NOTE TO USERS

Positional cloning of the *Legionella pneumophila*-resistance gene *Lgn1*

Eduardo Diez

Department of Biochemistry
McGill University
Montreal, Quebec, Canada

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Abstract

Legionella pneumophila is an intracellular bacterium that causes an acute form of pneumonia called Legionnaires' disease. Segregation analyses using macrophages from susceptible and resistant inbred mice previously indicated that a single genetic locus, named *Lgn1*, could determine permissiveness to intracellular replication of *L. pneumophila*. A positional cloning strategy was undertaken, which makes use of genetic and molecular biology techniques to identify the gene responsible for a particular phenotype, based mostly on its location within a chromosome. The work described in this thesis covers three aspects of *Lgn1*: (1) Building upon the work of others, the *Lgn1* genetic interval was narrowed to 0.32 cM within distal mouse chromosome 13. The corresponding 140 Kb *Lgn1* physical interval contains only two known transcripts: the Neuronal Apoptosis Inhibitor Protein (*Naip*) genes *Naip2* and *Naip5*. (2) The expression profile of the *Lgn1* candidates was investigated both at the mRNA and protein levels. Expression of both *Naip2* and *Naip5* in mouse macrophages strengthened their candidacy for the *Lgn1* locus. (3) Transfer of BAC clones from the critical interval into transgenic mice was successfully used to functionally complement the *Lgn1* susceptibility phenotype of A/J mice with cloned DNA from non-permissive 129X1 or C57BL/6J origins. Two independent rescuing BAC clones were identified, with a 56-Kb overlap where the entire *Lgn1* transcript must lie. The only known full-length transcript coded in this reduced genomic region is *Naip5*.

Thus, in our last publication we have proposed that *Naip5* (recently named *Birc1e*) is the gene within the *Lgn1* locus responsible for differential permissiveness to intracellular *L. pneumophila* replication in mice.

Résumé

La bactérie intracellulaire *Legionella pneumophila* peut causer chez l'humain une forme aigüe de pneumonie communément appelée "Maladie des légionnaires". Des analyses de ségrégation en utilisant des macrophages de souris innées susceptibles et résistantes ont indiqué un facteur génétique simple, appelé *Lgn1*, qui peut déterminer la permissivité à la réplication intracellulaire de *L. pneumophila*. Une stratégie de clonage positionel a été entreprise où des techniques de biologie moléculaire et de génétique ont été utilisées pour identifier le gène responsable du phénotype en question d'après son emplacement chromosomal. Le travail décrit dans cette thèse couvre trois aspects de *Lgn1*: (1) Donnant suite au travail entamé par d'autres, l'intervalle génétique *Lgn1* a été réduit à 0.32 cM dans la partie distale du chromosome 13 chez la souris. L'intervalle physique correspondant, de 140 Kb, contient seulement deux unités de transcription connues: les gènes Neuronal Apoptosis Inhibitor Protein (*Naip2* et *Naip5*). (2) Le profil d'expression des gènes candidats pour *Lgn1* a été étudié aux niveaux de l'ARN messager et de la protéine. L'expression de *Naip2* et de *Naip5* dans des macrophages de souris a renforcé leur candidature pour *Lgn1*. (3) Le transfert de clones génomiques (BAC) de l'intervalle *Lgn1* dans des souris transgéniques a été employé avec succès pour compléter fonctionellement le phénotype de susceptibilité des souris A/J avec de l'ADN provenant de souris résistantes telles 129X1 ou C57BL/6J. Deux clones genomiques indépendants ont été identifiés, capables de renverser le phenotype de susceptibilité à *Legionella*, avec un chevauchement de 56 Kb où l'unité de transcription *Lgn1* entière devait se trouver. La seule transcription intégrale connue codée dans cette région genomique réduite est *Naip5*.

Ainsi, nous avons proposé que *Naip5* (également appelé *Birc1e*) soit le gène correspondant à *Lgn1*, responsable de la permissivité différentielle à la réplication intracellulaire de *L. pneumophila* chez la souris.

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Preface

The work described in Chapters 2, 3 and 4 of this thesis have been published as follows:

Chapter 2. **Diez, E.**, Beckers, M. C., Ernst, E., DiDonato, C. J., Simard, L. R., Morissette, C., Gervais, F., Yoshida, S. I., and Gros, P. (1997). Genetic and physical mapping of the mouse host resistance locus *Lgn1*. Mamm Genome *8*, 682-685, © Springer. Reproduced here by permission from the publisher.

Chapter 3. **Diez, E.**, Yaraghi, Z., MacKenzie, A., and Gros, P. (2000). The neuronal apoptosis inhibitory protein (Naip) is expressed in macrophages and is modulated after phagocytosis and during intracellular infection with *Legionella pneumophila*. J Immunol *164*, 1470-1477, © The American Association of Immunologists, Inc. Reproduced here by permission from the publisher.

Chapter 4. **Diez, E.**, Lee, S. H., Gauthier, S., Yaraghi, Z., Tremblay, M., Vidal, S., Gros, P. (2003). *Birc1e* is the gene within the *Lgn1* locus associated with resistance to *Legionella pneumophila*. Nat Genet *33*, 55-60, © Nature Publishing Group. Reproduced here in an expanded form by permission from Nature Publishing Group (the publisher) and Philippe Gros (the corresponding author).

Contribution of co-authors

Chapter 2:

Shin-Ichi Yoshida designed the phenotyping assay for macrophage permissiveness to *L. pneumophila* replication and generated and phenotyped the first 182 animals of the ABA backcross panel. Céline Morissette and Francine Gervais performed most of the phenotyping of the backcross animals. Marie-Claire Beckers and Eric Ernst had previously defined the *Lgn1* genetic interval at 1.2 cM between markers *D13Mit146* and

D13Mit70. Christine J. DiDonato and Louise R. Simard provided the *E. coli* strains harboring BAC clones from the *Smn-Naip* genomic region.
I genotyped the ABA backcross panel with new genetic markers and performed segregation data analysis using Map Manager QT. I screened YAC library DNA pools by PCR, prepared intact high-molecular weight YAC DNA on agarose blocks, and sized the YAC clones by pulse-field gel electrophoresis and Southern blotting. I performed BAC DNA preparations and sized the clones by pulse-field gel electrophoresis. I determined the STS-content of all YAC and BAC clones by PCR and Southern blotting. I prepared the published manuscript with help and advice from my supervisor Dr. Philippe Gros.

Chapter 3:
The work described is essentially my own. Zari Yaraghi and Alex MacKenzie provided me with Naip antibody and *Naip1* cDNA. I prepared hybridization probes, performed RNA extractions, Northern hybridizations and RT-PCRs. I cultured cell lines, performed all cell line and macrophage protein preparations and all the Western blotting and semi-quantitative analyses shown. I performed all steps towards the isolation of mouse inflammatory peritoneal macrophages and cultured them. I performed all *L. pneumophila in vitro* infections and all non-specific phagocytosis experiments. I prepared the published manuscript with help and supervision from Dr. Philippe Gros.

Chapter 4:
The work described is largely my own. I identified BAC clones of interest and planned the breeding scheme of transgenic mice following advice from Drs. Philippe Gros and Michel Tremblay. Zari Yaraghi provided me with the *E. coli* strain harboring BAC clone 111p22. Seung-Hwan Lee and Silvia Vidal prepared microinjection-quality BAC DNA. Michel Tremblay supervised the generation of the BAC transgenic founder mice as a core service from the McGill Mouse Genetics Group. I coordinated the expansion and maintenance of the BAC transgenic mouse colony, which was performed mostly by Susan Gauthier. I performed all genomic DNA preparations, genotyping, and PCR- and Southern blotting-based STS-content analyses. I performed all the *L. pneumophila* permissiveness phenotyping. I performed the assays (RT-PCR followed by diagnostic

digests and/or subcloning and sequencing) for detection of C57BL/6J-derived *Birc1e* mRNA in BAC 164d12 transgenic mice, and Seung-Hwan Lee performed the assay (RT-PCR followed by diagnostic restriction digests) for detection of 129X1-derived *Birc1e* mRNA in BAC 227n6 transgenics. I prepared the published manuscript under the supervision of Dr. Philippe Gros.

Philippe Gros provided expert supervision and advice throughout the course of all studies.

Publications

Diez, E., Lee, S. H., Gauthier, S., Yaraghi, Z., Tremblay, M., Vidal, S., Gros, P. (2003). Birc1e is the gene within the Lgn1 locus associated with resistance to Legionella pneumophila. Nat Genet *33*, 55-60.

Fortin, A., **Diez, E.**, Rochefort, D., Laroche, L., Malo, D., Rouleau, G. A., Gros, P., Skamene, E. (2001). Recombinant congenic strains derived from A/J and C57BL/6J: a tool for genetic dissection of complex traits. Genomics *74*, 21-35.

Diez, E., Yaraghi, Z., MacKenzie, A., and Gros, P. (2000). The neuronal apoptosis inhibitory protein (Naip) is expressed in macrophages and is modulated after phagocytosis and during intracellular infection with Legionella pneumophila. J Immunol *164*, 1470-1477.

Yaraghi, Z., **Diez, E.**, Gros, P., and MacKenzie, A. (1999). cDNA cloning and the 5'genomic organization of Naip2, a candidate gene for murine Legionella resistance. Mamm Genome *10*, 761-763.

Diez, E., Beckers, M. C., Ernst, E., DiDonato, C. J., Simard, L. R., Morissette, C., Gervais, F., Yoshida, S. I., and Gros, P. (1997). Genetic and physical mapping of the mouse host resistance locus Lgn1. Mamm Genome *8*, 682-685.

Beckers, M. C., Ernst, E., **Diez, E.**, Morissette, C., Gervais, F., Hunter, K., Housman, D., Yoshida, S., Skamene, E., and Gros, P. (1997). High-resolution linkage map of mouse chromosome 13 in the vicinity of the host resistance locus Lgn1. Genomics *39*, 254-263.

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Special thanks go to Eddy Pereira for recommending to me the laboratory of Philippe Gros for my graduate studies and for putting out a good word for me. I want to thank past and present members of the laboratory who either helped me directly with my work or who simply made my days pleasant. In particular, Marie-Claire Beckers, who accepted my help with enthusiasm and really trusted my work during my first summer in the laboratory. I have interacted with so many wonderful people in the department, in affiliated institutes, and even in other cities: so little space to thank them all. I just hope that our paths will keep crossing in the future.

Without the support and love of my parents, without their sacrifices and exemplary hard work, I would not be a healthy, happy man writing a thesis and enjoying a feeling of intellectual achievement. ¡Grácias papá y mamá!

My final acknowledgement is for Isabelle. My laboratory colleague, my wife, my love. I thank her for her everyday understanding and support, and for the freshness and joy she brings to my life. She helped me greatly with the writing of this thesis. She not only provided important editorial help, she supported me financially and emotionally all throughout. She deserves my heartfelt gratitude.

Objectives of the Presented Work

Just before the start of this thesis project in 1996, the genetic locus controlling mouse macrophage permissiveness to *L. pneumophila* replication (*Lgn1*) had been localized within distal Chromosome 13 (Beckers et al. 1995). This was followed by extensive segregation analyses that effectively narrowed the *Lgn1* genetic interval to 1.2 cM (Beckers et al. 1997). However, the 17 recombinants that segregated the *Lgn1* locus from the closest distal marker (*D13Mit70*) indicated that the resolution power of the 1270-animal informative backcross used had not been fully exploited yet. With very little knowledge about the gene content of the potentially large genomic region, it was still not possible to propose candidates for the *Lgn1* gene.

The first objective of this thesis project was therefore to study the segregation pattern of additional genetic markers susceptible of narrowing the existing genetic interval (work described in Chapter 2).

After the genetic interval had been refined, it was necessary to identify and characterize cloned genomic DNA segments covering the entire *Lgn1* interval (Chapter 2). These genomic clones would thereafter be used to fulfill several objectives: to translate the genetic interval into a physical entity with a size that could be measured in number of nucleotides, to order co-segregating genetic markers with respect to each other, to reveal the genomic structure of the region (repeats), and eventually, to perform functional complementation studies using discrete portions of the *Lgn1* candidate region (Chapter 4).

Independent research aimed at identifying the gene responsible for Spinal Muscular Atrophy (SMA) in humans pointed out that the mouse *Lgn1* chromosomal region is syntenic with the human SMA candidate region that contained two known genes. In order for these genes to be considered as candidates for the *Lgn1* locus, they should be expressed within the cells that display the *L. pneumophila*-permissiveness phenotype. One important objective of my thesis work was therefore to characterize the mRNA and protein expression of candidate genes within mouse macrophages (Chapter 3).

The overall objective of this thesis project, encompasing all of the above, has been to identify the gene underlying the *Lgn1* phenotype.

Chapter 1

Introduction and Literature Review

Section 1.1

Legionella pneumophila bacterium

The research described throughout this thesis revolves around host resistance to a specific bacterium: *Legionella pneumophila*. The aim of this first section is to paint an overall portrait of this recently identified Prokaryote.

1.1.1 Microbiology

Legionella pneumophila is an aerobic, Gram-negative bacterium (McDade et al. 1977; Brenner et al. 1979). Structurally, *Legionella* cells are thin bacilli (0.3-0.9 × 2.0->20.0 μm), they possess pili and can be motile by means of a single, polar flagellum (Chandler et al. 1980). Long, filamentous forms develop under certain growth conditions, such as on agar surfaces (Katz et al. 1984). *Legionella* cells exhibit a bluish-white autofluorescence and can produce a diffusible brown pigment on tyrosine-containing media (Vickers and Yu 1984). *Legionella* are chemoorganotrophic, using amino acids (non-fermentatively) as carbon and energy sources; carbohydrates are generally not metabolized (Warren and Miller 1979; George et al. 1980). Some enzymatic characteristics of *Legionella* include presence of catalase activity, lack of urease activity and inability to reduce nitrates (Singleton and Sainsbury 1987).

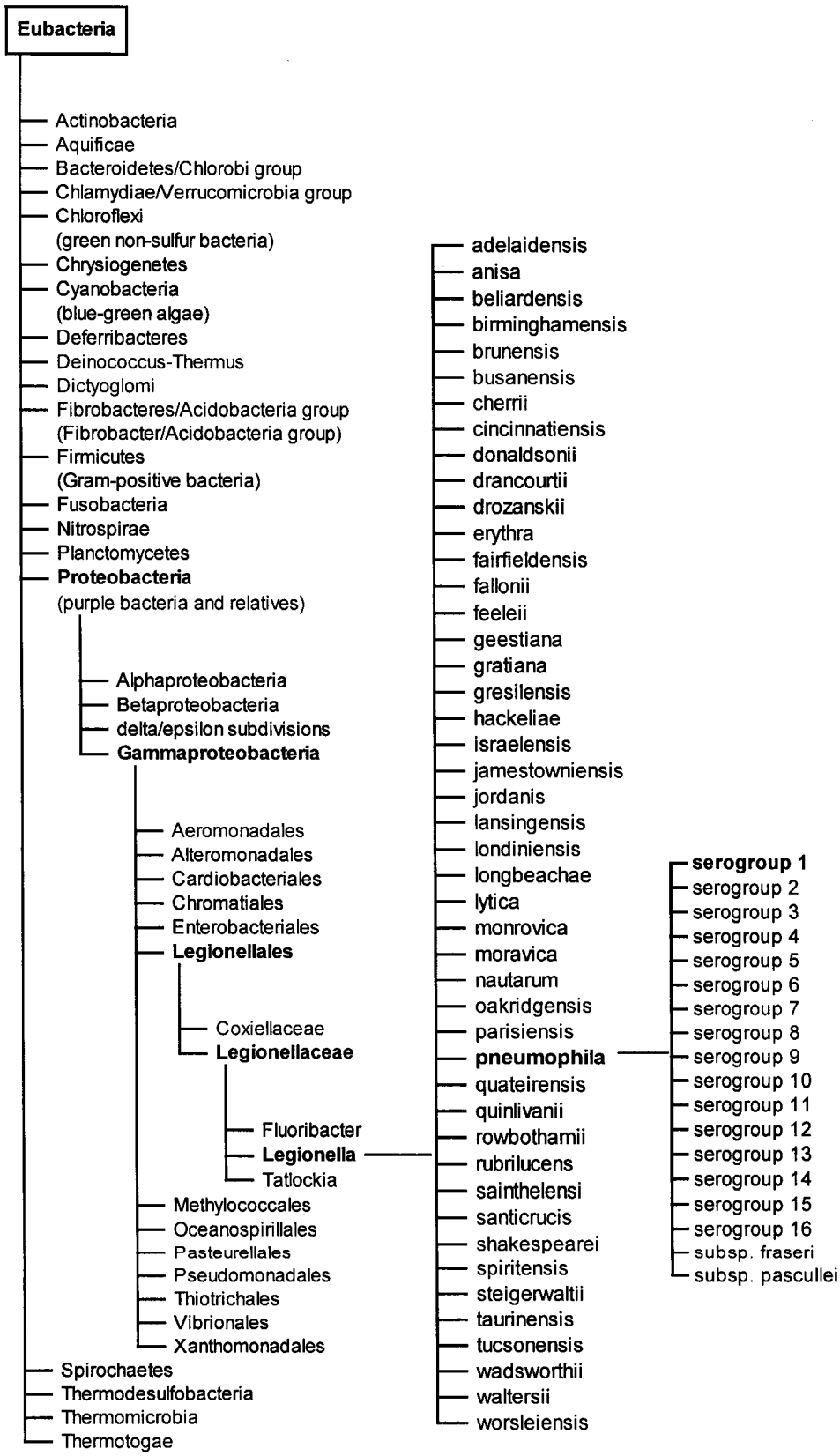
The pathogenic potential of *Legionella* species resides in their ability to replicate within a host cell (pathogenesis will be reviewed later). But *Legionella* are "facultative" intracellular pathogens since host-independent replication can be achieved when specific nutritional requirements are met (Warren and Miller 1979). L-cysteine and ferric iron (Fe^{3+}) are essential for *Legionella* growth. Other compounds can be added for optimal growth, making Buffered Charcoal Yeast Extract (BCYE) the media of choice when supplemented with L-cysteine and ferric iron (Feeley et al. 1979). The optimum replication temperature is 35-37°C. The usual tissue culture media, which are adequate to support the growth of human and animal cells, cannot support the growth of *Legionella* cells (Holden et al. 1984; Yoshida and Mizuguchi 1986).

Figure 1.1 depicts *Legionella pneumophila* within the current taxonomic trees. The discovery of this bacterium led to the creation, in 1979, of a new family (Legionellaceae) and order (Legionellales) within the Gammaproteobacteria (Brenner et al. 1979). The genus *Legionella* now has over 45 species, defined mainly by studies of DNA homology. Immunologic diversity within species is reflected in the creation of serogroups (Fig. 1.1). *Legionella pneumophila* holds the record, with over 16 distinct serologic types. The lipopolysaccharide is the major serogroup-specific antigen, which

FIGURE 1.1

How is *L. pneumophila* related to other bacteria.

This is a current taxonomic tree constructed from data accessible at the taxonomy browser of the National Center for Biotechnology Information (NCBI) web site (www.ncbi.nlm.nih.gov/Taxonomy/Browser). Only the taxonomic tree branches leading to the specific bacterium used throughout this research (*L. pneumophila* serogroup 1*)* have been expanded. The name of such branches is shown in bold fonts.


Eubacteria
Actinobacteria
Aquificae
Bacteroidetes/Chlorobi group
Chlamydiae/Verrucomicrobia group
Chloroflexi
(green non-sulfur bacteria)
Chrysiogenetes
Cyanobacteria
(blue-green algae)
Deferribacteres
Deinococcus-Thermus
Dictyoglomi
Fibrobacteres/Acidobacteria group
(Fibrobacter/Acidobacteria group)
Firmicutes
(Gram-positive bacteria)
Fusobacteria
Nitrospirae
Planctomycetes
Proteobacteria
(purple bacteria and relatives)
Alphaproteobacteria
Betaproteobacteria
delta/epsilon subdivisions
Gammaproteobacteria
Aeromonadales
Alteromonadales
Cardiobacteriales
Chromatiales
Enterobacteriales
Legionellales
Coxiellaceae
Legionellaceae
Fluoribacter
Legionella
Tatlockia
Methylococcales
Oceanospirillales
Pasteurellales
Pseudomonadales
Thiotrichales
Vibrionales
Xanthomonadales
Spirochaetes
Thermodesulfobacteria
Thermomicrobia
Thermotogae
adelaidensis
anisa
beliardensis
birminghamensis
brunensis
busanensis
cherrii
cincinnatiensis
donaldsonii
drancourtii
drozanskii
erythra
fairfieldensis
fallonii
feeleii
geestiana
gratiana
gresilensis
hackeliae
israelensis
jamestowniensis
jordanis
lansingensis
londiniensis
longbeachae
lytica
monrovica
moravica
nautarum
oakridgensis
parisiensis
pneumophila
quateirensis
quinlivanii
rowbothamii
rubrilucens
sainthelensi
santicrucis
shakespearei
spiritensis
steigerwaltii
taurinensis
tucsonensis
wadsworthii
waltersii
worsleiensis
serogroup 1
serogroup 2
serogroup 3
serogroup 4
serogroup 5
serogroup 6
serogroup 7
serogroup 8
serogroup 9
serogroup 10
serogroup 11
serogroup 12
serogroup 13
serogroup 14
serogroup 15
serogroup 16
subsp. fraseri
subsp. pascullei

can be analyzed using panels of monoclonal antibodies (Brenner et al. 1979; Brenner 1987). The division of the family Legionellaceae into three genera (*Fluoribacter*, *Legionella* and *Tatlockia*; Fig. 1.1) has been controversial. There are indeed biochemical and immunologic characteristics that can distinguish the genera (Vickers et al. 1981; Lema and Brown 1983; Fox et al. 1984). But recent 16S rRNA homology studies suggest that the segregation of the species into three genera is arbitrary and does not reflect their evolution (Brenner 1987; Fry et al. 1991). It is now common to find literature where the species *F. bozemanii*, *F. dumoffii*, *F. gormanii* or *T. micdadei* are identified as *Legionella* (Gao et al. 1999; Gerhardt et al. 2000; Flieger et al. 2001; Ogawa et al. 2001).

1.1.2 Ecology

Legionella pneumophila is a ubiquitous bacterium in natural and man-made aquatic environments (Tobin et al. 1980; Orrison et al. 1981; reviewed by Winn 1988). It is within aquatic biofilms that *Legionella* proliferates. *L. pneumophila* has been shown to replicate within protozoa from genera as evolutionarily distant as *Tetrahymena* ciliates (*T. pyriformis*, *T. thermophila*) (Fields et al. 1984; Kikuhara et al. 1994), *Hartmannella* amoebae (*H. vermiformis*) (King et al. 1991), *Acanthamoeba* (*A. polyphaga*, *A. castellanii*) (Holden et al. 1984; Kilvington and Price 1990), and *Dictyostelium* slime molds (*D. discoideum*) (Hagele et al. 2000). Except for specific laboratory media that can support extracellular growth, *Legionella* needs protozoan hosts to replicate (Holden et al. 1984). Protozoa do not only provide nutrients for the intracellular legionellae, but also represent a shelter when environmental conditions become unfavorable. Particularly inside *Acanthamoeba* cysts the bacteria are able to survive high temperatures, disinfecting procedures and drying (Kilvington and Price 1990). The highest numbers of *Legionella* are usually found in water samples with temperatures of 30-40°C (Fliermans 1983). Elevated temperature, inorganic and organic contents of the water and the presence of host protozoa thus play important roles in *Legionella* growth and spread. The concerted influence of these factors may explain why *Legionella* increases in density in artificial habitats such as man-made warm water systems (Fliermans 1983). Since *Legionella* is

ubiquitous in aquatic habitats, it seems difficult to prevent it from entering man-made water systems. Human infection can occur through inhalation of contaminated aerosols which can be produced by air conditioning systems, cooling towers, whirlpools, spas, fountains, ice machines, vegetable misters, dental devices and showerheads. In addition, the presence of dead-end loops, stagnation in plumbing systems and periods of non-use or construction have been shown to be technical risk factors (Ciesielski et al. 1984; Mermel et al. 1995). The material of the piping has also been shown to influence the occurrence of high bacterial concentrations. The use of copper as plumbing material may support lower numbers of *L. pneumophila* than plastic materials (Rogers et al. 1994). As an illustration of the everyday close contact of humans with *Legionella* species, we may quote an early study that took place in our own province. This study consisted in randomly collecting one hundred environmental water samples in the Quebec City area followed by direct immunofluorescent staining (DFA) for the presence of Legionellaceae. Forty three of the 100 samples were positive for Legionellaceae and 27 of those contained more than one serogroup and (or) species of Legionellaceae. *Legionella pneumophila* (serogroups 1 to 6) was the most frequent species seen by DFA (Joly et al. 1984). These results as well as those from similar studies (Ciesielski et al. 1984; Mermel et al. 1995; Patterson et al. 1997) clearly show that Legionellaceae can be frequent members of the freshwater microbial flora of a city. Hospitals are not spared from *L. pneumophila* contamination and this pathogen is widely recognized as a major etiological agent of nosocomial (hospital-acquired) pneumonia (reviewed by Kirby et al. 1980; Tobin et al. 1980; Neill et al. 1985).

The intracellular life cycle of *L. pneumophila* within protozoa is very similar to the one observed within mammalian macrophages (described in the next section). Therefore, it has been suggested that the interaction with protozoa is the driving force in the evolution of the pathogenicity of *Legionella* (Segal and Shuman 1999a; reviewed by Cianciotto 2001). It is also for this reason that *L. pneumophila* is considered to be an opportunistic human pathogen, or even an "aquatic microbe gone astray" (Steinert et al. 2002).

1.1.3 Legionnaire's Disease

Although *Legionella* is ubiquitous in the environment, its discovery (and the recognition of its importance as a human pathogen) had to wait until 1976, when a mysterious epidemic of pneumonia struck attendees of the Pennsylvania American Legion convention in Philadelphia. Out of 182 cases, 29 were fatal (Fraser et al. 1977). The disease was dubbed Legionnaire's disease by the press. Within six months, thanks to the efforts of many investigators from Pennsylvania and the Centers for Disease Control and Prevention in Atlanta, a bacterium had been isolated and definitively established as the agent (McDade et al. 1977). This bacterium was subsequently named *Legionella pneumophila* (Brenner et al. 1979). Retrospective studies have shown *Legionella* antigens in stored clinical samples from patients in previously unsolved outbreaks of respiratory disease as far back as 1943 (reviewed by Schurmann et al. 1988; Winn 1988). A general term for disease produced by *Legionella* species is legionellosis.

1.1.3.1 Symptoms and Diagnosis

Legionnaire's disease is the pneumonic form of legionellosis with an incubation time of 2-10 days. Legionnaire's pneumonia begins with a mild cough, malaise, muscle aches, low fever and gastrointestinal symptoms. The later manifestations of disease are high fever and a consolidating pneumonia which primarily involves the alveoli and terminal bronchioles; an intra-alveolar exudate is characteristic of the disease. Considerable lung damage with patchy infiltrated regions can be observed by X-ray radiography (reviewed by Winn 1988). Mortality rates may be high, particularly in immunocompromised individuals (Edelstein and Meyer 1984). Histological reports describe intra- and extracellular bacteria in phagocytes, fibroblasts and epithelial cells (reviewed by Fields 1996). Colonization and intracellular multiplication of the bacterium within alveolar macrophages correlates with the ability to cause disease (Cianciotto et al. 1989a).

Given the large number of pathogens able to inflict disease with very similar pneumonic symptoms, etiologic diagnosis of pneumonia on clinical grounds alone is almost impossible (reviewed by Steinert et al. 2002). Table 1.1 lists some of the organisms capable of causing pneumonia in man. The three organisms listed on the left column are responsible for approximately 60% of all pneumonias and are thus listed as "typical" agents (Singleton and Sainsbury 1987; Johnson et al. 2002). Of major concern is the fact that often, pneumonias are prescribed therapies adapted to the typical agents and that many of the atypical agents are not considered in initial diagnosis (Fass 1993). It is for this reason that although there are antibiotic compounds highly effective for treating legionellosis, the mortality rates remain high (reviewed by Sabria and Yu 2002).

Definitive diagnosis of Legionnaires' disease can be established through culture of the microorganism. Sputum should be examined for a predominant organism in any patient suspected to have a bacterial pneumonia; blood and pleural fluid (if present) should be cultured. However, *Legionella* does not grow in the standard bacteriological media used in most hospitals, and specialized selective media are needed. Unfortunately, in most hospitals, such media are not routinely used for patients with pneumonia (Fiore et al. 1999). For optimum culture of *Legionellae* in respiratory tract specimens, multiple media are required, including BCYE-alpha supplemented with antimicrobial agents (Stout and Yu 1997; Muder et al. 2000). The addition of dyes facilitates the visualization of the colonies, and pretreatment with acid or heat prevents overgrowth of competing bacterial microflora. The sensitivity of culture with multiple media and pretreatment has been calculated to be about 80% and specificity is presumed to be 100% (Ta et al. 1995; Leoni and Legnani 2001). The isolation of *Legionellae* also allows microbiological classification and subtyping by DNA studies to establish epidemiological links to water sources.

Table 1.1 Partial list of etiological agents of pneumonia in humans.

Typical agents	**Atypical agents**
Staphylococcus aureus	*Pseudomonas aeruginosa*
Streptococcus pneumonia	*Chlamydia pneumonia*
Haemophilus influenza	*Mycoplasma pneumonia*
	Legionella pneumophila
	Coxiella burnetii
	Klebsiella spp.
	Proteus spp.
	Mycobacterium spp.
	Yersinia spp.
	Viruses
	Fungi

Detection by urinary antigen has become the most widely used test for diagnosis of Legionnaires' disease (Wever et al. 2000; Formica et al. 2001). The urinary antigen appears early in the course of the disease and usually disappears within 2 months, although its excretion may be longer in patients receiving immunosuppressive treatment or corticosteroids (Sopena et al. 1999). Concentration of the urine specimen increases the sensitivity of the test (Dominguez et al. 1996). The major limitation of urinary antigen test is that it only detects the soluble antigen of *L. pneumophila* serogroup 1. Although serogroup 1 causes 92% of the cases of Legionnaires' disease in the community (Yu et al. 2002), the incidence drops to 80% in the hospital setting. The sensitivity and specificity of commercial kits for *L. pneumophila* serogroup 1 are about 70% and 99%, respectively (Binax, Portland, USA; Biotest AG, Dreieich, Germany; and Bartels, Washington, USA). A rapid immunochromatographic assay (Binax Now Legionella Urinary Antigen, Portland, USA) is now commercially available (Wever et al. 2000). The sensitivity and specificity of this test are similar to those obtained with ELISA (Dominguez et al. 1999), but it is more rapid than the ELISA test (15 minutes versus 2–3 hours) making it especially useful for small laboratories. Other rapid diagnostic tests, such as fluorescent-antibody tests have been developed for *Legionella*. Direct immunofluorescence (DFA) allows visualization of the microorganism in a specimen. But large numbers of *Legionellae* must be present before they can be readily visualized (Stout and Yu 1997).

Seroconversion is defined as an increase in antibody titers to *Legionella* of greater than or equal to fourfold. Maximum sensitivity of both IgG and IgM antibody seroconversion occurs at 90 days, convalescent serum samples drawn at 4–6 weeks may therefore give insignificant titers. Serological tests are useful for epidemiological studies but have limited utility in clinical practice (reviewed by Sabria and Yu 2002).

Molecular subtyping has proved useful in delineating the source of Legionnaires' disease. Techniques include monoclonal antibody typing, plasmid analysis, outer-membrane protein profiling, SfiI-macrorestriction analysis, amplified fragment length polymorphism (AFLP), and arbitrarily primed PCR (Jonas et al. 2000). Additionally, gas chromatographic mass spectrometry based on the unique 3-hydroxy and 2,3-dihydroxy fatty acids of the Legionella LPS has been described for complex microbial consortia (Walker et al. 1993). Fluorescence in situ hybridization (FISH) using probes targeting

regions of the 16S rRNA molecule, has been reported to be a valuable diagnostic tool for rapid and specific detection (Grimm et al. 1998; Grimm et al. 2001). This method allows detection of the bacteria without the need of cultivation. Therefore, this timesaving method also makes it possible to detect viable but non-culturable (VBNC) legionellae, which represent a large portion of the total Legionella population and may constitute an unrecognized reservoir for disease (Steinert et al. 1997). Since FISH can also be used to detect the protozoa hosts, it is expected that this method will improve the knowledge of the conditions that are conducive to *Legionella* growth (Grimm et al. 2001).

There is a benign flu-like form of legionellosis called Pontiac fever. It is a clinically distinct, self-limited and non-pneumonic disease (Glick et al. 1978). Pontiac fever patients seroconvert to *Legionella* (Kaufmann et al. 1981), however the microbe has never been isolated. It has been speculated that Pontiac fever is caused by VBNC forms of *Legionella* (Steinert et al. 1997). Other hypotheses to explain Pontiac fever include toxic or hypersensitivity reactions (Rowbotham 1986).

1.1.3.2 Epidemiology

The investigation of a number of epidemic and sporadic cases has shown that *L. pneumophila* is in fact a common cause of both community-acquired and nosocomial (hospital-acquired) pneumonia (Broome 1983).

The worst recorded outbreak of legionellosis occurred in the city of Murcia, Spain, in June 2001. Within a period of two weeks, 745 cases of pneumonia were reported; of which 315 were confirmed as Legionnaires' disease by the presence of *Legionella* antigen in urine (Navarro et al. 2001). Fortunately, only one person died, which is in sharp contrast with the previous worst outbreak: in February 1999, at the Westfriese Flora Show in the Netherlands, 231 people became ill and 21 died (Wijgergans 1999). Outbreaks of legionellosis make news headlines, but usually less than 5% of the community-acquired Legionnaires' Disease cases are due to large outbreaks (Marston et al. 1994). The most common form of legionellosis is sporadic Legionnaires' Disease, which often escapes diagnosis because of the difficulty in distinguishing this

disease from other forms of pneumonia and influenza. In the United States, it is estimated that legionellosis affects at least 20 000 persons annually (Marston et al. 1994). These high estimates are supported by serologic surveys, which show that many persons in an apparently healthy population often have antibodies against *Legionellae* (Foy et al. 1979). Current data, from a series of studies from North America and Western Europe, indicates that up to 15% of all community-acquired pneumonias that require hospitalization is associated with this pathogen (Muder et al. 1989; Marston et al. 1994).

Nosocomial legionellosis is often more severe, and its incidence more dramatic. According to data from the passive surveillance system of the Centers for Disease Control and prevention, 23% of the legionellosis cases reported from 1980 to 1989 may have been nosocomial (Broome 1983).

Epidemiological studies of Legionnaires' disease indicate that a robust immune response is sufficient to clear *L. pneumophila* infections (Fraser et al. 1977; reviewed by Stout and Yu 1997). For example, the hotel employees on duty during the 1976 Legionnaires' convention generally were seropositive for *L. pneumophila* antibodies, but asymptomatic (Fraser et al. 1977). Typically, those who become ill are of advanced age and have sustained damage to the host defenses that normally protect lungs from infection (Winn and Myerowitz 1981; Marston et al. 1994). Some of the most common risk factors for legionellosis are cigarette smoking, emphysema or other chronic lung diseases, lung and hematologic malignancies, and clinical immunosuppression or cytotoxic chemotherapy (Marston et al. 1994). Thus, *L. pneumophila* is a classic opportunistic pathogen. The case-mortality rate of Legionnaires' disease varies from 7% to 24% in the general population (Fliermans 1996). In nosocomial cases, the consequences of legionellosis are grave; fatality rates can approach 50% (Broome 1983). The observed differences in host susceptibility and bacterial virulence make it difficult to clearly define an infectious dose.

Among more than 45 species of the genus *Legionella*, over 90% of the isolates associated with Legionnaires' disease are *L. pneumophila* (Muder et al. 1989; Marston et al. 1994). Consequently, laboratory studies of *Legionella* pathogenesis have focused primarily on *L. pneumophila*. More specifically, *L. pneumophila* serogroup 1 was identified in 71.5% of legionellosis cases from 1980 to 1989 (Marston et al. 1994). This

particular serogroup is therefore very important not only historically for being the etiologic agent of the 1976 outbreak in Philadelphia, but also epidemically. It is for these reasons that *Legionella pneumophila* serogroup 1 was used throughout our own experiments in the quest to understand host resistance to *Legionella*.

Person-to-person transmission has never been observed. Thus, infection of the human lung represents a dead-end in the life cycle of *L. pneumophila*. Unlike many other human respiratory pathogens, the capacity of *L. pneumophila* to establish infection within the lung seems to be the consequence of selective pressure applied exclusively by its natural host: protozoa (review by Swanson and Hammer 2000).

1.1.3.3 Treatment and Prevention

Erythromycin has been the best antibiotic available against *L. pneumophila* for many years (Kirby et al. 1980; Muder et al. 1989; Johnson et al. 2002). However, erythromycin is no longer favored given its low solubility, the relatively high incidence of gastrointestinal side-effects as well as recorded cases of disease recurrence (Edelstein and Edelstein 1989; Sabria and Yu 2002). Moreover, time to apyrexia (recovery from fever) was longer and clinical complications more frequent for patients with Legionnaires' disease treated with erythromycin than in those treated with fluoroquinolones (Fass 1993; Edelstein 1995a). The newer macrolides (azithromycin, and particularly clarithromycin and roxithromycin) as well as fluoroquinolones are now the antibiotics of choice (Edelstein 1995a; Bryskier 1998; Celis et al. 2002; Johnson et al. 2002; Trubel et al. 2002).

Since person-to-person transmission has never been observed, prevention of Legionella infections concentrates on the elimination of the pathogen from water supplies. In high-risk areas, such as intensive care units, regular monitoring of Legionella concentrations is mandatory (reviewed by Steinert et al. 2002). Some methods for detecting *Legionella* have been described in section 3.1 of this chapter.

After detection of unacceptably high levels of *Legionellae*, effective decontamination and maintenance of water are critical for prevention of outbreaks of legionellosis. In general, actions need to be taken when the concentration of *Legionella*

exceeds 1 CFU/ml. Standards that are more restrictive apply for high-risk areas, including intensive care and transplantation units (reviewed by Steinert et al. 2002). In the recent years a number of methods for controlling the growth of legionellae in drinking water supply systems (heat flushing, ultraviolet light irradiation, ozonation, metal ionization, chlorination) and cooling towers (biocides) have been described (Kirby and Harris 1987; Patterson et al. 1997; Kool et al. 1998; Kool et al. 1999). Unfortunately, the decreased heat transfer and biocide penetration into biofilms as well as unused pipes of the water system often interferes with disinfection attempts (Ciesielski et al. 1984). In addition, the interaction of legionellae with amoebae hampers the disinfection in man-made water systems (Berk et al. 1998).

1.1.4 *L. pneumophila* Intracellular Life Cycle

As described in the previous sections, the natural host for *Legionella pneumophila* is protozoan cells. However, the clinical importance of this bacterium arises from its ability to infect human alveolar macrophages. Although some *L. pneumophila* virulence factors are host specific, there are many similarities, at both the phenotypic and the molecular levels, between the infection of mammalian and protozoan cells. Here is therefore, a generalized description of the intracellular life cycle of *L. pneumophila* within a permissive cell.

The intracellular life cycle of this bacterium shows several distinctive features (see Fig. 1.2) (Horwitz 1984) and can be described as a multistage process. *L. pneumophila* can be ingested by phagocytic cells in a unique manner termed 'coiling phagocytosis'. In this process, a phagocyte pseudopod coils around the bacterium as it internalizes. After entry, the bacterium is located in a phagosome that evades fusion with the endosomal compartments and lysosomes but interacts sequentially with smooth vesicles, mitochondria, and ribosomes. The bacteria multiply within an endoplasmic reticulum-derived and ribosome-studded vacuole called the 'replicative phagosome'.

FIGURE 1.2

Compilation of transmission electron micrographs that illustrate distinctive steps in the *Legionella pneumophila* intracellular life cycle.

A Uptake of a Philadelphia 2 strain of *L. pneumophila* by *A. castellanii* through coiling phagocytosis at 30 min of coincubation. Bar, 0.5 μm.

B *H. vermiformis* infected with *L. pneumophila* (Lpn) AA100 for 30 min.

C Thin section cut near the surface of a U937 macrophage fixed after five minutes of infection with *L. pneumophila* at an MOI of 20. Within the phagosome is a *L. pneumophila* bacterium. Attached to the basal surface of the phagosomal membrane (70 Å thick) are a series of vesicles of the ER (60 Å thick).

D *H. vermiformis* infected with *L. pneumophila* (Lpn) for 2.5h. Arrowheads indicate mitochondria.

E Transverse section through a *L. pneumophila* bacterium enclosed in a vacuole. The U937 cells were exposed to *L. pneumophila* for 30 minutes then washed free of unattached bacteria and incubated for an additional 5.5 hours before fixation. The surface of the phagosome has reduced numbers of attached ER vesicles by this time. In their place are ribosomes that are directly attached to the phagosome.

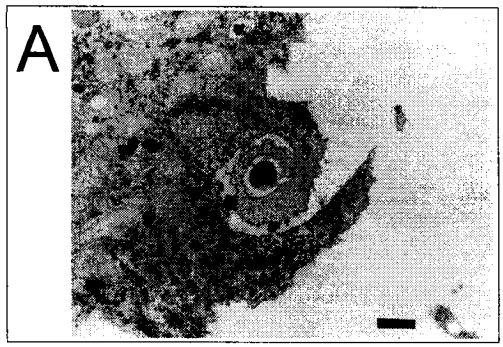
F *H. vermiformis* infected with *L. pneumophila* (Lpn) for 5 h. Arrows indicate the ribosome-studded multilayer phagosomal membrane.

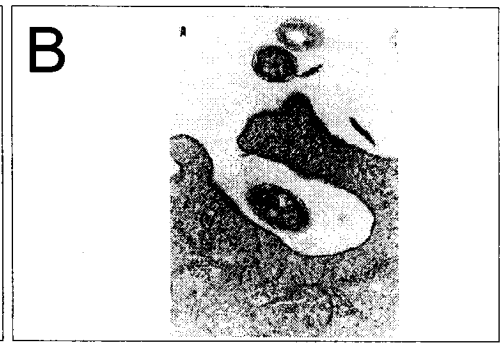
G *A. castellanii* infected with a virulent Phil 2 strain of *L. pneumophila* for 12 h. Arrow indicates *L. pneumophila* located within a ribosome-studded phagosome. Bar, 0.25 μm.

H *H. vermiformis* infected with *L. pneumophila* (Lpn) for 8 h. Arrows indicate the ribosome-studded multilayer phagosomal membrane.

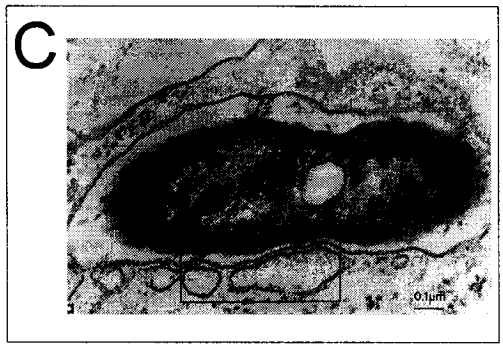
I *H. vermiformis* infected with *L. pneumophila* AA100 for 20 h.

