chemokine which bind to the CXCR3 receptor and specifically targets T-lymphocytes and natural killer (NK) cells [115]. The production of IP-10 by various cell types is associated with diseases in which IFN-γ production is increased. IP-10 also is a part of the innate immune response to bacterial infection, probably by

attracting CXCR3 positive Th1 cells to the site of inflammation [116]. The role of IP-10 in *L. pneumophila* has not been examined thus far. The ERL-containing CXC chemokines, KC (keratonocyte-derived chemokine), MIP-2 (macrophage inflammatory protein 2) and LIX (lipopolysaccharide-induced CXC chemokine), induce neutrophil chemotaxis and stimulate neutrophil activation in inflammatory responses. Tateda et al demonstrated that blocking the receptor for ERL-positive CXC chemokines (CXCR2) dramatically sensitized mice to *L. pneumophila* and decreased the levels of IL-12 [117].

Resolution of *L. pneumophila*: cell types involved

Early recruitment of PMNs was found to be crucial for survival after pulmonary *L. pneumophila* infection in mice and determined the subsequent Th1/Th2 cytokine ratio by producing IL-12 [97,117]. Although mononuclear cells can not be activated by IFN-γ to kill intracellular *L. pneumophila,* IFN-γ and/or TNF-α can activate PMNs to kill extracellular *L. pneumophila* [118]. It is unknown whether the phagocytic function of PMN contribute to the resolution of Legionella pneumonia.

T-lymphocytes are required for the cell-mediated immune response to Legionella infection. Cytokines as IL-12 and IFN-γ promote the development of a Th1 response. Legionella antigens presented on antigen presenting cells are readily accessible to MHC class II molecules, leading to activation of $CD4^+$ T cells. Depletion of CD4+ (T helper cells) in A/J mice resulted in an increase of infection lethality [93]. This is probably the result of an impaired production of cytokines (IFN-γ) and the supportive role of T-cells on humoral immunity and specific T cell mediated immunity.

Several studies showed that delayed-type hypersensitivity and cell mediated immune reactions of lymphoid cells from either immunized animals or patients who had recovered from LD could be readily detected [119,120].

$CD8^+$ T cells (cytotoxic T-cells) recognize antigens that are processed and presented on cell surfaces in a MHC class I restricted fashion, which then bind to the T-cell. The precise role of $CD8^+$ T cells in Legionella infection is not clear, but mice depleted of $CD8^+$ T cells had an impairment of bacterial clearance from the lungs [93].

Natural killer (NK) cells probably play a dual role in *L. pneumophila* infection. In addition to killing harmful targets these cells are also important early sources of cytokines. It has been shown that NK-cells respond to *L. pneumophila* antigen stimulation *in vitro* and *in*

vivo by producing IFN-γ and by becoming activated to kill *L. pneumophila*-infected macrophage targets [121,122].

Humoral immunity probably plays a role as a second line of defense since specific antibodies are produced late in the course of the disease [123]. In addition, the immunopathological role of antibodies in Legionella infection is not clear, since antibodies promote uptake in macrophages rather than promote elimination.

In summary, studies in human and animals suggest that the host response to *L. pneumophila* contains features of both innate and CMI. Legionella bacteria are phagocytosed by alveolar macrophages and they survive in a phagosome, which does not fuse with lysosomes and therefore evades the anti-microbial defenses of mononuclear cells. The bacteria multiply in this phagosome and ultimately destroy the host cell via necrosis and apoptosis and subsequently parasite newly recruited cells. Legionella antigens induce a non-specific inflammatory response, resulting in activation of macrophages and restriction of intracellular multiplication. Th1 cells, that produce Th1 class cytokines such as IFN-γ are pivotal in resolution of Legionella pneumonia. However, besides activation of macrophages by cytokines that help to restrict intracellular multiplication, the lysis of infected macrophages by cytotoxic cells may also be an effective way of elimination of bacteria and exposing the bacteria to other bactericidal cells such as neutrophils.

8. Outline of the thesis

In March 1999 one of the largest outbreaks of Legionnaires' disease since the first described outbreak in Philadelphia [6] occurred in the Netherlands. The outbreak originated at the Westfrisian Flora, an annual flower show combined with a consumer products exhibition. A case-control study, an environmental investigation and a serological cohort study among exhibitors identified the source of the outbreak: a whirlpool spa (**Chapter 2**). The size of the outbreak provided the unique opportunity to determine which clinical factors on hospital admission predict ICU admission or mortality. We also evaluated whether the rapid urinary antigen test can help identify those patients with LD for whom adequate antibiotic therapy cannot be delayed (**Chapter 3**).

One year after the outbreak many patients still had health complaints and in **Chapter 4 and 5** we describe the persistence of symptoms, the health related quality of life and the presence of posttraumatic stress disorder in survivors of the outbreak. The persistence of pulmonary radiological and functional abnormalities was also evaluated.

The urinary antigen test combines reasonable sensitivity and high specificity with rapid test results. To assess the value of the urinary antigen test in an outbreak situation, we evaluated three urinary antigen tests with the urine specimens from the patients and determined the influence of clinical severity of disease on test sensitivity (**Chapter 6**).

Predisposing factors that increase susceptibility to *L. pneumophila* infection have been identified earlier but impairments in cytokine mediated immune responses leading to increased susceptibility have not yet been studied. We measured *in vitro* cytokine production after whole blood stimulation with aspecific and specific stimuli in patients recovered from LD and in controls (**Chapter 7**). *L. pneumophila* has a definite requirement for iron and the protective role of IFN-γ is at least partly mediated via limitation of intracellular iron. In **Chapter 8** we determined whether serum iron status, haptoglobine phenotype and mutations in the haemochromatosis gene influenced susceptibility for and outcome in LD patients compared to two control groups.

An effective host defense requires the detection of foreign pathogens and the Toll-like receptors have been identified as the link between pathogens and the mobilization of innate immune responses. We used a mouse model to determine the role of TLR4 in the host defense against *L. pneumophila* infection (**Chapter 9**). Legionella bacteria are intracellular pathogens that have macrophages as their primary host cell. However, macrophages are also the first line of defense. This raises questions as to the exact role of macrophages and macrophage

apoptosis during Legionella infection. In **Chapter 10** we studied in a mouse model the role of alveolar macrophages and apoptosis of these cells in the pulmonary host response to infection. All findings are discussed in **Chapter 11**.

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