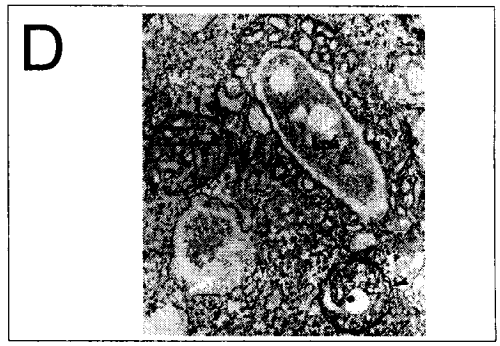
Coiling phagocytosis and Macropinocytosis **0-30 min**



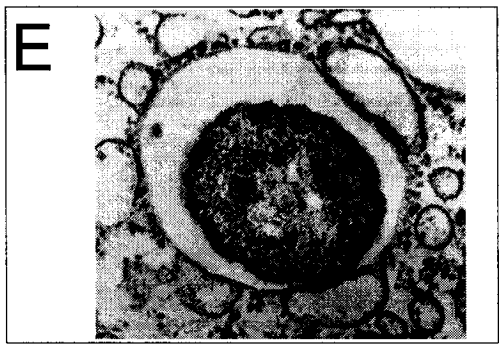


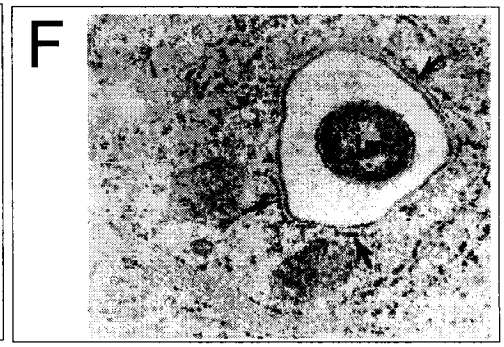
Interaction with smooth vesicles and mitochondria **5 min-3 h**



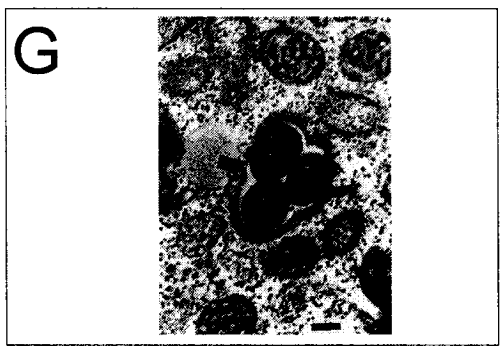


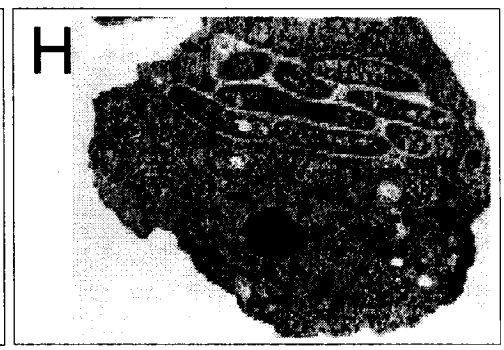
Formation of rER-derived replication niche **5-6 h**



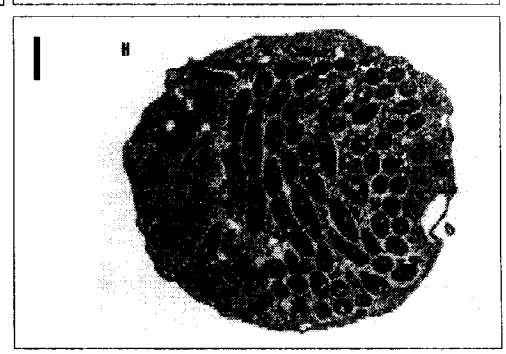


Replication within ribosome-studded vacuole **8-12 h**





Post-exponential phase *L.p.* ready to exit host **20-24 h**



Following bacterial replication, the monocyte is actively lysed, releasing the progeny bacteria for a new round of infection. Strains of *L. pneumophila* unable to evade fusion with lysosomes or to associate with host endoplasmic reticulum are avirulent (Horwitz 1987; Berger and Isberg 1993).

1.1.4.1 Attachment to host and entry.

Opsonization of *L. pneumophila* with specific antibody and complement enhances by threefold its attachment to human monocytes, compared to adherence in the presence of complement alone (Horwitz and Silverstein 1981). However, the mechanism of binding does not appear to influence significantly the intracellular fate of *L. pneumophila*. Indeed, bacteria that are phagocytosed in the presence of specific antibody and complement, or in the presence of complement alone, replicate as efficiently as untreated bacteria (Horwitz and Silverstein 1981).

When monocytes are incubated with *L. pneumophila* in the presence of serum, phagocytosis occurs via CR1 and CR3, complement receptors that are present on the surface of macrophages and several other mammalian cell lines (Payne and Horwitz 1987). Virulent *L. pneumophila* strains are resistant to complement-mediated lysis. Complement component C3 present in immune and nonimmune sera fixes primarily to the major outer membrane protein (MOMP), encoded by *ompS* (Hoffman et al. 1992), on the *L. pneumophila* surface (Bellinger-Kawahara and Horwitz 1990). In fact, C3 opsonization of purified MOMP reconstituted in liposomes induces phagocytosis by monocytes, suggesting that a MOMP-C3 complex ligand is sufficient to mediate uptake of *L. pneumophila* via the macrophage CR1 and CR3 receptors. MOMP may also have a complement-independent function: this abundant outer membrane protein also enhances bacterial binding to U937 cells in the absence of serum, and it increased the virulence of *L. pneumophila* in chick embryo assays (Krinos et al. 1999). Ultimately, the construction of a *L. pneumophila ompS* mutant and assessment of its virulence phenotype in phagocyte and animal models of infection will provide a more detailed understanding of the role of this dominant surface protein in *L. pneumophila* pathogenesis.

Because complement levels in the human lung are normally low, it is likely that, at least early in infection, *L. pneumophila* attach to phagocytes by another mechanism (Reynolds and Newball 1974). In fact, in the absence of antibody or complement, this pathogen still binds phorbol ester-treated U937 cells, monocytic cells that express Fc, CR1, and CR3 receptors (Rodgers and Gibson 1993). Also, preincubation of these phagocytes with monoclonal antibodies (mAbs) directed against CR1 and CR3 does not inhibit binding of *L. pneumophila*. Furthermore, *L. pneumophila* need not enter macrophages by a complement-mediated route to establish an intracellular replication niche: bacterial growth after complement-independent attachment has been observed in guinea pig alveolar macrophages, phorbol ester-treated U937 cells, and MRC5 cells (Rodgers and Gibson 1993; Gibson et al. 1994). Complement binding was also excluded in our own infection experiments with elicited mouse peritoneal macrophages *ex-vivo* by using heat-inactivated serum. A bacterial protein associated with lipids or carbohydrates may mediate binding to carbohydrates on the host plasma membrane. This hypothesis arises because bacterial attachment to U937 cells is inhibited after treatment of *L. pneumophila* with several proteolytic enzymes and after both the bacterial and host cells are treated with lipase and a carbohydrate-oxidizing agent (Gibson et al. 1994).

Complement-independent mechanisms must also promote phagocytosis of *L. pneumophila* by aquatic amoebae. It is within these model host systems that the most data to describe complement-independent attachment has been gathered. The opsonin-independent entry process of *L. pneumophila* is much less well characterized for mammalian host systems (Stone and Abu Kwaik 1998; reviewed by Steinert et al. 2002). *L. pneumophila* attachment to and invasion of the protozoan *Hartmannella vermiformis* is mediated by a protozoan 170-kDa lectin that is inhibited by galactose/*N*-acetylgalactosamine (Petri et al. 1987; Venkataraman et al. 1997; Abu Kwaik et al. 1998b; Harb et al. 1998). It is interesting that *Entamoeba histolytica* also encode a 170-kDa lectin that mediates its attachment to mammalian epithelial cells. Inhibition studies demonstrated the functional similarity of these lectins: *L. pneumophila* attachment to and invasion of *H. vermiformis* was decreased in a dose-dependent manner by two mAbs specific to the 170-kDa protein of *E. histolytica* (Ravdin et al. 1986). The bacterial ligand(s) responsible for lectin binding have yet to be identified. Invasion of *H.*

vermiformis by *L. pneumophila* requires host protein synthesis, as eukaryotic protein synthesis inhibitors (cycloheximide and emetine) block the entry process (abu Kwaik et al. 1994). Furthermore, specific *H. vermiformis* proteins are induced by wild-type *L. pneumophila* but not by an attenuated mutant strain of this bacterium (abu Kwaik et al. 1994). Once *L. pneumophila* engages the receptor, a rapid and dramatic dephosphorylation of several prominent tyrosine-phosphorylated proteins of *H. vermiformis* occurs. The dephosphorylated proteins include the 170 kDa receptor (Venkataraman et al. 1997; Abu Kwaik et al. 1998b) and the cytoskeletal-associated proteins, paxillin, pp125 FAK and vinculin (Venkataraman et al. 1998). Entry of *L. pneumophila* into *H. vermiformis* is not inhibited by microfilament inhibitors such as cytochalasin D and colchicine (King et al. 1991; Harb et al. 1998). Instead, entry is prevented by methylamine, an inhibitor of receptor-mediated phagocytosis (King et al. 1991). However, Legionellae have evolved heterogeneous mechanisms of attachment and entry into their protozoan host cells. Infection of *Acanthamoeba* by *L. pneumophila* occurs through an apparently different mechanism. *L. pneumophila* invasion of *A. polyphaga* is not inhibited by galactose or N-acetylgalactosamine (Harb et al. 1998). In addition, the 170 kDa galactose/N-acetylgalactosamine-inhibitable lectin is only mildly dephosphorylated in *A. polyphaga* upon attachment of *L. pneumophila* (Harb et al. 1998). Furthermore, host protein synthesis by *A. polyphaga* is not required for invasion by *L. pneumophila* (Harb et al. 1998). Interestingly, the uptake process of *L. pneumophila* by *A. polyphaga* is not inhibited by cytoskeleton-disrupting agents (Harb et al. 1998). Thus, *L. pneumophila* has evolved diverse mechanisms to invade different protozoa and may possess different ligands for attachment to different host cells. The *L. pneumophila* ligand(s) involved in the invasion process has(ve) not been identified, although several candidates exist. Mutants of *L. pneumophila* that fail to express type IV pili are partially defective in attachment to *A. polyphaga*, indicating that these pili may be involved in the attachment process (Stone and Abu Kwaik 1998). Other potential ligands may include the heat shock protein (Hsp60) and the major outer membrane protein (MOMP) of *L. pneumophila*, which play a role in the attachment to mammalian cells (Bellinger-Kawahara and Horwitz 1990; Garduno et al. 1998c; Krinos et al. 1999). Mutants of *L. pneumophila* that are defective in attachment to protozoa should be useful for the

identification of bacterial factors involved in the invasion process (Gao et al. 1997; Harb et al. 1998; Venkataraman et al. 1998).

Ultrastructural studies have described two modes of entry for *L. pneumophila* until recently: "coiling" and "conventional phagocytosis". Coiling phagocytosis, in which a long pseudopod literally coils around the bacterium, appears to be an occasional finding with both macrophages and amoebae (Horwitz 1984; Bozue and Johnson 1996; Venkataraman et al. 1998). However, this unusual mode of entry does not appear to be necessary or sufficient for intracellular survival of *L. pneumophila* in professional phagocytes. Heat-killed, fixed, and some avirulent *L. pneumophila* are also ingested within coiled phagosomes, but these particles are delivered to the endosomal compartment (Horwitz 1983b; Horwitz 1984; Horwitz 1987; Bozue and Johnson 1996). Conversely, *L. pneumophila* that have been opsonized with specific antibody form conventional phagosomes, but evade lysosomes (Horwitz 1984). Coiling phagocytosis also was not observed for the virulent Knoxville 1 strain of *L. pneumophila* nor for *L. micdadei* (Rechnitzer and Blom 1989). Coiling phagocytosis has been observed for a number of other microbes, including *Leishmania donovani, Borrelia burgdorferi*, various spirochetes, trypanosomatids, and yeasts (Chang 1979; Rittig et al. 1998a; Rittig et al. 1998b). Based on their detailed ultrastructural studies of coiled and conventional phagosomes, Rittig and colleagues (Rittig et al. 1998a) have proposed that coiling phagosomes are a direct consequence of a perturbation to conventional circumferential phagocytosis. According to this model, when the membranes of pseudopods that surround a particle fail to fuse, whorls of closely apposed plasma membrane form. Since both heat-killed and formalin-fixed *L. pneumophila* form coiled phagosomes, it is presumably due to a passive inhibitory factor on the bacterial surface (Amer and Swanson 2002). Within minutes of formation, the coiling phagosome resolves to a vacuole with a single membrane (Horwitz 1983a).

It has been noted that the composition of newly formed *L. pneumophila* phagosomes differs markedly from plasma membrane. Although these phagosomes contain the plasma membrane protein 5'-nucleotidase (Clemens and Horwitz 1992), they lack other protein residents of the plasma membrane, including MHC class I and class II molecules and alkaline phosphatase (Clemens and Horwitz 1992; Clemens and Horwitz

1995). Accordingly, Clemens & Horwitz (Clemens and Horwitz 1995) postulated that during phagocytosis of *L. pneumophila*, membrane proteins are sorted rapidly in such a manner that the membranes that surround the bacterium are markedly different from the plasma membrane. There is evidence that *L. pneumophila* might enter the host cell associated with lipid rafts (Watarai et al. 2001). Lipid rafts are discrete patches on the plasma membrane with characteristic protein and lipid content: they are rich in cholesterol, glycosphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins, and physically exclude a number of common plasma membrane molecules/receptors (early reviews: Fielding and Fielding 1997; Harder and Simons 1997). Association with such lipid domains could thus enable the pathogen to effectively evade recognition by the host phagocyte. A strikingly similar strategy has been proposed for the intracellular bacterium *Brucella abortus* (Kim et al. 2002; Watarai et al. 2002), as well as an increasing number of unrelated pathogens (Scheiffele et al. 1997; Samuel et al. 2001; Duncan et al. 2002; Nguyen and Taub 2002). Interestingly, association of *L. pneumophila* with lipid rafts was described as being followed by a macropinocytic uptake of the bacterium by permissive mouse macrophages (Watarai et al. 2001). Macropinosomes can occur spontaneously in cells and originate as ruffles at the cell margins that fold back on themselves, internalizing extracellular medium and solutes (Swanson 1989). Watari and colleagues described the formation of large, fluid-filled, spacious phagosomes around the *L. pneumophila* bacterium. These vacuoles were morphologically similar to macropinosomes and their formation appeared to occur during, rather than after, the closure of the plasma membrane about the bacterium, since a fluid-phase marker preloaded into the macrophage endocytic path failed to label the bacterium-laden macropinosome (Watarai et al. 2001). Importantly, macropinosome formation was correlated with intracellular survival and replication of *L. pneumophila* (Watarai et al. 2001). This subject is further discussed in chapter 5 of this thesis as it begs for further research that is directly related to the cloning of *Lgn1*.

1.1.4.2 Modulation of the phagosome maturation process.

As seen in the previous section, it is right from the time of entry that *L. pneumophila* reprograms the maturation pathway of the phagosome. *L. pneumophila*-containing vacuoles aged 5–60 min do not interact with the early endosomal compartment, as judged by the absence of transferrin receptors (Clemens and Horwitz 1995; Swanson and Hammer 2000) and their failure to accumulate the endocytic tracers Texas Red-ovalbumin, the lipid dye CM-DiI, or Alexa Fluor-streptavidin, markers that were readily detected in phagosomes containing polystyrene beads (Swanson and Isberg 1996a; Swanson and Isberg 1996b; Sturgill-Koszycki and Swanson 2000; Swanson and Hammer 2000). More evidence that young vacuoles containing virulent *L. pneumophila* are completely isolated from the endosomal compartment: the majority of phagosomes aged 5–90 min lack LAMP-1 (lysosome-associated membrane protein) and Rab7, a monomeric GTP-binding protein that acts as a positive regulator of fusion between the early and late endosomal compartments (Feng et al. 1995; Soldati et al. 1995; Swanson and Isberg 1996b; Roy et al. 1998). Therefore, to survive in macrophages, *L. pneumophila* appears to employ a strategy reminiscent of that of *Toxoplasma gondii*, which triggers formation of a vacuole that is completely separate from the endocytic network (Mordue and Sibley 1997). As they age, *L. pneumophila* phagosomes lose some host proteins. The majority of the *L. pneumophila* phagosomes lack 5'-nucleotidase activity 1 h after formation and have reduced levels of CR3 (Clemens and Horwitz 1992). The vacuoles that harbor *L. pneumophila* differ from conventional phagosomes in two other important respects: they do not acidify or fuse with lysosomes (Horwitz 1983b; Horwitz and Maxfield 1984). Since the early studies of Horwitz, several laboratories, using a variety of methods, have established clearly that *L. pneumophila* phagosomes aged 5 min to 8 h do not acquire lysosomal markers. Electron microscopic studies indicated that lysosomes labeled by acid phosphatase cytochemistry or electron-dense colloids do not fuse with *L. pneumophila* phagosomes (Horwitz 1983b; Berger and Isberg 1993; Clemens and Horwitz 1995). Cryosection immunogold localization of CD63, LAMP-1, LAMP-2, and cathepsin D demonstrated that bacterial phagosomes do not acquire these late endosomal and lysosomal proteins (Clemens and Horwitz 1995). Finally, fluorescence microscopic

assays confirmed that *L. pneumophila* phagosomes do not contain LAMP-1 and demonstrated further that they do not acquire Texas Red-ovalbumin preloaded into lysosomes by pinocytosis (Swanson and Isberg 1996b). Similarly, after ingestion by *A. castellanii*, virulent *L. pneumophila* reside in vacuoles that do not acquire lysosomal characteristics, including host acid phosphatase and ferritin that had been delivered by endocytosis to the lysosomal compartment (Bozue and Johnson 1996).

Virulent *L. pneumophila* blocks maturation of its own phagosome with no apparent effect on phagolysosome formation elsewhere within the phagocyte. Vacuoles harboring *L. pneumophila* remain at a neutral pH, whereas neighboring erythrocyte-containing phagosomes acidify below pH 5 (Horwitz and Maxfield 1984). In addition, after infection with *L. pneumophila*, macrophages continue to deliver *Saccharomyces cerevisiae* to phagolysosomes (Coers et al. 1999). Therefore, the *L. pneumophila* virulence factors that prevent its delivery to lysosomes must act locally, most likely by altering the phagosomal membrane.

The following are important ultrastructural features of the modified phagosome maturation process. After entry into protozoan cells and macrophages (15-60 min after phagocytosis), *L. pneumophila* is localized within a membrane-bound vacuole that interacts with mitochondria and smooth vesicles (Horwitz 1983a; Holden et al. 1984; Newsome et al. 1985; Fields et al. 1986; Abu Kwaik 1996; Bozue and Johnson 1996; Gao et al. 1997). 4 h after entry, the *L. pneumophila* phagosome is surrounded by a ribosome-studded multilayered membrane that seems derived from the rough endoplasmic reticulum (rER) (Horwitz 1983a; Fields et al. 1986; Swanson and Isberg 1995; Abu Kwaik 1996; Gao et al. 1997). Discovery of the 'replicative phagosome' being associated with membranes derived from the host endoplasmic reticulum has led some researchers to suggest that *L. pneumophila* exploits the autophagy machinery of macrophages to establish a replication niche (Swanson and Isberg 1995). Autophagy is a process for the degradation of unwanted organelles and cellular components. It can also be viewed as a critical mechanism for cellular homeostasis. When stressed, such as by nutrient deprivation or elevated temperature, cells increase their rate of autophagy. Portions of the cytoplasm, including organelles, are sequestered within vacuoles derived from the ER, called autophagosomes. Next, these vacuoles merge with the lysosomal compartment,

wherein the contents are degraded (Dorn et al. 2002). By this process, an eukaryotic cell presumably reduces its metabolic load and liberates molecules needed for vital cellular activities. Although autophagosomes do resemble *L. pneumophila* replicative phagosomes structurally, there are notable differences between the two. For example, autophagosomes fuse quickly (within 1 h) with the lysosomal compartment and do not recruit mitochondria or ribosomes (Dorn et al. 2002). More convincingly, new knowledge on the *L. pneumophila* replicative phagosome biogenesis points to a mechanism different from autophagy. Under the electron microscope, ER membrane cross-sections can be distinguished from other membranes including the plasma membrane. Due to their characteristic lipid composition (lack of cholesterol, sphingolipids and glycolipids), ER and mitochondrial membranes are 60±2 Å thick versus the usual 72±2 Å for other membranes (Tilney et al. 2001). It was observed that the host vesicles that attach to nascent phagosomes within 5 min after entry are thin-walled and therefore derived from the ER. These vesicles flatten along the surface of the phagosome within 15 min and tiny "hairs" connect the two. The thickness of the phagosomal membrane becomes similar to ER within an additional 15 min (Tilney et al. 2001). It is still not clear whether it is fusion with the vesicles (Roy and Tilney 2002) or an exchange of lipid bilayer (Tilney et al. 2001) that occurs. Very recent studies have shed light on the precise nature of the vesicles that attach to the *L. pneumophila* phagosome (Kagan and Roy 2002). First, it was observed that the maturation of *Legionella*-containing phagosomes (LCPs) into ER-derived organelles was biphasic, as the acquisition of two different resident ER proteins did not happen simultaneously. Indeed, a hybrid marker protein with a yellow fluorescent protein (YFP) domain attached to a KDEL motif for ER localization was enriched on LCPs within 30 minutes of uptake of the bacterium (Kagan and Roy 2002). From earlier studies, the YFP-KDEL protein is known to be present in the ER lumen and also cycles between the ER and Golgi within early secretory vesicles (Pelham 1996; Roderick et al. 1997). In contrast, calnexin, a resident integral membrane protein in the rough ER, was not detected in substantial quantities within LCPs until 10 hours after uptake of the bacterium (Kagan and Roy 2002). The different kinetics of acquisition of calnexin and YFP-KDEL could be explained if LCPs interacted with early secretory vesicles before being transported to the ER (Kagan and Roy 2002). The normal process of forming early

secretory vesicles at exit sites within transitional ER (tER) is dependent on the sequential action of two proteins Sar1 and ARF1, which are small GTPases that regulate the formation of COPII- and COPI-coated vesicles respectively (Aridor et al. 1995). Dominant interfering variants of Sar1 and ARF1 blocked early secretory vesicle formation as well as enrichment of LCPs for YFP-KDEL and replicative organelle formation by *L. pneumophila* (Kagan and Roy 2002). Blocking ARF-dependent vesicular traffic from the ER with brefeldin A (BFA) (Donaldson et al. 1992) gave similar results (Kagan and Roy 2002). Evidence was also given that the intercepted early secretory vesicles never reach the Golgi apparatus before being shunt back to the ER. Interestingly, although ARF1 was shown to be recruited to LCPs about 30 min after uptake of the bacterium in a Dot- and Ralf-dependent manner (see virulence factor section that follows), this recruitment did not seem to play a critical role in replicative organelle formation. ARF1 function was therefore only seen to be critical for early secretory vesicle formation just before the infection with *L. pneumophila* (Kagan and Roy 2002). One more important finding was that LCP resistance to fusion with the late endosomal compartment right from the time of phagocytosis is dependent on the *L. pneumophila* Dot machinery but independent of host ARF1 function. Still, the interception of ARF1-dependent early secretory vesicles was shown to be necessary to keep LCPs separate from late endosome compartments beyond a 30-min timepoint in the infection (Kagan and Roy 2002). The overall conclusion of the study described here was that the *L. pneumophila* phagosome intercepts early secretory vesicles exiting from the transitional endoplasmic reticulum (tER) (Kagan and Roy 2002); however, the underlying mechanism remains to be elucidated.

1.1.4.3 Replication within an ER-derived vacuole.

Following the initial 4 h after entry, *L. pneumophila* begins to replicate within its ribosome-studded phagosome (Abu Kwaik 1996). Theoretically, pathogens could exploit one or more of the activities of the ER to obtain nutrients. In addition to its protein and phospholipid biosynthetic enzymes, protein-conducting channels, and peptide pores, the

ER participates in autophagy, which can result in an increased local supply of nutrients (Dorn et al. 2002). Genetic and kinetic studies of *L. pneumophila* infections have correlated ER association and intracellular replication (Horwitz 1983a; Swanson and Isberg 1995; Swanson and Isberg 1996b). In none of these cases has a direct role for ER in pathogen survival or growth been demonstrated (nor for the earlier-associating mitochondria). Mitochondria also associate with vacuoles containing *T. gondii*, and both *T. gondii* and *B. abortus* replicate in vacuoles decorated with rough ER (Anderson and Cheville 1986; Detilleux et al. 1990).

Interestingly, the phagosome harboring *L. micdadei* is morphologically distinct from the *L. pneumophila* phagosome. The *L. micdadei* phagosome is not surrounded by the rER (Weinbaum et al. 1984; Abu Kwaik et al. 1998b; Gao et al. 1999). This suggests that *L. pneumophila* and *L. micdadei* have evolved different strategies for intracellular survival. Knowing this, perhaps is it not surprising that *L. micdadei* intracellular replication is not controlled by the host *Lgn1* gene as will be discussed in later sections (Miyamoto et al. 1996). Additionally, as *L. pneumophila* is more pathogenic to humans than *L. micdadei*, it may be inferred that *L. pneumophila* has evolved better mechanisms of intracellular survival. This is supported by the recent findings that *L. micdadei* does not replicate significantly within *A. polyphaga*, is avirulent in the A/J mouse model and does not possess a pore-forming toxin activity (Gao et al. 1999). Furthermore, a comparative study of *L. pneumophila* and *L. micdadei* has indicated that the two bacteria do not share common virulence mechanisms (Joshi and Swanson 1999). It should be noted however that despite all those differences *L. micdadei* remains the second most common *Legionella* species that causes Legionnaires' disease in humans (Gao et al. 1999).

Cellular markers such as CD63, LAMP-1, LAMP-2, lysosomal cathepsin D, transferrin receptors and Rab7 are still excluded from the phagosome during the early course of intravacuolar growth of *Legionella* (Clemens and Horwitz 1995). Replication in the ribosome-studded vacuole goes on for a total of about 20 h. At mid-log phase, *Legionella* replicates, by binary fission, with a doubling time of approximately 2 h (reviewed by Swanson and Hammer 2000).

Importantly, *L. pneumophila* undergoes a dramatic phenotypic modulation upon replication within protozoan (Cirillo et al. 1994) and mammalian cells (Abu Kwaik and

Engleberg 1994; Abu Kwaik and Pederson 1996; Fernandez et al. 1996; Abu Kwaik et al. 1997; Harb and Abu Kwaik 1999; Kwaik and Harb 1999). Conditions within the phagocyte vacuole as well as growth phase clearly influence the *L. pneumophila* phenotype. "Replicative" *L. pneumophila* lose a number of traits (such as osmotic resistance and flagella) known to promote survival in the environment and transmission to a new host (Barker et al. 1992; Abu Kwaik et al. 1993; Barker et al. 1995). Interestingly, during the late replicative phase at 16 to 20 h after uptake of the bacterium, the *Legionella* phagosome merges with lysosomes without detrimental consequences for the enclosed bacteria (reviewed by Swanson and Hammer 2000). Presumably, this class of vacuolar pathogens exploits the period when delivery to lysosomes is blocked to convert to a replicative form which not only tolerates, but thrives within the acidic and hydrolytic lysosomal compartment (Sturgill-Koszycki and Swanson 2000).

Indeed, during the time when the yield of *L. pneumophila* colony forming units typically increases 10-fold, a significant proportion of the bacterial vacuoles acquires lysosomal characteristics (Sturgill-Koszycki and Swanson 2000). In particular, by 18 h post-infection, 70% of the vacuoles contain LAMP-1, a late endosomal and lysosomal membrane glycoprotein, and 50% contain the lysosomal enzyme cathepsin D, as judged by fluorescence microscopic assays. Additionally, 50% of the replication vacuoles accumulate the fluorescent endocytic probes Texas Red-ovalbumin and fluorescein-dextran. Finally, whereas nascent *L. pneumophila* phagosomes remain a neutral pH, by 16 to 20 h after infection, replication vacuoles are acidic, averaging pH 5.5 (Sturgill-Koszycki and Swanson 2000). Thus, as they mature, *L. pneumophila* replication vacuoles appear to merge with the lysosomal compartment. Although some macrophage pathogens, such as *Mycobacterium* (Crowle et al. 1991), *Toxoplasma* (Jones and Hirsch 1972), and *Chlamydia* (Friis 1972) species, replicate within compartments which remain separate from the lysosomes, *Leishmania* does not (reviewed by Swanson and Hammer 2000). Similar to *L. pneumophila*, the growth phase of *Leishmania* determines its competence to inhibit phagosome-lysosome fusion (Turco and Descoteaux 1992; Desjardins and Descoteaux 1997).

1.1.4.4 Killing of host and exit.

The growth phase has therefore a dramatic effect on the phenotype of *L. pneumophila* cultured in phagocytes and in broth. By microscopic observation of infected amoebae, Rowbotham (Rowbotham 1986) first noted that the intracellular life cycle of *L. pneumophila* consists of two distinguishable phases. After a period of replication, *L. pneumophila* enter an "active infective phase", marked by their synchronous conversion to highly motile short and thick rods that were observed to escape lysed host cells and disperse in culture. This paradigm has later been supported by phenotypic and molecular studies of *L. pneumophila* cultured in broth and in macrophages (Byrne and Swanson 1998; Hammer and Swanson 1999). Unlike replicating cells, bacteria obtained from post-exponential phase cultures of *L. pneumophila* express a number of traits that have been correlated with virulence, including sodium-sensitivity, cytotoxicity, osmotic resistance, motility, and the capacity to evade phagosome-lysosome fusion. Post-exponential *L. pneumophila* are also characterized by a smooth, thick cell wall, a higher ß-hydroxybutyrate content, different staining properties, and expression of a different array of proteins and genes (Rowbotham 1986; Abu Kwaik et al. 1993; Cirillo et al. 1994; Abu Kwaik and Pederson 1996; Edelstein et al. 1999). In addition, compared to replicative bacteria, *A. polyphaga*-grown cells have a different composition of membrane fatty acids, profile of lipopolysaccharide and outer membrane proteins, and susceptibility to proteinase K (Barker et al. 1993). Post-exponential *L. pneumophila* have also been shown to be more resistant to biocides and antibiotics (Barker et al. 1992; Barker et al. 1995), more invasive for mammalian cells, and more virulent in mouse models of infection (Cirillo et al. 1994; Brieland et al. 1997; Cirillo et al. 1999). Finally, after replicating for 10–12 hours within monocytic U937 cells, *L. pneumophila* begin to express stress proteins (Abu Kwaik et al. 1993). It is likely that multiple environmental signals determine the phenotype of intracellular *L. pneumophila*. Amino acid limitation appears to be a major inducer of the virulent phenotype, because exponential phase cells convert to the virulent phenotype when incubated in post-exponential phase culture supernatant. Phenotype conversion does not take place when the supernatant is supplemented with amino acids (Byrne and Swanson 1998). Accordingly, it has been postulated that when nutrient levels

and other conditions are favorable, *L. pneumophila* replicates within its specialized vacuole. When amino acids become scarce, intracellular bacteria coordinately express several traits that facilitate escape from the depleted cell and transmission to a new host (Byrne and Swanson 1998; Hammer and Swanson 1999).

A fundamental step in the life cycle of an intracellular pathogen is its ability to exit the host cell after termination of intracellular replication in order to infect a suitable new host. There is a rapid induction of necrosis by *L. pneumophila* upon entry into the post-exponential phase of growth (Byrne and Swanson 1998). Necrotic killing by *L. pneumophila* has been shown to be essential for subsequent release of the intracellular bacteria from *A. polyphaga* (Gao and Kwaik 2000). This growth phase-dependent cytotoxicity of *L. pneumophila* is mediated by temporal expression of a pore-forming activity (Byrne and Swanson 1998; Kirby et al. 1998; Alli et al. 2000; Gao and Kwaik 2000). Wild-type intracellular *L. pneumophila* causes necrosis-mediated cytolysis of *A. polyphaga* within 48 h after infection, and the intracellular bacteria are released into the tissue culture medium. In contrast, mutant strains of *L. pneumophila* defective in the pore-forming activity replicate as well as the parental strain in *A. polyphaga*, but are severely defective in killing and lysis of *A. polyphaga* and remain `trapped' within the amoebae (Gao and Kwaik 2000). A similar mechanism is also used to egress mammalian cells (Gao et al. 1999; Molmeret et al. 2002a; Molmeret et al. 2002b).

Interestingly, *L. pneumophila* has been shown to induce apoptosis (programmed cell death) in human host cells that included HL-60 (Muller et al. 1996) and U937 (Gao and Abu Kwaik 1999b) cell lines differentiated into macrophage-like cells, peripheral blood monocytes (Hagele et al. 1998) and the WI-26 alveolar epithelial cell line (Gao and Abu Kwaik 1999b). This programmed cell death was initially characterized by condensation of chromatin at the nuclear boundary and interchromosomal DNA cleavage (Muller et al. 1996; Gao and Abu Kwaik 1999a; Gao and Abu Kwaik 1999b). DNA fragmentation was typically detected by agarose gel electrophoresis and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL). Also, flow cytometry was successfully used to detect annexin V binding to surface-exposed phosphatidylserine on monocytes undergoing *L. pneumophila*-induced apoptosis (Hagele et al. 1998). Apoptosis was invariably detected during the early stages of infection: within

3 h of initiation of the infection and prior to intracellular replication (Gao and Abu Kwaik 1999b). Induction of apoptosis was found to be independent of the bacterial growth phase at the time of uptake by the host (Gao and Abu Kwaik 1999a), but it was dependent on the multiplicity of infection (Hagele et al. 1998). Evidence was provided showing that *L. pneumophila*-induced apoptosis in human host cells did not require intracellular bacterial replication and that extracellular *L. pneumophila* were capable of inducing apoptosis (Gao and Abu Kwaik 1999b). It was demonstrated that the induction of apoptosis by *L. pneumophila* in human macrophages is mediated through the activation of caspase 3 (Gao and Abu Kwaik 1999a). The enzymatic activity of caspase 3 to cleave a specific synthetic substrate *in vitro* was detected in *L. pneumophila*-infected macrophages at 2 h after infection and was maximal at 3 h, with over 900% increase in activity. The activity of caspase 3 to cleave the specific substrate poly(ADP-ribose) polymerase, or PARP, *in vivo* was also detected at 2 h and was maximal at 3 h postinfection. The activity of caspase 3 to cleave the synthetic substrate *in vitro* and PARP *in vivo* was blocked by a specific inhibitor of caspase 3. It was noted that the kinetics of caspase 3 activation correlated with that of *L. pneumophila*-induced nuclear apoptosis, and that inhibition of caspase 3 activity blocked *L. pneumophila*-induced nuclear apoptosis and cytopathogenicity during early stages of the infection (Gao and Abu Kwaik 1999a).

It is not clear what role the induction of apoptosis plays in the infection of human cells by *L. pneumophila*. A correlation has been pointed out between the ability of *Legionella* strains to induce apoptosis and their cytopathogenicity to host cells (Gao and Abu Kwaik 1999b). It should be noted, however, that the same group later determined that it is the pore-forming ability of the bacterium that enables it to kill the host cell (Alli et al. 2000).

Contrary to human host cells, it was proposed early on that *L. pneumophila* does not induce apoptosis in its protozoan host (Hagele et al. 1998). More recently, it has been shown that *A. polyphaga* is capable of undergoing apoptosis upon stimulation by actinomycin D, as evidenced by classic internucleosomal DNA fragmentation. However, no such induction of DNA cleavage was observed during *L. pneumophila* infection (Gao and Kwaik 2000). Induction of apoptosis by *L. pneumophila* has not been observed in

mouse macrophages either (Diez et al. 2003; Wright et al. 2003); the importance of which will become apparent on subsequent sections of this thesis.

Taken together, current knowledge of *L. pneumophila* virulence regulation and replication vacuole biogenesis support the following multistage model for the *L. pneumophila* life cycle. When ingested by amoebae or macrophages, *L. pneumophila* modify the nascent phagosome and separate it completely from the endosomal pathway. Next, endoplasmic reticulum engulfs the isolated phagosome, forming a "replicative vacuole". Within this protected niche, *L. pneumophila* converts to a replicative form that is acid tolerant and does not express several virulence traits, including those factors which block fusion with the lysosomal compartment. Consequently, the pathogen is delivered to the lysosomal compartment, a harsh but nutrient-rich environment where the bacteria are now able to survive. Once the bacterial progeny have depleted the local amino acid supply, expression of traits important for transmission of *L. pneumophila* to a new phagocyte is triggered. In particular, a cytotoxin promotes escape from the spent host, osmotic resistance increases survival in the extracellular environment, motility facilitates dispersal and contact with a new host cell, and the capacity to evade phagosome-lysosome fusion promotes survival within the next phagocyte, where the cycle repeats.

1.1.5 *L. pneumophila* Virulence Factors

Over the past 15 years, several genetic loci of *L. pneumophila* that are required for its intracellular survival and replication have been identified. *Legionella* strains bearing a mutation within these virulence genes are invaluable tools for dissecting the interaction of this pathogen with its host.

1.1.5.1 Type IV pili

Type IV pili, which mediate host cell attachment by pathogenic *Neisseria* species (Meyer et al. 1984), *Pseudomonas aeruginosa* (Johnson et al. 1986), and other bacterial species (Strom and Lory 1993), may also function as *L. pneumophila* adhesins. An insertional mutation in the putative *L. pneumophila* pilin structural gene, $pilE_L$, reduced the bacterial adherence to *A. polyphaga* and the mammalian monocytic U937 and epithelial HeLa cell lines by about 50% (Stone and Abu Kwaik 1998). Interestingly, intracellular replication was not affected in any of the hosts studied (Stone and Abu Kwaik 1998). Because adherence of the $pilE_L$ mutant was attenuated in both mammalian cells and amoebae, *L. pneumophila* pili may contribute to the observed complement-independent binding (Stone and Abu Kwaik 1998). Future research will no doubt focus on finding the host receptor responsible for binding to type IV pili. A strong candidate in protozoa is the Gal/GalNAc lectin of *H. vermiformis* (Venkataraman et al. 1997).

1.1.5.2 Type II secretion

The pilBCD genes of *L. pneumophila* are homologous to the well-studied PilBCD system of *P. aeruginosa* and are involved in type II secretion (Liles et al. 1998). Type II secretion systems enable animal pathogens, such as *Vibrio cholerae* and *P. aeruginosa*, to secrete toxins and proteases, and plant pathogens, like *Erwinia chrysanthemi* and *Xanthomonas campestris*, to secrete cellulases and pectinases (reviewed by Russel 1998). In order for proteins to enter the type II secretion pathway, they have to first translocate across the cytoplasmic membrane. For that, those proteins contain an amino terminal signal sequence that directs their delivery into the periplasm by the Sec secretion machinery. The proteins then fold into a translocation competent conformation and subsequent transport through the outer membrane is then achieved by the type II secretion machinery, a complex of at least 14 proteins (Russel 1998).

L. pneumophila is the first intracellular pathogen found to carry a chromosomal locus encoding a type II secretion system (Liles et al. 1998; Liles et al. 1999). Expression of the pilBCD genes is not required for growth of *L. pneumophila* in culture medium (Liles et al. 1998). A role for type II secretion in *L. pneumophila* pathogenesis was demonstrated by analysis of a nonpolar *pilD:kanR* insertion mutant strain. The pilD locus encodes a prepillin peptidase that processes proteins destined for secretion by the type II system. Interestingly, pilD mutants of *L. pneumophila* are defective in intracellular replication within both macrophages and *H. vermiformis* (Liles et al. 1998; Liles et al. 1999). In addition, this mutant is also defective in the secretion of a Zn^{21}-metalloprotease, also known as the major secreted protein (MSP) and type IV pili biogenesis (Stone and Abu Kwaik 1998; Liles et al. 1999). Further, mutants in other components of the type II secretion apparatus of *L. pneumophila*, the *lsp* (*Legionella* secretion pathway) *FGHIJK* locus, are defective in infection of *A. polyphaga* and secretion of the MSP protease (Hales and Shuman 1999a; Aragon et al. 2000). However, type IV pili and the MSP protease are not required for intracellular replication of *L. pneumophila* within mammalian and protozoan cells (Szeto and Shuman 1990; Moffat et al. 1994; Stone and Abu Kwaik 1998; Hales and Shuman 1999a). Thus, it is likely that the type II secretion apparatus may be involved in the secretion of other proteins involved in the virulence of *L. pneumophila* to both mammalian and protozoan cells.

Other proteins transported through the type II secretion system of *L. pneumophila* include two phosphatases, an RNAse, mono-, di- and triacylglycerol lipases, phospholipase A, a lysophospholipase A and a *p*-nitrophenyl phosphorylcholine hydrolase (Hales and Shuman 1999a; Aragon et al. 2000; Aragon et al. 2001; Flieger et al. 2001). The relative contribution of each of these substrates to the overall ability of *L. pneumophila* to survive and replicate within host cells remains to be elucidated.

1.1.5.3 Hsp60

Besides type IV pili, further attachment factors include the 60-kDa heat shock protein Hsp60 (Hoffman et al. 1990). Although bacterial heat shock proteins typically serve as cytoplasmic chaperones (Ellis 1987), *L. pneumophila* Hsp60 belongs to a large family of immunodominant protein antigens, termed "common antigens", many of which share cross-reactive epitopes and appear to be extracellular (Thole et al. 1988). Hsp60 was first shown to be implicated in attachment and entry of *L. pneumophila* to HeLa epithelial cells (Hoffman et al. 1989; Hoffman et al. 1990; Garduno et al. 1998b). Several other pathogens, including *Haemophilus ducreyi* (Frisk et al. 1998), *Helicobacter pylori* (Dunn et al. 1997), *Mycobacterium avium* (Rao et al. 1994) and *S. typhimurium* (Ensgraber and Loos 1992), appear to release proteins homologous to Hsp60 that have been implicated in virulence. It remains to be determined whether the extracellular localization of Hsp60 proteins is a consequence of its release from cells in certain growth conditions, *bona fide* secretion, or, as hypothesized for the Hsp60 homolog in *H. pylori*, bacterial cell lysis (Phadnis et al. 1996). *L. pneumophila* Hsp60, encoded by *htpB*, is induced during growth in macrophages and *in vitro* in response to H_2O_2, heat, and osmotic shock (Hoffman et al. 1989; Hoffman et al. 1990; Abu Kwaik et al. 1993; Garduno et al. 1998b). In *L. pneumophila* cultured in broth, immunogold labeling of Hsp60 indicated both cytoplasmic and surface locations; heat-shock increased the amount of surface-exposed Hsp60 epitopes modestly (Garduno et al. 1998a). In infected HeLa cells, extracellular Hsp60 protein can be detected lying free within replication vacuoles (Garduno et al. 1998b). Hsp60-specific antibody inhibits invasion by wild-type *L. pneumophila* and purified Hsp60 protein stimulates uptake of latex beads by HeLa cells (Garduno et al. 1998b). The closely related human HSP60 and Chlamydial Hsp60 proteins have been shown to elicit a strong proinflammatory response in cells of the innate immune system with Toll-like receptor (TLR) 2, and TLR4 as mediators of signaling (Vabulas et al. 2001; Bulut et al. 2002; Habich et al. 2002; Zanin-Zhorov et al. 2003). Although it is tempting to speculate that the same signalling pathway might be at work for *Legionella*, specific host receptors for the *Legionella* Hsp60 protein have not been formally identified yet.

1.1.5.4 Mip

The *mip* (macrophage infectivity potentiator) gene was the first cloned gene from *L. pneumophila* that exhibited a role in virulence (Cianciotto et al. 1989b). Immunogold techniques have shown that the Mip protein is exposed on the cell surface of extracellular grown *L. pneumophila*. In *Acanthamoeba* infected with *Legionella* the Mip protein was also detected on host membranes which exhibited a multilamellar structure (Helbig et al. 2001). The 24-kDa Mip is constitutively expressed and the 2.4-Å crystal structure has been described. Each monomer of the homodimeric protein consists of an N-terminal dimerization module, a long 65-Å connecting α-helix and a C-terminal peptidyl-prolyl *cis/trans* isomerase (PPIase) domain (Riboldi-Tunnicliffe et al. 2001).

During the initial infection period, 10-fold fewer viable *mip*-mutant cells associate with human monocytic U937 cells and alveolar macrophages compared with wild-type bacteria (Cianciotto et al. 1989b). More dramatically, after infection of *A. castellani*, the yield of *mip* mutants is 50- to 100-fold lower than that of the wild type (Wintermeyer et al. 1995). In a guinea pig model of infection, *mip* null mutants are also less virulent than wild type, as determined by lower morbidity and mortality (Cianciotto et al. 1990). Thus, judging by a variety of criteria, Mip contributes to *L. pneumophila* virulence. However, the intracellular growth rate of the *mip* mutants of *L. pneumophila* that do enter the host cell has been shown to be comparable with that of the wild-type strain (Cianciotto et al. 1989b; Cianciotto and Fields 1992; Cianciotto et al. 1995). Thus, Mip appears to promote efficient establishment of infection rather than intracellular replication *per se*.

The Mip protein exhibits peptidyl-prolyl-*cis/trans* isomerase (PPIase) activity, as measured by cleavage of synthetic substrates and this activity is inhibited by the immunosuppressant macrolide FK506 (Fischer et al. 1992). Because peptidyl prolyl isomerases are characteristic of eukaryotes, Mip may target a host protein substrate, as documented for the *Yersinia* YopH virulence protein (Guan and Dixon 1990). Yet, no bacterial or host substrate for Mip has been identified. Curiously, truncated Mip proteins defective for enzymatic activity abrogated virulence in a guinea pig model of infection but not within *A. castellanii* (Kohler et al. 2003). Thus, the mode of action of this virulence factor in the early stages of infection by *L. pneumophila* remains to be determined.

1.1.5.5 Dot/Icm Type IV Secretion System

The *dot* (defect in organelle trafficking, Berger and Isberg 1993; Andrews et al. 1998; Vogel et al. 1998) and *icm* (intracellular multiplication, Brand et al. 1994; Segal and Shuman 1997; Purcell and Shuman 1998; Segal et al. 1998) loci refer to the same 24 genes of *L. pneumophila* and were identified independently by two different laboratories. All of the *dot/icm* genes are located within either of two *L. pneumophila* chromosomal locations. Membership of the *dot/icm* genes in the family of type IV transport systems was defined originally by their collective ability to transfer DNA to a recipient cell. Unlike type III secretion systems which have co-opted the flagellar assembly pathway (Nguyen et al. 2000), and type II secretion systems which double as pilin extrusion machinery (Sandkvist 2001), type IV systems are encoded by chromosomal loci homologous to operons dedicated to conjugal transfer of plasmid DNA (Cao and Saier 2001). Analysis of the predicted amino acid sequences of the *dot/icm* genes has revealed several characteristics that indicate a role in conjugation. Indeed, fourteen of the *dot/icm* genes share detectable homology to the *tra/trb* genes of col1b-P9 plasmid, a member of the IncI class of conjugal plasmids (Segal and Shuman 1997; Vogel et al. 1998; Segal and Shuman 1999b). The ability of the *dot/icm* genes to mediate conjugal transfer of DNA has been confirmed (Segal et al. 1998; Vogel et al. 1998). For example, the plant pathogen *A. tumefaciens* transfers RSF1010 plasmids by a process that requires a functional type IV secretion apparatus (Stahl et al. 1998). In a similar manner, *L. pneumophila* transfer of the same RSF1010 plasmids to bacterial recipients depends on a functional set of *dot/icm* genes (Segal and Shuman 1997; Vogel et al. 1998).

The majority of the *dot/icm* genes are predicted to encode membrane-associated proteins. DotA stands out as an integral cytoplasmic membrane protein with eight membrane-spanning domains (Roy and Isberg 1997), and IcmW is a small, soluble protein that resides in the cytoplasm (Zuckman et al. 1999). An increasing number of studies strengthen the view that establishment of the intracellular niche of *L. pneumophila* requires the type IV conjugational transfer system (Segal and Shuman 1998b; Segal et al. 1999). How the *L. pneumophila* type IV secretion system contributes to bacterial

pathogenesis and the identity of its substrates is the focus of a great deal of research. Mutants defective in this secretion apparatus are defective in early events required for proper maturation of the *L. pneumophila* phagosome in mammalian cells, inhibition of phagosome-lysosome fusion, induction of apoptosis and pore formation-mediated cytotoxicity (Kirby et al. 1998; Segal and Shuman 1998a; Vogel et al. 1998; Gao and Abu Kwaik 1999a). Also, the Dot/Icm secretion system is required for intracellular replication within protozoa (Gao et al. 1997; Segal and Shuman 1999a). The Dot/Icm type IV secretion system appears to act during phagocytosis to establish the *L. pneumophila* replication vacuole. Every mutant of the *dot/icm* family that has been examined is defective for evasion of the endocytic pathway (Horwitz 1987; Marra et al. 1992; Berger et al. 1994; Swanson and Isberg 1996b; Segal and Shuman 1997; Andrews et al. 1998; Roy et al. 1998; Vogel et al. 1998; Wiater et al. 1998; Zuckman et al. 1999). Each of these mutants is mistargeted to the endosomal pathway within the earliest period examined, in some cases 5-30 min after infection (Roy et al. 1998; Wiater et al. 1998). For example, phagosomes containing *dotA* mutants acquire the late endosomal and lysosomal marker LAMP-1 within 5 min of uptake (Roy et al. 1998). Thus, to evade delivery to the lysosomes, *L. pneumophila* must alter its phagosome immediately, and the Dot/Icm type IV secretion system must be transporting effector macromolecules that can act almost instantly within the host cell.

L. pneumophila is one of a growing list of extracellular and intracellular bacterial pathogens that exploit a type IV secretion system for virulence. The phytopathogen *Agrobacterium tumefaciens* (Christie 1997) and the animal pathogens *Brucella suis* (O'Callaghan et al. 1999), *H. pylori* (Censini et al. 1996), and *B. pertussis* (Weiss et al. 1993) all encode type IV secretion loci. Although related to conjugal DNA transfer complexes, type IV secretion systems also export proteins that are effectors of virulence (Winans et al. 1996). For example, *A. tumefaciens* VirE2 protein accompanies T-DNA (tumor DNA) during transfer (Winans et al. 1996), and pertussis toxin is a protein substrate of the *B. pertussis* secretion system (Weiss et al. 1993). As described above, the Dot/Icm complex must act during phagocytosis to divert phagosome maturation. Therefore, the putative effector molecule is not likely to be DNA. By analogy to other

type IV systems, the *L. pneumophila* Dot/Icm conjugation complex is postulated to deliver virulence proteins to phagocytes, to establish a protective replication vacuole.

The Dot/Icm complex is required by *L. pneumophila* to insert pores into the host plasma membrane (Kirby et al. 1998). Besides residing in vacuoles that acquire endocytic markers, *dot* and *icm* mutants are noncytotoxic, and several mutants have been shown specifically to lack pore-forming activity. Accordingly, one model postulates that delivery of a small number of pores is sufficient to retard phagosome maturation (Kirby and Isberg 1998). In addition, at a high level of infection, insertion of a large number of pores into the host plasma membrane causes rapid lysis of the phagocyte (Kirby and Isberg 1998). A related model postulates that the Dot/Icm-dependent pore serves as the conduit for the effector molecules that modify the nascent phagosomal membrane to alter its course (Zuckman et al. 1999). Accordingly, mutants lacking such effectors are predicted to retain cytotoxicity but fail to evade the endosomal compartment. By these criteria, IcmW was an attractive candidate effector. However, cellular fractionation experiments indicate that this small, soluble protein resides in the bacterial cytoplasm. Therefore, instead of acting as a substrate for type IV secretion, IcmW may regulate Dot/Icm activity, directly or indirectly (Zuckman et al. 1999). It is important to note however, that the *icmW* mutant phenotype indicates that although pore-formation may be required by *L. pneumophila* to establish an isolated phagosome, it is not sufficient.

RalF is the first *L. pneumophila* protein shown to be exported through the type IV secretion apparatus (Nagai et al. 2002). The protein ADP ribosylation factor-1 (ARF1), a highly conserved small GTP-binding protein, acts as an important regulator of vesicle traffic from ER to Golgi. ARF1 is found on about 30% of the phagosomes that contain wild-type *L. pneumophila* but not *dot/icm* mutants (Nagai et al. 2002). These data suggest that a protein injected through the type IV secretion system may be required for ARF1 recruitment. Nagai and colleagues searched the *L. pneumophila* genome for proteins that have homology to ARF-specific guanine nucleotide exchange factors (GEFs). A protein was identified that has a sec7-homology domain, known to be sufficient to stimulate the exchange of GDP for GTP (Nagai et al. 2002). The protein was named RalF (recruitment of ARF to the *Legionella* phagosome). Indeed, RalF has been shown to be injected through the phagosomal membrane by a process that requires the Dot/Icm system (Nagai

et al. 2002). Phagosomes containing *ralF* mutants do not recruit ARF1. However, these mutants are still able to evade fusion to lysosomes and the bacteria replicate intracellularly within macrophages and amoebae (Nagai et al. 2002). Thus, RalF is not essential for transport of *L. pneumophila* to the ER, replicative organelle biogenesis or intracellular replication (Kagan and Roy 2002; Nagai et al. 2002). So why would *Legionella* inject an exchange factor for ARF into eukaryotic hosts during infection? An interesting theory was put forward in a recent review (Roy and Tilney 2002). It is likely that the function of RalF is to stimulate normal host processes that *Legionella* subvert during biogenesis of an ER vacuole. Accordingly, RalF may play a role in the creation or transport of ER vesicles that associate with *Legionella* phagosomes shortly after uptake. The reason RalF function is not required by *Legionella* during infection of host cells cultured in the laboratory may be that ER vesicles that transport cargo to the Golgi are created constitutively in healthy cells growing in nutritionally rich medium. However, in nature *Legionella* is likely to encounter protozoan hosts that are conserving energy and are less active metabolically. Under these conditions, host ARF–GEFs are likely down-regulated, reducing vesicular transport between the ER and Golgi. By injecting their own ARF exchange factor during infection, *Legionella* may be able to stimulate the creation of the ER–Golgi transport vesicles these bacteria require to remodel their phagosomes (Roy and Tilney 2002). Anyhow, in addition to RalF, *Legionella* must be injecting additional proteins into macrophages that bind ER vesicles and promote phagosome remodeling.

A second protein that is secreted through the Dot/Icm machinery has recently been discovered. A group of researchers hypothesized that some translocated proteins also function to maintain the integrity of the bacterial membrane (Conover et al. 2003). Mutations that destroy this function are predicted to result in a Dot/Icm complex that poisons the bacterium, resulting in reduced viability. To identify such mutants, strains were isolated that showed reduced viability on bacteriological medium in the presence of an intact Dot/Icm apparatus, but which had high viability in the absence of the translocator. Several such mutants were analyzed in detail to identify candidate strains that may have lost the ability to synthesize a translocated substrate of Dot/Icm. Two such strains had mutations in the *lidA* (*l*owered viability *i*n the presence of *dot*) gene. The LidA protein is indeed a translocated substrate of Dot/Icm and it can associate with the

cytoplasmic face of the phagosome (Conover et al. 2003). *LidA* mutants retain wild-type pore-mediated cytotoxicity as measured by ethidium bromide permeability of the infected macrophages (Conover et al. 2003). About 30% of the *lidA* mutants survive intracellularly and present no defects in replicative vacuole formation and overall intracellular growth (Conover et al. 2003). Further research will be necessary to enlighten how the LidA protein performs its duty as a gatekeeper and protects *L. pneumophila* from degradation by its own secretion apparatus.

Once the vacuole provides conditions for the bacteria to grow, genes of the *dot/icm* family become dispensable (Coers et al. 1999). For example, by using an inducible promoter to control *dotA* transcription, Roy and coworkers (Roy et al. 1998) found that *L. pneumophila* which express DotA before contact with macrophages but not after still replicate during the primary infection cycle. The hypothesis that Dot/Icm function is dispensable during the replication period is consistent with the observation that virulence traits are not expressed by both *in vitro*- and *in vivo*-grown *L. pneumophila* during the exponential phase of growth (Byrne and Swanson 1998; Hammer and Swanson 1999). It is worth noting that when a *dotA* mutant resides within the same phagosome as a wild-type bacterium, it can replicate (Coers et al. 1999). Furthermore, those studies have indicated that the effector molecules involved in intracellular trafficking of *L. pneumophila* and subversion of phagolysosomal fusion within mammalian cells are limited to the phagosome harboring the bacterium, where they exert a cis-acting effect that does not alter the biology of the rest of the cell (Coers et al. 1999).

Recently, Segal and colleagues (Segal et al. 1999) identified a second *L. pneumophila* secretion apparatus related to type IV systems that is distinct from the Dot/Icm complex. Designated *Lvh* (for *Legionella vir* homologues), this locus is dispensable for intracellular growth but can cooperate with the Dot/Icm complex to transfer RSF1010 plasmids by conjugation (Segal et al. 1998; Segal et al. 1999). Disruption of several of the *dot/icm* genes completely abolishes conjugation, indicating that the *lvh* locus itself cannot confer conjugation. However, components of the *lvh* system may be able to replace some Dot/Icm factors for conjugation, as judged by comparing the phenotype of particular single and double mutant strains. Deletion of the *lvh* locus in the wild-type JR32 strain modestly reduces conjugation efficiency, 10-fold. In

a similar manner, *dotB* and *icmE* mutants donate plasmid at a somewhat reduced efficiency. On the other hand, double mutants carrying a *lvh* deletion and a *dotB* or an *icmE* mutation are completely defective for conjugation. Thus, components of the *lvh* system may substitute for *dotB* and *icmE* functions that are important for conjugation, but not virulence (Segal et al. 1999).

1.1.5.6 *Pmi* and *mil* loci

Gao and colleagues (1997) identified 89 mutants of *L. pneumophila* that are not cytotoxic to and fail to grow within both U937 macrophage-like cells and *Acanthamoeba polyphaga*. As all of these mutants exhibit similar defects in both host cells, the disrupted loci were designated as *p*rotozoan and *m*acrophage *i*nfectivity (*pmi*) loci (Gao et al. 1997). Interestingly, 12 of the *pmi* mutants contain insertions in the *dot/icm* genes (Gao et al. 1997). Thus, the type IV (Dot/Icm) secretion system of *L. pneumophila* is required for infection of both mammalian and protozoan cells. These observations were later substantiated by the finding that nine *icm* genes are required for intracellular growth within human macrophages and *A. castellanii* (Segal and Shuman 1999a).

The similarity in the defects of the *pmi* mutants as well as other identified virulence loci in macrophages and *A. polyphaga* revealed that *L. pneumophila* uses many of the same genes to invade and survive within its evolutionarily distant hosts (Gao et al. 1997; Abu Kwaik et al. 1998a). It is believed that legionellae may have been primed within protozoa for the infection of mammalian cells.

However, several loci, such as the macrophage infectivity loci (*mil*) of *L. pneumophila*, seem to be only required for the infection of mammalian cells (Gao et al. 1998a; Gao et al. 1998b). Gao and colleagues screened a bank of transposon insertion mutants of *L. pneumophila* for potential mutants that exhibited defective phenotypes of cytopathogenicity and intracellular replication within macrophage-like U937 cells but not within *A. polyphaga* (Gao et al. 1998a). Twenty-six mutants were identified, with various degrees of defects in cytopathogenicity, intracellular survival, and replication within human macrophages, but wild-type phenotypes within protozoa (Gao et al. 1998a). The

growth kinetics of many mutants was also examined, and these were shown to have a similar defective phenotype in peripheral blood monocytes and a wild-type phenotype within another protozoan host, *Hartmannella vermiformis*. Transmission electron microscopy of *A. polyphaga* infected by the *mil* mutants showed that they were similar to the parental strain in their capacity to recruit the rough endoplasmic reticulum (RER) around the phagosome. In contrast, infection of macrophages showed that the mutants failed to recruit the RER around the phagosome during early stages of the infection (Gao et al. 1998a).

One of the *mil* loci studied further has been identified as a HtrA stress-induced protease/chaperone homologue (Pedersen et al. 2001). Amino acid substitutions of two conserved residues in the trypsin-like protease catalytic domain and in-frame deletions of either or both of the two conserved PDZ domains of HtrA were shown to abolish its function (Pedersen et al. 2001). A promoterless *lacZ* fusion to the *htrA* promoter was used to probe the phagosomal microenvironment harboring *L. pneumophila* within macrophages and within *A. polyphaga* for the exposure to stress stimuli. Expression through the *htrA* promoter is induced by 12 000- to 20 000-fold throughout the intracellular infection of macrophages but its induction is 120- to 500-fold within protozoa compared to *in vitro* expression (Pedersen et al. 2001). Data derived from confocal laser scanning microscopy revealed that in contrast to the parental strain, phagosomes harboring the *htrA* mutant within U937 macrophages colocalize with the late endosomal-lysosomal marker LAMP-2, similar to killed *L. pneumophila* (Pedersen et al. 2001). Coinfection experiments showed that in communal phagosomes harboring both the parental strain and the *htrA* mutant, replication of the mutant is not rescued. In contrast, replication of a *dotA* mutant control, which is normally trafficked into a phagolysosome, is rescued by the parental strain (Pedersen et al. 2001). The *L. pneumophila* stress response that is mediated by HtrA is therefore indispensable for intracellular replication within mammalian but not the protozoan cells studied (Pedersen et al. 2001).

The presence of *mil* loci raises the possibility that *L. pneumophila* has evolved mechanisms to invade mammalian cells that are independent of invasion of protozoa (Gao et al. 1998a). It should be noted, however, that *L. pneumophila* is able to invade more than 15 very divergent species of protozoa (Fields 1996; Abu Kwaik et al. 1998a; Abu

Kwaik et al. 1998b). Therefore, a likely hypothesis is that virulence mechanisms may be differentially required for invasion of different protozoa, some of which are also required for the invasion of mammalian cells (Gao et al. 1998a).

The identity of several of the *pmi* and *mil* loci is still under investigation (reviewed by Harb et al. 2000).

1.1.5.7 Iron Acquisition

Once established in the replication vacuole, iron acquisition and assimilation appears to be critical for intracellular growth of *L. pneumophila*. Interfering with the supply of intracellular iron, either by addition of chelators or by γ-interferon activation of macrophages, inhibits intracellular bacterial replication (Byrd and Horwitz 1989; Pope et al. 1996). The growth of *L. pneumophila* within human monocytes has been documented to be iron dependent as well. In the case of an aberrantly low expression of transferrin receptor in human monocytes, no infection by *Legionella* occurs (Byrd and Horwitz 2000).

However, *Legionella* does not use transferrin or lactoferrin directly (Johnson et al. 1991; Goldoni et al. 2000). Instead, the pathogen utilizes secreted and cell-associated factors as well as heme-containing compounds of the host as iron sources (O'Connell et al. 1996). The iron acquisition genes are regulated by the transcriptional regulator Fur (Hickey and Cianciotto 1997). Fur (ferric uptake regulation) is known to repress expression of iron acquisition genes (among others) when ferrous iron is present (Bagg and Neilands 1987; Hickey and Cianciotto 1994). Therefore, this intracellular pathogen likely responds to iron limitation in part by altering its pattern of gene expression. The *L. pneumophila*-specific Fur-regulated *frgA* gene encodes a protein that has homology with the aerobactin synthetases IucA and IucC (iron uptake chelate) of *E. coli*. A *frgA* mutant exhibited an 80-fold reduced intracellular growth in U937 cells (Hickey and Cianciotto 1997).

The non-classical siderophore legiobactin as well as a methyltransferase (*iraA*), a putative iron peptide transporter (*iraB*), the inner-membrane cytochrome *c* biogenesis system (*ccmC*), periplasmic and cytoplasmic Fe^{3+} reductases are known to contribute to iron assimilation (Poch and Johnson 1993; Pope et al. 1996; Liles et al. 2000; Viswanathan et al. 2000). For example, an *L. pneumophila iraAB* mutant, identified originally in a screen for strains defective for intracellular iron acquisition and assimilation, replicates poorly in U937 cells following a prolonged lag phase (Pope et al. 1996). An *iraAB* mutant is also defective for replication in a guinea pig model of lung infection (Viswanathan et al. 2000).

1.1.5.8 Growth phase and the Stringent-response

Amino acid depletion leads to the transition of *L. pneumophila* from a replicative to an infectious phase (Hammer and Swanson 1999). The conversion involves a stringent response-like mechanism, a developmental pathway thoroughly studied in *Escherichia coli* that promotes long-term survival in adverse conditions (reviewed by Swanson and Hammer 2000). Uncharged tRNAs activate RelA, a guanosine 3',5'-bispyrophosphate synthetase (Hammer and Swanson 1999). The following accumulation of ppGpp then induces the stationary-phase regulon and coordinates the entry of bacteria into a stationary and infectious phase characterized by a rapid arrest of growth and of protein and stable RNA molecule synthesis. By this mechanism, bacteria alter their physiology to tolerate a nutrient-poor environment. By analogy to *E. coli* (Gentry et al. 1993), it has also been speculated that the accumulation of ppGpp increases the amount of alternative sigma factor RpoS in *Legionella*. In support of this hypothesis, it has been observed that expression of RpoS increases during the stationary phase of *Legionella* and apparently coordinates the expression of virulence traits (Hales and Shuman 1999b). RpoS is required for maximal virulence of several pathogens, including *Salmonella* (Fang et al. 1992), *Shigella flexneri* (Waterman and Small 1996), toxigenic *E. coli* (Small et al. 1994), and phytopathogenic *Erwinia carotovora* (Mukherjee et al. 1998). An *rpoS* transposon insertion mutant strain of *L. pneumophila* replicated as well as wild-type *L. pneumophila*

within monocytic HL60 and THP-1 cells, but it was attenuated for virulence in *A. castellanii* cultures (Hales and Shuman 1999b). According to the stringent-response paradigm, RpoS functions primarily to coordinate entry into stationary phase. Consequently, in *L. pneumophila*, RpoS may be dispensable for replication but important for efficient transmission to a new phagocyte or for survival in fresh water, traits that may be critical for efficient infection in amoebae experimental models (Hales and Shuman 1999b). Therefore, it is of interest to determine whether *rpoS* null mutants express postexponential phase activities implicated in *L. pneumophila* transmission, including cytotoxicity, osmotic resistance, motility, and evasion of phagosome-lysosome fusion (reviewed by Swanson and Hammer 2000).

The stringent-response mechanism has been adopted by a large variety of organisms to respond to a changing environment according to their particular lifestyles. For *Myxococcus xanthus*, ppGpp accumulation initiates the formation of a multicellular fruiting body that subsequently differentiates into hardy myxospores (Harris et al. 1998). *Bacillus subtilis* that is starved for amino acids accumulates ppGpp, which induces expression of stress response proteins that may promote sporulation (Harris et al. 1998). In *Streptomyces coelicolor*, ppGpp accumulation plays a role in antibiotic production and the pigmentation characteristic of mature spores (Chakraburtty and Bibb 1997). For *L. pneumophila*, when nutrients are limited within its host cell, transmission to a new phagocyte is paramount.

Section 1.2

Host Resistance to *L. pneumophila*

This introductory chapter has thusfar reviewed some factors that affect the virulence of the pathogen *Legionella pneumophila.* The following section describes the interaction between *L. pneumophila* and its host from a different perspective; it provides an overview of the mechanisms by which potential hosts can resist infection. A particular emphasis is given to the host resistance factors that are under genetic control, as it is the search for one of these resistance loci that has driven the research described within the next chapters of this thesis.

1.2.1 Host Resistance to bacterial infections

A fundamental goal of host resistance research is to define the elements of a host-parasite interaction that a specific pathogen subverts to its own advantage. The normal course of events in a host-parasite interaction should therefore be known. This subsection is a rough summary of defense mechanisms that have become "common knowledge" in the immunology field, with a particular emphasis on intracellular bacteria among all possible pathogens. The facts stated can be found in most current biology/physiology/immunology textbooks. Except for very specific and new data that will be referenced in-text, here is an acknowledgement of the textbook that helped structure this section (Sleigh and Timbury 1998) and two textbooks with which the information was complemented (Vander et al. 1994; Baron 1996).

1.2.1.1 Defense mechanisms of the host

The potential host has a number of defense mechanisms with which to counteract bacterial aggression. There are two categories of defense mechanisms: nonspecific, which are not directed at a particular organism and are non-immunological, and specific mechanisms.

Among the nonspecific defenses, we may point out the skin, the normal bacterial flora of the host, lysozymes, flushing actions as well as low pH. The skin represents one of the most important barriers of the body to the microbial world. When this barrier is breached, infection is frequent. In addition to the skin, other portals through which bacteria can gain access to the body include the mucous membranes of the respiratory, gastrointestinal, and urogenital systems. Like the squamous epithelial cells of the skin, the mucosal epithelial cells divide rapidly. In the intestine for example, as the cells mature, they are pushed laterally toward the intestinal lumen and shed. The entire process is reported to require only 36-48 hours for complete replacement of the epithelium, which diminishes the number of bacteria associated with it. Also, the pores and crevices of the body are colonized by the "normal bacterial flora", which by competition can make it difficult for exogenous pathogens to establish themselves. Normal flora also produce

substances with antibacterial activity, such as fatty acids produced by skin flora from glycerides in sebum and by intestinal anaerobes from the contents of the colon. Other hostile substances to microbial colonization, which either kill bacteria or restrict their growth, include protective levels of lactoferrin, lactoperoxidase, and lysozyme, an enzyme found in tears and other body fluids that lyses the mucopeptide of the cell wall of bacteria. Tears contain lysozyme, but their flushing action is also important to keep the surface of the eye sterile. The respiratory tract mucus traps bacteria and constantly moves them upwards, away from the lungs, propelled by cilia on the cells of the epithelium. Urine also helps to flush out bacteria that have gained entry to the bladder. Low pH environments, such as found in the stomach, significantly help to destroy ingested bacteria. Vaginal secretions also have acid pH due to lactobacilli, which metabolize glycogen present in the epithelium because of circulating oestrogens. The lactic acid produced prevents access of harmful bacteria. Another mechanism of restricting growth of bacteria that penetrate the skin and mucous membranes is competition for iron. Typically, the amount of free iron in tissues and blood available to bacteria is very low, since plasma transferrin binds virtually all iron in the blood. Similarly, hemoglobin in the erythrocytes binds iron. Without free iron, bacterial growth is restricted unless the bacteria synthesize siderophores or receptors for iron-containing molecules that compete for transferrin-bound iron.

A higher level of complexity is involved in the non-specific defense provided by phagocytic cells and complement molecules. Phagocytosis is a powerful defense mechanism, mediated by scavenger cells that ingest invading organisms and destroy them intracellularly by enzyme action. Phagocytic function can be divided into four stages. The first step is chemotaxis, or the attraction of phagocytes to the site of infection. The second step is attachment of the bacterium to the membrane of the phagocyte. The third stage is the ingestion of the microbe, in which the phagocytic cell extends pseudopods to envelop the bacterium. The pseudopods then fuse to form a pouch or phagosome. Fourth and last stage is the intracellular killing of the ingested bacterium. Lysosomes containing hydrolytic enzymes and other bactericidal substances migrate towards the phagosome, and fuse with its membrane to form a phagolysosome. Most bacteria are killed within a

few minutes of phagocytosis, although the degradation of the bacterial cell may take several hours.

There are two types of phagocytes. Neutrophil polymorphonuclear leukocytes (polymorphs), also known as microphages, are produced in the bone marrow and, when mature, circulate in the bloodstream for 6 to 7 hours. These short-lived cells arrive rapidly at the scene of infection, attracted by chemotactic substances elaborated during the inflammatory process. Polymorphs, which are part of the early defense against infection, are the "pus cells" seen in the exudate from acute infections. The second type of phagocytes is macrophages of the mononuclear phagocyte system. Also produced in the bone marrow, they travel as monocytes in the bloodstream to become distributed as free macrophages in lung alveoli, peritoneum and inflammatory granulomas, or fixed macrophages, integrated into the tissues, like in lymph nodes, spleen, liver (Kupffer cells), CNS (microglia) and connective tissue (histiocytes). Phagocytosis by these long-lived cells can be either nonspecific or promoted by antibody and complement.

Complement molecules can act as opsonins: substances that bind to bacteria and increase their susceptibility to phagocytosis. Complement refers to a family of proteins present in serum. These proteins interact sequentially in a proteolytic cascade, following activation of the first stage with a bacterial or other antigen. The sequential reaction liberates fragments that attract phagocytic cells by chemotaxis, and promote subsequent phagocytosis (opsonization).

It is important to point out that phagocytes and complement also have important roles in the specific immune responses described in the next paragraphs. During the interaction of bacterial cells with macrophages, T cells, and B cells, specific immunity develops to protect against reinfection. There are two main mechanisms by which the host mounts a specific immune response against bacterial infection: the humoral (antibody) response and the cell-mediated response.

Antibodies are proteins in the bloodstream produced in response to infection by microorganisms. They are specifically directed against the antigens of the microorganism or its component parts, which are usually proteins or carbohydrates. When an antigen, e.g. on a bacterium, encounters B- (bone marrow derived) lymphocytes in the secondary lymphoid organs (spleen, lymph nodes), the lymphocytes are activated and transformed

into antibody-secreting plasma cells. The antigen is presented by macrophages and the involvement of T-lymphocytes, especially T-helper cells, is required to initiate the immune response to some antigens. Antibodies are protein molecules of high molecular weight also known as immunoglobulins (Ig). Their structure is Y-shaped and consists of an Fc fragment (the stem of the Y) and two Fab fragments (the arms of the Y). The Fab fragments contain the combining sites for specific antigens and, in antibodies to different antigens, show highly variable amino acid sequences. The Fc fragment of different antibodies, on the other hand, has a relatively constant amino acid composition and is the site for the attachment of complement. In addition, the Fc fragment is recognized by specific receptors in the membrane of phagocytic cells during opsonophagocytosis. Although there are five types of immunoglobulins, only three are critical in the response to bacterial infections. IgM, a pentamer of IgG, is the first antibody produced. It appears approximately one week after infection and persists only for about two weeks. IgG, a monomer, is the main antibody produced. It appears about two weeks after infection, and persists for longer periods of time, about six weeks. IgA, is a monomer in blood, but present as a dimer in body secretions like saliva, respiratory and alimentary mucus, tears and colostrum. IgA in extracellular fluids (secretory IgA) is coupled to a protein secretory piece, which is not found on serum IgA. Antibodies are a powerful defense mechanism against viruses, because they neutralize viral infectivity. They are much less effective on their own (i.e. without complement) against bacteria, but are nevertheless important in combating bacterial infection by the following mechanisms: neutralization of toxins, promotion of phagocytosis (antibody-coated bacteria are more readily phagocytosed than those coated with complement alone), and bacterial lysis (certain Gram-negative bacilli, such as strains of *Escherichia coli*, are lysed in the presence of antibody and complement).

The second mechanism for mounting a specific immune response is a cell-mediated response. Delayed hypersensitivity, or cell-mediated immunity, was first described for tuberculosis in the late 19th century. Delayed hypersensitivity develops slowly over 24-48 h, and is especially important in infections due to organisms which persist or multiply intracellularly, such as the bacteria which cause tuberculosis, leprosy and brucellosis, and viruses. Many of the functions described are initiated and regulated

by a variety of chemical mediators (e.g. interleukins, tumor necrosis factor) collectively known as cytokines. T-lymphocytes are a population of leucocytes that have undergone maturation in the thymus. Responsible for cell-mediated immunity, they comprise the majority of the circulating lymphocytes in humans, the rest being bone marrow derived B-lymphocytes described previously for their role in humoral immunity. Macrophages are among a group of cells that can process bacterial antigens and present them to lymphocytes to stimulate a specific immune response. When sensitized, or primed, T-lymphocytes become activated and release a variety of cytokines (lymphokines), which in turn recruit other inflammatory cells to mount the cell-mediated immune reaction. The activities of these lymphokines include chemotactic attraction of lymphocytes, macrophages and polymorphonuclear leukocytes to the site of infection, increased capillary permeability and mitogenic activity (stimulation of lymphocytes to divide and differentiate). In delayed hypersensitivity, the inflammatory lesion is heavily infiltrated with sensitized T-lymphocytes and macrophages. Other T-lymphocytes, called helper and suppressor cells, regulate the immune response. The overall effect of delayed hypersensitivity is to limit the size of the lesion and to localize the organism within it: although initially protective, there is some risk of unwanted tissue damage. In general, bacteria that can enter and survive within eukaryotic cells are shielded from humoral antibodies and can be eliminated only by a cellular immune response. Certainly, the capacity of bacteria to survive and multiply within host cells has great impact on the pathogenesis of the respective infections. Fortunately, most bacteria in the environment are relatively benign to individuals with normal immune systems. However, in patients who are immunosuppressed, such as individuals receiving cancer chemotherapy or who have AIDS, opportunistic microbial pathogens can establish life-threatening infections.

1.2.1.2 Defense mechanisms critical for *Legionella*

Risk factors for Legionnaire's disease include conditions that compromise both the specific and non-specific defenses. The fact that smokers as well as patients with chronic lung disease are at increased risk of developing serious *Legionella* pneumonia (Pedro-

Botet et al. 1995; Tkatch et al. 1998) suggests that the integrity of physical clearance mechanisms, such as the mucociliary escalator of the tracheobronchial tree, is an important element of the defenses. Nonimmunologic antibacterial factors normally found in respiratory secretions, such as lactoferrin or lysozyme, may also play a role (Hambleton et al. 1982; Bortner et al. 1986).

Inflammatory cell defenses play both positive and negative roles. The human alveolar macrophage and its relative, the recruited blood monocyte, fail in their normal roles as primary antibacterial defenses in *Legionella* infections: instead of destroying the invading bacteria, they serve as a replication niche (reviewed by Cianciotto 2001). Polymorphonuclear leukocytes, on the other hand, do not support bacterial growth *in vitro* (Davis et al. 1983). Neutropenia (diminished number of circulating neutrophils) is therefore a potential risk factor for legionellosis (Hollander et al. 1991).

The most impressive risk factors for human disease are various types of immunosuppression. In a small outbreak of disease caused by contaminated nebulizers, pneumonia developed most often in patients being treated with corticosteroids (Schlossberg and Bonoan 1998). This points out that since human phagocytes do not clear *Legionella* from the alveoli, we rely mainly on the secondary, specific immune responses to put a check on the pathogen's progress.

Attention has focused on cell-mediated immunity because *Legionella* is a facultative intracellular pathogen. In contrast to naive alveolar macrophages, which are permissive for intracellular bacterial growth, activated alveolar macrophages or peripheral blood monocytes have been shown to restrict *Legionella* multiplication *in vitro* (Nash et al. 1984). The macrophages can be activated by treatment with lymphokines produced by specifically stimulated lymphocytes. Indeed, lymphocytes appear in the air spaces of experimentally infected animals about 5 days after an acute infection (Susa et al. 1998). Therefore, infected patients undergo a cell-mediated immune response that can be detected by measuring lymphocyte blastogenesis (Plouffe and Baird 1982). Gamma interferon, which can substitute for the lymphokines, is an important mediator (reviewed by Friedman et al. 1998). Depletion of gamma interferon makes experimental animals vulnerable to infection (Heath et al. 1996; Shinozawa et al. 2002). Restricting the availability of iron (an important growth factor for *Legionella*) in the *Legionella*

phagosome has also been shown to be an important factor to inhibit intracellular growth (Byrd and Horwitz 1989; Marra et al. 1990; Byrd and Horwitz 1991; Gebran et al. 1994; Gebran et al. 1995; Byrd and Horwitz 2000).

The role of humoral immunity is less clear. Antibodies in all immunoglobulin classes are made after human or experimental infection with *Legionella* (reviewed by Ehret 1992; Yoon et al. 2002). This antibody serves an opsonizing function *in vitro*, facilitating the phagocytosis of bacteria by polymorphonuclear leukocytes, macrophages, and monocytes (Horwitz and Silverstein 1981). Antibody does not kill most strains of *Legionella* however, so that the outcome of the interaction depends on the capabilities of the phagocytic cell (Horwitz and Silverstein 1981). The classic pathway of the complement system is activated by *L. pneumophila*, enhancing phagocytosis still further (Verbrugh et al. 1985; Mintz et al. 1992). *Legionella micdadei* activates the alternative complement pathway as well, so that opsonization of this species can occur even before an immunologically specific antibody response is mounted (Steffensen et al. 1985). One can construct scenarios from *in vitro* data in which antibody is deleterious as well as helpful. Experimental studies with animals support a protective role for antibody (Breiman and Horwitz 1987; Blander and Horwitz 1989; Spitsyn et al. 1990; Weeratna et al. 1994).

Our primary defense mechanisms being defective, a large pressure is exerted on our secondary immune response. The lag of time required for a specific response to enter in effect is enough that we do not have one *Legionella* cell to combat, but thousands of freshly replicated daughter cells. For healthy humans, the condition is not necessarily life threatening, but it is for the growing percentage of the population that because of old age and/or parallel disease, fail to mount an effective immune response. Should phagocytes succeed in killing *Legionella* cells from the first encounter, this pathogen would not be as big a threat for humans. A striking proof of this is the resistance of mice to experimental *Legionella* pneumonia, attributed to the fact that their alveolar macrophages do not support intracellular bacterial growth (Yoshida and Mizuguchi 1986). This has been a major driving force in our own investigation of macrophage resistance to *Legionella* replication, as will be seen in detail throughout the rest of this thesis.

1.2.2 Experimental models of legionellosis.

In order to dissect and analyze the host-parasite interactions involved in legionellosis, model systems of the disease can be very helpful. Indeed, the study of cases of legionellosis in humans can reach a high level of complexity due to a multitude of factors. Some of these variability factors are environmental, such as infection dosage and infection route; other factors include the possible genetic heterogeneity of both the host and the pathogen. Most of these variables can be easily controlled in an experimental setting. Model systems for the study of *Legionella*-host interactions include *in vivo* infection of animals, *ex vivo* infection of cells explanted from humans and a variety of animals, *in vitro* experimentation with cell lines, and infection of natural protozoan hosts grown in the laboratory.

1.2.2.1 *In vivo* infection of animals.

Legionella can infect several laboratory animals, including rats (Davis et al. 1982; Yoshida and Mizuguchi 1986) and hamsters (Katz and Poropatich 1986; Yoshida and Mizuguchi 1986), but the guinea pig remains the most susceptible animal known. Guinea pigs exposed to aerosols of *Legionella pneumophila* develop pyrexia and pneumonia, with high mortality within three days after exposure (Baskerville et al. 1981). Accompanied by weight loss and fever, histopathological changes indicative of an acute fibrinopurulent pneumonia can be observed, with widespread fibrin exudation and accumulation of neutrophils and macrophages in alveolar lesions (Baskerville et al. 1981). Overall, infected guinea pigs exhibit symptoms which closely resemble *Legionella* pneumonia in man (Baskerville et al. 1981; Davis et al. 1982) and have become, by far, the most widely used animal model in the study of legionellosis. A closer look at the infected animals yielded important knowledge about the interaction between *Legionella* and host cells (Katz and Hashemi 1982). Within neutrophils, *Legionella pneumophila* typically displayed degenerating forms, suggesting that this intracellular environment is

somewhat hostile to the bacterium. By contrast, macrophages tended to contain intact forms, located within organelles morphologically identical with rough endoplasmic reticulum. Some bacteria were replicating at this site. These findings suggested for the first time that *Legionella pneumophila* is an intracellular parasite of macrophages (Katz and Hashemi 1982). Subsequent experimentation with lower infection doses enabled to study the resolution of the disease in this very susceptible host (Davis et al. 1983). *Legionella pneumophila* still produces pneumonia at low doses of intranasal infection. Bronchoalveolar lavage was used to sample airspace cells, secretions, and bacteria during developing infection. An influx of polymorphonuclear neutrophils followed exponential bacterial growth during the initial three days of infection and coincided with limitation of the increase in bacteria recovered. Again, most viable *L. pneumophila* organisms were associated with alveolar macrophages, whereas most of the bacteria associated with polymorphonuclear neutrophils were nonviable. A macrophage influx occurred around day five. Bacteria were eliminated from the lung by 11 days after exposure. Thus, it became clear that recruited, and possibly immune, defenses are required for successful resolution of *Legionella* pneumonia (Davis et al. 1983). This was later confirmed (Breiman and Horwitz 1987). Guinea pigs sublethally infected with *L. pneumophila* by the aerosol route develop strong humoral and cell-mediated immune responses to this pathogen, are able to clear the bacteria from their lungs, and are protected against subsequent lethal aerosol challenge (Breiman and Horwitz 1987). Experimentally induced *Legionella* pneumonia in guinea pigs has been crucial to the study of antibacterial agents efficient against *Legionella in vivo* (Kohno et al. 1988; Edelstein 1995b). Also, several studies used this *in vivo* model to identify *Legionella* strains (Fitzgeorge et al. 1983; Fields et al. 1990) or mutants (Blander et al. 1990; Cianciotto et al. 1990; Tully et al. 1992; Moffat et al. 1994; Edelstein et al. 1999; Higa and Edelstein 2001; Kohler et al. 2003) displaying reduced virulence. Besides the relevant intra-nasal and intra-tracheal routes of infection used in many of these studies, direct intraperitoneal injection of the bacteria has also been used successfully (Elliott and Johnson 1982; Hambleton et al. 1982; Katz and Hashemi 1982). Guinea pigs were also used to study the fate of orally ingested *L. pneumophila* (Plouffe et al. 1986). Guinea pigs were fed *L. pneumophila* through an orogastric tube. Gastric acid was rapidly cidal to the organisms. Serial

necropsies demonstrated the killed organisms in the colon and blood stream at 1 hr. Guinea pigs fed large doses of *L. pneumophila* seroconverted. Previously fed guinea pigs were then challenged with a lethal intraperitoneal dose of *L. pneumophila* and were protected in a dose-dependent fashion (Plouffe et al. 1986). *L. pneumophila* antigens with potential as vaccines have usually been first tested for efficacy in guinea pigs (Blander and Horwitz 1989; Weeratna et al. 1994).

Rhesus monkeys (*Macaca mulatta*) given low doses of bacteria have been shown to display slight fever and mild histological lesions in the lungs (Baskerville et al. 1981). Thus, aerosol infection of these animals offers a suitable experimental model of legionnaires' disease (Fitzgeorge et al. 1983) and certainly represents the closest parallel to the human disease. However, there are obvious ethical, practical and economical factors that limit the use of monkeys in most *Legionella*-specific research projects.

Most inbred mouse strains have been shown to be highly resistant to *L. pneumophila* infection, their 50% lethal dose of *L. pneumophila* being at least 1000-fold higher than in the susceptible guinea pig model (Yoshida and Mizuguchi 1986). The resistance observed in mice can be correlated to the non-permissiveness of their macrophages (Yoshida and Mizuguchi 1986). This is in contrast to rats and hamsters which display a certain level of resistance to *Legionella* pneumonia relative to guinea pigs even though their macrophages are clearly permissive to *L. pneumophila* replication (Yoshida and Mizuguchi 1986). The non-permissive nature of the inflammatory peritoneal macrophages from several mouse inbred strains (BDF1, AKR, DBA/2, C3H/HeN, C57BL/6, and BALB/c) has been clearly shown (Yoshida and Mizuguchi 1986; Yamamoto et al. 1988). Interestingly, although mice are resistant to induction of disease by *Legionella* aerosols, the organisms persist in the lungs for at least 4 days (Fitzgeorge et al. 1983). This already pointed out to a bacteriostatic rather than a bactericidal activity of mouse macrophages. Important exceptions to the overall resistance of inbred mice to *L. pneumophila* infections will be presented in subsequent sections of this thesis.

1.2.2.2 *Ex vivo* models.

In order to study the cellular and subcellular aspects of Legionnaires' disease, human polymorphonuclear neutrophils, alveolar macrophages, peripheral blood monocytes and epithelial cells are widely used *ex vivo*. These explanted cells are not only convenient to use as hosts for *L. pneumophila* replication in an experimental setting, they also remain highly relevant to the human disease. A recurrent use for explanted human cells is to confirm observations made in other model systems and to affirm their relevance to the human disease (Mody et al. 1993; Cirillo et al. 2001; Higa and Edelstein 2001). For example, alveolar macrophages were used succesfully to demonstrate the loss of infectivity of *Legionella Mip* mutants (Cianciotto et al. 1990); a phenotype previously reported in cell lines (Cianciotto et al. 1989b).

In addition to the human cells described, *L. pneumophila* readily infects and multiplies in mononuclear phagocytes of the guinea pig *ex vivo* (Horwitz and Silverstein 1980; Kishimoto et al. 1981), and these have been widely used as experimental models for legionellosis (Elliott and Winn 1986; Yoshida et al. 1987; Edelstein and Edelstein 1989; Miyamoto et al. 1993; Miyamoto et al. 1995; Rajagopalan-Levasseur et al. 1996). The relative availability of guinea pig cells serves well such large-scale experiments as the screening of transposon mutants of *L. pneumophila* for loss of virulence-associated characteristics (Tully et al. 1992). In this example, hundreds of mutants were screened and three were found with a greatly reduced ability to multiply within guinea pig alveolar macrophages *in vitro* (Tully et al. 1992). Specific *L. pneumophila* mutants, such as for the Zn-metalloprotease encoded by the *proA* gene (Moffat et al. 1994) and *ptsP* (phosphoenolpyruvate phosphotransferase) (Higa and Edelstein 2001), have also been tested for their ability to replicate within explanted guinea pig alveolar macrophages. The replication defects observed within the guinea pig macrophages *ex vivo* were then verified for their ability to cause disease *in vivo* (Tully et al. 1992; Moffat et al. 1994; Higa and Edelstein 2001). Guinea pig peritoneal macrophages provide researchers with an additional level of convenience, as they are plentiful and very easy to collect. Both resident and elicited (either with proteose peptone or thioglycollate medium) guinea pig

peritoneal macrophages are permissive to *L. pneumophila* replication (Yoshida et al. 1987).

1.2.2.3 *In vitro* experimentation with cell lines.

Cell lines derived from human leukemias like the phagocytic U937 and HL60 cells, or non-phagocytic HeLa, Vero and WI-26 cells have the advantage of being immortal and thus constantly replicating and available for laboratory experiments. U937 and HL-60 cells are immature monocyte-like cells that can be terminally differentiated into macrophages upon treatment with the phorbol ester PMA (phorbol myristate acetate). The macrophage monolayers obtained are permissive to *L. pneumophila* replication (Cianciotto et al. 1989b; Marra et al. 1990) and have been used extensively since their acceptance as suitable host models (Marra et al. 1990; King et al. 1991; Abu Kwaik et al. 1993; Wintermeyer et al. 1995; Muller et al. 1996; Gao et al. 1997; Segal and Shuman 1997; Hales and Shuman 1999a; Viswanathan et al. 2000; Helbig et al. 2003). Experimentation with cell lines being faster and cheaper than with primary cells or live animals, the immortalized cells are usually used first, and the data is then confirmed with other models of legionellosis. Such was the case for the loss of infectivity of *Mip* mutants, first seen in U937 cells (Cianciotto et al. 1990). Non-phagocytic epithelioid HeLa cells, derived in 1952 from a human cervical carcinoma, have been extensively used to study *Legionella* adherence and invasiveness into the host cell (Dreyfus 1987; Hoffman et al. 1990; Garduno et al. 1998b; Garduno et al. 1998c; Goldoni et al. 1998; Stone and Abu Kwaik 1998). Fibroblast-like Vero cells, derived from the kidney of an African green monkey, have been successfully used to study the morphology of different *Legionella* species throughout their intracellular life cycle (Ogawa et al. 2001) as well as to study the cytolytic activity of the bacteria (Hacker et al. 1991; Wintermeyer et al. 1991). Epithelial cells, which constitute the majority of the alveolar wall surface, have been recognized as an important replication niche for *L. pneumophila* (Gao et al. 1998b). The WI-26 (Wistar Institute 26) cell line, an SV40-transformed type-II epithelial cell from a human lung, is therefore being used in an increasing number of studies (Cianciotto et al. 1995; Stone and Abu Kwaik 1998; Gao and Abu Kwaik 1999b; Harb and Abu Kwaik 2000). A549 cells,

derived from a human lung carcinoma, which also display some properties of type-II epithelial cells have also been successfully infected to study a *Legionella* mutant deficient for intracellular replication (Higa and Edelstein 2001).

1.2.2.1 Infection of natural protozoan hosts.

Axenically grown protozoa of the *Acanthamoeba*, *Hartmannella*, *Naegleria* and *Tetrahymena* genera have been shown to support intracellular *Legionella pneumophila* replication and are good model systems (Rowbotham 1980; Fields et al. 1984; King et al. 1991; Fields 1996). *Acanthamoeba*, a genus of amoebae (order amoebida) is the most extensively studied protozoan host of *L. pneumophila*. These amoebae are widespread and common in soil and fresh water, where they prey on bacteria and yeasts. Two species of *Acanthamoeba* are equally popular model systems in the literature: *A. castellanii* (Holden et al. 1984; Bozue and Johnson 1996) and *A. polyphaga* (Kilvington and Price 1990; Gao et al. 1997). Some milestones in the knowledge obtained through the use of *Acanthamoebae* as model hosts of *L. pneumophila*: *Legionella* replicates within these amoebae within rough endoplasmic reticulum-derived vacuoles lined with ribosomes just as within mammalian macrophages, but different attachment and invasion processes are used within the different hosts (Harb et al. 1998). Also, it was discovered that there are many *L. pneumophila* genes that are equally important for succesfull intracellular replication within mammalian and protozoan cells (Gao et al. 1997; Segal and Shuman 1999a; Fettes et al. 2000). There are, however, some *L. pneumophila* genes such as those involved in the stress response of the bacterium that seem to be differentially required for replication witihin macrophages and amoebae (Gao et al. 1998a; Hales and Shuman 1999b; Pedersen et al. 2001). Amoebae of the genus *Hartmannella* are also popular for the study of *L. pneumophila* within a natural host. *H. vermiformis* is the most recurrent species in the literature (Abu Kwaik 1996). Major discoveries using *H. vermiformis* include the modulation of host protein levels after contact with *L. pneumophila* (abu Kwaik et al. 1994), the Gal/GalNAc lectin that serves as a receptor for the bacterium (Venkataraman et al. 1997), and the tyrosine dephosphorylation cascade that is induced

by *L. pneumophila* upon attachment to its host (Venkataraman et al. 1998). As mentioned previously, *L. pneumophila* replicates not only within amoeba but all sorts of different protozoa, and a good amount of research has been conducted on the interaction of *L. pneumophila* with *Tetrahymena* hosts, mainly *T. pyriformis* (Fields et al. 1984; Fields et al. 1986). *Tetrahymenas* are a genus of ciliate protozoa (order hymenostomatida) which occur in various freshwater habitats (Singleton and Sainsbury 1987).

Recently it was found that simple model organisms like *Caenorhabditis elegans* can reveal how bacteria infect cells (Mahajan-Miklos et al. 1999; Tan et al. 1999; Labrousse et al. 2000). This encouraged the development of genetically manipulatable host systems for *Legionella*. *Dictyostelium* is a genus of soil-living slime moulds and *D. discoideum* is the best-known species as it is easily cultured in the laboratory. During the feeding stage of the life cycle, the amoebal *Dictyostelium discoideum* has a haploid genome and feeds mainly on bacteria. Upon starvation, *D. discoideum* aggregates and differentiates into pluricellular fruiting bodies. Besides its amenability to genetic manipulation, *D. discoideum* expresses highly conserved cellular markers, and its cell-signaling pathways are well characterized. Moreover, sequencing of the *D. discoideum* 34 Mb, 6-Chromosome haploid genome will soon be complete (information conveniently available at http://dictybase.org). Consequently, future strategies with the *Legionella–Dictyostelium* model will rely on a two-sided genetic approach. By using single cell stages of the amoeba *Dictyostelium discoideum* as host cells, researchers have already begun a molecular analysis of host cell functions and targets during *Legionella* infection (Hagele et al. 2000; Solomon et al. 2000; Duhon and Cardelli 2002).

1.2.3 A genetic approach to the study of host resistance

Components of defense to infection can manifest themselves as genetic determinants conferring innate resistance or susceptibility to infection in human populations (Hill 1998) or in experimental animal models of infection, such as the laboratory mouse (Skamene 1983). Characterizing the genes and proteins implicated in these natural resistance phenomena and elucidating the mechanisms involved can provide insight into host interactions with pathogens as well as shed light on basic cellular functions.

The past decade has witnessed a rapid transition from the first positional cloning of an infectious disease susceptibility gene in the mouse (*Slc11a1*, also called *Nramp1*), to genome-wide scans in human multicase families and the identification of potential disease-causing genes by simple inspection of the public human genome databases. Nevertheless, the search for individual genes that control variable disease outcome in humans remains often difficult since many traits do not follow simple Mendelian genetics and the number of affected cases and well-matched controls is often limiting (Lander and Schork 1994; Skamene et al. 1998).

A simpler approach is to first identify genes important in mouse models of disease, and then to determine their involvement in disease onset, progression and outcome in human populations (Qureshi et al. 1999b). Genetic analysis of mouse models of infectious diseases provides many advantages when compared with gene discovery in humans. Animal studies facilitate establishment of uniform and controlled experimental conditions with respect to strain, dose, time, and route of inoculation of the pathogen, which minimizes phenotypic heterogeneity due to non-genetic factors. Similarly, the effects of environmental conditions and prior exposure or vaccination can be ruled out. Genetically, inbred strains of mice provide an unlimited number of identical individuals that are homozygous at each locus. When differences in resistance or susceptibility to infection between different mouse strains are identified, informative crosses can be set up in a prospective, directed fashion to identify and eventually isolate the loci involved. This is in direct contrast to genetic studies in humans, where linkage analysis is usually retrospective. Another significant advantage of using the mouse as a model system is the

feasibility of introducing germ line mutations, which allows direct assessment of infection and resulting disease in presence or absence of the chosen gene product. Finally, once host resistance genes have been identified in mice, their human counterparts can easily be isolated, and their relevance in human disease examined. The identified candidate genes can be tested in association or linkage studies in human populations from endemic areas of disease.

Genetic studies of mouse models of disease have led to the mapping of numerous loci involved in host resistance to infection with various microbes, including bacteria, protozoa and viruses (Table 1.2). However, the number of loci for which the corresponding gene has been identified and cloned has remained small until recently. Since many of the resources and tools that facilitate these studies are still evolving, the number of host resistance genes cloned is expected to increase substantially within the next few years (Blackwell 2001). The identification and characterization of the murine natural resistance-associated macrophage protein 1 (*Nramp1*) serves as a paradigm for the successful use of this approach to gain novel insight into the mechanisms of host defense.

The *Nramp1* gene was isolated by positional cloning: a technique to pinpoint a gene and identify it on the basis of its location in the genome. The *Nramp1* genetic locus has been variously known in earlier studies as *Ity*, *Lsh* and *Bcg* for its role in controlling innate resistance and susceptibility to *Salmonella typhimurium*, *Leishmania donovani* or *Mycobacterium bovis* BCG infection, respectively. The ability to clone the gene on the basis of its map location was vitally dependent on the ability to accurately predict the genotype of individual mice according to their phenotypic response to infection. For all three infections (Bradley 1974; Plant and Glynn 1974; Gros et al. 1981), segregation of disease phenotypes in the F2 and backcross progeny made between resistant and susceptible inbred mouse strains followed perfect Mendelian inheritance. Progeny mice fell clearly into non-overlapping resistant or susceptible phenotypes on the basis of liver or spleen pathogen counts, with resistance behaving as a dominant trait. After a number of Mendelian segregation analyses and genetic mapping studies (Plant and Glynn 1976; Bradley 1977; Bradley et al. 1979; Plant and Glynn 1979; Forget et al. 1981), it was concluded that resistance to *Salmonella* (*Ity* locus), *Leishmania* (*Lsh* locus) and

Table 1.2 Examples of host resistance loci discovered using mouse models of disease.

Infectious agent	Locus	Mapping Ref.	Gene cloned	Cloning Ref.
Xenotropic and polytropic murine leukemia viruses (MuLV).	*Rmc1* (Chr.1)	(Kozak 1983; Lyu and Kozak 1996)	Xenotropic and polytropic retrovirus receptor; *Xpr1*	(Tailor et al. 1999)
Herpes simplex virus type 1 (HSV-1)	*Hrl* (Chr. 6)	(Lundberg et al. 2003)		
Friend leukemia retrovirus	*Fv1* (Chr. 4)	(Rowe and Sato 1973; Stoye et al. 1995)	Fv1 endogenous retroviral sequence	(Best et al. 1996)
	Fv2 (Chr. 1)	(Lilly 1970)	Stem cell kinase receptor; *Stk*	(Persons et al. 1999)
West Nile flavivirus	*Wnv* or *Flv* (Chr. 5)	(Sangster et al. 1994)	2'-5'-oligoadenylate synthetase; *Oas1*	(Mashimo et al. 2002; Perelygin et al. 2002)
Murine cytomegalovirus (MCMV)	*Cmv1* (Chr. 6)	(Scalzo et al. 1995)	Killer cell lectin-like receptor; *Klra8* (formerly *Ly49h*)	(Lee et al. 2001)
Neuroadapted sindbis virus	*Nsv1* (Chr. 2)	(Thach et al. 2001)		
Influenza A virus	*Mx* (Chr. 16)	(Arnheiter et al. 1976) (Staeheli et al. 1986)	Myxovirus resistance 1; *Mx1*	(Hug et al. 1988; Staeheli et al. 1988)
Gram-negative bacteria	*Lps* (Chr. 4)	(Watson et al. 1978b)	Toll-like receptor 4; *Tlr4*	(Poltorak et al. 1998b; Qureshi et al. 1999a)
Mycobacterium tuberculosis	*Sst1* or *Trl-1* (Chr. 1)	(Kramnik et al. 2000; Mitsos et al. 2000)		
	Trl-2 (Chr. 3)	(Mitsos et al. 2000)		
	Trl-3 (Chr. 7)			
	Trl-4 (Chr. 19)	(Mitsos et al. 2003)		
Salmonella typhimurium	*Ity* (Chr. 1)	(Plant and Glynn 1979)	Solute carrier family 11a member 1; *Slc11a1* (previously *Nramp1*)	(Vidal et al. 1993)
Mycobacteria	*Bcg* (Chr. 1)	(Gros et al. 1981; Brown et al. 1982)		
Leishmania donovani	*Lsh* (Chr. 1)	(Bradley et al. 1979)		
Plasmodium chabaudi	*Char1* (Chr. 9)	(Fortin et al. 1997)		
	Char2 (Chr. 8)			
	Char3 (Chr. 17)			
	Char4 (Chr. 3)	(Fortin et al. 2001)	Pyruvate kinase; *Pklr*	(Min-Oo et al. 2003)

Mycobacteria (*Bcg* locus) was conferred by one single gene on mouse Chromosome 1, with a somewhat general role in controlling resistance to intracellular infections (Brown et al. 1982). The inheritance of resistance to infection in comparison to the inheritance of known genetic markers throughout the genome in close to 1500 backcross and recombinant inbred mice was followed to generate a high-resolution linkage map in the vicinity of the *Bcg* locus (Malo et al. 1993b). This genetic map was then converted to a physical map by isolation and characterization of overlapping DNA fragments which covered the entire genetic interval (Malo et al. 1993a). Exon trapping and analysis of tissue expression profiles of candidate exons or genes within the narrowed-down interval known to contain the gene finally identified the macrophage-expressed *Nramp1* gene as the most likely candidate for the *Bcg* locus (Vidal et al. 1993). Subsequent transgenesis (Govoni et al. 1996) and gene targeting (Vidal et al. 1995b) experiments confirmed that *Nramp1* controls natural resistance to *Mycobacterium*, *Salmonella* and *Leishmania*.

Once the host resistance gene has been properly identified, further research is necessary to understand the molecular basis to disease, and ideally, to develop new therapeutic interventions. Such has been the case for *Nramp1*. Although there were overall features of the molecule that suggested that Nramp1 was a transporter, there was no match to other sequences in the public databases when the gene was first positionally cloned (Vidal et al. 1993). The Nramp1 protein is an integral membrane protein expressed exclusively in the lysosomal compartment of monocytes and macrophages (Gruenheid et al. 1997; Searle et al. 1998). After phagocytosis, Nramp1 is targeted to the membrane of the microbe-containing phagosome, where it may modify the intraphagosomal milieu to affect microbial replication. Eventually, sequence identity to two yeast orthologues SMF1/2 (West et al. 1992) was recognized (Blackwell et al. 1994). Functional characterization of SMF1/2 (Supek et al. 1996) further suggested divalent cation transport as the function of Nramp1. A second member of the mammalian *Nramp* family, *Nramp2*, was discovered (Gruenheid et al. 1995; Vidal et al. 1995a) and shown to be mutated in animal models of iron deficiency (Fleming et al. 1997; Fleming et al. 1998). This, with other studies (Gunshin et al. 1997; Pinner et al. 1997) determined that Nramp2 is the major transferrin-independent iron uptake system of the intestine. The most recent studies

indicate that Nramp1 (now renamed solute carrier family 11a member 1; Slc11a1) may control intracellular microbial replication by actively removing iron or other divalent cations from the phagosomal space (Jabado et al. 2000; Goswami et al. 2001). *Nramp* homologues have now been identified in many other animal species and actually define a protein family conserved from bacteria to humans (Cellier et al. 1995; Cellier et al. 1996). Allelic variants at the human *Nramp1* homologue have recently been found to be associated with susceptibility to tuberculosis (Bellamy et al. 1998; Greenwood et al. 2000; Awomoyi et al. 2002) and leprosy (Abel et al. 1998) in humans. The cloning of *Nramp1* thus provides strong support for the use of murine models of disease in identifying disease susceptibility genes that will be important in man. However, it also demonstrates that gene identification could represent only the first step in a long process of determining the underlying molecular basis to disease susceptibility.

1.2.4 Genetic control of resistance to *Legionella pneumophila* in mice

Most inbred mouse strains have been shown to be highly resistant to *L. pneumophila* infection, their 50% lethal dose (LD_{50}) of *L. pneumophila* being at least 1000-fold higher than in the susceptible guinea pig model. In the first publication to demonstrate this, intraperitoneal injection of the Philadelphia-1 strain of *L. pneumophila* into 25 Guinea pigs of the Hartley strain determined an LD_{50} per animal of 7.6×10^4 CFU, compared to the 6.7×10^7 CFU necessary with the BALB/c strain of mice (Yoshida and Mizuguchi 1986). The difference in susceptibility became even more obvious when the LD_{50} was related to the body weight of the infected animal (LD_{50}/g body weight): 1.5×10^2 CFU for the guinea pig versus 3.3×10^6 CFU for the mouse (Yoshida and Mizuguchi 1986). The resistance observed in mice was correlated to the non-permissiveness of their macrophages. Inflammatory peritoneal macrophage monolayers from several mouse inbred strains (BDF1, AKR, C3H/HeN, C57BL/6 and BALB/c) never supported more than a 10-fold increase in *L. pneumophila* CFUs during a three-day infection period, whereas at least a 3-log replication could be observed within guinea pig macrophages

(Yoshida and Mizuguchi 1986; Yamamoto et al. 1987). It was tempting to investigate further the mechanism behind the observed difference in permissiveness between mouse and guinea pig macrophages to *L. pneumophila* intracellular replication. First, it was determined that the initial uptake of *L. pneumophila* by macrophages from both guinea pigs and mice appeared to be almost the same on the basis of CFU per macrophage (Yamamoto et al. 1987). Also, guinea pig peritoneal macrophages readily supported the growth of *L. pneumophila* at infectivity ratios ranging from two bacteria per macrophage (2:1) to as little as 0.002:1 (Yamamoto et al. 1987). Mouse peritoneal macrophages derived from the BDF1 strain, on the other hand, restricted growth of the bacteria at all infectivity ratios tested: as low as 0.006:1 and as high as 65:1 (Yamamoto et al. 1987). Therefore, bacterial load did not affect the innate ability of the macrophage to restrict intracellular replication. Further experiments showed that cell populations permissive for *L. pneumophila* could be transformed to nonpermissive by products from stimulated lymphocytes. Before being infected with *L. pneumophila*, guinea pig macrophage monolayers were preincubated for 24 h with culture supernatants from guinea pig spleen cells activated by ConA. Within 2 days of infection, growth of *L. pneumophila* in the treated cultures was markedly restricted (Yamamoto et al. 1987). However, opsonization of the bacteria with immune serum did not induce the restriction of growth within guinea pig macrophages (Yamamoto et al. 1987). Unfortunately, there are many biochemical, morphological, and functional differences between lymphokine-treated and nontreated guinea pig macrophages (Yamamoto et al. 1987); pointing to the need for further comparative analyses of permissive and nonpermissive macrophage populations to define molecular mechanisms responsible for the restriction of *L. pneumophila* growth. As will be seen in the next two subsections, a major breakthrough came up when exceptions to the overall resistance of inbred strains of mice to *L. pneumophila* infection were discovered. The availability of permissive and nonpermissive mouse strains enabled to implement, for the first time, a genetic approach to the study of host susceptibility to *Legionella*.

1.2.4.1 *Lgn1* Locus

In the quest for a mouse model that might be permissive for *L. pneumophila* growth, Yamamoto and colleagues found one when they performed an *in vitro* infection of thioglycolate-elicited peritoneal macrophages from the A/J strain (Yamamoto et al. 1988). Indeed, over the course of a three-day infection, A/J macrophage monolayers supported a greater than 100-fold replication of the bacterium (Fig. 1.3a), a permissive phenotype reminiscent of the one observed for guinea pig macrophages. This study also added DBA/2 to the growing list (BDF1, AKR, C3H/HeN, C57BL/6, BALB/c) of mouse strains whose inflammatory peritoneal macrophages were shown to restrict *L. pneumophila* replication (Yoshida and Mizuguchi 1986; Yamamoto et al. 1987; Yamamoto et al. 1988). A closer look at their results revealed that the initial uptake of the bacterium was similar in all macrophage cultures tested, permissive and non-permissive. *Legionella* growth in A/J macrophages was then tested at different infectivity ratios. 0.02, 0.2 and 2 bacteria per macrophage were added to the monolayers, and at all three ratios, A/J macrophages readily supported the growth of *L. pneumophila* (Yamamoto et al. 1988). At lower infectivity ratios, however, a smaller percentage of macrophages were infected during the initial phagocytosis period (as little as 1/1000 infected macrophages). This resulted in replication curves that do not plateau before the three-day infection period (Fig. 1.3b). Thus, greater than 1000-fold bacterial replication could be observed before exhausting the availability of uninfected macrophages for *L. pneumophila*. This is because the intracellular life cycle of *L. pneumophila* consists in lysis of the host macrophage and reinfection of neighboring macrophages by newly replicated bacteria every 24 hours approximately under these *in vitro* conditions (Gao et al. 1999; Molmeret et al. 2002b).

Researchers seeked to ascertain whether elicited peritoneal macrophages from the A/J strain had a generalized functional defect. IL-1 production following *in vitro* stimulation with *L. pneumophila* vaccine or *E. coli* endotoxin by A/J macrophages was perfectly normal when compared directly with the other three strains tested: BDF1, C57BL/6 and BALB/c (Yamamoto et al. 1988). Also, A/J macrophages were shown to be able to kill the extracellular bacterium *Staphylococcus epidermidis*

FIGURE 1.3

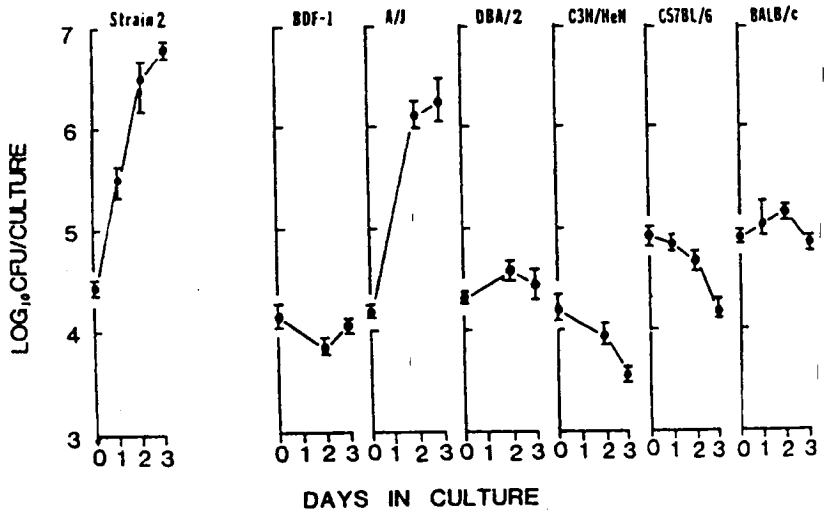
A/J mouse TGC-elicited peritoneal macrophages are permissive for intracellular replication of *L. pneumophila*.

A *Legionella* growth in TGC-elicited peritoneal macrophage cultures from guinea pigs and various inbred mouse strains. Macrophage monolayers (approximately 10^6 cells per well) were infected with 1×10^6 (guinea pig strain 2 and mouse strains BDF1, A/J, DBA/2, and C3H/HeN) or 2×10^6 (mouse strains C57BL/6 and BALB/c) bacteria for 30 min at 37°C, washed, and then incubated for appropriate periods. The number of CFU in the macrophage cultures was then determined. Each point represents the mean CFU ± the standard deviation (SD) of triplicate cultures from three (guinea pig) or five (mouse) animals per group.

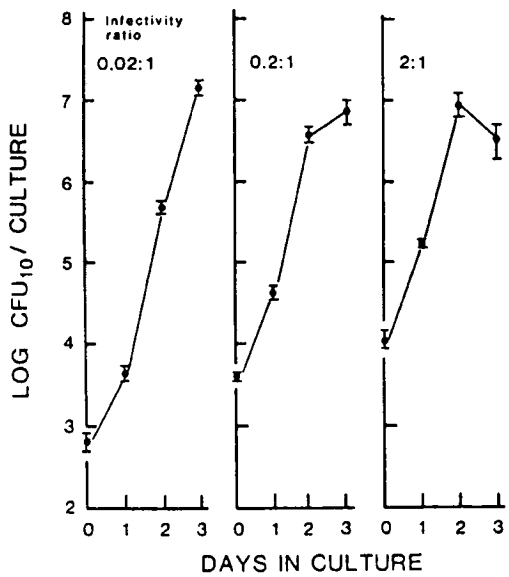
B *Legionella* growth in elicited peritoneal macrophage cultures from A/J mice at different infectivity ratios. Elicited peritoneal macrophage monolayers (approximately 10^6 cells per well) were infected with 2×10^4, 2×10^5, or 2×10^6 bacteria, resulting in infectivity ratios of 0.02:1, 0.2:1, and 2:1, respectively. Each point represents the mean CFU ± SD of triplicate macrophage cultures.

C *Legionella* growth in resident peritoneal macrophage cultures from A/J and BDF1 mice. Resident peritoneal macrophages were cultured (approximately 10^6 cells per well) and infected with 2×10^6 bacteria. The numbers of CFU were determined at 24 and 48 h after infection. Each point represents the mean CFU ± SD of triplicate macrophage cultures.

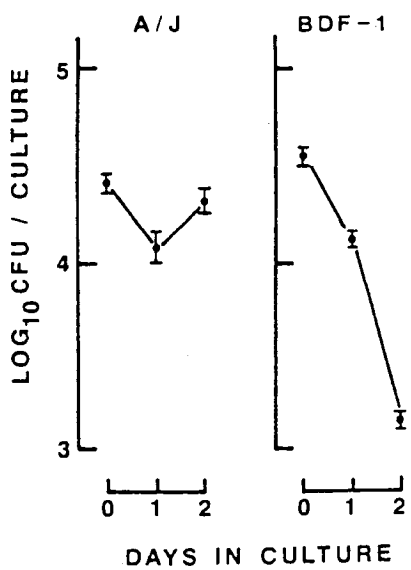
A


Strain 2
BDF-1
A/J
DBA/2
C3H/HeN
C57BL/6
BALB/c
LOG10CFU/CULTURE
DAYS IN CULTURE

B


Infectivity ratio
0.02:1
0.2:1
2:1
LOG CFU10/ CULTURE
DAYS IN CULTURE

C


A/J
BDF-1
LOG10 CFU / CULTURE
DAYS IN CULTURE

(Yamamoto et al. 1988), as well as the temperature sensitive mutant (C5/TS1/1) of *Salmonella typhimurium* (Yamamoto et al. 1992b). The A/J strain was also known to be resistant to infections with the intracellular bacteria *Mycobacterium bovis* (Gros et al. 1981), *Mycobacterium lepraemurium* (Skamene et al. 1984), and virulent *Salmonella typhimurium* (Plant and Glynn 1976). These findings suggested that A/J macrophages share functional capabilities with cells from other mouse strains, and that there is a difference in growth restriction capacity that seems specific for *L. pneumophila.*

Moreover, the susceptibility of A/J mice to *Legionella* infection *in vivo* was higher than for other inbred mouse strains. A 50% lethal intraperitoneal dose below 5×10^6 bacteria was calculated for A/J mice whereas about 5×10^7 bacteria were necessary to kill mice from the other strains tested (Yamamoto et al. 1988). These data suggested that a pivotal role for macrophages in the early stages of *Legionella* infection does influence the overall outcome of the disease. When compared with guinea pigs though, A/J mice do not appear as susceptible to *L. pneumophila.* Apparently (the data was not shown), pretreatment of A/J mice with thioglycolate did not substantially change their 50% lethal dose. Thus, it appeared that early growth of *Legionella* in macrophages was subsequently restricted by other resistance mechanisms (Yamamoto et al. 1988), probably lymphocyte-mediated mechanisms as reviewed in previous sections of this thesis. The A/J mouse was therefore presented as a useful animal model for the study of *Legionella* infection and immunity; mimicking the progress of legionellosis in immunocompetent humans with permissive macrophages but effective cell-mediated acquired immunity (Yamamoto et al. 1988).

Interestingly, resident macrophages from the peritoneal cavity of A/J mice were shown to be non-permissive to *L. pneumophila* replication (Fig. 1.3c); CFUs remained fairly constant during the 48-hour infection that was performed (Yamamoto et al. 1988). This *in vitro* finding was extended to *in vivo* experiments: prior injection of mice with thioglycolate resulted in an enhanced recovery of viable bacteria from the peritoneal cavity 24 and 48 hours after intraperitoneal infection (Yamamoto et al. 1988). These observations, however, did not in any way undermine the phenotypic difference observed between thioglycolate-elicited cells of A/J mice and other mouse strains with respect to bacteriostatic activity against *L. pneumophila.* It had previously been reported that

thioglycolate-elicited peritoneal macrophages in general, display reduced bactericidal activity (Baker and Campbell 1980). Indeed, BDF1 resident cells rapidly killed the added *L. pneumophila* bacteria (Fig. 1.3c) instead of merely restricting their growth (Fig. 1.3a) as had been observed with the elicited macrophages (Yamamoto et al. 1988). A later publication by the same group showed that macrophages elicited with casein did not behave like thioglycolate-elicited cells when infected with *L. pneumophila*; their phenotype was reminiscent of that of resident peritoneal macrophages (Yamamoto et al. 1992b). Casein-elicited macrophages from A/J mice restricted *L. pneumophila* growth during a 48-hour infection period (Fig. 1.5a), whereas BALB/c macrophages started killing the bacteria instead of just preventing their replication (Yamamoto et al. 1992b). The phenotype of casein-elicited macrophages will be further described in a later paragraph.

The next step was to characterize the genetic control underlying the susceptibility of A/J thioglycolate-elicited macrophages. For that purpose, Yamamoto and colleagues set up a number of diagnostic crosses (Fig. 1.4a) between A/J mice and the closely related, but non-permissive to *L. pneumophila* replication, BALB/c strain (Yamamoto et al. 1991). The growth of *Legionella* in the macrophages from F1 mice prepared from a cross between A/J and BALB/c parents (ACF1 mice) was restricted and essentially the same as when *Legionella* were cultured in macrophages from non-permissive parental BALB/c animals. However, macrophages from about one-third of the ACF2 mice obtained by brother/sister inbreeding of ACF1 mice showed some permissiveness for growth of *Legionella* (about 30%). This suggested a dominance of the phenotype in the F2 mice in terms of non-permissiveness for growth of *Legionella*. Indeed, the predicted percentage of permissive individuals among segregating progeny for a recessive trait under monogenic control was of 25% for the ACF2 mice. This simple model of inheritance was further validated with backcross populations. When the ACF1 mice were backcrossed with either parental A/J or BALB/c mice, there was a segregation of permissiveness of growth of *Legionella*. For example, macrophages from about half of the ACF1 × A/J mice were permissive for *Legionella* growth (precisely 57%), whereas macrophages from all backcrossed ACF1 × BALB/c mice were non-permissive. The conclusion from this study was therefore that the permissive nature of mouse

FIGURE 1.4

Evidence for the genetic control of *L. pneumophila* intracellular replication.

A Fate of *L. pneumophila* in thioglycolate-elicited macrophages from individual A/J, BALB/c, ACF1 (A/J×BALB/c), ACF2 (ACF1×ACF1), and backcrossed (ACF1×A/J) and (ACF1×BALB/c) mice. Each point represents the results obtained from an individual mouse. The dotted line shows 95% confidence limit for typing individual animals as permissiveness (above line) or nonpermissiveness (below line) of macrophages to *Legionella* growth. Predicted percentages of permissiveness of individuals among segregating progeny are those for a trait under monogenic control.

This panel was reproduced, with permission from the publisher, from the following publication (Yamamoto et al. 1991), © Society for Experimental Biology and Medicine, 1991.

B Genetic analysis of macrophage resistance and susceptibility to *L. pneumophila* for A/J and C57BL/6 mice. Intracellular growth of the organism was assayed in TGC-elicited peritoneal macrophages obtained from parental, F1 and F2 hybrid, and backcross mice. Bacterial increase is shown as the $\log_{10}$ increase in numbers of CFU on day 3 of *in vitro* culture. Data were analyzed by using the chi-square test, and the expected percentages are the percentages expected for a trait controlled by a single gene with a dominant resistance allele. The stippled area shows the 99% level of confidence for typing of the strains as resistant or susceptible. A, A/J; B, C57BL/6; S, number of susceptible mice.

This panel was reproduced, with permission from the publisher, from the following publication (Yoshida et al. 1991b), © American Society for Microbiology, 1991.

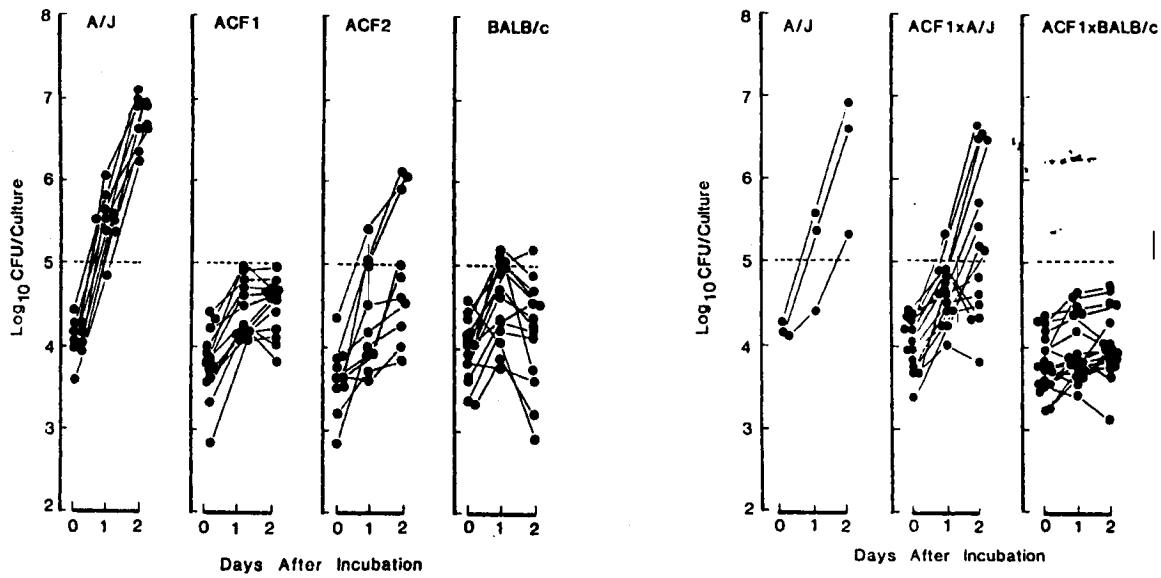
A

Percent of permissiveness for Legionella growth

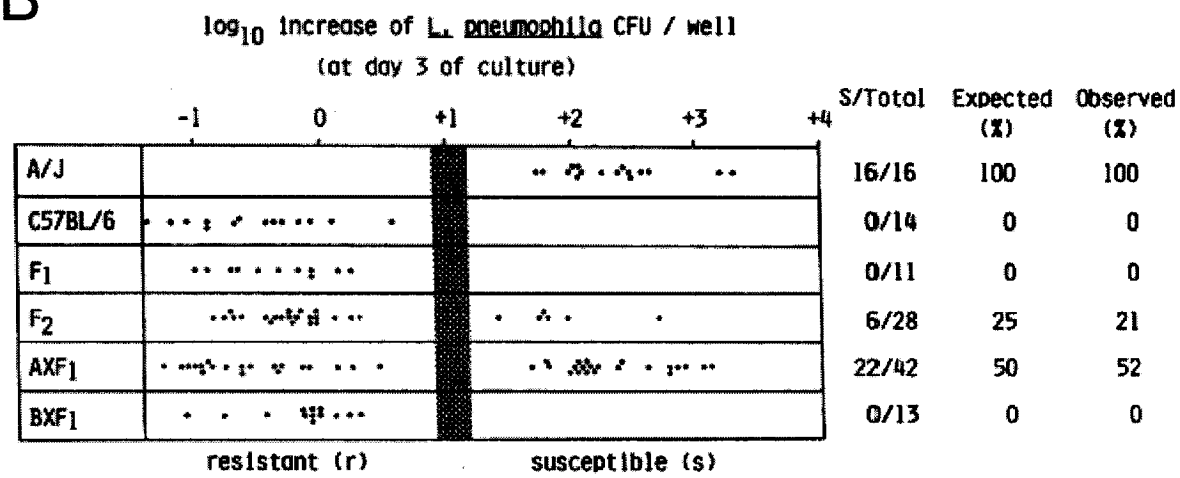
Predicted:	100	0	25	0
Observed:	100	0	30	7

Percent of permissiveness for Legionella growth

Predicted:	100	50	0
Observed:	100	57	0



B



	S/Total	Expected (%)	Observed (%)
A/J	16/16	100	100
C57BL/6	0/14	0	0
F_1	0/11	0	0
F_2	6/28	25	21
AXF_1	22/42	50	52
BXF_1	0/13	0	0

macrophages for *Legionella* growth is a recessive feature of the animals that may be controlled by a single gene or tightly clustered gene family (Yamamoto et al. 1991). Independently, Yoshida and colleagues came to the same conclusion using crosses between A/J and the non-permissive C57BL/6J strain (Yoshida et al. 1991b). The percentages of F1 hybrid, F2 hybrid, and backcross mice that were observed to be susceptible were particularly close to the expected percentages for a trait controlled by a single, recessive gene (Fig. 1.4b). This gene was named *Lgn1* (Yoshida et al. 1991b).

A/J mice were already known to display weak inflammatory responses when compared with many other inbred strains (Stevenson et al. 1984; Czuprynski et al. 1985; Stevenson et al. 1986). Relevant to these experiments, this translates into fewer inflammatory macrophages being recruited to the peritoneum of A/J mice following thioglycolate injection. In view of this, Yamamoto and colleagues explored a possible correlation between the number of recruited macrophages and permissiveness to *L. pneumophila* replication. The number of recruited macrophages was shown to segregate among progeny from different crosses between A/J (weak recruitment) and the non-permissive strain BALB/c (strong recruitment) (Yamamoto et al. 1992b). The F1 mice that resulted from crossing the two strains had relatively high numbers of recruited cells in their peritoneum, similar to parent BALB/c mice. F2 mice showed a large spread in their phenotype, albeit with overall lower recruitment numbers than observed in BALB/c and F1 mice. When ACF1×A/J backcrosses were studied, the number of peritoneal exudate cells obtained from the mice was essentially similar to that observed in the parent A/J mice. The number of peritoneal exudate cells of ACF1×BALB/c backcross mice was almost the same as that of parental BALB/c mice. The permissive nature of these peritoneal cells for *L. pneumophila* growth was investigated (Yamamoto et al. 1992b). As described before, some of the peritoneal macrophages from ACF2 mice were permissive for the growth of *L. pneumophila*, but some of these permissive mice yielded high numbers of peritoneal exudate cells. Similarly, for the ACF1×A/J backcross, the mice that showed the low number of peritoneal exudate cells did not always show permissiveness of their cells to *L. pneumophila* replication. In conclusion, the number of inflammatory cells in the peritoneal cavity of mice induced by thioglycolate did not correlate with

permissiveness versus nonpermissiveness of macrophages from these mice to *L. pneumophila* (Yamamoto et al. 1992b).

Another interesting set of experiments by the same group showed that interferon-γ can activate macrophages from A/J mice to inhibit the growth of, and even kill, *L. pneumophila* (Klein et al. 1991). Thus, the genetic control of resistance versus susceptibility of macrophages for growth of *Legionella in vitro* seemed to be abrogated by activation with a cytokine. It was verified that the cytokine treatment did not inhibit the phagocytosis of *Legionella* by the treated macrophages. Also, restriction of bacterial replication was not mediated by an oxygen metabolite, as oxygen scavenger (catalase and superoxide dismutase) treatment of the macrophage monolayers did not abolish the protective effect of IFN-γ (Klein et al. 1991).

Was the genetic control abrogated by the addtion of a cytokine, or did the activation of macrophages simply shift the growth curves without changing the innate difference in susceptibility between A/J mice and other strains? As mentioned in a previous paragraph, Yamamoto and colleagues showed in 1992 that both resident and casein-elicited peritoneal macrophages from the A/J strain restricted *L. pneumophila* growth (Yamamoto et al. 1992b). And that the same cells collected from the BALB/c strain showed active killing of the bacteria instead of just inhibiting their replication (Fig. 1.5a). Therefore, the bactericidal phenotype of casein-elicited macrophages could be followed in diagnostic crosses between A/J and BALB/c mice, exactly as described for the bacteriostatic phenotype of thioglycolate-elicited macrophages. This bactericidal phenotype was quantitated as % killing of *L. pneumophila*: the number of bacteria phagocytosed (time 0), minus the number of bacteria still present in the monolayers 48 hour later, divided by the number of phagocytosed bacteria, times 100 (Fig. 1.5b). None of the A/J mice tested showed significant killing of the bacteria whereas most of the BALB/c mice had killed at least 80% of the phagocytosed bacteria by the second day of infection. All of the ACF1 mice showed some level of bactericidal activity against *L. pneumophila*, just as did ACF1×BALB/c backcross mice. About 20% of the ACF2 mice displayed a failure to clear *L. pneumophila* from their macrophages, which is close to the 25% expected from a single autosomal recessive gene controlling susceptibility to *Legionella*. And about half (60% precisely) of the ACF1×A/J mice were able to kill *L.*

FIGURE 1.5

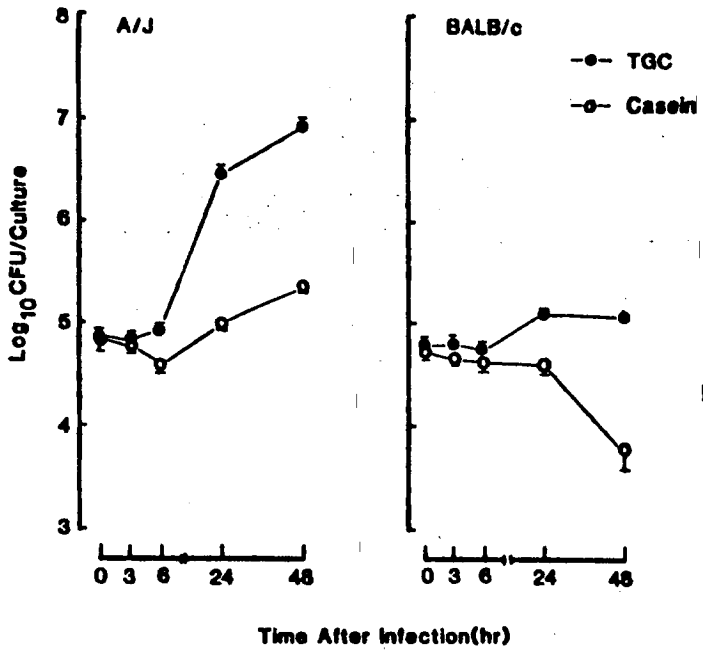
The bactericidal activity of casein-elicited peritoneal macrophages is also under the control of *Lgn1*.

A Fate of *L. pneumophila* in A/J and BALB/c mouse macrophages elicited with TGC or casein. Macrophage monolayers (approximately 1×10^6 cells per well, 24-well plates) were infected with 2×10^7 bacteria for 30 min at 37°C, washed, supplied with 1.0 ml of 15% FCS-RPMI 1640 medium, and then incubated for appropriate periods. Macrophage lysates were prepared at 0, 3, 6, 24 and 48 h after infection and the number of viable bacteria determined by the plate count method. Each point represents the mean CFU ± S.D. of triplicate macrophage cultures.

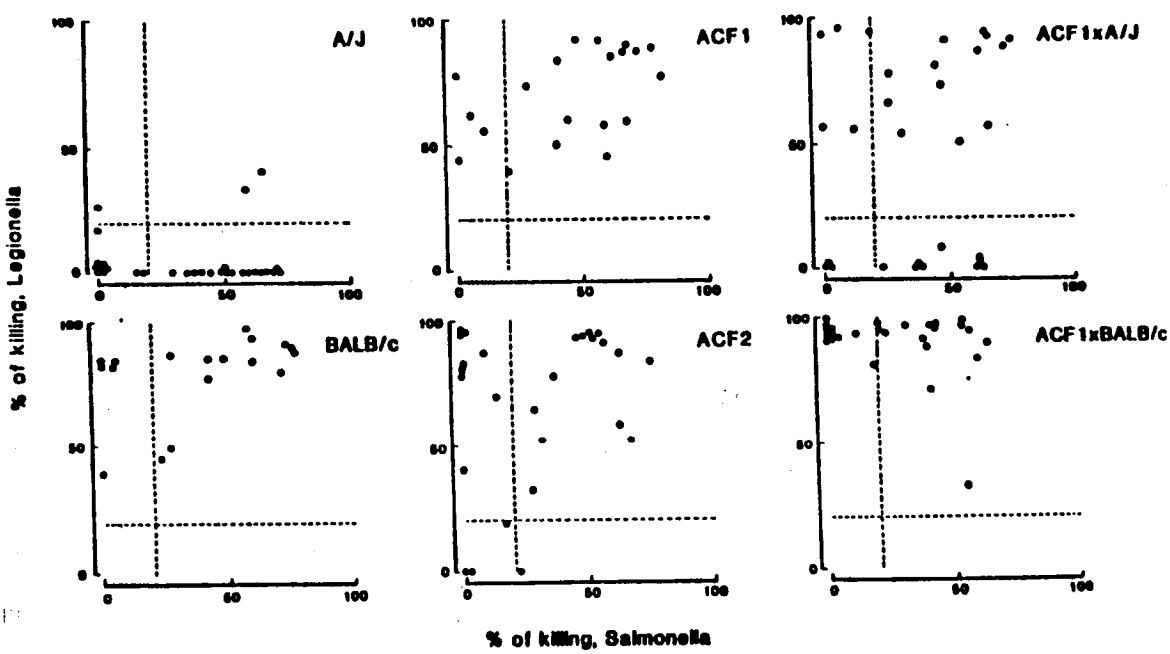
B Comparison of intracellular killing activities of casein-induced macrophages obtained from individual inbred and hybrid mice against *L. pneumophila* and *S. typhimurium*. Casein-induced macrophage monolayers (approximately 1×10^6 cells per well) were infected with 2×10^7 *Legionella* or *Salmonella* for 30 min at 37°C, washed, supplied with 15% FCS-RPMI 1640 medium, and then incubated for 2 h in the case of *S. typhimurium* and 48 h for *L. pneumophila*, respectively. Macrophage lysates were prepared at 0 time and either 2 h or 48 h of incubation, and the number of viable bacteria in lysates was determined by the plate count method. Percentage of killing of *Legionella* or *Salmonella* was calculated as follows: % of killing = (No. of CFU at 0 time - No. of CFU at 2 h (*Salmonella*) or 48 h (*Legionella*)) / No. of CFU at 0 time × 100. Each point represents the result obtained from an individual mouse.

Both panels are reproduced, with permission from the publisher, from the following publication (Yamamoto et al. 1992b), © Elsevier, 1992.

A


A/J
BALB/c
TGC
Casein
Log10CFU/Culture
8
7
6
5
4
3
0 3 6 24 48
0 3 6 24 48
Time After Infection(hr)

B


A/J
ACF1
ACF1xA/J
BALB/c
ACF2
ACF1xBALB/c
% of killing, Legionella
% of killing, Salmonella

pneumophila. Therefore, the bactericidal phenotype of casein-elicited macrophages follows the same segregation pattern as the bacteriostatic phenotype of thioglycolate-elicited macrophages (Yamamoto et al. 1992b). Both phenotypes were then assumed to be controlled by the same gene, *Lgn1*. Thus, the work by Yamamoto suggests that the activation state of macrophages does not necessarily abrogate the genetic control exerted by *Lgn1*, and it may also provide an alternative phenotype to follow *Lgn1* segregation. However, a formal demonstration that bactericidal activity of casein-induced macrophages is under *Lgn1* control remains to be presented. Why has this important work been overlooked ever since? Perhaps because the data was not presented clearly (Fig. 1.5b). Confusing, two-dimensional graphs were used to show a lack of correlation between susceptibility to *L. pneumophila* and killing of *S. typhimurium*. % killing of *L. pneumophila* was plotted on the y-axis, and % killing of *S. typhimurium* was plotted on the x-axis. In fact, the dominant message from their graphs is that the *S. thyphimurium* infection was not carried out properly: pure inbred A/J as well as BALB/c mice displayed a large range of susceptibilities to *Salmonella* that is obviously not under genetic control. Perhaps if the *S. typhimurium* infection had been carried out for more than six hours instead of just two, their numbers would have been more homogeneous, as suggested by the growth curves shown in the same report. In any case, their poor *Salmonella* data basically eclipsed their very interesting *Legionella* results.

The studies described thus far, pertaining to the genetic control of *Legionella* replication within A/J macrophages, used the *L. pneumophila* species exclusively. Yoshida and colleagues used the Philadelphia-1 strain (serogroup 1) (Yoshida et al. 1991b), and Yamamoto and colleagues (Yamamoto et al. 1988; Yamamoto et al. 1991; Yamamoto et al. 1992b) used a clinical isolate of *L. pneumophila* (serogroup 1 as well). Their choice is well justified, as *L. pneumophila* (serogroup 1 precisely) was not only the first *Legionella* discovered (at the 1976 American Legion convention epidemic), it also remains the most often associated with outbreaks of legionellosis (Yu et al. 2002). However, there are many *Legionella* species, and within the *L. pneumophila* species, there are many known strains and serogroups. Does the *Lgn1* gene control the intracellular replication of all these different *Legionellae*? It was not until 1996, when the positional cloning of *Lgn1* was well under way, that this question was looked into

(Miyamoto et al. 1996). Different *Legionellae* were studied for their ability to replicate within A/J and C57BL/6J thioglycolate-elicited peritoneal macrophages; two mouse strains that display differential replication of the *L. pneumophila* Philadelphia-1 bacterium, as controlled by the *Lgn1* gene (Yoshida et al. 1991b). The results are summarized in Table 1.3. 15 different strains of the *L. pneumophila* species, chosen to represent six different serogroups, were tested. 13 of them behaved exactly as the Philadelphia-1 strain: they replicated more than 100-fold within a 48-hour infection period in A/J macrophages, but their growth was restricted by C57BL/6J macrophages. There were two strains however, Togus-1 (serogroup 2) and Bloomington-2 (serogroup 3), that replicated within C57BL/6J macrophages as well as they did in A/J macrophages (Miyamoto et al. 1996). The bacteriostatic activity of *Lgn1* is therefore not specific to the *L. pneumophila* species as a whole.

To determine whether the bacteriostatic activity of *Lgn1* is specific to certain serogroups of *L. pneumophila*, the intracellular growth of the GIFU 10073 strain, a clinical isolate of *L. pneumophila* that belongs to the same serogroup as Bloomington-2 (serogroup 3), was examined (Miyamoto et al. 1996). The bacterial growth in C57BL/6 macrophages was clearly suppressed, which is in contrast with the observed growth of the Bloomington-2 strain, suggesting that the bacteriostatic activity of *Lgn1* is not specific to serogroups of *L. pneumophila.*

Five additional *Legionella* species were tested: *L. bozemanii*, *L. dumoffii*, *L. feeleii*, *L. micdadei* and *L. oakridgensis* (Miyamoto et al. 1996). Albeit more rare than *L. pneumophila*, these species have also been associated with disease in humans (Korvick et al. 1987; Fang et al. 1989; Yu et al. 2002). *L. bozemanii*, *L. dumoffii*, *L. feeleii* and *L. oakridgensis* replicated decisevily within both A/J and C57BL/6J macrophage cultures, with a greater than 100-fold replication within 48-hours. The *L. micdadei* strain showed a reduced ability to replicate within mouse macrophages (not more than 20-fold replication in 48 hours), but the same replication was achieved in both mouse strains nevertheless. Consistent with this result, a subsequent publication showed that *L. micdadei* does not replicate within the lungs of A/J mice *in vivo* (Gao et al. 1999). Although there are many more *Legionella* species and strains known, making this study far from exhaustive, it is clear that the *Lgn1* gene does not control the intracellular replication of all *Legionella*

Table 1.3. Spectrum of *Legionella* species and strains whose intracellular multiplication in murine macrophages is genetically controlled by *Lgn1*.
Summary of results presented by Miyamoto and colleagues (Miyamoto et al. 1996).

Strain	Serogroup	Controlled by *Lgn1*?
L. pneumophila		
Philadelphia-1	1	**Yes**
Nagasaki 80-045	1	**Yes**
GIFU 9799	1	**Yes**
GIFU 9888	1	**Yes**
GIFU 10067	1	**Yes**
GIFU 10068	1	**Yes**
GIFU 12438	1	**Yes**
Togus-1	2	No
Bloomington-2	3	No
GIFU 10073	3	**Yes**
GIFU 11491	4	**Yes**
GIFU 10841	4	**Yes**
GIFU 11395	5	**Yes**
GIFU 12293	5	**Yes**
KO 127	6	**Yes**
L. micdadei TATLOCK		No
L. dumoffii Tex-KL		No
L. bozemanii WIGA	1	No
L. oakridgensis OR-10		No
L. feeleii WO-44C	1	No

species. And although apparently specific for the *L. pneumophila* species, it does not control the species as a whole either (Miyamoto et al. 1996).

In the same report, a mouse strain congenic for the *Lgn1* locus is described (Miyamoto et al. 1996). Construction of the congenic (A.B *Lgn1*) was performed as follows. The resistance-susceptibility phenotype of macrophages from the progeny of a (A/J × C57BL/6) × A/J backcross was determined. The progeny that exhibited less than a 1.0 $\log_{10}$ increase in *L. pneumophila* Philadelphia-1 CFU per well after 3 days were selected as possessing the resistance allele ($Lgn1^r$) of C57BL/6 mouse origin. The progeny bearing $Lgn1^r$ were then backcrossed with the A/J mouse. The procedures of backcrossing the progeny and of typing their macrophages for resistance or susceptibility to *L. pneumophila* Philadelphia-1 were performed for 18 generations. The backcross progeny with the $Lgn1^{r/r}$ genotype were used as congenic-strain mice (A.B *Lgn1*) in their study (Miyamoto et al. 1996). The congenic strain held approximately a 15-centimorgan gene fragment of C57BL/6 origin on chromosome 13. Macrophages of the congenic strain (A.B *Lgn1*) showed the same pattern of resistance-susceptibility as macrophages of C57BL/6 when challenged with all the different *Legionella* strains. This supported the theory that the strain difference shown between A/J and C57BL/6 mice is controlled by *Lgn1* and that the seven *Legionella* strains which grew in the macrophages of the congenic strain are not controlled by *Lgn1* (Miyamoto et al. 1996).

Although macrophages of the congenic mouse strain did not permit bacterial growth, bacteria could survive in these macrophages. This observation further suggested that the *Lgn1* gene product has a bacteriostatic activity rather than a bactericidal activity and that A/J mouse macrophages may be defective in their ability to inhibit bacterial growth. It was further proposed that since the genetic defect can be overcome by gamma interferon activation of A/J macrophages (Klein et al. 1991), the *Lgn1* gene product may affect a very early step of the macrophage-*Legionella* interaction, such as macrophage signal transduction in response to the infecting bacterium (Miyamoto et al. 1996). Although Miyamoto and colleagues did not possess much evidence to support this last remark, several years of research have elapsed since and it still holds a likely hypothesis.

There are numerous reports suggesting that diverse intracellular growth mechanisms are used by *Legionella* strains and species (Weinbaum et al. 1984;

Rechnitzer and Blom 1989; Neumeister et al. 1997; Abu Kwaik et al. 1998b; Gao et al. 1999; Joshi and Swanson 1999; Gerhardt et al. 2000; Neumeister et al. 2000; Ogawa et al. 2001). It is therefore reasonable to speculate that expression of the bacteriostatic activity controlled by the *Lgn1* locus may reflect the development of adaptive mechanisms that enable *Legionella* strains and species to grow intracellularly (Miyamoto et al. 1996). Knowing the spectrum of bacterial strains that are or are not influenced by the *Lgn1* locus should help to point out the bacterial gene products on which the *Lgn1* gene acts. This could therefore help to shed light on *Lgn1*'s mechanism of action.

1.2.4.2 *Lps* Locus

Lipopolysaccharide (LPS) is an abundant glycolipid present in the outer membrane of Gram-negative bacteria. It can provoke a generalized pro-inflammatory response in the infected host (Raetz et al. 1991) which has been shown to be under genetic control of the *Lps* locus (Qureshi et al. 1999a). Variation in inflammatory response in inbred strains after challenge with purified LPS was noted many years ago. Of particular interest is the C3H/HeJ mouse strain, which is resistant to a lethal challenge with LPS and is generally hyporesponsive to LPS *in vitro*, as measured by polyclonal mitogenic response of splenic B cells (Sultzer 1968; Vas et al. 1973). Further characterization showed that this LPS-unresponsiveness extended to fibroblasts (Ryan and McAdam 1977), T-lymphocytes (Koenig et al. 1977) and macrophages (Glode et al. 1977; Ruco and Meltzer 1978). This phenotype, expressed by multiple cell types, is controlled by a single autosomal gene designated *Lps* on mouse chromosome 4 (Watson and Riblet 1974; Watson et al. 1978a). There is a second mouse strain, C57BL/10Sc, which is also hyporesponsive to LPS according to the same criteria (Coutinho et al. 1977; McAdam and Ryan 1978). The inability of the C57BL/10Sc mouse to become stimulated by endotoxin was also linked to mouse chromosome 4. F1 progeny resulting from a cross of C57BL/10Sc mice with C3H/HeJ were equally refractory to LPS as the parents, indicating that the same chromosome 4 locus was defective in both strains (Coutinho et al. 1977). At the molecular level, the mutant phenotype of C3H/HeJ and C57BL/10Sc mice has been attributed to defective recognition of the lipid A moiety of LPS by lymphocytes and macrophages

(McAdam and Ryan 1978). Interestingly, mice homozygous for the *Lps* defective alleles are also highly susceptible to infection with *S. typhimurium* and other Gram-negative bacteria (O'Brien et al. 1980).

In 1991, while mapping the *Lgn1* gene, Yoshida and colleagues infected macrophages from the two known *Lps*-defective strains with *L. pneumophila* (Yoshida et al. 1991a). Thioglycollate-induced peritoneal macrophages obtained from LPS-low responder C3H/HeJ mice (J) permitted a moderate intracellular growth of *L. pneumophila* after *in vitro* phagocytosis, while macrophages of high-responder C3H/HeN mice (N) did not. It should be noted, however, that bacterial replication in the C3H/HeJ strain plateaus at about 10-fold by the second day of infection and is therefore nowhere near the one seen in A/J (over 100-fold in 3 days). Intracellular growth of the bacterium in macrophages of (J x N) F1 progeny was intermediate between the parental strains, showing that the traits were co-dominantly expressed (Yoshida et al. 1991a). The co-dominant nature of the *Lps* alleles had been observed previously (Coutinho et al. 1978; Rosenstreich et al. 1978; McGhee et al. 1979). But the mechanism behind the co-dominance has only been elucidated in a recent article (Pereira et al. 2003) that shows monoallelic expression of the *Lps* gene. Correlation between intracellular bacterial growth in macrophages and LPS response of spleen cells was then examined. A negative correlation was found between the two factors in F2, (J × F1) backcross and (N × F1) backcross progeny. Meaning that mice with a low LPS response were more susceptible to *L. pneumophila* replication than LPS high-responder littermates. This result further implied that the *Lps* gene, and not some other mutation in the C3H/HeJ strain, controls the innate resistance of murine macrophages against the bacteria. Gene complementation analysis of A/J and C3H/HeJ mice made clear that the genetic defect in C3H/HeJ with respect to *L. pneumophila* replication differs from that of the A/J strain: bacterial growth in A/J×C3H/HeJ F1 macrophages was completely suppressed. Therefore, the work by Yoshida and colleagues strongly suggested that the *Lps* gene also controls the natural resistance of murine macrophages against *L. pneumophila.*

Additionally, they tested the permissiveness to *L. pneumophila* replication of the C57BL/10Sc mouse strain, which is also known to be defective at the *Lps* locus (Coutinho et al. 1977; McAdam and Ryan 1978; Qureshi et al. 1999a). Yoshida states that

C57BL/10Sc macrophages were defective in controlling the bacterial growth when compared to *Lps*-normal C57BL/10 mice (Yoshida et al. 1991a). Although there is a statistically significant difference in bacterial replication between the two strains, a critical eye would point out that they are both highly resistant to *L. pneumophila*. Unfortunately, no one has pursued the *in vitro* infection of macrophages derived from *Lps*-defective strains with *L. pneumophila* ever since.

Lps was recently identified by positional cloning as the Toll-like receptor 4 gene (*Tlr4*) (Poltorak et al. 1998b; Qureshi et al. 1999a). Just like the cloning of *Nramp1*, this is a great example of a successful approach to identify the gene product responsible for a long-known phenotype. In 1996, a high-resolution genetic map of the chromosomal region surrounding the *Lps* locus was published (Qureshi et al. 1996). LPS-induced spleen cell proliferation was the phenotype assayed to follow inheritance of the Lps locus between the responder strains C57BL/6J and DBA/2J and the non-responder C3H/HeJ. Analysis of a total of 1604 backcross mice with microsatellite markers, anonymous DNA probes from microdissected libraries and cDNA probes allowed the positioning of the *Lps* gene to within a 1.1 cM interval. Subsequently, DNA physically spanning this genetic region was isolated in the form of a contig comprising YAC, BAC, and P1 clones. cDNA selection, exon trapping and direct sequencing of selected clones were used to identify novel transcription units within the candidate region (Poltorak et al. 1998b; Qureshi et al. 1999a). Following shotgun sequencing of the interval, Poltorak and colleagues proposed *Tlr4* as a candidate for the *Lps* gene (Poltorak et al. 1998a; Poltorak et al. 1998b). At approximately the same time, Qureshi using comparative mapping substantiated the candidacy of *Tlr4* as the *Lps* gene (Qureshi et al. 1999a). The Tlr4 protein is an 835 amino acids polypeptide with an extracellular domain containing 22 leucine-rich repeat motifs connected by a single transmembrane domain to an intracellular signalling domain that shares homology to the interleukin-1 receptor. C3H/HeJ mice exhibit an H712P substitution within the highly conserved Toll/Interleukin-1 Receptor (TIR) signalling domain (Poltorak et al. 1998a; Qureshi et al. 1999a). In C57BL/10Sc, the *Tlr4* gene is completely deleted resulting in no *Tlr4* mRNA expression (Poltorak et al. 1998a; Qureshi et al. 1999a). The identification of distinct mutations involving the same gene at the *Lps*

locus in two different hyporesponsive inbred mouse strains strongly supported the hypothesis that altered *Tlr4* function is responsible for endotoxin tolerance.

Toll-like receptors (TLR) are an ancient family of proteins showing high sequence and structural homology to *Drosophila* Toll. Toll is a protein with dual function. In embryos, Toll controls dorso-ventral polarity through activation of the transcription factor dorsal (Hashimoto et al. 1988). In the adult fly, Toll is necessary to induce the expression of the anti-fungal peptide, drosomycin (Lemaitre et al. 1996). Interestingly, another *Drosophila* TLR, 18-wheeler, stimulates an anti-bacterial response (Williams et al. 1997). The *18-wheeler* mutant flies show decreased expression of the anti-bacterial genes, attacin, cecropin, and to a lesser extent, diptericin in response to infection (Williams et al. 1997). The Toll signalling pathway implicated in embryonic dorsal-ventral patterning is fully characterized (Anderson 1998). Activation of Toll is achieved following the binding of proteolytically-cleaved Spaetzle (Morisato and Anderson 1994). This process leads, via the interaction of the Toll cytoplasmic domain with the adaptor protein Tube and the kinase protein Pelle, to the degradation of Cactus and the release of Dorsal. Dorsal, a member of the Rel/ NF-κB family of transcription factors, translocates to the nucleus where it activates and represses transcription of target genes. The immune response leading to the Toll-induced transcription of antibacterial genes in the adult *Drosophila* shares the same signalling molecules described above (Lemaitre et al. 1996). The ability of Dorsal-mutant flies to produce drosomycin lead to the discovery that, in addition to Dorsal, the other known Rel/NF-κB transcription factors, Relish and Dif, are involved in the induction of immune genes (Manfruelli et al. 1999; Meng et al. 1999).

Human TLR4 was the first isolated mammalian homologue of Drosophila Toll. As mentioned before, TLR4 contains a cytosolic Toll/Interleukin-1 receptor (TIR) signalling domain (Medzhitov et al. 1997). Like Toll and the IL-1R (interleukin-1 receptor), Medzhitov and coworkers showed, using *in vitro* transfection studies, that TLR4 signalling also leads to the induction of NF-κB-mediated genes (Medzhitov et al. 1997). The complete cascade of signalling events involved in TLR4 signalling are identical to those implicated in IL-1R signalling (Medzhitov et al. 1998; Muzio et al. 1998). The intracellular domain of TLR4 interacts with MyD88 which subsequently recruits the IL-1R associated kinase (IRAK) (Cao et al. 1996a; Muzio et al. 1997; Wesche et al. 1997; Burns

et al. 1998; Medzhitov et al. 1998; Muzio et al. 1998). This is followed by the sequential interaction with TRAF6, NIK and the IKK complex, which phosphorylate I-κB and activate NF-κB (Cao et al. 1996b; DiDonato et al. 1997c; Mercurio et al. 1997; Regnier et al. 1997; Woronicz et al. 1997; Zandi et al. 1997; Medzhitov et al. 1998; Muzio et al. 1998). As suggested by the cloning of the murine *Tlr4* as a candidate for the *Lps* locus, transient transfection of HEK293 cells with the human TLR4 cDNA demonstrated NF-κB activation as a result of stimulation with LPS (Chow et al. 1999).

The mammalian Toll-like receptor family has at least 10 members described to date. Several of these members play important roles in the defense against pathogens by acting as pattern-recognition molecules on immune cells such as macrophages (Rock et al. 1998; Takeuchi et al. 1999a; Takeuchi et al. 1999b; Aderem and Ulevitch 2000; Medzhitov 2001). For example, TLR2 is essential for the induction of NF-κB driven genes upon stimulation with peptidoglycan and lipotechoic acid from the cell wall of gram-positive bacteria as well as to cell wall preparations from *S. aureus*, *S. pneumoniae*, *L. monocytogenes*, *M. tuberculosis* and *avium*, and *M. jermentans* (Means et al. 1999a; Means et al. 1999b; Takeuchi et al. 1999a; Underhill et al. 1999; Yoshimura et al. 1999; Flo et al. 2000).

Biochemical studies have indicated that Tlr4 participates directly in binding to bacterial LPS with the co-operation of several proteins including LBP (LPS-binding protein), and co-receptors CD14 and MD-2 (Lien et al. 2000; Poltorak et al. 2000; da Silva Correia and Ulevitch 2002). Lipopolysaccharide sensing by Tlr4 leads to activation of NF-κB and MAP kinases (JNK, p38) (reviewed by (Medzhitov 2001). Which ultimately leads to induction of a number of host defense genes including pro-inflammatory cytokines IL-1, IL-6, IL-8, and IL-12, chemokines, co-stimulatory molecules CD80 and CD86, MHC Class II molecules, and *Nos2* (Medzhitov et al. 1997; Schnare et al. 2001). Induction of these molecules by Tlr4 helps initiate adaptive immunity. Thus, the study of the *Lps* mutation has contributed significantly to a better understanding of the role of Tlr molecules in macrophage activation and resistance to infections. Knowledge of the mechanism of action of the Tlr proteins is relevant to the research described in this thesis, as Tlr4 was shown to control intracellular replication of *L. pneumophila*.

Section 1.3

Positional cloning of the *Lgn1* Locus

As shown in the previous section, the A/J mouse strain displays a deficiency in restriction of *L. pneumophila* replication within its thioglycolate-elicited peritoneal macrophages as compared with several other mouse strains. The permissive phenotype of A/J mice is inherited as a recessive trait that seems under the control of a single autosomal gene, or very tightly linked group of genes. This putative gene was named *Lgn1*. Researchers quickly determined that *Lgn1* is not allelic with the Natural Resistance-Associated Membrane protein (*Nramp1*) that controls infection with other intracellular pathogens. The *Lgn1* phenotype was not linked to Major Histocompatibility antigens, as the B10.A mouse strain was able to restrict *L. pneumophila* replication. Neither sex nor coat color could be correlated with the trait either (Yoshida et al. 1991b). With no obvious candidate for the *Lgn1* gene to work with, no biochemical assay for the *Lgn1* gene product, and no obvious cytogenetic alteration associated with the *Lgn1* susceptibility allele of the A/J strain, it was decided to identify the gene through a positional cloning approach. *Lgn1* would therefore be cloned solely based on its position within the mouse genome. The first step towards positional cloning of a gene is called genetic mapping. For that purpose, it was necessary to follow the inheritance of the *Lgn1* permissiveness phenotype and compare it to the inheritance of other genes and genetic markers whose position in the genome is already known. Genes that are very close to one another on a given parental chromosome have more chances of being passed-on together to progeny than if they were further apart; simply because there is a smaller chance of them being separated by homologous recombination between the two parental chromosomes.

1.3.1 Genetic mapping: chromosomal assignment.

Given two mouse strains that display a phenotypic difference, there are several different breeding schemes that can be followed in order to assess the inheritance of genes within their progeny. Indeed, there will be many different crosses between A/J and other mouse strains described throughout subsequent sections of this thesis. Yoshida and colleagues made use, in 1991, of what is still today a very powerful tool for mapping genetic traits in mice: recombinant-inbred (RI) mouse strains. RI sets are derived by inbreeding F2 progeny from two different inbred strains of mice. Each brother-sister mating at the F2 generation is the beginning of an individual RI strain. Repeated brother-sister mating for many generations results in inbreeding and each newly created strain contains, due to the overall randomness of homologous recombination, a unique combination of alleles from each of the progenitor strains. On average, 50% of the genome of each resulting RI strain is derived from each of the two parent strains. Each genetic marker that is polymorphic between the progenitor strains has a characteristic strain distribution pattern (SDP), which can be used to rapidly and efficiently map any polymorphic locus. Inbreeding is key to the power of this genetic tool, each RI strain representing an unlimited source of genetically identical individuals with fixed homologous recombination events that can thus be studied by any researcher, anywhere in the world, anytime. When these strains are phenotyped for a certain trait, or their DNA genotyped for a certain polymorphism, the results can be directly compared to the SDP of all previously mapped genes/markers. When two genes produce very similar SDPs, it means that they are often inherited together, thus, they can be assumed to map close to one another.

Yoshida and colleagues used RI strains derived from the A/J (A) and C57BL/6J (B) strains (Yoshida et al. 1991b). These RI strains were initially constructed by Dr. M. Nesbitt beginning in 1975 (Nesbitt and Skamene 1984). Half of the strains were constructed using A as the maternal parent (A×B) and half used a reciprocal cross, with B as the maternal parent (B×A). The Jackson Laboratory currently maintains these strains. A total of 20 (A×B) and (B×A) RI strains were phenotyped for the ability of their

thioglycolate-elicited peritoneal macrophages to suppress *L. pneumophila* replication. Each of the RI strains tested was clearly either permissive to *L. pneumophila* replication or non-permissive. This binomial segregation of the phenotype was expected from previous work with crosses between A/J and C57BL/6J mice, pointing to a single gene controlling the phenotype. In 1991, there were not that many genes that had been mapped on the RI strains. This translated into large gaps between the mapped genes: large portions of chromosomes where no gene had been identified yet, and therefore, a potential for some homologous recombination events not being detected. With the data available at that time, a correlation was detected between the SDP of A/J and C57BL/6J alleles of the gene encoding the Legionella-susceptibility phenotype (*Lgn1*) and the SDPs of four genes previously located on the proximal part of chromosome 15. The four genes were namely, *Pol-5* (15 of 20 SDPs matched), *Xmmv* 54 (14 of 18 matched), *Sis* (15 of 20 matched), and *Ly-6* (15 of 20 matched). No correlation with other allelic markers on the remaining 18 chromosomes was detected. Thus, the results predicted that the gene for macrophage susceptibility to *L. pneumophila* would be located within 8 to 14 centimorgans of the above-mentioned cluster of chromosome 15 genes. A retrospective look at the data reveals that the xenotropic-MCF leukemia virus 54 (*Xmmv54*) had been wrongfully mapped to chromosome 15, as it is in fact within distal mouse chromosome 6 (Douville and Carbonetto 1992; Taylor and Reifsnyder 1993). In any case, Dr. Yoshida was quick to point out that the chromosomal assignment of the *Lgn1* gene should be verified by formal backcross linkage analysis (Yoshida et al. 1991b).

By the year 1994, there were many more genes that had been mapped using the recombinant inbred strains. But more importantly, genetic mapping had been revolutionized by the isolation, at the Massachussetts Institute of Technology (MIT), of over 4000 simple sequence length polymorphisms (SSLPs) scattered throughout the mouse genome (Copeland et al. 1993; Dietrich et al. 1994). Not only are these genetic markers, known as "MIT markers", fast to analyze as they rely on a simple PCR reaction to detect alleles of different lengths, their shear quantity reduced the gaps between known genetic markers in all mouse chromosomes. Immediately, Dietrich and colleagues reanalyzed the *Lgn1* SDPs published by Yoshida (no new phenotyping was performed) and compared it to the updated SDPs available in the Jackson Laboratory databases for

the A×B and B×A RI strains (Dietrich et al. 1995). By this analysis, the *Lgn1* phenotype showed close linkage to *Pmv9* (polytropic murine leukemia virus 9) on distal chromosome 13, having only one discordance out of 16 informative genotypes (Dietrich et al. 1995). Although some of the strains phenotyped by Yoshida in 1991 had become extinct since then, the linkage observed to the chromosome 13 marker was much more convincing than the ones published in 1991. To further establish the linkage of *Lgn1* to chromosome 13, the SDP for 12 additional polymorphic genetic markers (MIT markers) from distal chromosome 13 was determined. This analysis demonstrated very close linkage of the *Legionella* susceptibility SDP (Yoshida *et al.,* 1991) to the SDP for *D13Mit146* (0/16 discordance) (Dietrich et al. 1995). *Lgn1* mapped consistently within the chromosome 13 linkage group: the SDP introduced no isolated double crossovers and had closely spaced flanking markers proximally and distally (Dietrich et al. 1995).

The map position of *Lgn1* was then confirmed using a 39 animal F2 cross between A/J and C57BL/6J (Dietrich et al. 1995). Bone marrow-derived macrophages were chosen as host cells for *in vitro* infection with *L. pneumophila*, as a higher yield of cells can be obtained from each mouse. The bacterial strain used (Lp02) was also different from the ones used previously. The Lp02 strain of *L. pneumophila* is a streptomycin-resistant, thymine-auxotrophic laboratory derivative of the Philadelphia-1 strain (Berger and Isberg 1993). Despite these two major technical differences, the phenotypic difference between A/J and C57BL/6J mice with respect to permissiveness to the bacterium remained as published by the other groups (Yoshida et al. 1991b; Yamamoto et al. 1992b; Dietrich et al. 1995). The F2 animals were then typed for 72 genetic markers spanning the entire mouse genome, including 21 markers from chromosome 13. By comparing the inheritance of these genetic markers with the inheritance of permissiveness to *Legionella* infection under a single gene model, it was confirmed that *Lgnl* maps to chromosome 13, consistent with the results of the RI analysis. The statistical support for this mapping result was strong: the Lod score for linkage of *Lgnl* to *D13Mit146* was 9.9. A Lod score (log of odds) is a measure of the likeliness that linkage was not obtained just by chance; a lower Lod score of 6 is normally considered to represent highly significant linkage ($p<0.001$). The trait showed no linkage to markers spanning the entire length of chromosome 15. The *Lgn1* genetic interval had therefore been convincingly mapped to a

maximum of 7.8 centimorgans between markers *D13Mit128* and *D13Mit70* within distal mouse chromosome 13. It was also noted that the homologous region of the human genome is contained within 5q, suggesting that the human homolog for *Lgn1* should map there.

In the meantime Dr. Beckers, a post-doctoral fellow in the laboratory of Dr. Gros, collaborated with Dr. Yoshida in the task of verifying the localization of the *Lgn1* gene by using a 182-animal (A/J×C57BL/6J)×A/J backcross. Dr. Yoshida phenotyped the animals and the genotyping was done by Dr. Beckers. Just a few PCRs with chromosome 15 SSLP genetic markers were needed to realize that the *Lgn1* phenotype was not at all linked to that chromosome. This prompted the group to phenotype all 25 available RI strains once more to make sure that no mistakes had been done and to take advantage of the new SSLP markers available to refine the SDP analysis in those mice. At first, the new strain distribution pattern (SDP) of resistance/susceptibility to *L. pneumophila* of the AXB/BXA strains was compared with the database of SDPs for previously typed polymorphic markers. This database had grown considerably since 1991. The analysis did not identify unambiguously a single chromosomal location for *Lgn1*. It suggested two possible locations, one again on Chr 15 (linkage to *Iap1s1-39*, two recombinants in 17 strains, 99% confidence), and a new one on distal Chr 13 (strong linkage to *Pmv9*, no recombinant in 15 strains, 99% confidence). As mentioned before, the chromosome 15 linkage had already been dismissed by genotyping a small set of 60 *Lgn1*-phenotyped (A/J×C57BL/6J) ×A/J interspecific backcross progeny for five polymorphic dinucleotide repeat markers (*D15Mit31*, *D15Mit22*, *D15Mit67*, *D15Mit147*, and *D15Mit42*) dispersed over the entire Chr 15. Indeed, all of the Chr 15 markers tested displayed recombination frequencies near 50% with respect to *Lgn1*, excluding a Chr 15 location. The search was then focused on Chr 13. The SDP of the AXB/BXA RIS set for *Pmv9* and a total of 10 polymorphic dinucleotide repeat markers spanning approximately 30 cM on the distal half of this chromosome was established. Comparison of these SDPs with those obtained for *Lgn1* identified strong linkage with markers *D13Mit128*, *D13Mit194* and *D13Mit70*, with two recombinants each in 25 RIS tested (Beckers et al. 1995). Also, complete concordance of *Lgn1* and *Pmv9* alleles was noted in the 22 RIS for which *Pmv9* allele was known (95% confidence interval 0.0-5.0 cM; (Silver 1985)). Unfortunately, strain

AXB3 was now extinct; the only strain that had previously been shown to bear a recombination event between *Pmv9* and *Lgn1* (Yoshida et al. 1991b). A combined analysis of the SDPs, minimizing the number of double crossovers, produced the most likely gene order *D13Mit231-D13Mit193-D13Mit194/D13Mit128-Lgn1/Pmv9-D13Mit70-D13Mit73-D13Mit53*-D13Mit32-D13Mit77-D13Mit78 (Beckers et al. 1995). This gene order was in agreement with that presented by the MIT Genome Center (Dietrich et al. 1994) and the Chromosome Committee Report (Mouse Genome Encyclopedia) at the time.

To further confirm a Chr 13 assignment for *Lgn1* and to accurately determine the genetic interval defined by the two most tightly linked flanking markers, additional linkage analyses were carried out on their entire 182-animal (A/J×C57BL/6J)×A/J informative backcross. Genomic DNA from those mice was analyzed for allelic combinations at the 10 Chr 13 polymorphic SSLP markers previously analyzed in the A×B/B×A RIS set. The analysis confirmed that *Lgn1* maps on Chr 13, with 5/182 recombinants detected with *D13Mit128* and 4/180 recombinants detected with *D13Mit70*. It also provided an unambiguous marker order for the region (no double crossovers detected), with the following Lod score observed to *Lgn1*: *D13Mit231* (26), *D13Mit193* (36), *D13Mit194* (41), *D13Mit128* (45), *Lgn1*, *D13Mit70* (47), *D13Mit73* (34), *D13Mit53* (23), *D13Mit32* (23), *D13Mit77* (21), *D13Mit78* (20) (Beckers et al. 1995). The gene order obtained from the analysis of these 182 backcross mice was identical to that suggested by the independent analysis of the RIS SDPs.

The linkage data obtained from the 25 A×B/B×A RIS and from the 182 segregating backcross mice were pooled and used to calculate combined pairwise genetic intervals for the region. The locus order and interlocus distances (in cM) for the region were found to be: *D13Mit231*-(5.5±1.5)-*D13Mit193*-(2.2±0.9)-*D13Mit194*-(1.1±0.6)-*D13Mit128*-(2.6±1.0)-*Lgn1*-(2.2±0.9)-*D13Mit70*-(3.9±1.3)-*D13Mit73*-(7.2±1.7)-*D13Mit53*-(0.7±0.5)-*D13Mit32*-(0.7±0.5)-*D13Mit77*-(0.7±0.5)-*D13Mit78*. This analysis unambiguously positioned *Lgn1* within a 4.8-cM interval delineated on the proximal side by marker *D13Mit128*, and on the distal side by marker *D13Mit70* (Beckers et al. 1995).

The microsatellite markers delineating the minimal genetic interval of *Lgn1* map on a segment of distal mouse Chr 13 to which several genes with known polypeptide

products had been mapped before. These genes included dihydrofolate reductase (*Dhfr*), hexosaminidase B (*HexB*), an activator of p21 Ras (*Rasa*), microtubule-associated protein 5/1B (*Mtap5*), and several others (Beckers et al. 1995). The human homologs of these genes, including Rasa (RASA) and Mtap5 (MAP1B) which flank *Lgn1* on either side, all map to the proximal portion of the long arm of human Chr 5 (5q11-5q13). This strongly suggested that a human *Lgn1* homolog would map to that portion of human Chr 5q (Beckers et al. 1995). All in agreement with the independent report by Dietrich and colleagues (Dietrich et al. 1995).

1.3.2 High-resolution genetic mapping.

It was at this point, in the summer of 1995, that I had the opportunity to provide technical help for Dr. Beckers and colleagues. Still an undergraduate in the McGill Biochemistry department, this was my first experience working in a research laboratory. At first, my help was limited to sample preparations and sequencing-gel casting. Eventually, sets of markers were confided to me for characterization and mapping (particularly RFLP-mapping of cDNAs and *D13Hun*-series markers), which earned me authorship in the next publication of the group.

To positionally clone the *Lgn1* gene, it was necessary to increase the resolution of the linkage map of the *Lgn1* chromosomal region through extensive segregation analyses (Beckers et al. 1997). For this, the number of informative (A/J×C57BL/6J)×A/J (ABA) backcross mice segregating the resistance allele of C57BL/6J and the susceptibility allele of A/J (Beckers et al. 1995) was increased to a total of 1270 animals. These animals were used to determine the order and distance of numerous genetic markers with respect to the *Lgn1* locus (Beckers et al. 1997). Three additional panels of backcross animals were used in this study. An interspecific (C57BL/6J×*Mus spretus*)×C57BL/6J backcross (BSB-1) panel consisting of 281 animals that the group had previously described (Schurr et al. 1989), and two commercially available mapping panels derived from the same parents (BSB-2, BSS), each consisting of 94 animals (Jackson Laboratories, Bar Harbor, ME).

These latter two panels had been typed for over 700 (BSB) and 1900 polymorphic loci (BSS) (Rowe et al. 1994). Although the three interspecific crosses were not informative for *Lgn1*, their high polymorphic content made them valuable to rapidly assign novel markers to the roughly defined subchromosomal interval, order them with respect to each other, and estimate intergene distances (Beckers et al. 1997). Combined linkage analyses in independent backcross panels could provide the added advantage of minimizing the effect of possible recombination distortion sometimes observed in a specific cross for a particular interval, possibly introducing bias in the final estimate of the genetic interval (Carson and Simpson 1991; Reeves et al. 1991; Malo et al. 1993b). A total of 39 DNA markers were mapped within an interval of approximately 30 cM overlapping the *Lgn1* region. These markers included 10 cDNA probes corresponding to known genes which were mapped by RFLP (restriction fragment length polymorphism) analysis, 24 PCR-based dinucleotide repeats of the Mit series, 4 newly generated *D13Hun* markers or interspersed repeat sequence probes (IRS-PCR) and 1 marker typed by SSCP (single strand conformation polymorphism) analysis. The following mapping strategy was used: genetic markers and cDNAs corresponding to genes mapping to the distal portion of mouse Chr 13 or to the syntenic portion of human Chr 5 were systematically mapped with respect to *Lgn1* (Beckers et al. 1997). First, gene order and position with respect to the minimal *Lgn1* interval previously defined (*D13Mit128* to *D13Mit70*) was determined for these markers in the BSB-1 cross, and in a subset of 182 animals of the ABA cross. Markers falling within the *D13Mit128* to *D13Mit70* interval were analyzed further in the BSB-2 and BSS panels, and their position with respect to *Lgn1* was established in an additional 1088 mice of the ABA cross for a total of 1270 informative meioses. In the ABA cross, animals showing recombination events within the *D13Mit128* to *D13Mit70* interval were typed for *Lgn1* by *in vitro* infection of their macrophages. The eight cDNA probes tested were polymorphic in the interspecific crosses, while only four (*Hexb, Ctla3, Itga1 and Itga2*) were polymorphic in the ABA cross and could be mapped with respect to *Lgn1*. All cDNA probes tested were found to map outside the above-mentioned genetic interval for *Lgn1*, with the exception of *Mtap5* (microtubule-associated protein 5).

TABLE 1.4

Combined Analysis of Recombination Fractions (a) in Intra- and Interspecific Backcross Offspring for Chromosome 13 Markers in the Vicinity of the *Lgn1* Locus

	ABA[b]	BSB-1[b]	BSB-2[b]	BSS[b]	Genetic distance (cM ± SE)
D13Mit231					
	10/182	9/276			4.15 ± 0.93
D13Mit193					
		0/276			
Dhfr/Sell	4/179				
		7/280			
D13Mit108	3/179				1.68 ± 0.96
Hexb	1/180	1/280			0.43 ± 0.31
D13Mit111,110		0/281			
D13Hun34	1/80	0/281	3/94		0.80 ± 0.46
D13Hun33		0/281	0/94		
D13Mit128					
	20/1270	4/281	0/94	0/94	1.38 ± 0.28
D13Mit194	1/1270				0.08 ± 0.08
D13Mit147	12/1270	1/281	2/94		0.94 ± 0.27
D13Mit36				3/94	
D13Hun35[c]					
	12/1270	0/281	0/94		
Mtap5/D13Gor4/D13Mit30, D13Mit72, 195, 203					
		0/281	0/94	0/94	
D13Mit146	2/1268				0.16 ± 0.11
Lgn1		0/281	0/94	0/93	
	0/1268				0
D13Mit37	17/1270	1/281	0/94	0/93	1.03 ± 0.24
D13Mit70	3/182				1.65 ± 0.94
D13Mit287		5/281			
Gpcr18/Htr1/D13Mit112, 71, 47	5/182				
D13Mit73		15/276			
	13/182				7.14 ± 1.91
Ctla3/D13Mit53, 32	0/182	3/276			0.65 ± 0.38
Itga2/Itga1/D13Hun39	2/182	1/270			0.66 ± 0.38
D13Mit77					
	2/182				1.10 ± 0.77
D13Mit78					

Note. Boxes indicate that only the distal and proximal markers at the boundaries of the boxed segment were typed, with intervening markers nonpolymorphic in this cross. Brackets indicate that no marker order can be inferred for these markers.

[a] Recombination fractions are expressed as the number of recombinants divided by the number of backcross animals tested.

[b] ABA, BSB-1, BSB-2, and BSS represent the (C57BL/6J X A/J) X A/J cross, *(Mus spretus X* C57BL/6J) X C57BL/6J cross previously described (Schurr *et al.*, 1989), and the (M. spretus X C57BL/6J) X C57BL6J and (C57BL/6J X *M. spretus*) X *M. spretus* crosses generated at The Jackson Laboratory, respectively.

[c] The position of this group of markers with respect to *Lgn1* cannot be established, although they map within the *D13Mit194* to *D13Mit70* interval.

Herein, is a complete review of the gene order obtained for markers mapping within the *Lgn1* interval previously defined by *D13Mit128* and *D13Mit70* (see Table 1.4). 47 crossovers were identified between *D13Mit128* and *Lgn1* amongst 1270 animals of the ABA cross analyzed, placing it at a genetic distance of 3.7 cM (2.7-4.7; 95% confidence interval) proximal to *Lgn1*. Amongst these 47 recombinants, 27 were between *D13Mit194* and *Lgn1*, 26 between *D13Mit147* and *Lgn1*, 14 between *D13Mit36* and *Lgn1* and 2 between *D13Mit 146* and *Lgn1*. Therefore, the gene order for these markers was unambiguous and *D13Mit146* defined the new proximal boundary of the *Lgn1* interval, mapping at 0.2 cM (0.0-0.6) proximal to *Lgn1*. *Lgn1* cosegregated with *D13Mit37* in 1268 meioses tested (the polymorphic variants of *D13Mit37* are defined by SSCP for A/J and C57BL/6J) and are considered to lie between 0.0 and 0.2 cM of each other, at 95% confidence interval. 17 crossovers were detected between *D13Mit70* and *Lgn1/D13Mit37* in 1270 mice from the ABA cross, placing it distal to *Lgn1/D13Mit37* at a map distance of 1.3 cM (0.8-2.1), therefore representing the distal boundary of the *Lgn1* interval (Beckers et al. 1997). In the BSB-1, BSB-2 and BSS crosses, a total of 10 crossovers in 468 meioses were identified between *D13Mit128* and *D13Mit37* (tightly linked to *Lgn1*): 5 in BSB-1, 2 in BSB-2 and 3 in the BSS cross. Among these 10 recombinants, 4 crossovers were detected between *D13Mit128* and *D13Mit194*, and 6 between *D13Mit194* and a large cluster of markers formed by *D13Hun35, Mtap5, D13Gor4, D13Mit30, 72, 195, 203* that cosegregated with *D13Mit146* and *D13Mit37*. Finally, a single crossover was detected on the distal side between *D13Mit37* and *D13Mit70* in 468 informative animals from the BSB-1, BSB-2 and BSS crosses. Table 1.4 shows the analysis of recombination frequencies from the ABA, BSB-1, BSB-2 and BSS crosses for all markers tested. The combined pedigree analyses for the chromosomal region overlapping *Lgn1* indicated the locus order and the interlocus distances (in cM): ***D13Mit128*** - (1.4) - *D13Mit194* - (0.1) - *D13Mit147* - (0.9) - *D13Mit36*- (0.9) - *D13Mit146* - (0.2) - ***Lgn1****/D13Mit37* - (1.0) - ***D13Mit70.*** Thus the minimum genetic interval defining the *Lgn1* locus was delineated at 1.2 cM between the new proximal (*D13Mit146*) and distal (*D13Mit70*) boundaries. In addition, marker *D13Mit37* was found to segregate with *Lgn1* in 1268 meioses tested, and additional genetic linkage studies of markers not informative in the A/J×C57BL/6J cross positioned *D13Mit30, 72, 195, 203,*

D13Gor4, *D13Hun35*, and *Mtap5* as in the immediate vicinity of the *Lgn1* locus (Beckers et al. 1997). The marker density and resolution of this genetic linkage map was deemed sufficient to proceed with the next step in the positional cloning process, physical mapping: the cloning and characterization of large pieces of genomic DNA spanning the subchromosomal region that contains the *Lgn1* gene.

At that point, no obvious candidate gene for the *Lgn1* mutation was identified. *Mtap5* was the cloned gene most tightly linked to *Lgn1* (O'Brien et al. 1995; Beckers et al. 1997), but was an unlikely candidate. Indeed, the pattern of expression of this microtubule associated protein isoform is tissue specific and restricted to neuronal axons, dendrites and glial cells (Riederer et al. 1986; Schoenfeld et al. 1989), clearly incompatible with the putative expression of *Lgn1* in cells of the reticuloendothelial system.

1.3.3 Physical mapping.

While the high-resolution genetic mapping report was in press (Beckers et al. 1997), the physical mapping of the *Lgn1* chromosomal region was started. The first step to create a physical map was to use the genetic markers most closely associated with *Lgn1* to probe yeast artificial chromosome (YAC) libraries for sets of overlapping clones that may span the entire *Lgn1* genetic region.

In the spring of 1996, Dr. Beckers met Dr. L.R. Simard and her student C.J. DiDonato at the Canadian Genetic Diseases Network annual meeting. These researchers were interested in cloning and characterizing the genomic region surrounding the mouse homolog of the human SMN (Survival of Motor Neuron) gene, believed to be involved in the onset of Spinal Muscular Atrophy. Interestingly, the mouse *Smn* gene mapped to distal Chr. 13 (DiDonato et al. 1997a), not far from marker *D13Mit37* that our group had found to cosegregate with *Lgn1*. This group had isolated bacterial artificial chromosome (BAC) clones that contained some of the genetic markers that we had mapped in close proximity to *Lgn1*. A collaboration was quickly set up in which both groups could have access to the full set of isolated YACs and BACs without duplicating work.

Colocalization of the SMA and *Lgn1* genomic regions brought two new candidate genes for the *Lgn1* locus: the Survival of Motor Neuron (*Smn*) and the Neuronal Apoptosis Inhibitor Protein (*Naip*) genes.

In the summer of 1996, Dr. Beckers left behind the cloning of *Lgn1* to pursue other projects. From that point in time, the positional cloning of *Lgn1* became my own Ph.D. project, and is thus described in the following three chapters of this thesis; chapters 2, 3 and 4, corresponding to publications in recognized scientific journals in which I had primary authorship.

While I was busy isolating genomic clones, defining their sequence-tagged site (STS) content, and setting a rare-cutter restriction map of the *Lgn1* region, a report appeared in the literature (Scharf et al. 1996) describing several of the findings that we were hoping would form the basis for our own next publication. This group from Boston had been studying the genetic basis for human SMA and had been hampered in their evaluation of candidate genes by an intricate genomic structure that included repeated sequences and expressed pseudogenes (Selig et al. 1995) surrounding the SMA locus on human Chr 5q13. Just like our new collaborators, this group turned to the mouse syntenic region in the hope that it would contain the same genes on a simpler genomic background. The mouse model would also provide an opportunity to perform crucial gene-deletion studies. In their report, the SMA region was indeed mapped to mouse chromosome 13 and to the critical interval for *Lgn1* (Scharf et al. 1996). In collaboration with the laboratory of Dr. W.F. Dietrich, a mouse YAC contig was generated across the *Lgn1/Sma* interval and the two flanking gene markers for the human SMA locus, MAP1B and CCNB1, were mapped onto the contig (Scharf et al. 1996). In addition, the two SMA candidate genes, SMN and NAIP, were localized to the *Lgn1* critical region, making these two genes candidates for the *Lgn1* phenotype. Upon subcloning of the YAC contig into P1s and BACs, a large, low copy number repeat was detected that contained at least one copy of *Naip* exon 5 (Scharf et al. 1996). Thus, it seemed that the mouse SMA/*Lgn1* region would not prove to be simpler in structure than its human counterpart. On a brighter note, it was proposed that the identification of the *Lgn1* gene could eventually provide a novel function for either SMN or NAIP (Scharf et al. 1996).

With this report in our hands, it was decided to drop the detailed restriction map of the *Lgn1* physical interval in favor of publishing immediately the most significant clones that we had isolated. Unlike the group in Boston, the genomic clones that we had isolated seemed to span the entire *Lgn1* interval. We could therefore make an estimate of the physical distance separating the new proximal and distal boundaries of the *Lgn1* candidate region. Also, a refined genetic interval could be shown now that there were new genetic markers available and that we had a much larger number of meioses to analyze than the group in Boston. This publication (Diez et al. 1997) forms the basis for chapter 2 of this thesis. With rather small *Lgn1* genetic and physical intervals and two candidate genes within them, our aim was to test without delay the candidacy of *Smn* and *Naip* for *Lgn1*. The first requirement was that the *Lgn1* gene product must be expressed within cells of the reticuloendothelial system, macrophages most specifically. From previous reports, both the *Smn* and *Naip* genes seemed rather ubiquitously expressed (Liston et al. 1996; Bergin et al. 1997). However, Northern blot analysis had shown higher *Naip* expression levels in spleen compared with brain (Yaraghi et al. 1998). Therefore, a priority was assigned to verifying *Naip* expression within mouse peritoneal macrophages (Diez et al. 2000); work which is described within chapter 3.

Section 1.4

The *Birc1* (*Naip*) Gene

The *Naip* gene has been identified as a strong candidate for the *Lgn1* locus through our own research (described further in chapters 2, 3, and 4 of this thesis) as well as the work of other groups. This section gives an overview of what is already known about the *Naip* gene.

1.4.1 A candidate modulator of SMA disease severity.

Spinal muscular atrophy (SMA) is a common autosomal recessive disorder characterized by degeneration of lower motor neurons of the spinal cord, leading to progressive paralysis with muscular atrophy. Three different forms of childhood SMA have been recognized on the basis of age at onset and clinical course: Werdnig-Hoffmann disease (type-I), the intermediate form (type-II) and Kugelberg-Welander disease (type-III). For all three types of SMA, a genetic factor was mapped to the human chromosome 5q13 region. In a search for the gene causing SMA, Roy and colleagues isolated a gene on chromosome 5q13.1, of which the first 2 coding exons were deleted in approximately 67% of type I SMA chromosomes compared with 2% of non-SMA chromosomes (Roy et al. 1995a; Velasco et al. 1996). One model of SMA pathogenesis invokes an inappropriate persistence of motor neuron apoptosis, which is a normally occurring phenomenon in development. Consistent with this hypothesis, the novel gene was labeled 'neuronal apoptosis inhibitory protein' (NAIP) and its function was supported by the finding that it contains domains with sequence similarity to IAPs, baculovirus proteins that inhibit virally induced insect cell apoptosis (Crook et al. 1993; Roy et al. 1995a; Liston et al. 1996). However, a neighboring gene termed 'survival of motor neuron' (SMN) has been later recognized as the disease-causing gene in SMA (Souchon et al. 1996; Campbell et al. 1997; Chang et al. 1997; Taylor et al. 1998; Cusco et al. 2001; Wong and Chan 2001; Savas et al. 2002; Bouhouche et al. 2003). Still, Roy and colleagues had raised the possibility that NAIP functions in concert with SMN mutations in causing spinal muscular atrophy, or more likely, affecting the severity of the disease. Importantly, NAIP protein is present in motor neurons and other neuronal populations affected in type I SMA (Xu et al. 1997b). Although NAIP deletions are more frequently observed in patients affected by the acute form of SMA, it has not been possible to establish an unambiguous correlation between deletion size and clinical severity (Brahe and Bertini 1996; Burlet et al. 1996; Samilchuk et al. 1996; Wang et al. 1997; Al Rajeh et al. 1998; Gambardella et al. 1998; Jordanova et al. 1998; Bouhouche et al. 2003). The very latest reports argue that different deletions/mutations in the SMN genes can account for disease variability (Cusco et al. 2001; Savas et al. 2002).

1.4.2 What does the *Naip* gene encode for?

The NAIP coding region spans 4212 nucleotides and encodes for a 1403-amino acid 150-kDa protein with strong homology to the baculoviral inhibitor of apoptosis proteins (IAPs) Cp-IAP and Op-IAP (Liston et al. 1996). The IAP protein family has since then been defined; its members containing one to three imperfect repeats of a motif called baculovirus inhibitor of apoptosis repeat (BIR) domain (Liston et al. 1996). The growing IAP protein family currently includes NAIP, HIAP2, HIAP1, XIAP, SURVIVIN, KIAA1289, and LIVIN (now called BIR-Containing proteins -1 through -7 respectively) (Liston et al. 1996; Farahani et al. 1997). The BIR domains are defined by a $CX_2CX_{16}HX_{6-8}C$ consensus sequence (Liston et al. 1996) and form zinc coordination structures that have been implicated in protein-protein interactions (Hozak et al. 2000; Mercer et al. 2000; Verdecia et al. 2000; Wu et al. 2000; Kasof and Gomes 2001; Vucic et al. 2002). Specifically, the third BIR domain of human NAIP has been shown to interact with the neuron-restricted, calcium-binding Hippocalcin protein (Mercer et al. 2000; Lindholm et al. 2002). Importantly, the downstream caspases 3 and 7 have been shown to interact tightly with the BIR domain of human LIVIN (Kasof and Gomes 2001). And the pro-apoptotic Smac/DIABLO protein has also been shown to bind human XIAP (Wu et al. 2000) and LIVIN (Vucic et al. 2002) BIR domains. Additionally, BIR domains have been shown to cause homo-oligomerization of baculoviral Op-IAP (Hozak et al. 2000) and dimerization of human SURVIVIN (Verdecia et al. 2000). Immediately following the three N-terminal NAIP BIR domains (Roy et al. 1995a; Liston et al. 1996), there is a putative ATP/GTP binding site, followed by a long carboxy-terminal region with no known functional motifs or domains other than a leucine-rich region. Notably absent is the RING zinc-finger motif that is found in most other human IAPs (Liston et al. 1996) and is believed to control subcellular localization (Kasof and Gomes 2001).

1.4.3 The mouse "Naip swamp".

The NAIP gene exists in two copies in the human genome as a result of an inverse duplication within the SMA region of Chr 5q13 that includes the SMN gene as well (Roy et al. 1995a; Campbell et al. 1997). In a recent report, it was shown that both NAIP copies possess a functional promoter region and are indeed transcribed (Xu et al. 2002). It was hoped that the syntenic chromosomal region in the mouse would provide a simpler genomic structure to study. Unfortunately, the complexity of the mouse SMA/*Lgn1* region would soon earn it the nickname of "*Naip* swamp" by the researchers trying to make sense of it, including us. The first indication that the mouse genome encodes for several copies of the *Naip* gene was observed during physical mapping of the region by Scharf and colleagues (Scharf et al. 1996). Upon subcloning of their YAC contig into smaller P1s and BACs, it was found that non-overlapping clones seemed to contain the same *Naip* exons as well as flanking genetic markers. A large, low copy number repeat that included at least one copy of *Naip* exon 5 was suggested (Scharf et al. 1996). Further proof of the complexity of the mouse *Naip* region was provided by DiDonato and colleagues (DiDonato et al. 1997a). Fluorescence in-situ hybridization (FISH), Southern blotting, and single-strand conformation polymorphism analysis (SSCP) were used to show that the mouse *Smn* gene, unlike its human counterpart, was present as one single copy. The same techniques, however, detected at least four copies of the *Naip* exon 5 that was used as a probe. These *Naip* copies seemed tightly clustered, just distal to *Smn* (DiDonato et al. 1997a). More detailed physical mapping by Yaraghi and colleagues (Yaraghi et al. 1998) indicated the existence of a minimum of six distinct *Naip* loci in the 129/SvJ mouse strain, designated Naip1-6. The coding region of one of the potentially functional loci (*Naip1*) demonstrated 77% homology to NAIP at the nucleotide level and 68% identity at the amino acid level (Yaraghi et al. 1998). The mouse *Naip* loci not only showed some sequence differences at the cDNA level, but also a divergence both in size and sequence of introns. This observation suggested that mice possess different, full-length *Naip* loci representing a family of genes. One possible reason for this genomic redundancy was postulated to be tissue-specific expression of each member of the gene family (Yaraghi et al. 1998). In order to assess the possibility of tissue-specific expression

of each one of the *Naip1, 2,* and *3* loci, RT-PCR analysis of mouse C57BL/6J brain and spleen RNA was carried out with copy-specific primer pairs (Yaraghi et al. 1999). *Naip2* was identified as a spleen-transcribed locus and *Naip1* as a brain-transcribed locus (Yaraghi et al. 1999). This, however, did not exclude the possibility that all loci might be expressed in each tissue, albeit at levels too low for detection in standard RT-PCR reactions. Nevertheless, these observations provided the foundation for our own assessment of *Naip2* as a good candidate gene for murine *Legionella* resistance. A further characterization of the *Naip2* locus was undertaken (Yaraghi et al. 1999). Sequence analysis of cDNA clones from a mouse C57BL/6J spleen cDNA library showed that *Naip2* encodes a protein of 1447 amino acids with a predicted size of 164 kDa. The *Naip2* coding region revealed 90.4% nucleotide sequence homology to *Naip1* and 77% to human NAIP. Both *Naip1* and *Naip2* show similar levels of amino acid identity to human NAIP (68.4% and 68.9% respectively), while sharing an 86.6% identity. The three BIR domains encoded by exons 2-8 are highly conserved between *Naip1* and *Naip2* (91% amino acid identity), as is the potential ATP/GTP binding site originally identified in the human NAIP (Roy et al., 1995). A number of different *Naip2* transcripts produced by a combination of two transcription-start sites and alternative splicing of non-coding 5' exons were identified. The presence of multiple 5'UTR exons in *Naip2,* in contrast to the one 5'UTR exon observed in *Naip1,* was proposed to reflect differences in the regulation of translation of these genes (Yaraghi et al. 1999). Later on, meticulous work by Growney and colleagues determined that mice of the 129 haplotype harbor seven intact and three partial *Naip* transcription units arranged in a closely linked direct repeat (Growney et al. 2000). An evolutionary model for the expansion of the *Naip* gene array from a single progenitor *Naip* gene was constructed (Endrizzi et al. 2000). This model predicted the presence of two distinct families of *Naip* paralogs: *Naip1/2/3* and *Naip4/5/6/7*. Unlike the divergences among the other *Naip* paralogs, the splits among *Naip4*, *Naip5*, *Naip6*, and *Naip7* appeared to have occurred relatively recently (Endrizzi et al. 2000). Importantly, differences in the structure of the *Naip* array among commonly used inbred mouse strains were identified. Namely, a physical map of the region employing clones of the C57BL/6J haplotype confirmed that there are fewer copies of *Naip* in this strain than are in the physical map of the 129 haplotype (Growney and Dietrich 2000). Unfortunately, these

gross structural differences did not correlate with differences in *L. pneumophila* permissiveness (Growney and Dietrich 2000).

1.4.4 Functional studies of the Naip protein.

An antiapoptotic effect of NAIP and other members of the human IAP family was first shown in a cell culture system: NAIP overexpression in mammalian cells inhibited apoptosis induced by a variety of signals (Liston et al. 1996). Importantly, following ischemic damage, apoptotic rat hippocampal neurons have been rescued by stereotactic microinjection of NAIP-expressing adenovirus, suggesting that the antiapoptotic activity of NAIP shown *in vitro* extends to the *in vivo* situation (Xu et al. 1997a). Similarly, adenoviral delivery and expression of NAIP within peripheral motoneurons was shown to protect them significantly from apoptotic death following sciatic axotomy (Perrelet et al. 2000). In addition to supraphysiologic levels of NAIP conferring neuronal protection, the loss of endogenous mouse *Naip1* has been shown to result in enhanced neuronal vulnerability (Holcik et al. 2000). Mice were generated with a targeted deletion of *Naip1*. These *Naip1*-deleted mice developed normally. However, the survival of hippocampal neurons after kainic acid-induced limbic seizures was greatly reduced in the *Naip1* knockout animals. Thus, although *Naip1* does not seem to be necessary for normal development of the murine central nervous system, it is required for neuronal survival in pathological conditions. (Holcik et al. 2000). And finally, a direct inhibition of effector caspases by NAIP BIR domains has been shown (Maier et al. 2002) and thus provided a mechanistic explanation for the cytoprotective effect of NAIP and its function as an important regulator of neuronal apoptosis. In this last study, recombinant N-terminal NAIP protein containing BIR domains was overexpressed, purified, and tested for direct caspase inhibition potential. It was demonstrated that inhibition of caspases is selective and restricted to the effector group of caspases, with K(i) values as low as approximately 14 nM for caspase-3 and approximately 45 nM for caspase-7. Additional investigations with NAIP fragments containing either one or two NAIP BIRs revealed that the second

BIR and to a lesser extent the third BIR alone are sufficient to mediate caspase inhibition (Maier et al. 2002).

Available data indicates that the large part of the cell death protective effects of the IAPs can be ascribed to their ability to bind and inhibit caspases (Roy et al. 1997; Maier et al. 2002). Recently, however, it has been shown that NAIP can protect cell from apoptosis through an alternate mechanism that depends on the selective activation of the mitogen-activated protein (MAP) kinase JNK1 (Sanna et al. 2002). In this study, a short NAIP-BIR 1-3 was shown to activate JNK1 selectively (as witnessed by a 10-fold increase in phosphorylation of the ATF2 substrate of JNK1). JNK2 was also activated by the NAIP BIR domains, but to a lower extent. JNK3 was not activated. Furthermore, expression of catalytically inactive JNK1 blocked NAIP protection against ICE- (caspase 1) and TNF-alpha-induced apoptosis, indicating that JNK1 activation is necessary for the antiapoptotic effect of NAIP. The MAP3 kinase TAK1, instead of the usual map kinase kinases MKK4 or MKK7, appears to be an essential component of this antiapoptotic pathway since IAP-mediated activation of JNK1, as well as protection against TNF-alpha- and ICE-induced apoptosis, was inhibited when catalytically inactive TAK1 was expressed. In addition, both NAIP and JNK1 bind to TAK1, as seen by immunoprecipitation of cotransfected, tagged proteins. Importantly, NAIP did not bind to JNK1 directly, but it did bind TAB1, a protein previously known to be able to stimulate TAK1. The conclusion was therefore that TAK1 and TAB1 mediate the functional interaction between NAIP and JNK1 (Sanna et al. 2002). These data suggested that NAIP's antiapoptotic activity is achieved by two separate mechanisms: one requiring TAK1-dependent JNK1 activation and the second involving caspase inhibition (Sanna et al. 2002).

Besides the role described for the Naip protein in protecting cells from apoptosis, Gotz and colleagues described one additional function for Naip, as discovered by experimenting with overexpression of mouse Naip2 within the rat PC12 cell line (Gotz et al. 2000). PC12 cells differentiate, in the presence of nerve growth factor (NGF), into cells that resemble sympathetic neurons. The expected NGF-induced neurite outgrowth was impaired in PC12 cells that overexpressed Naip. The BIR motifs of Naip (residues 1-345) were not required for this effect (Gotz et al. 2000). However, the same study showed

that the BIR domains of Naip were essential to prevent apoptosis in PC12 cells after NGF deprivation or tumor necrosis factor-alpha receptor (TNFAR) stimulation, as expression of full-length but not BIR-deleted Naip protected against cell death. This correlated with reduced activity of the cell death effector protease, caspase-3, in lysates of Naip-PC12 cells (Gotz et al. 2000). The authors hypothesized that both dysregulation of cellular differentiation and caspase suppression may contribute to motoneuron dysfunction and cell death in spinal muscular atrophy when NAIP is mutated (Gotz et al. 2000).

Figure 1.6 shows a selection of proteins with domain architectures similar to Naip. ATP/GTP binding sites and leucine-rich repeats on their own do not help much in elucidating the function of the Naip protein as there are proteins with very diverse functions that contain such conserved motifs. It is the combination of both motifs within the same protein, and their spatial arrangement within the protein, rather than a conventional nucleotide or amino acid sequence alignment, that have pointed out possible functional relatives of Naip. In fact, NBS-LRR (nucleotide-binding site and leucine-rich repeat) proteins form a newly defined family involved in intracellular recognition of microbes and their products (Chamaillard et al. 2003). The other known class of pattern-recognition molecules, the Toll-like receptors (TLRs), are involved in detection of microbes in the extracellular compartment rather than intracellularly. NBS-LRR proteins are characterized by three structural domains: a C-terminal leucine-rich repeat (LRR) domain able to sense a microbial motif, an intermediary nucleotide binding site (NBS) essential for the oligomerization of the molecule that is necessary for the signal transduction induced by different N-terminal effector motifs, such as a pyrin domain (PYD), a caspase-activating and recruitment domain (CARD) or a baculovirus inhibitor of apoptosis protein repeat (BIR) domain. Two of these family members, Nod1 and Nod2, play a role in the regulation of pro-inflammatory pathways through NF-kappaB induced by bacterial ligands. Recently, it was shown that Nod2 recognizes a specific peptidoglycan motif from bacteria, muramyl dipeptide (MDP). A surprising number of human genetic disorders have been linked to NBS-LRR proteins. For example, mutations

FIGURE 1.6

Schematic representation of Naip (Birc1) protein conserved domains and a selection of proteins with similar domain architectures.

These protein domains were detected using the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd). Seven types of domain architectures most closely related to that of Birc1 proteins are depicted here. For each type of domain architecture, one representative protein is drawn to scale (shown at the bottom of the figure) with colored boxes depicting conserved protein domains:

BIR domain (Baculoviral Inhibition of apoptosis protein Repeat; smart00238; cd00022; red box). In higher eukaryotes, BIR domains inhibit apoptosis by acting as direct inhibitors of the caspase family of protease enzymes. In yeast, BIR domains are involved in regulating cytokinesis. This novel fold is stabilized by zinc tetrahedrally coordinated by one histidine and three cysteine residues and resembles a classical zinc finger.

NACHT domain (named after some of the proteins in which it is found: NAIP, CIITA, HET-E and TP1; pfam05729; blue box). A putative NTPase domain.

LRR domain (Leucine-Rich Repeats; cd00116; green box). Repeated structural units consisting of a beta strand (LxxLxLxxN/CxL conserved pattern) and an alpha helix.

CARD domain (CAspase Recruitment Domain; pfam00619; smart00114; yellow box). Mediates homodimerisation. Structure consists of six antiparallel helices arranged in a topology homologous to the DEATH domain.

PYD domain (PYrin Domain, also named PAAD/DAPIN/Pyrin domain; pfam02758; orange box). Predicted to contain 6 alpha helices and to possess a DEATH (pfam00531) domain-like fold thus being a protein-protein interaction domain.

RING domain (Really Interesting New Gene-finger domain; cd00162; grey box). Specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc and probably mediates protein-protein interactions.

The represented groups of proteins with common conserved domain architectures are:

C2ta (class II transactivator; NP_031601 shown), Nalp5 (Q9R1M5) and NALP4 (AAL35293) NACHT-LRR proteins. Molecular function: ATP-binding. Biological process: defense response to pathogens.

Cias1 (cold autoinflammatory syndrome 1; Q8R4B8 shown) and NALP1 (Q9C000) PYD-NACHT-LRR proteins. Molecular function: caspase activator activity. Biological process: signal transduction, induction of apoptosis and defense response to pathogens.

NOD1 (nucleotide-binding oligomerization domain 1; Q9Y239 shown) and CARD12 (Q9NPP4) are CARD-NACHT-LRR proteins. Molecular function: ATP binding and protein binding. Biological process: signal transduction, positive regulation of I-kappaB kinase/NF-kappaB cascade, regulation of apoptosis and defense response to pathogens.

Birc1 human and mouse proteins (baculoviral IAP repeat-containing 1; BIR-BIR-BIR-NACHT-LRR proteins) are represented here by the C57BL/6J mouse-derived Birc1e protein (AAN60207).

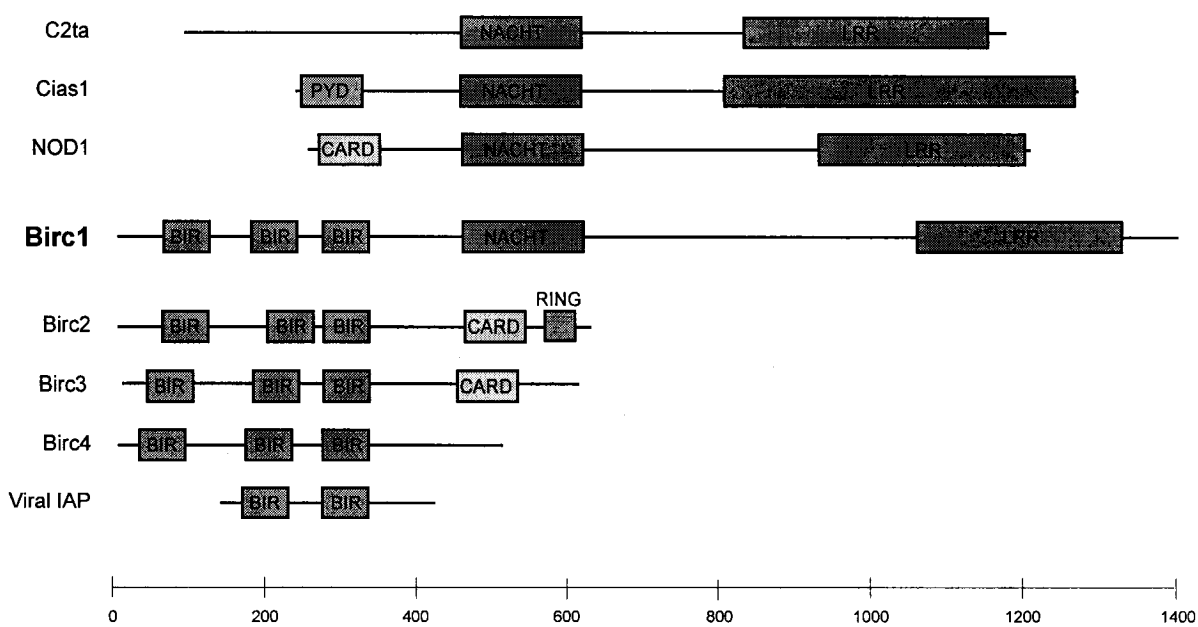
Birc2 (baculoviral IAP repeat-containing 2; NP_031491) has a BIR-BIR-BIR-CARD-RING structure. Molecular function: protein binding. Biological process: signal transduction, positive regulation of I-kappaB kinase/NF-kappaB cascade.

Birc3 (baculoviral IAP repeat-containing 3; NP_031490) has a BIR-BIR-BIR-CARD structure. Molecular function: protein binding. Biological process: signal transduction.

Birc4 (baculoviral IAP repeat-containing 4; NP_033818) has a BIR-BIR-BIR structure. Molecular function: caspase inhibitor activity. Biological process: anti-apoptosis.

Viral inhibitors of apoptosis such as IAP1 (inhibitor of apoptosis protein 1; NP_932645 shown), IAP3 (NP_932639) and IAP5 (NP_891953) have a BIR-BIR structure.

Gene ontologies can be found at the mouse genome informatics site (MGI; http://www.informatics.jax.org) or directly at (http://www.godatabase.org).


C2ta
NACHT
LRR
Cias1
PYD
NACHT
LRR
NOD1
CARD
NACHT
LRR
Birc1
BIR
BIR
BIR
NACHT
LRR
Birc2
BIR
BIR
BIR
CARD
RING
Birc3
BIR
BIR
BIR
CARD
Birc4
BIR
BIR
BIR
Viral IAP
BIR
BIR
0
200
400
600
800
1000
1200
1400

in Nod2, which render the molecule insensitive to MDP and unable to induce NF-kappaB activation when stimulated, are associated with susceptibility to a chronic intestinal inflammatory disorder, Crohn's disease. Conversely, mutations in the NBS region of Nod2 induce a constitutive activation of NF-kappaB and are responsible for Blau syndrome, another auto-inflammatory disease. Nalp3, which is an NBS-LRR protein with an N-terminal Pyrin domain, is also implicated in rare auto-inflammatory disorders. Therefore, NBS-LRR molecules appear as intracellular receptors that regulate bacterial-induced inflammation (Chamaillard et al. 2003).

The *Naip* gene being a strong candidate for *Lgn1* (see following chapters), future research will focus on determining which of the aforementioned interactions and cellular functions attributed to the Naip protein (or other ones not yet discovered) are important in the context of macrophage permissiveness to intracellular bacterial replication.

Chapter 2

Genetic and physical mapping of the mouse host resistance locus *Lgn1*

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Diez, E., Beckers, M. C., Ernst, E., DiDonato, C. J., Simard, L. R., Morissette, C., Gervais, F., Yoshida, S. I., and Gros, P. (1997). Genetic and physical mapping of the mouse host resistance locus Lgn1. Mamm Genome *8*, 682-685, © Springer.

2.1 Abstract

We have previously generated a large backcross panel consisting of 1270 (A/J × C57BL/6J)F_1 × A/J mice (ABA) segregating the *Lgn1* gene and have used a series of informative polymorphic markers to delineate the genetic interval of *Lgn1* to 1.2 cM: *D13Mit146*-(0.2 cM)-*Lgn1*/*D13Mit37*-(1 cM)-*D13Mit70* (Beckers et al. 1997). Additional mapping studies in 281 (*Mus spretus* × C57BL/6J) F_1 × C57BL/6J backcross mice (not informative for *Lgn1*) positioned *D13Mit30, -72, -195* and *-203, D13Gor4, D13Hun35,* and *Mtap5* in the immediate vicinity of *Lgn1* (Beckers et al. 1997). Independently, (Scharf et al. 1996) described new informative markers near *Lgn1* and used 466 animals from an ABA backcross panel to reduce the *Lgn1* genetic interval to 1.0 cM, as *D13Mit146*/*D13Die6*-(0.6 cM)-*Lgn1*/*D13Mit37*-(0.4 cM)-*D13Die7a*/*D13Die1b*/*D13Die3*-(1.2 cM)-*D13Mit70.* The nonrecombinant interval for *Lgn1* is homologous to the human Spinal Muscular Atrophy (SMA) gene region and is composed of a complex repeated unit that includes several copies of the Neuronal apoptosis inhibitor protein (*Naip*) gene (exon 5; (Scharf et al. 1996; DiDonato et al. 1997a), the Survival motor neuron (*Smn*) gene (DiDonato et al. 1997a; Viollet et al. 1997), as well as multiple copies of certain polymorphic markers used for mapping (*D13Mit37, D13Die1, D13Die7;* (Scharf et al. 1996). A set of genomic clones overlapping this region was identified, and physical mapping of these clones was used to propose a genetic and physical organization of the *Lgn1* locus. In the current study, we have used these novel markers to re-evaluate the *Lgn1* genetic interval, using our large ABA cross, and report on an independent set of YAC and BAC clones that cover the entire *Lgn1* region.

2.2 Introduction

Legionella pneumophila is a strict intracellular pathogen that causes an acute form of pneumonia in humans, called Legionnaires' disease (Fraser et al. 1977; McDade et al. 1977). *L. pneumophila* enters human macrophages through a unique "coiling phagocytosis" mechanism (Horwitz 1984) and replicates within the phagosome (Horwitz and Silverstein 1980; Kishimoto et al. 1981) by inhibiting fusion to endosomes and lysosomes (Horwitz 1987; Berger and Isberg 1993). These replicative phagosomes are morphologically distinct and are associated with endoplasmic reticulum membranes and dotted with ribosomes (Swanson and Isberg 1995). The mechanism by which *L. pneumophila* modulates the fusogenic properties of the phagosome and survives intracellularly remains unknown. In contrast to their human and guinea pig counterparts, mouse macrophages are not permissive to *L. pneumophila* replication (Yoshida and Mizuguchi 1986) with the exception of mice of the A/J strain, where inflammatory peritoneal macrophages and alveolar macrophages are highly permissive to *L. pneumophila* replication in vitro. Indeed, A/J macrophages can support a 1000-fold increase in viable bacteria during a 72-h infection (Yamamoto et al. 1988; Yoshida et al. 1991b; Fujio et al. 1992). The characteristics of *L. pneumophila* replication observed in A/J macrophages, including the unique inhibition of phagosome maturation, are similar to those observed in human cells (Yamamoto et al. 1988; Brieland et al. 1994; Yamamoto et al. 1994a). Segregation analyses in informative backcross populations derived from susceptible (A/J) and resistant (C57BL/6J) progenitors have indicated that permissiveness of A/J macrophages to intracellular replication of *L. pneumophila* is determined by a single recessive gene designated *Lgn1* (Yoshida et al. 1991b), which maps on Chromosome (Chr) 13 (Beckers et al. 1995; Dietrich et al. 1995). Understanding the molecular mechanism of action of *Lgn1* may provide important clues on the intracellular survival strategy of *L. pneumophila,* and possibly on a new antimicrobial mechanism of the macrophage effective against *L. pneumophila.*

2.3 Results

2.3.1 Genetic Mapping

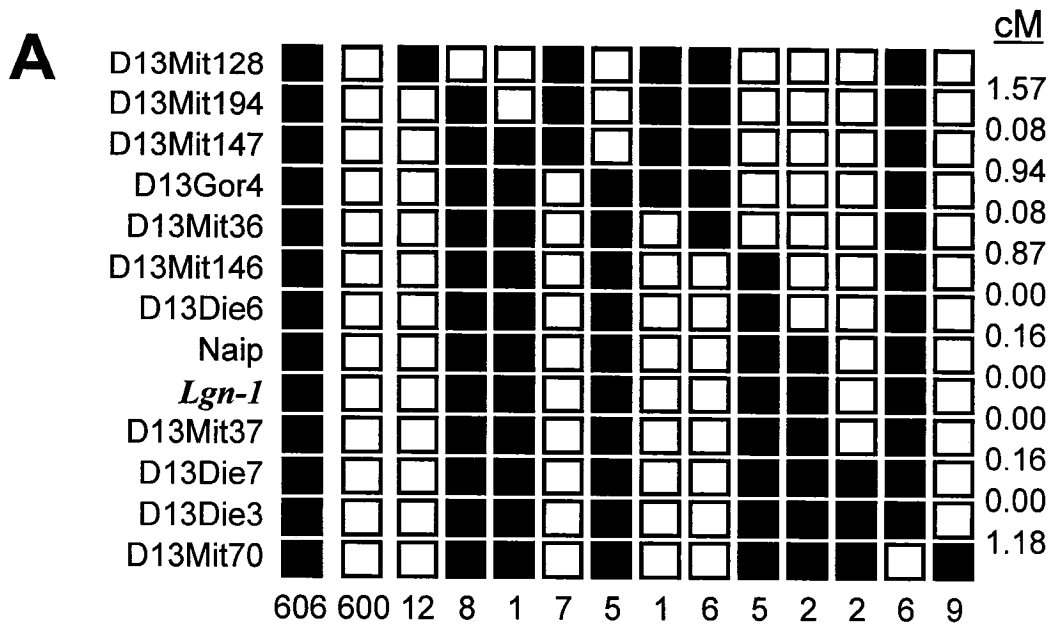
Among the 1270 mice from our ABA cross, we previously identified 64 animals with recombination events between *D13Mit128* and *D13Mit70* (5-cM interval delineating *Lgn1*). These 64 animals were genotyped for the following polymorphic markers: the newly derived *D13Die3, -6* and *-7* markers (Scharf et al. 1996), the *D13Lsd1* informative dinucleotide repeat located 5' of *Naip* exon 5 (DiDonato et al. 1997b), and a new anonymous marker *D13Gor4* (Xu et al. 1996) (435-bp probe detects an *Eco*RI RFLP; A/J, 8 kb; C57BL/6J, 3.2 kb). Genotypes for these markers were combined with previously established ones (Beckers et al. 1997) for additional *D13Mit* markers from the region to derive a composite genetic map (Fig. 2.1). *D13Gor4* mapped about 1 cM proximal to the *Lgn1* interval. In the immediate vicinity of *Lgn1, D13Die6* co-segregated with *D13Mit146,* and together they defined the proximal boundary of *Lgn1* at two crossovers (progenies #3 and #442; 2/1270, 0.16 cM). *D13Die7* co-segregated with *D13Die3,* and these two markers defined the new distal boundary of *Lgn1* at two crossovers (progeny #142 and #1178; 2/1270, 0.16 cM). The *Naip*-associated dinucleotide repeat marker, *D13Lsd1,* co-segregated with *D13Mit37,* and neither recombined with *Lgn1* in all informative animals (0/1270). Although synteny mapping in humans and linkage mapping in mice (interspecific cross) indicate that the *Smn* gene is closely linked to the *Naip* cluster (Scharf et al. 1996; DiDonato et al. 1997a), we were unable to identify informative polymorphisms for *Smn* in our ABA cross, and thus could not map it relative to *Lgn1.* The combined locus order and inter-locus distances (in cM ± SE) for the *Lgn1* gene region deduced from our analysis is *D13Mit128*-(1.58 ± 0.25)-*D13Mit194*-(0.08 ± 0.06)-*D13Mit147*-(0.95 ± 0.19)-*D13Gor4*-(0.08 ± 0.06)-*D13Mit36*-(0.87 ± 0.18)-*D13Mit146/D13Die6*-(0.16 ± 0.08)-*Lgn1/D13Lsd1*(*Naip*)/*D13Mit37*-(0.16 ± 0.08)-*D13Die7/D13Die3*-(1.19 ± 0.21)-*D13Mit70.* Therefore, the analysis of 1270 animals segregating *Lgn1* in this study has enabled us to narrow the genetic interval for

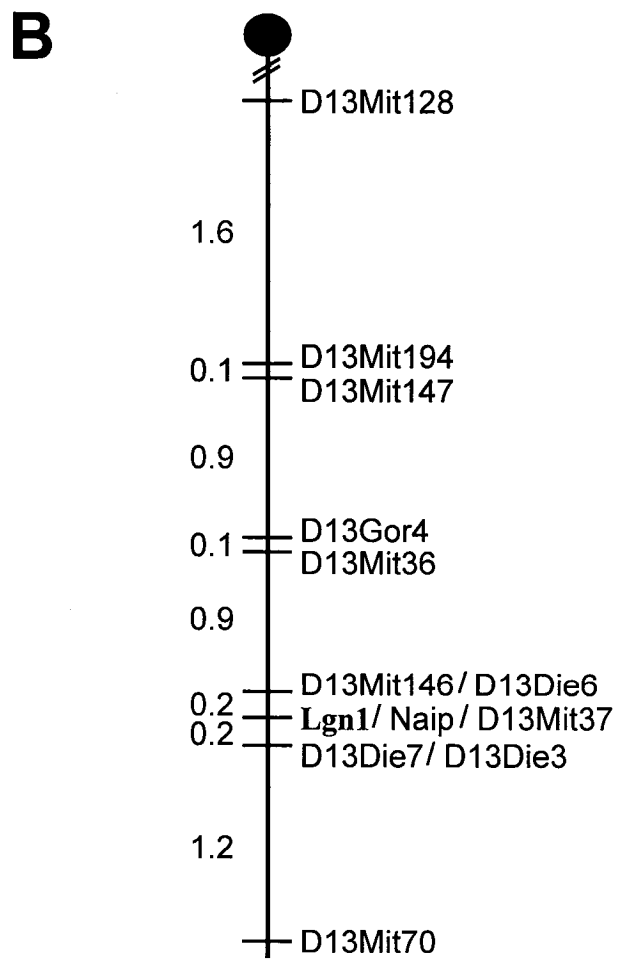
FIGURE 2.1

Genetic Mapping of the *Lgn1* Locus.

(A) Haplotype analysis of (C57BL/6J × A/J) F_1 × A/J (ABA) backcross progeny for Chr 13 markers. Each column represents a specific chromosomal haplotype detected in the ABA backcross. Solid boxes represent C57BL/6J alleles, while open boxes represent A/J alleles. The number of animals possessing a specific haplotype is indicated at the bottom of each column. Experimental conditions for detecting informative polymorphisms at the listed markers have been previously described (Beckers et al. 1995; Scharf et al. 1996; Beckers et al. 1997) or are described in the text for *D13Gor4.* The *Naip* gene was mapped by using the previously described *D13Lsd1* informative DNA marker (DiDonato et al. 1997b). Marker order for the interval was deduced by minimizing the number of crossovers, as previously described (Green 1981; Beckers et al. 1995). The pairwise inter-locus distances (in cM) shown to the right were calculated from the recombination fraction measured between the two loci.

(B) Genetic linkage map of mouse Chr 13 near the *Lgn1* locus. The genetic map was deduced from the haplotype analysis in **A** and shows locus order and inter-locus distances in cM. The centromere is identified by a solid dot on top.


A
cM
D13Mit128
D13Mit194
D13Mit147
D13Gor4
D13Mit36
D13Mit146
D13Die6
Naip
Lgn-1
D13Mit37
D13Die7
D13Die3
D13Mit70
1.57
0.08
0.94
0.08
0.87
0.00
0.16
0.00
0.00
0.16
0.00
1.18
606 600 12 8 1 7 5 1 6 5 2 2 6 9


B
D13Mit128
1.6
0.1
D13Mit194
D13Mit147
0.9
0.1
D13Gor4
D13Mit36
0.9
D13Mit146/ D13Die6
0.2
Lgn1/ Naip/ D13Mit37
0.2
D13Die7/ D13Die3
1.2
D13Mit70

Lgn1 to less than 0.4 cM (95% confidence interval), down from the 1.0 cM proposed by us (Beckers et al. 1997) as well as by Scharf and colleagues (Scharf et al. 1996).

Unfortunately, this relatively small genetic interval contains a genomic segment of great complexity, with the nonrecombinant marker *Naip* present at as many as six almost identical copies (exon 5; (Scharf et al. 1996). Similarly, anonymous markers tightly linked to the *Lgn1* gene, *D13Mit37* and *D13Die7,* are present at several tightly linked copies as detected by SSCP (Scharf et al. 1996) and by SSLP analyses ((Scharf et al. 1996); Diez et al. data not shown). This situation makes it very difficult to distinguish individual A/J and C57BL/6J alleles for a specific copy of the marker from independent but tightly linked copies. Consequently, we were unable to further map the four recombination events that define the proximal and distal boundaries of the *Lgn1* locus. As previously proposed by Scharf and associates (1996), we used the single-copy *D13Die6* and *D13Die3* markers to define the proximal and distal boundaries of *Lgn1,* respectively (Fig. 2.1).

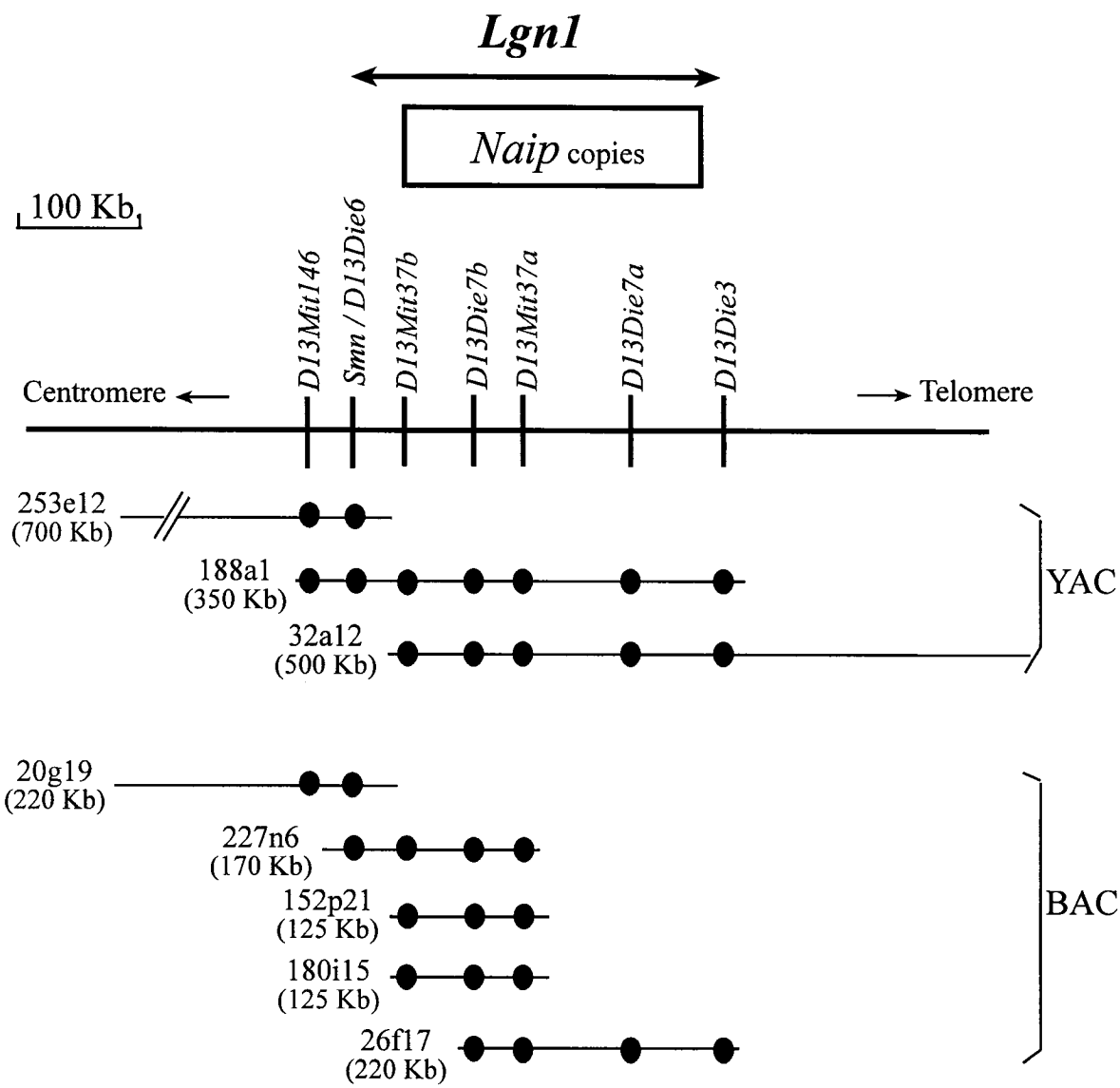
2.3.2 Physical Mapping

Physical mapping of genomic clones overlapping the minimal genetic interval for *Lgn1* may help elucidate the marker order and overall organization of the repeat unit, including the size of the minimal physical interval for *Lgn1.* For this, markers *D13Mit146, D13Mit37,* and *Smn* were used as entry points in commercially available genomic libraries constructed in YAC (Research Genetics, Huntsville, Ala.) or BAC vectors (Genome Systems Inc, St-Louis, Mo.), resulting in the identification of three overlapping YACs and several BAC clones covering the entire *Lgn1* region. Figure 2.2 shows the arrangement of a representative subset of clones overlapping the region. Individual clones were further analyzed for the presence of additional markers from the region (*D13Mit146, D13Mit37, D13Die3, D13Die6, D13Die7, Naip/D13Lsd1,* and *Smn*)

FIGURE 2.2

Physical mapping of the *Lgn1* region on mouse Chr 13.

Primer pairs corresponding to anonymous markers *D13Mit146* and *D13Mit37,* and to the *Smn* gene (exons 2b and 3; (DiDonato et al. 1997a) were used as entry points in genomic YAC and BAC libraries. The clones were sized by gel electrophoresis, and the presence of various genetic markers from the region in these clones was established by PCR-based typing (STS content). This information, together with the genetic map of the region, was used to organize the contig of genomic clones and to define a marker order within the repeated unit. Individual BAC and YAC clones are identified and are drawn to scale. Filled dots indicate presence of the markers in the clones. We could not segregate in this analysis *Smn* from *D13Die6, D13Die7b* from *D13Mit37a,* or *D13Die7a* from *D13Die3,* which segregated together in all clones tested; the order presented for these markers is based on that proposed by Scharf and coworkers (1996). The location of the multiple copies of the *Naip* genes is shown, together with the boundaries of the *Lgn1* interval.


Lgn1
Naip copies
100 Kb
D13Mit146
Smn / D13Die6
D13Mit37b
D13Die7b
D13Mit37a
D13Die7a
D13Die3
Centromere
Telomere
253e12
(700 Kb)
188a1
(350 Kb)
32a12
(500 Kb)
YAC
20g19
(220 Kb)
227n6
(170 Kb)
152p21
(125 Kb)
180i15
(125 Kb)
26f17
(220 Kb)
BAC

by PCR amplification and gel electrophoresis (STS content). In addition, two allele systems could be defined by SSLP for markers *D13Mit37* (a, b) and *D13Die7* (a, b), and were independently analyzed in our clones. Analysis of the STS content of overlapping BAC and YAC clones generated the following marker order for the region: *D13Mit146-Smn/D13Die6-D13Mit37b-D13Die7b/D13Mit37a-D13Die7a/D13Die3* (Fig. 2.2). Although none of our clones allowed segregation of the *Smn/D13Die6, D13Die7b/D13Mit37a,* and the *D13Die7a/D13Die3* pairs, the marker order obtained by this analysis is in good agreement with that deduced from the genetic map (Fig. 2.1) and from independent studies by others (Scharf et al. 1996). Finally, determination of the insert sizes of the BAC and YAC clones suggested minimal and maximal sizes for the physical interval of *Lgn1.* Indeed, BAC clones 152p21 and 180i15 are approximately 125 kb in length each and are entirely comprised within the proximal (*D13Die6*) and the distal (*D13Die3*) boundaries of the region (minimal interval), while YAC clone 188a1 (350-kb insert) spans the entire region and contains both markers defining the proximal and distal boundaries of the locus (maximal interval). Therefore, this set of genomic clones suggests a *Lgn1* interval between 125 and 350 kb in size. This estimate is tentative as it is based solely on the size and STS content of genomic clones from the region, and not on the physical map of the corresponding genomic DNA. Although our coverage of this segment is between three- and six-fold, we cannot exclude the presence of internal deletions or illegitimate duplications within our clones possibly affecting the organization of the interval. Finally, the BAC clones identified for this region are derived from 129/SvJ genomic DNA, while the YAC clones are derived from a C57BL/6J library. We cannot eliminate the possibility that the organization of the *Lgn1* region, in particular the *Naip* repeated unit, may be different in the two strains. Additional physical mapping experiments based on genomic DNA will be required to validate the physical map.

2.4 Discussion

The presence of a complex repeated unit within the nonrecombinant genetic interval of *Lgn1* renders difficult the high-resolution linkage mapping of the gene. In particular, the presence of multiple copies of polymorphic dinucleotide repeat markers used to narrow the interval such as *D13Die7* and *D13Mit37* is particularly confounding, as it is difficult to assign a particular PCR amplification product to a specific allele (strain specific) of a particular copy of the marker. Therefore, one must rely on markers located outside this repeated unit together with a large number of informative meioses to get an accurate estimate of the genetic interval defining *Lgn1*. By analyzing a total of 1270 informative backcross mice with newly derived "single copy" markers *D13Die6* and *D13Die3,* we have been able to reduce the genetic interval for *Lgn1* from 1 cM (Beckers et al. 1995; Scharf et al. 1996) to less than 0.4 cM, a distance clearly amenable to positional cloning. The presence of multiple copies of structural genes and polymorphic markers also complicates the physical mapping of the region by standard methods such as PFGE and Southern blotting. In particular, the cross-hybridization of *Naip* cDNA probes and other cloned probes from the region to multiple clones and to non-overlapping restriction fragments of the same clone is a difficult problem. The high degree of sequence similarity observed among *Naip* gene copies (exon 5; Scharf et al. 1996) and the relatively small amount of nucleotide sequence available for these genes have so far rendered difficult the discrimination between individual gene copies. Therefore, one has to rely on both the genetic map and the STS content mapping of multiple overlapping clones to derive a marker order and repeat structure for the region. The accuracy of the map will depend in part on the depth of the BAC/YAC contig, and our study presents a new set of clones overlapping the *Lgn1* locus independent from that obtained by Scharf and colleagues (1996). Within the repeated unit, we have detected at least two copies of *D13Mit37;* this was based on restriction mapping and Southern blotting data of independent clones overlapping this region, with a repeat-free 103-bp probe derived from *D13Mit37* but mapping outside the $(CA)_n$ repeat (oligonucleotides used: 5'-AGCAGAAGTGGTGGTCTTAC-3' and 5'-GGAAGGAGACAGTTCTCCATA-

3'). Similar hybridization experiments using a cloned *Naip* cDNA (kind gift of R. Korneluk and A. McKenzie) as a hybridization probe on digested YAC clones 32a12 and 188a1 have suggested up to three independent *Naip*-hybridizing fragments in each clone (data not shown), reinforcing evidence for *Naip* being present as multiple copies within the C57BL/6J mouse *Lgn1* genomic interval.

Lgn1 maps within the intensely studied syntenic human Spinal Muscular Atrophy (SMA) chromosomal region, in which several transcription units and ORFs have been identified (Lefebvre et al. 1995; Pizzuti et al. 1995; Roy et al. 1995a; Roy et al. 1995b; van der Steege et al. 1995). Such transcription units may be considered candidates for *Lgn1*. These include multiple ORFs with high sequence similarity to the beta-glucuronidase gene (Pizzuti et al. 1995; Roy et al. 1995a; van der Steege et al. 1995) as well as to the Br-Cadherin gene (Selig et al. 1995), a cofilin pseudogene, the p44 subunit of the transcription factor BTF2 (BTF2p44) and another ORF showing sequence similarity to an integral membrane glycoprotein (RNINMEGLA) (van der Steege et al. 1995). Additional transcripts expressed in liver (pL7), during neurogenesis (pGA1; Roy et al. 1995a), and in adult spinal cord (OL-1, CB-1, and FS-1) have been identified in this region (Pizzuti et al. 1995). *Smn* (the SMA-determining gene; (Bussaglia et al. 1995; Lefebvre et al. 1995) and the *Naip* family (a putative SMA-modifying gene; Roy et al. 1995b) map directly within the minimal genetic and physical intervals of *Lgn1* (Figs. 2.1 and 2; Scharf et al., 1996), thereby making these genes attractive candidates for *Lgn1*. Indeed, it is well documented that many pathogenic bacteria and fungi modulate or take advantage of the natural apoptotic pathways of their host cells to establish a replicative niche. More specifically, it was recently observed that virulent but not avirulent strains of *L. pneumophila* can induce an apoptotic response in human HL-60 cells differentiated along the monocytic pathway (Muller et al. 1996). We are currently testing the intriguing possibility that the genetic advantage at the *Lgn1* locus may involve differential induction of apoptosis in macrophages, a process in which the *Smn* and/or *Naip* genes may be implicated.

2.5 Materials and methods

Molecular Probes. The *D13Mit36, D13Mit37, D13Mit70, D13Mit128, D13Mit146, D13Mit147* and *D13Mit194* microsatellite markers had been previously described (Dietrich et al. 1994) and mapped within our 1270-animal (A/J × C57BL/6J) × A/J backcross (Beckers et al. 1997). Oligonucleotide primer pairs defining these simple sequence length polymorphisms (SSLP) were purchased from Research Genetics (Huntsville, AL), and used as recommended by the supplier. Marker *D13Gor4* had been described before (Xu et al. 1996; Beckers et al. 1997) but the following new procedure enabled its mapping in the ABA cross: AvaII digestion of the PCR amplification product obtained from *Mus spretus* yielded a 435-bp fragment that was used as a probe to detect the following *Eco*RI RFLP (A/J, 8 kb; C57BL/6J, 3.2 kb). Other genetic markers used in this study included the newly derived *D13Die3, -6* and *-7* dinucleotide-repeat markers (Scharf et al. 1996), and the *D13Lsd1* informative dinucleotide repeat located 5' of *Naip* exon 5 simply referred to as *Naip* in our genetic maps (DiDonato et al. 1997b). The mouse *Smn* cDNA clone used to hybridize BAC libraries had been described before (DiDonato et al. 1997a). A 2.3 Kb *Naip* cDNA clone used for hybridizations was a kind gift from Drs. A. MacKenzie and R. Korneluk.

Genomic DNA Preparation. Genomic DNA was prepared from tail tips and/or spleens of individual backcross mice for PCR-based typing of SSLPs by incubation (16h, 55°C) in 700 µl of a buffer (100 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) containing 0.5 mg/mL Proteinase K, followed by RNAseA treatment (0.3 mg/mL ; 2h at 37°C). DNA was purified by phenol/chloroform/isoamyl alcohol extraction(s), precipitated with isopropyl alcohol and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA). Working aliquots of all DNA samples were adjusted to a final concentration of 4 ng/µl in 10 mM Tris pH 8.0, 1 mM EDTA and they were stored at 4°C.

PCR Amplification. For PCR amplification of SSLPs from either genomic or cloned DNA, a 20 ng aliquot of DNA dissolved in PCR buffer (50mM KCl, 10mM Tris-

HCl, pH 9.0, 1.5mM $MgCl_2$ and 0.1% Triton X-100) was added to a final 10 µl volume in a buffer containing sequence-specific oligonucleotide primers (100 nmol), dNTPs (200 µM each), and 1 unit of *Thermophylus aquaticus* (Taq) DNA polymerase (BIOCAN, Montreal). One of the two oligonucleotide primers was labeled with [γ ^{32}P]-ATP using T4 polynucleotide kinase, and 20 nmol of the [^{32}P]-labeled primer was added to the PCR reaction. The thermocycling program was one initial denaturation at 94°C for 3 min, 30 three-step cycles at 94°C for 30s, 55°C for 30 s and 72°C for 30 s, followed by a final cycle at 72°C for 7min. [^{32}P]-labeled PCR products were diluted two-fold in 100 % formamide, denatured 5 min at 90°C and electrophoresed in denaturing 8 % polyacrylamide gels containing 8 M urea and TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8). For SSCP analysis, [^{32}P]-α-dATP or [γ^{32}P]-labeled primer was added to the PCR reaction and the [^{32}P]-labeled PCR products were electrophoresed on a 4.5% non-denaturing polyacrylamide gel containing TBE buffer with or without 5% glycerol. Electrophoresis of glycerol-free gels was carried out at 4˚C according to a protocol previously described (Orita et al. 1989). Gels were autoradiographed using Kodak XAR films for a period of 12-16 h.

Statistical Analysis. Genetic linkage was determined by segregation analysis. Gene order was deduced by minimizing the number of crossovers between the different loci within the linkage group (Green 1981). The mapping data were analyzed with Map Manager QT version b10 (Silver 1985; Neumann 1990).

Isolation and Processing of genomic BAC and YAC Clones. BAC clones were isolated by hybridization of probes to high-density library filters from a 129/Sv-derived BAC library (Genome Systems Inc, St-Louis, Mo.). The library was initially screened with mouse Naip cDNA (exon 5), and mouse Smn cDNA (exons 6-8) (DiDonato et al. 1997a). Radioactive labeling of probes used a standard random-primed method. Markers *D13Mit146* and *D13Mit37* were used as entry points in the PCR-based screening of DNA pools from a commercially available C57BL/6J-derived genomic library constructed in YAC vectors (Research Genetics, Huntsville, Ala.). Individual BAC and YAC clones

were further analyzed for the presence of additional markers from the region (*D13Mit146, D13Mit37, D13Die3, D13Die6, D13Die7, Naip/D13Lsd1,* as well as a primer set that amplified *Smn* exon 2b (DiDonato et al. 1997a)) by PCR amplification and gel electrophoresis (STS content). Two allele systems could be defined by SSLP for markers *D13Mit37* (a, b) and *D13Die7* (a, b), and were independently analyzed in our clones.

BAC DNA was prepared by standard alkaline lysis methods from 5 ml of overnight culture and resuspended in 40 µl of TE buffer. Miniprep DNA (5 µl) was digested immediately in a total volume of 20 µl with 5 units of *Not*I enzyme (New England Biolabs, Inc., Beverly, MA) for 2 hr at 37°C. Samples were loaded on a 1% agarose gel in 0.5% Tris-borate-EDTA (TBE) and subjected to PFGE (Bio-Rad CHEF DR II) for 22 hr at 6 V/cm, 15°C with a switching interval of 15 sec. BAC insert sizes were assigned from ethidium bromide-stained gels.

YAC DNA was prepared from 50 ml liquid yeast cultures grown to an OD600 of 1.0, pelleted and resuspended in an equal volume (500 µl) of low-melt agarose. The agarose blocks were then zymolyase- and proteinase K-treated. Samples were loaded on a 0.8% agarose gel in 0.5% Tris-borate-EDTA (TBE) and subjected to PFGE (Bio-Rad CHEF DR II) for 22 hr at 6 V/cm, 15°C with a switching interval of 100 sec. The yeast chromosomes were then blotted onto nylon membranes and YAC insert sizes were assigned by hybridization with YAC vector probes (derived from PBR-322 vector).

Southern Hybridization. DNA probes were gel-purified and labeled to high specific activity (1 X 10^9 cpm/µg DNA) with [^{32}P]α-dATP by random priming. Either high molecular weight genomic or cloned DNA was digested to completion with a ten-fold excess (10U/µg) of restriction endonucleases under conditions recommended by the supplier (Pharmacia, Montreal, Canada). Five µg of restricted genomic DNA was electrophoresed in 0.8 % agarose gels containing TAE buffer (40mM Tris-acetate, 20mM sodium acetate, 20mM EDTA, pH 7.6) and transferred by capillary blotting onto nylon membranes (Hybond N, Amersham) in 10 × SSC (1 × SSC is 0.15M sodium chloride, 0.15M sodium citrate). Southern blots were prehybridized for 16h at 42°C and then hybridized with [^{32}P]-radiolabeled DNA probes (1 × 10^6 cpm/ml) for 16h at 42°C in the

same mixture composed of 50% formamide, 5 × SSC, 1 % SDS, 10 % dextran sulfate, 20mM Tris-HCl, pH 7.5, 1 × Denhardt's solution (100 × is 2% bovine serum albumin, 2% Ficoll, 2% polyvinylpyrrolidone) and 200 μg/ml heat-denatured salmon sperm DNA. The membranes were washed to a final stringency of 0.1 × SSC, 0.5% SDS at 65°C for 45 min. Autoradiography was to Kodak XAR film for 1 to 4 days at -80°C with an intensifying screen.

2.6 Acknowledgements

The authors are indebted to Drs. A. MacKenzie and R. Korneluk (Children's Hospital of Eastern Ontario, Ottawa, Canada) for the kind gift of Naip cDNA. This work was supported by grants to P. Gros from the Network of Centers of Excellence (Canadian Genetic Diseases Network). P. Gros is supported by a Senior Scientist Award from the Medical Research Council of Canada and is an International Research Scholar of the Howard Hughes Medical Institute.

Chapter 3

The neuronal apoptosis inhibitory protein (Naip) is expressed in macrophages and is modulated after phagocytosis and during intracellular infection with *Legionella pneumophila*

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Diez, E., Yaraghi, Z., MacKenzie, A., and Gros, P. (2000). The neuronal apoptosis inhibitory protein (Naip) is expressed in macrophages and is modulated after phagocytosis and during intracellular infection with *Legionella pneumophila*. J Immunol 164, 1470-1477, © The American Association of Immunologists.

3.1 Abstract

Legionella pneumophila is an intracellular pathogen that causes Legionnaires' disease in humans. Inbred mouse strains are uniformly resistant to *L. pneumophila* infection with the notable exception of A/J, where the chromosome 13 locus *Lgn1* renders A/J macrophages permissive to *L. pneumophila* replication. The mouse *Lgn1* region is syntenic with the spinal muscular atrophy (SMA) locus on human chromosome 5 and includes several copies of the neuronal apoptosis inhibitory protein (*Naip*) gene. We have analyzed a possible link among *Lgn1*, *Naip,* and macrophage function. RNA expression studies show that *Naip* (mostly copy 2) mRNA transcripts are expressed in macrophage-rich tissues, such as spleen, lung, and liver and are abundant in primary macrophages. Immunoblotting and immunoprecipitation analyses identify Naip protein expression in mouse macrophages and in macrophage cell lines RAW 264.7 and J774A. Interestingly, macrophages from permissive A/J mice express significantly less Naip protein than their nonpermissive C57BL/6J counterpart. Naip protein expression is increased after phagocytic events. Naip protein levels during infection with either virulent or avirulent strains of *L. pneumophila* increase during the first 6 h postinfection and remain elevated during the 48-h observation period. This enhanced expression is also observed in macrophages infected with *Salmonella typhimurium.* Likewise, an increase in Naip protein levels in macrophages is observed 24 h after phagocytosis of Latex beads. The cosegregation of *Lgn1* and *Naip* together with the detected Naip protein expression in host macrophages as well as its modulation after phagocytic events and during intracellular infection make it an attractive candidate for the *Lgn1* locus.

3.2 Introduction

Legionella pneumophila is a facultative intracellular parasite that in humans can cause an acute form of pneumonia called Legionnaires' disease (McDade et al. 1977). *L. pneumophila* enters macrophages through a unique coiling phagocytosis mechanism (Horwitz 1984) and replicates within maturation-defective phagosomes (Horwitz and Silverstein 1980), which do not fuse to endosomes or lysosomes (Horwitz 1987). These replicative phagosomes are morphologically distinct and are associated with endoplasmic reticulum membranes and dotted with ribosomes (Swanson and Isberg 1995). Although certain *Legionella* proteins (dot/icm) have recently been shown to play an important role in the inhibition of phagosome-lysosome fusion (Segal and Shuman 1997; Segal et al. 1998; Vogel et al. 1998), the molecular mechanisms underlying successful intracellular survival and replication of *L. pneumophila*, in particular the host proteins targeted for inhibition, remain largely unknown.

In contrast to their human and guinea pig counterparts, mouse macrophages are not permissive to *L. pneumophila* replication even though the bacteria still rapidly inhibit phagosome-lysosome fusion soon after phagocytosis (reviewed in Yamamoto et al. 1994b). The A/J strain is an exception, however, as A/J inflammatory peritoneal macrophages are highly permissive to *L. pneumophila* replication *in vitro*, resulting in a 1000-fold increase in viable bacteria during a 72-h infection, compared with macrophages from non-permissive mouse strains such as C57BL/6J, C3H, and DBA/2J (Yamamoto et al. 1988; Yoshida et al. 1991b). The permissiveness of A/J macrophages to *L. pneumophila* replication provides a unique experimental system to study the parallel human disease (Brieland et al. 1994; Yamamoto et al. 1994b). Linkage studies have indicated that a single autosomal, recessive gene, designated *Lgn1* (Yoshida et al. 1991b), determines macrophage permissiveness to intracellular replication of *L. pneumophila*. *Lgn1* maps to the distal mouse chromosome 13 (Beckers et al. 1995; Dietrich et al. 1995; Scharf et al. 1996; Beckers et al. 1997; Endrizzi et al. 1999), within a genetic interval of 0.32 centiMorgan (95% confidence interval), defined distally by the genetic marker *D13Die3* and proximally by *D13Die6/D13Die26*. Physical mapping

studies and assembly of a cloned contig of BAC and YAC (bacterial and yeast artificial chromosome) clones for the region suggest a minimal physical interval for *Lgn1* of 350 kb (Dietrich et al. 1995; Scharf et al. 1996; Diez et al. 1997; Endrizzi et al. 1999).

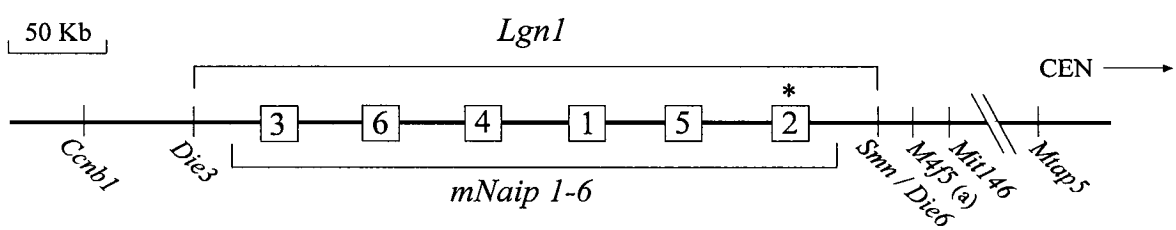
The murine chromosome 13 *Lgn1* region is syntenic with the spinal muscular atrophy (SMA) locus on human chromosome 5, which includes the survival motor neuron SMN gene and the neuronal apoptosis inhibitory protein NAIP gene (Lefebvre et al. 1995; Roy et al. 1995a). There is one functional copy of NAIP in the human genome and approximately two-thirds of type I SMA cases are associated with its homozygous deletion (Velasco et al. 1996). Although it was later shown that the closely linked SMN gene is the SMA-determining gene (Lefebvre et al. 1997), NAIP remains a strong candidate as a phenotypic modifier of SMN mutations. The NAIP protein has been shown to inhibit apoptosis of neurons and other cell types both *in vitro* and *in vivo* (Liston et al. 1996; Xu et al. 1997a). In addition, NAIP has been shown to inhibit the proapoptotic cysteine proteases known as caspases; in particular, caspases 3 and 7 have been shown to interact with NAIP (A. MacKenzie, unpublished observations). The mouse *Lgn1* locus includes the *Smn* gene as well as six copies of the *Naip* gene (Fig. 3.1). Analysis of mouse brain RNA and other tissues has revealed that at least three of the *Naip* copies (*Naip1*, *Naip2*, and *Naip3*) encode full-length mRNA and possibly functional proteins (Yaraghi et al. 1998; Huang et al. 1999). The tissue- and cell-specific expression of these *Naip* mRNAs and proteins remain largely unknown.

It has recently been observed that live *L. pneumophila* induce apoptosis in human macrophages *in vitro*, whereas heat-killed bacteria or avirulent mutants do not (Muller et al. 1996; Gao and Abu Kwaik 1999a; Gao and Abu Kwaik 1999b). Of particular interest, *in vitro* and *in vivo* studies have shown that *L. pneumophila*-induced apoptosis is mediated by activation of caspase 3 (Gao and Abu Kwaik 1999a). These studies have suggested that induction of apoptosis may be an important pathogenicity determinant of *L. pneumophila* for intracellular survival in host macrophages. These observations together with the genetic and physical colocalization of *Naip* genes within the minimal interval of *Lgn1* and the demonstrated role of Naip proteins in inhibition of apoptosis in neuronal tissues have prompted us to analyze a possible link among *Lgn1*, Naip protein, and macrophage function.

FIGURE 3.1

Schematic representation of the *Lgn1* region on distal mouse chromosome 13.

Genetic mapping has defined a 0.32-centiMorgan minimal interval for *Lgn1* delineated proximally by *D13Die6* (*Die6*) and distally by *D13Die3* (*Die3*; 2/1270 recombinations each). Physical mapping has suggested that the size of the *Lgn1* locus is between 125–350 kb and contains six copies of the *Naip* gene (*mNaip*, shown as squares). Copy 2 is the most closely linked to *Smn*, followed distally by copy 5 (Yaraghi et al. 1998). Although a preliminary order has been proposed all of the *Naip* copies by Scharf et al. in 1996, the order of the remaining four *Naip* copies has not been established with certainty. *Smn* and *M4f5* have recently been segregated from *Lgn1* (Endrizzi et al. 1999). The asterisk identifies the *Naip2* copy most abundantly expressed in macrophages. The data shown were obtained from maps in the reports by (Scharf et al. 1996), (Diez et al. 1997), (Yaraghi et al. 1998), and (Endrizzi et al. 1999).


50 Kb
Lgn1
CEN
*
3
6
4
1
5
2
Ccnb1
Die3
mNaip 1-6
Smn / Die6
M4f5 (a)
Mit146
Mtap5

3.3 Results

3.3.1 Naip mRNA expression in macrophages

The *Lgn1* gene region on mouse chromosome 13 contains a minimum of six closely homologous copies of the *Naip* gene (designated copies 1–6; Fig. 3.1) (Scharf et al. 1996; Yaraghi et al. 1998).To date, full-length cDNA sequences have been reported for copies 1, 2, and 3 (GenBank accession nos. AF007769, AF102871, and AF135492), while partial sequences of single exons have been reported for additional *Naip* copies (Scharf et al. 1996; Yaraghi et al. 1998; Endrizzi et al. 1999; Huang et al. 1999). Recent hybridization studies and sequencing of genomic clones suggest that only three of the six *Naip* gene copies (copies 1, 2, and 3) encode mRNAs that have the 5' sequences required for translation in normal tissues (Yaraghi et al. 1998).

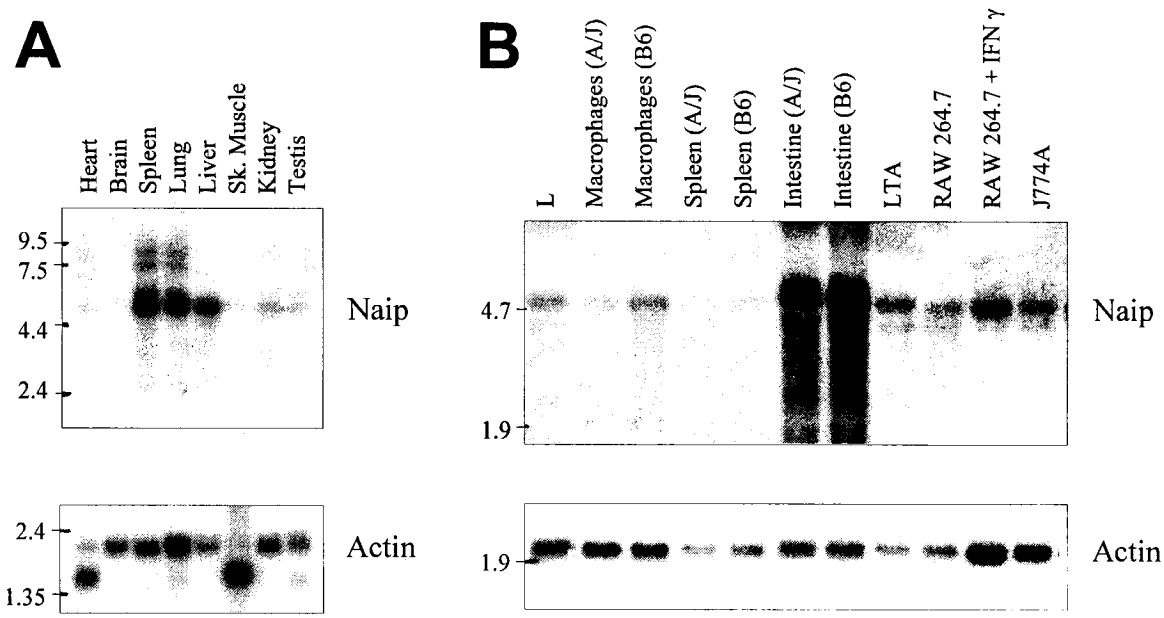
To examine a possible association between *Naip* and the *Lgn1* locus, we first investigated possible *Naip* mRNA expression in macrophages, the cell population known to phenotypically express the genetic difference at *Lgn1* (Diez et al. 1997). As isoform-specific *Naip* hybridization probes have not been described and as full-length sequence data are not yet available for the six *Naip* loci, hybridization probes that are expected to cross-react with most, if not all, *Naip* copies were used for this Northern blotting analysis (Fig. 3.2). The two hybridization probes used overlap the baculovirus inhibition of apoptosis protein repeat (BIR) domains, which are highly conserved in the sequenced *Naip copies* (93% identity) (Yaraghi et al. 1998). The *Naip* mRNA expression was most abundant in the intestinal tract (Fig. 3.2B). Hybridization of a Northern blot containing poly(A)+ mRNA identified readily detectable *Naip* expression in macrophage-rich tissues (spleen, lung, and liver), with lower expression in kidney and testis, while expression was below detection levels in brain, heart, and skeletal muscle. Using Northern blots containing total cellular RNA, *Naip* expression was easily detected in primary, thioglycolate- (TGC) elicited macrophages (Fig. 3.2B). The *Naip* mRNA was expressed in macrophage cell lines J774A and RAW264.7 and was also present in two mouse fibroblast cell lines (L and LTA; Fig. 3.2B). Treatment of RAW264.7 macrophages with

FIGURE 3.2

Northern blot analysis of *Naip* mRNA expression.

A, A blot containing polyadenylated RNA (2 μg/lane) from different mouse tissues was hybridized to a 32P-labeled *Naip* cDNA subfragment (*Naip1*; see Materials and Methods) under high stringency conditions (upper panel). The same blot was rehybridized to an actin cDNA probe (bottom panel). The hybridizing *Naip* (5 kb) and actin species (2 kb) are identified, and the positions of molecular size markers (in kilobases) is indicated to the left of the blot.

B, Total cellular RNA (10 μg/lane) from mouse fibroblast cell lines (L and LTA), from macrophage cell lines RAW 264.7 (treated or not with IFN-γ), and J774 and from spleen, intestine, and TGC-elicited peritoneal macrophages from mouse strains A/J and C57BL/6J (B6) were separated on a denaturing formaldehyde gel and transferred to a hybridization membrane. The blot was then probed with a *Naip2* cDNA probe overlapping exon 2 to exon 5 as described in Materials and Methods (top panel). The same blot was then rehybridized to a control actin cDNA probe (bottom panel). The position of molecular size markers (in kilobases) is indicated to the left of the blot.


A
Heart
Brain
Spleen
Lung
Liver
Sk. Muscle
Kidney
Testis
9.5
7.5
4.4
2.4
Naip
2.4
1.35
Actin
B
L
Macrophages (A/J)
Macrophages (B6)
Spleen (A/J)
Spleen (B6)
Intestine (A/J)
Intestine (B6)
LTA
RAW 264.7
RAW 264.7 + IFN γ
J774A
4.7
1.9
Naip
1.9
Actin

IFN-γ did not affect *Naip* mRNA expression levels. Taken together, these results indicate that *Naip* mRNA is expressed at readily detectable levels in macrophage-rich organs, in elicited (TGC) macrophages, and in two murine macrophage cell lines. In these cells, *Naip* is detected as a 5- to 5.5-kb hybridizing species, a size compatible with the known full-length sequence of *Naip* copies 1, 2, and 3 (Yaraghi et al. 1998; Huang et al. 1999; Yaraghi et al. 1999). Interestingly, we consistently noted on independent Northern blots a lower level of *Naip* RNA expression in peritoneal macrophages from A/J mice (Lgn-s) compared with B6 (Lgn-r) mice; this was by a factor of 2.5-fold (Fig. 3.2B).

RT-PCR was used to determine which of the *Naip* copy mRNAs are expressed in macrophages. The following strategy was applied. Macrophage RNA was transcribed into total cDNA using random hexamers and reverse transcriptase. Oligonucleotide primers corresponding to perfectly conserved sequences in *Naip* copies 1–6 flanking exon 2 and exon 4 were then used to amplify these portions of all *Naip* transcripts present. These products were eluted from gel as a single band and cloned, and the nucleotide sequences of 20 such clones were determined; previously published copy-specific single nucleotide polymorphisms (Scharf et al. 1996; Yaraghi et al. 1998) were then used to identify which *Naip* isoforms are expressed in macrophages. This analysis revealed that 10 of the clones sequenced corresponded to *Naip2* (50%), five to *Naip1* (25%), and two to *Naip3* (10%), with additional single clones corresponding to *Naip4*, *Naip5*, and *Naip6*. We also screened a macrophage cDNA library with a highly conserved *Naip* hybridization probe and characterized the positive clones by restriction mapping and partial nucleotide sequencing. Using this approach, we also noted that the majority of clones analyzed corresponded to *Naip2*. Together, these results suggest that although multiple *Naip* RNA isoforms are expressed by macrophages, *Naip2* appears to represent the majority of *Naip* transcripts produced in these cells. This is in keeping with the tissue expression results of Yaraghi et al. (1999) and the cDNA cloning experiments of Huang et al. (1999).

3.3.2 Naip protein expression in tissues and macrophages

The Naip protein expression was next analyzed in tissues and cell types positive for *Naip* mRNA expression. For this, we used a rabbit anti-Naip polyclonal antiserum (antiserum 1.7) directed against a fusion protein consisting of GST fused to a large central portion of the predicted Naip1 protein. The immunoblotting results in Fig. 3.3 show that this antiserum detects abundant Naip protein expression in intestinal extracts prepared from either the ileum or the colon (sites of known mRNA expression; Fig. 3.2). The Naip protein is also expressed in soluble tissue extracts from spleen and is enriched in similar extracts from mature macrophages (Fig. 3.3). The immunoreactive Naip species migrates at 150 kDa, a molecular mass compatible with that expected from the predicted amino acid sequence of *Naip* cDNAs (Yaraghi et al. 1999). The relative levels of Naip protein detected in Fig. 3.3 by immunoblotting are comparable to the levels of *Naip* mRNAs detected in the same tissues by Northern blotting (Fig. 3.2, A and B).

The relative expression of Naip protein was compared in peritoneal macrophages from susceptible A/J (Lgn-s) and resistant B6 (Lgn-r) mice (Figs. 3.4-3.6). Results from immunoblotting experiments showed that A/J macrophages express considerably less Naip protein than their B6 counterparts (Fig. 3.4B). This difference was not due to unequal loading of proteins on the gel, as very similar immunoreactive signals were obtained in these samples with an anti-actin antiserum (Fig. 3.4B, bottom panel). Independently, immunoprecipitation using metabolically labeled macrophage extracts also showed lower Naip protein expression in A/J compared with B6 macrophages (Fig. 3.4A). Thirdly, several 2-fold dilutions of A/J and B6 macrophage extracts (10, 20, and 40 μg) were separated by SDS-PAGE and analyzed by Western blotting (Fig. 3.5A), and the intensity of the immunoreactive band (indexed optical density, IOD) was quantitated using an Imaging station (BioImage). The IOD of the Naip band vs the IOD of actin band was calculated for each sample (Fig. 3.5B) and was used to calculate a relative Naip expression ratio in A/J and B6 populations. A 4-fold difference in Naip protein expression was observed for B6 vs A/J macrophages (six independent experiments; $p < 0.01$, by Student's t test). Finally, the reduced levels of Naip protein seen in A/J macrophages compared with B6 cells are in agreement with differences in Naip mRNA

FIGURE 3.3

Naip protein expression in mouse tissues.

Mouse intestinal segments corresponding to the ileum and colon as well as spleen were dissected and homogenized to isolate a total soluble protein fraction. Likewise, TGC-elicited mouse peritoneal macrophages were harvested and lysed, and a total soluble protein extract was prepared. Proteins (60 μg) were separated by SDS-PAGE on a 7.5% acrylamide gel and transferred to a nylon membrane. The immunoblot was incubated with a polyclonal rabbit anti-Naip antiserum (1.7; used at a 1/2000 dilution) and was revealed by a secondary goat anti-rabbit antiserum. The size of the major immunoreactive band detected (150 kDa) is in agreement with the predicted size of the Naip protein (GenBank accession nos. AF007769, AF102871, and AF135492). The positions of the molecular mass markers are indicated to the left of the blot.

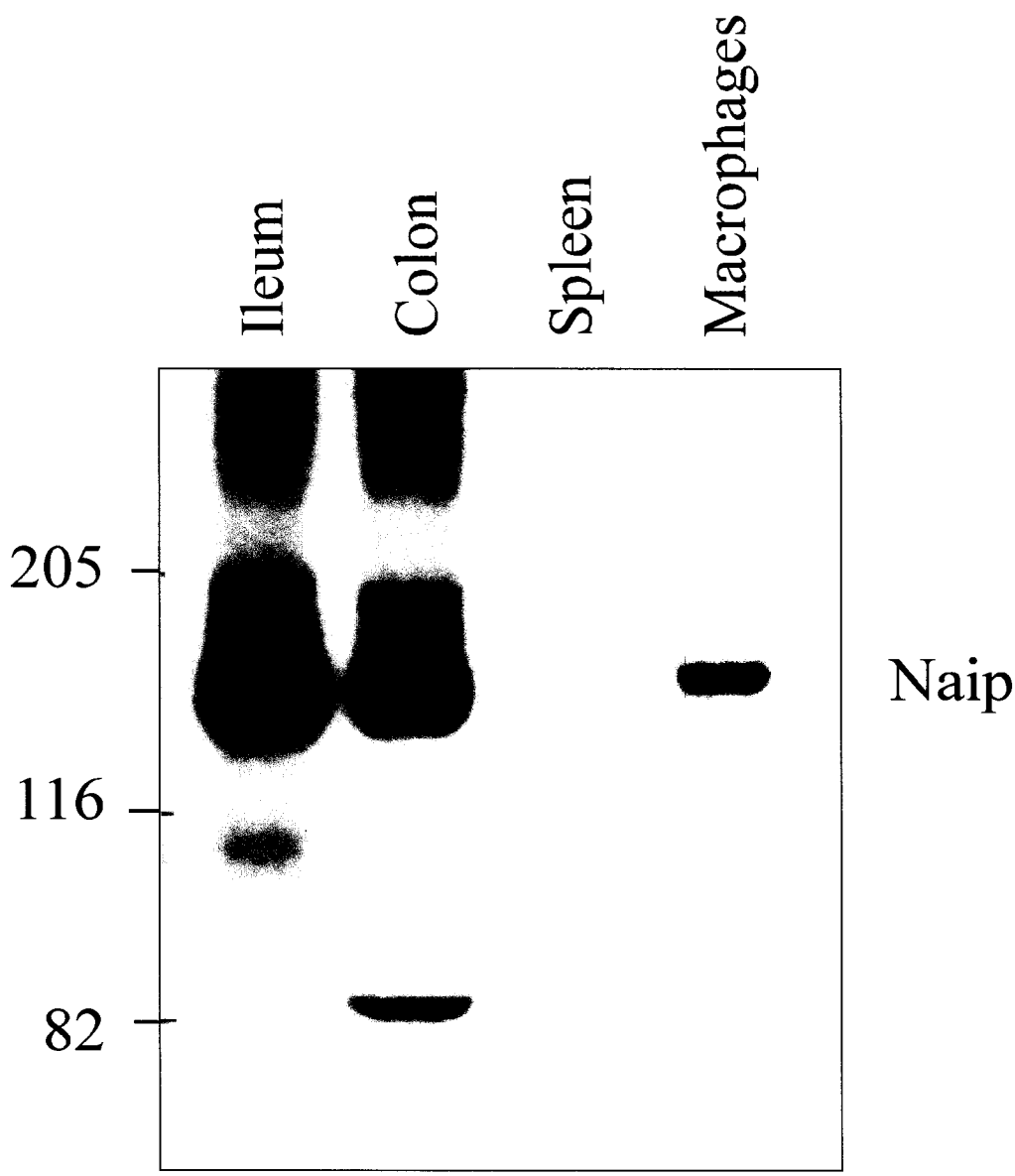

Ileum
Colon
Spleen
Macrophages
205
116
82
Naip

FIGURE 3.4

Naip protein expression in mouse macrophages.

A, Mouse TGC-elicited peritoneal macrophages from A/J and B6 strains were metabolically labeled with [35S]methionine for 16 h in methionine-free medium supplemented with 10% dialyzed FBS, and total cell lysates were prepared in detergent-containing immunoprecipitation buffer (see Materials and Methods). Lysates were precleared by incubation with preimmune rabbit serum, followed by incubation with anti-Naip polyclonal Ab 1.7 (used at a 1/200 dilution). Immune complexes were recovered by incubation with protein A/protein G-Sepharose beads and were separated by SDS-PAGE on a 7.5% acrylamide gel, followed by autoradiography. The immunoreactive Naip protein migrates as a single band of apparent molecular mass 150 kDa.
B, Total cell lysates from TGC-elicited peritoneal macrophages from A/J and B6 and from macrophage cell lines RAW 264.7 and J774A (60 μg/lane) were separated by SDS-PAGE and analyzed by immunoblotting as described in Fig. 3.3. The top panel shows the immunoblot probed with the anti-Naip Ab, and the bottom panel shows the same blot probed with an anti-actin Ab. The positions and sizes of protein molecular mass markers are indicated to the left of the blot.

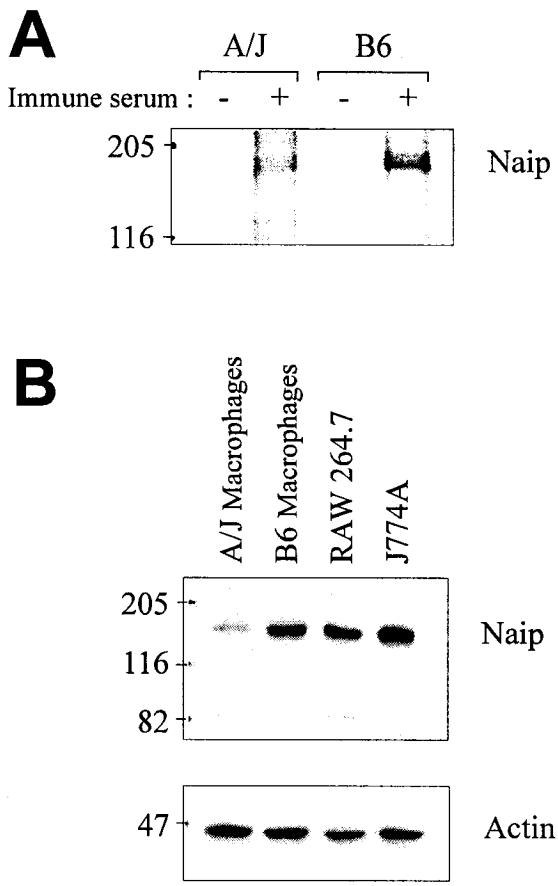
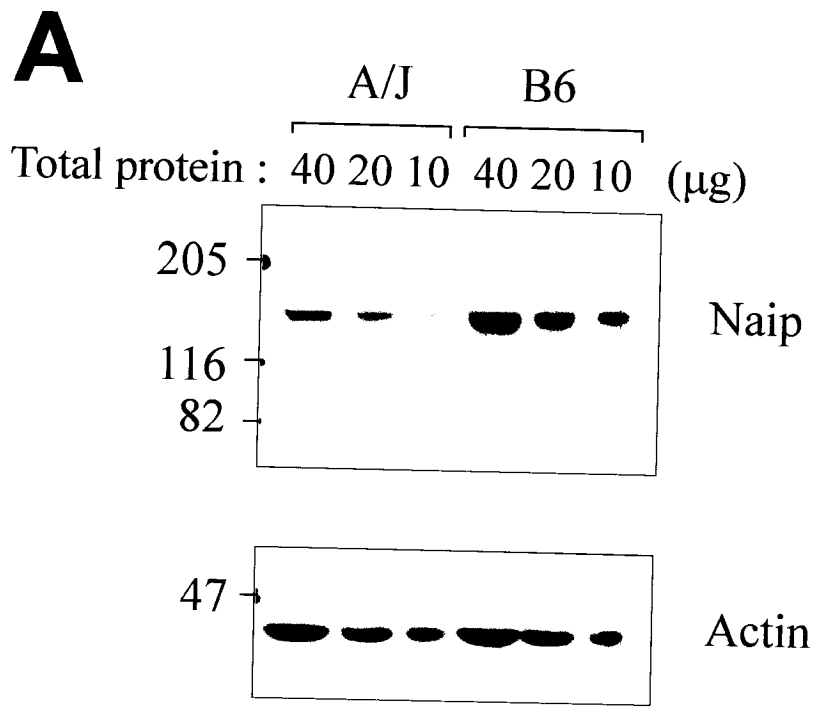

A
A/J
B6
Immune serum : - + - +
205
116
Naip
B
A/J Macrophages
B6 Macrophages
RAW 264.7
J774A
205
116
82
Naip
47
Actin

FIGURE 3.5

Comparison of Naip protein expression in A/J and B6 macrophages.

A, Immunoblotting of 2-fold serial dilutions of soluble protein extracts from A/J and B6 macrophages (10, 20, and 40 μg/lane), using either polyclonal anti-Naip ab (top panel) or anti-actin Ab (bottom panel). Conditions for immunoblotting were described in Fig. 3.3 and Materials and Methods.

B, The intensities of the immunoreactive Naip and actin signals were quantitated using a Bioimaging station. An IOD value was determined for each lane by calculating the ratios of the Naip to actin signals. Results from six independent experiments were pooled and used to calculate an average of the relative Naip expression value for B6 compared with A/J macrophages (set at 1). The mean and SE are shown for B6, indicating that Naip protein levels are significantly higher in B6 than in A/J macrophages (by Student's t test, $p < 0.01$).


A
A/J
B6
Total protein : 40 20 10 40 20 10 (μg)
205
116
82
Naip
47
Actin

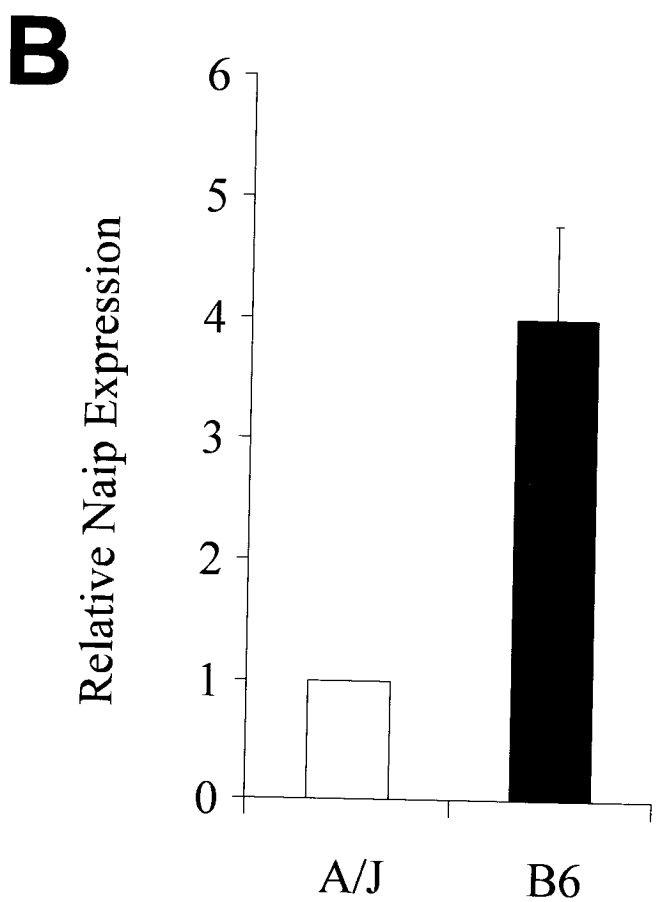
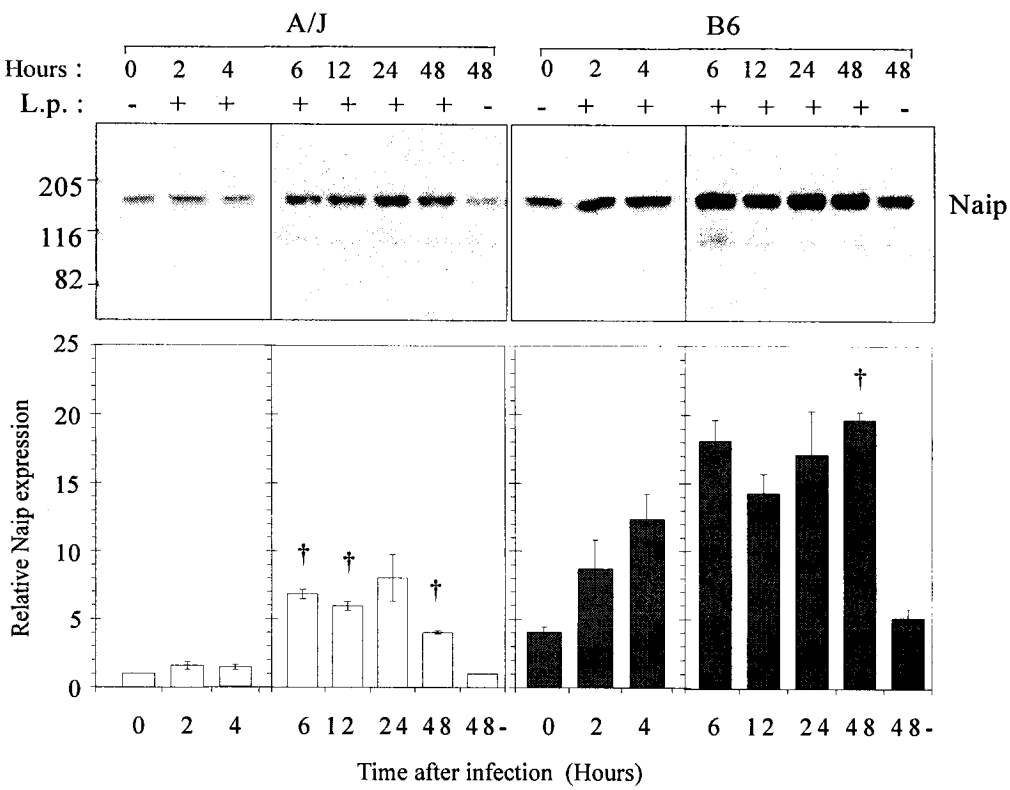

B
Relative Naip Expression
6
5
4
3
2
1
0
A/J
B6

FIGURE 3.6

Naip protein expression in A/J and B6 macrophages during infection with *L. pneumophila*.

Peritoneal macrophages from A/J and B6 mice were infected with *L. pneumophila* Philadelphia 1 with a multiplicity of infection (MOI) of two bacteria per cell (see Materials and Methods). At 2, 4, 6, 12, 24, and 48 h postinfection, protein extracts were prepared, separated by SDS-PAGE, and analyzed by immunoblotting for Naip protein expression (top panel). The relative Naip expression level was calculated as described in Fig. 3.5, using actin as an internal standard (bottom panel). The relative expression is further expressed as the increase above the Naip expression level (±SE) measured in A/J macrophages at the zero time point, before infection. These data represent four independent experiments. Statistically significant differences are indicated: †, $p < 0.001$ vs uninfected macrophages of the same strain.


A/J
B6
Hours : 0 2 4 6 12 24 48 48 0 2 4 6 12 24 48 48
L.p. : - + + + + + + - - + + + + + + -
205
116
82
Naip
Relative Naip expression
25
20
15
10
5
0
†
0 2 4 6 12 24 48 48- 0 2 4 6 12 24 48 48-
Time after infection (Hours)

expression levels detected for these two populations by Northern blotting (Fig. 3.2B).

Together, these results indicate that Naip is expressed at significant levels in mouse macrophages and macrophage cell lines. Interestingly, macrophages from susceptible A/J mice express less Naip protein than their resistant B6 counterpart.

3.3.3 Modulation of Naip protein expression in macrophages

The level of Naip protein expression was monitored in macrophages after phagocytic events and during infection with intracellular parasites. In the first experiment, macrophages from A/J and B6 mice were infected *in vitro* with a wild-type strain of *L. pneumophila* (MOI of 2) for 2 h at 37°C. Following extensive washing, cells were harvested at predetermined time points, and soluble protein extracts were prepared and analyzed for Naip protein expression by Western blotting. A representative experiment is shown in Fig. 3.6, but similar results were obtained in four independent experiments. During *L. pneumophila* infection, Naip protein expression was increased, with a progressive increase during the first 6–12 h, at which point it peaked and remained constant over the 48-h observation period. A maximum induction of 4.5- to 5-fold was seen in B6 macrophages, as quantitated by imaging and comparison to control immunoreactive signals obtained for actin. The induction was specifically due to *L. pneumophila* infection, because this increased Naip expression was not seen in control, noninfected cultures similarly incubated for 48 h. Finally, although the absolute level of Naip expression was lower in A/J than in B6 macrophages at all time points, we noted a comparable induction of Naip expression in A/J macrophages. These results indicate that Naip protein expression in macrophages is increased following *L. pneumophila* infection. To determine whether this modulation of Naip expression was an active process mediated by live, intracellular, and replicating *L. pneumophila* cells, similar experiments were performed with an avirulent dotA *L. pneumophila* mutant that does not inhibit phagosome maturation and thus does not replicate intracellularly (Sadosky et al. 1993). The results shown in Fig. 3.7A show that Naip protein expression was also up-regulated in B6 macrophages after infection with the avirulent dotA mutant by a factor of 4-fold.

These results suggest that increased Naip expression in macrophages is not in response to active intracellular replication of *L. pneumophila*.

Additional experiments were conducted to determine whether enhanced Naip expression during *L. pneumophila* infection was specific to this bacterium or was also seen with another unrelated intracellular parasite, *S. typhimurium*. As *S. typhimurium* causes a severe destructive infection in primary macrophages from B6 mice, it was not possible to assess the effect of wild-type S. typhimurium infection on protein expression at 24 or 48 h. Thus, for these experiments we used a temperature-sensitive, replication-defective mutant of *S. typhimurium* (TS27) that does not replicate in primary macrophages or macrophage cell lines at 37°C (Govoni et al. 1999). Twenty-four hours after infection of B6 macrophages with the TS27 mutant, Naip induction was readily detected in these cells, and after normalizing to actin expression level, this induction was 3-fold (Fig. 3.7A). Finally, we also tried to determine whether increased Naip expression detected during *S. typhimurium* and *L. pneumophila* infection was specific to intracellular bacteria or whether it may be part of a more general macrophage response to phagocytic events. Thus, B6 macrophages were fed a meal of inert Latex beads, and the level of Naip expression was monitored at 0 and 24 h postphagocytosis (Fig. 3.7B). We noted little if any Naip induction immediately following phagocytosis, while increased expression was detected after 24 h (Fig. 3.7B) by a factor of 3-fold.

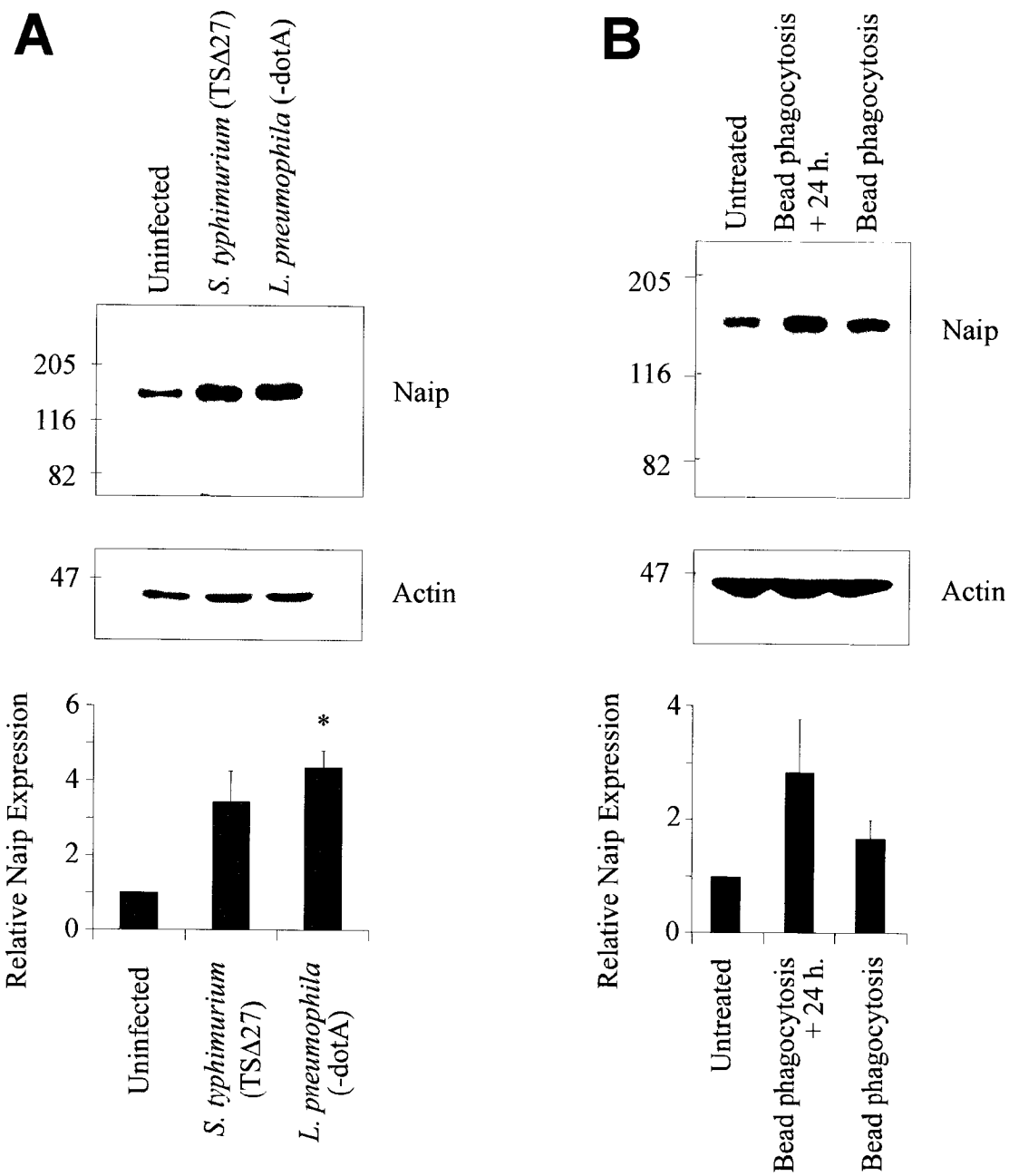
Together, these results indicate that Naip protein expression can be increased in macrophages in response to ingestion of live bacteria or inert particles.

FIGURE 3.7

Naip protein expression after infection with avirulent bacteria and after phagocytosis of inert particles.

A, Peritoneal macrophages from B6 mice were infected with either a temperature-sensitive replication defective mutant of *S. typhimurium* (TS27) or an avirulent dotA mutant of *L. pneumophila*, as described in Materials and Methods. Twenty-four hours after infection, cell extracts were prepared, separated by SDS-PAGE, and analyzed for Naip (top panel) and actin (middle panel) protein expression by immunoblotting. The relative Naip expression was quantitated as described in Fig. 3.5, using the actin signal as an internal standard, and this is shown in the bottom panel. The Naip expression levels are expressed as a mean compiled from two independent experiments ± SE. The asterisk denotes a statistically significant increase ($p < 0.001$) over levels in uninfected macrophages.

B, Peritoneal macrophages from B6 mice were fed a meal of Latex beads for 2 h at 37°C, and protein extracts were prepared at that point or after washing the cells and further incubation for 24 h. The results are presented as described in **A**.


A
Uninfected
S. typhimurium (TSΔ27)
L. pneumophila (-dotA)
205
116
82
Naip
47
Actin
Relative Naip Expression
6
4
2
0
*
Uninfected
S. typhimurium (TSΔ27)
L. pneumophila (-dotA)
B
Untreated
Bead phagocytosis + 24 h.
Bead phagocytosis
205
116
82
Naip
47
Actin
Relative Naip Expression
4
2
0
Untreated
Bead phagocytosis + 24 h.
Bead phagocytosis

3.4 Discussion

Apoptosis of phagocytes in response to intracellular infection has been described for a number of pathogens (Hilbi et al. 1997). Apoptosis of infected macrophages may be an advantageous strategy for a multicellular host, where this would have a net effect of limiting infection; on the other hand, from the parasite's perspective, host cell death may be required to release intracellular organisms. Thus, a number of intracellular pathogens have developed intracellular survival strategies that are based on activation (*Shigella flexneri*) or inhibition (*Chlamydia trachomatis*, *Rickettsia rickettsii*) of host macrophage apoptotic responses (Hilbi et al. 1997; Clifton et al. 1998). Successful intracellular survival and replication of *L. pneumophila* also appear to involve modulation of host macrophage apoptosis. *L. pneumophila* induces apoptosis during infection of permissive, HL-60-derived human macrophages (Muller et al. 1996), but also in the human macrophage line U937 and the alveolar epithelial cell line WI-26 (Gao and Abu Kwaik 1999b). *L. pneumophila*-induced apoptosis occurs within 1–2 h of infection, can take place in the absence of intracellular replication, and can also be induced by extracellular bacteria. Induction of apoptosis in *L. pneumophila*-infected macrophages is mediated by activation of the caspase pathway (Gao and Abu Kwaik 1999b) and does not require a functional TNF- pathway (Hagele et al. 1998). Induction of apoptosis by *L. pneumophila* is through the activation of caspase 3, which is detectable 2 h after infection and is maximal at 3 h (9-fold increase in activity) (Gao and Abu Kwaik 1999a). Avirulent *L. pneumophila* mutants cannot induce either apoptosis or caspase 3 activation. Specific inhibition of caspase 3 activity can block both *L. pneumophila*-induced apoptosis and cytopathogenicity (Gao and Abu Kwaik 1999a). Whether the nonpermissive nature of mouse macrophages (vs human cells) to *L. pneumophila* infection is linked to resistance of murine cells to *L. pneumophila*-induced apoptosis is currently not known. Likewise, it remains to be determined whether the differential response of susceptible A/J and resistant B6 macrophages to *L. pneumophila* infection involves different macrophage apoptotic responses in these two strains.

We have used a positional cloning approach to clone the *Lgn1* locus on mouse chromosome 13. Combined genetic and physical mapping studies by us (Beckers et al. 1997; Diez et al. 1997; Yaraghi et al. 1999) and others (Dietrich et al. 1995; Scharf et al. 1996; Endrizzi et al. 1999) have narrowed the interval for *Lgn1* to a chromosome segment that includes up to six copies of the *Naip* gene (Fig. 3.1). *Naip* is a very interesting candidate for *Lgn1* for the following reasons. 1) The *Naip* gene cluster does not recombine with *Lgn1* in the large number of meioses studied thus far; 2) infection and replication of *L. pneumophila* in permissive human cells are associated with activation of caspase 3 (Gao and Abu Kwaik 1999a) and induction of apoptosis (Muller et al. 1996; Gao and Abu Kwaik 1999b); 3) Naip protein expression prevents apoptosis in a number of cell types (Liston et al. 1996; Xu et al. 1997a); and 4) we have shown NAIP to be a potent inhibitor of apoptosis largely and possibly exclusively through the direct inhibition of caspase 3 with an IC_{50} in the range of 20 nM (A. Mackenzie et al., unpublished observations). Thus, we have studied the possible expression of *Naip* mRNA and protein in macrophages.

In the current study we have shown that *Naip* mRNA is indeed expressed in macrophage-rich tissues, in particular in primary macrophages derived from them as well as in macrophage cell lines. Screening of a macrophage cDNA library with a *Naip* cDNA probe suggests a frequency of 0.03% of total cellular mRNA (data not shown), suggesting that *Naip* mRNA is actually quite abundant in macrophages. Results from RT-PCR studies, nucleotide sequencing, and cDNA cloning from macrophages indicate that *Naip2* is the most abundantly expressed *Naip* copy (>50%) followed by *Naip1*. These results are in agreement with recent tissue expression studies of the mouse *Naip* isoforms in normal tissues and macrophages (Huang et al. 1999; Yaraghi et al. 1999). Using a polyclonal anti-Naip antiserum, we show that Naip protein is expressed in macrophages (Figs. 3.3–3.7), the cell population phenotypically expressing the genetic difference at *Lgn1*, strengthening the candidacy of *Naip* for *Lgn1*. In addition, we have observed that Naip protein expression in macrophages can be further up-regulated during a 48-h infection with *L. pneumophila* (Fig. 3.6) at low MOI. This induction of Naip expression does not require intracellular bacterial replication, because it still occurs when an avirulent *dotA L. pneumophila* mutant or an unrelated replication-defective *S. typhimurium* mutant is used

for infection (Fig. 3.7). Interestingly, this Naip protein induction is also seen 24 h after phagocytosis of inert Latex particles by macrophages. Should Naip also act as an inhibitor of apoptosis in macrophages, then this response may increase the lifespan of macrophages, possibly enhancing their net antimicrobial activity. Thus, the genetic mapping data, the known function of Naip, and the role proposed for apoptosis in *L. pneumophila* infection together with the expression of Naip protein detected in cells phenotypically expressing the genetic difference at *Lgn1* and the modulation of Naip protein expression observed in macrophages during phagocytosis of inert particles or live bacteria combine to make Naip an attractive candidate for *Lgn1*. In such a model, successful infection of macrophages by *L. pneumophila* is dependent on the induction of apoptosis. In mouse macrophages, constitutive or inducible Naip expression may play a protective role by preventing induction of apoptosis. This Naip-mediated inhibition of *L. pneumophila* replication would be lost in A/J cells by a loss-of-function mutation.

Southern blotting analyses of genomic DNA and RT-PCR analysis of *Naip* transcripts from A/J and B6 macrophages failed to detect a major genomic deletion of part of the *Naip* cluster in A/J mice that would result in the absence of expression of individual *Naip* copies. We did detect a small, but reproducible, 2- to 3-fold reduction in *Naip* mRNA levels in A/J compared with B6 macrophages. It is difficult to conclude with certainty that this difference is due to reduced expression of a specific Naip copy in A/J as opposed to lower transcription of the whole locus. The levels of constitutive and inducible Naip protein expression were also analyzed in A/J ($Lgn1^s$) and B6 ($Lgn1^r$) macrophages. It was consistently observed that both constitutive and inducible Naip protein expression levels were reduced by at least 4-fold in macrophages from A/J vs B6 mice (Figs. 3.5 and 3.6). The reduced protein expression in A/J macrophages may be a result of decreased protein expression of a single or multiple Naip isoforms in A/J macrophages compared with B6. This reduced Naip expression may result in enhanced ability of *L. pneumophila* to induce apoptosis (activation of caspase 3) and thus increased permissiveness to infection. Additional experiments are required to resolve this issue.

In conclusion, the expression of Naip protein in macrophages, both at rest and after phagocytosis, reported in this study suggests that Naip may play a key role in macrophage function, possibly by contributing to the apoptotic response of these cells.

The possible participation of *Naip* in this process opens a new window for understanding the regulation of the apoptotic response in macrophages. These results also make the *Naip* cluster an attractive candidate for the host resistance locus *Lgn1*. A formal demonstration of this point awaits the creation of mouse mutant strains bearing loss- or gain-of-function mutations at this locus.

3.5 Materials and methods

Animals. Inbred mouse strains A/J and C57BL/6J (B6) were initially purchased from The Jackson Laboratory (Bar Harbor, ME), and subsequently maintained as breeding colonies in our laboratories. Maintenance and experimental manipulation of the animals were performed according to the guidelines and regulations of the Canadian Council on Animal Care.

Isolation of thioglycolate(TGC)-elicited peritoneal macrophages. TGC-elicited inflammatory macrophages were obtained from the peritoneal cavity as previously described (Yamamoto et al. 1988). Macrophages were elicited by i.p. injection of 1 ml of sterile 3% thioglycolate broth, and peritoneal exudate cells were obtained 72 h later by washing the peritoneal cavity with 10 ml of RPMI 1640 medium supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies, Burlington, Canada). The TGC-elicited cells ($2–3 \times 10^7$ cells) were plated in 80-cm^2 flasks in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Life Technologies) and incubated for 16 h at 37°C, at which point nonadherent cells (mostly neutrophils) were removed by washing with PBS. Cells prepared in this way were used for RNA isolation, protein determination, and bacterial infections.

RNA expression. For Northern blotting experiments, a Clontech mouse multiple tissue poly$(A)^+$ RNA blot (2 μg/lane) was hybridized with a partial *Naip* cDNA (clone ms6) (Yaraghi et al. 1998) from which 3'-untranslated sequences had been removed. Clontech ExpressHyb solutions were used, and the hybridization conditions were as

recommended by the supplier. In other experiments total cellular RNA was extracted from normal mouse tissues and cultured cells using 6 M guanidium hydrochloride, and purified by sequential ethanol precipitations and phenol-chloroform extractions. Equal amounts of RNA (10 μg/lane) were separated on a 1% agarose gel containing 0.66 M formaldehyde in MOPS buffer (40 mM morpholinopropanesulfonic acid, 10 mM sodium acetate, and 10 mM EDTA, pH 7.2) and blotted by capillarity onto a nylon membrane (GeneScreen Plus, New England Nuclear) in 10×SSC (1×SSC is 0.15 M NaCl/0.015 M sodium citrate). Following transfer, the RNA was cross-linked to the blot by UV irradiation and by baking (2 h, 80°C). The blots were then prehybridized overnight at 65°C in 0.75 M NaCl, 1% SDS, 10% dextran sulfate, and denatured salmon sperm DNA (200 μg/ml). Hybridization was performed for 20 h at 65°C in the same hybridization solution without salmon sperm DNA. The probe used on total RNA blots was a *Naip* cDNA subfragment (1.1-kb *Eco*RI fragment encompassing exons 5–10 of *Naip2*, from cDNA clone 8; see below). Hybridization probes were labeled with [α^{32}P]dATP (sp. act., 3000 Ci/mmol; DuPont-NEN, Boston, MA) by the random priming method (Beckers et al. 1995). Blots were washed at a final stringency of 0.5× SSC/0.1% SDS at 65°C for 30 min followed by autoradiography (Kodak Biomax MS film, Eastman Kodak, Rochester, NY) at -80°C with an intensifying screen for 1–8 days. Blots were stripped of probe by incubation in 0.1× SSC/0.1% SDS (90°C, three times for 15 min each time) and rehybridized to an actin cDNA control probe following the same procedure.

For RT-PCR amplification of *Naip* cDNA sequences, cDNA synthesis and PCR amplification conditions were as previously described (Epstein et al. 1991). Reverse transcriptase-directed first-strand cDNA synthesis was conducted using 2 μg of total cellular RNA, 100 ng of random hexamers, and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). The hexamer/RNA mixture was first incubated for 5 min at 65°C, followed by addition of enzyme and further incubation at 37°C for 90 min. Exon 2 sequences from all *Naip* transcripts were PCR-amplified using primer pairs corresponding to sequences in exon 2 that are conserved in all *Naip* isoforms, according to (Scharf et al. 1996) (exon 2F, 5'-GCTCTAGATCATGGACGCCACAGGAGATG-3'; exon 2R, 5'-CCGCTCGAGATGTCCCATGGGCATAAAATGGC-3'). Exon 4 sequences from all *Naip* transcripts were PCR-amplified using primer pairs corresponding to

sequences in exons 3 and 5 that are conserved in all *Naip* isoforms (exon 3, 5'-GCTCTAGAGTAAAAGGGACACTGTGCAG-3' and a reverse primer on exon 5 5'-CCGCTCGAGTAATTCTCTTCTGACCCAGG-3'). Amplification products were gel-purified and subcloned in plasmid vector pBluescript, using restriction enzyme sites included in the oligonucleotide primers (underlined). The nucleotide sequence of 20 independent clones from each PCR amplification was determined and used to identify the *Naip* transcripts expressed in macrophages, using diagnostic sequence polymorphisms in exons 2 and 4 unique to each *Naip* copy and reported by (Scharf et al. 1996) and (Yaraghi et al. 1998).

The presence and identity of *Naip* mRNA transcripts expressed in macrophages were also investigated by screening a cDNA library. For this, a mouse macrophage cDNA library in bacteriophage vector gt11 (oligo(dT)-primed; Clontech, Palo Alto, CA) was screened with a *Naip* cDNA (ms6 clone, without the 3'-untranslated region) (Yaraghi et al. 1998). Positive phage clones were plaque-purified, and their inserts were characterized by restriction enzyme digestion, by hybridization to different isoform-specific and nonspecific *Naip* cDNA probes and ultimately by nucleotide sequencing of cDNA inserts positive for exon 2 or exon 4.

Immunoblotting. Cultured cells and primary macrophages were collected and resuspended in a buffer consisting of 20 mM HEPES (pH 7.6), 150 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.2 mM EGTA, 25% glycerol, and protease inhibitors (2 μg/ml aprotinin, 4 μg/ml leupeptin, 2 μg/ml pepstatin A, and 100 μg/ml PMSF) (Vidal et al. 1996). Cells were then lysed by sonication (20 s, on ice), and unbroken cells and nuclei were eliminated by centrifugation (5 min, 2000 × *g*). The protein concentration was measured using a commercial reagent based on BCA staining (Pierce, Rockford, IL), using BSA as an internal standard. For protein extracts from tissues, organs were removed immediately after death, frozen in liquid nitrogen, and ground to a fine powder using mortar and pestle. The tissue powder was then resuspended in 10 ml/g of tissue of a solution consisting of 0.25 M sucrose and 0.03 M histidine (pH 7.2) supplemented with 2 mM EDTA and protease inhibitors. Tissues were homogenized using a glass potter with a tight-fitting Teflon pestle rotated at 1300 rpm. The homogenate was then centrifuged at

6000 × *g* for 15 min, and the supernatant corresponding to the soluble fraction was collected. Equal amounts of cellular protein were resolved on SDS-7.5% polyacrylamide gels, followed by electroblotting onto a nitrocellulose membrane (Schleicher & Schuell/Xymotech, Montreal, Canada). The blots were blocked overnight at 4°C in a solution containing 5% nonfat skim milk in 10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween-20. Membranes were then probed with the polyclonal anti-Naip antiserum 1.7 (used at a 1/2000 dilution). This rabbit polyclonal antiserum is directed against a mouse Naip/GST fusion protein containing 1.7 kb (nucleotides 2000–3660) of the *Naip1* cDNA from clone ms6 (Yaraghi et al. 1998). The isoform specificity of the 1.7 antiserum has not yet been characterized, although high sequence conservation among Naip protein isoforms suggests that this antiserum may recognize several Naip isoforms (Yaraghi et al. 1998). Alternatively, blots were analyzed with an anti-actin polyclonal antiserum (Sigm-Aldrich, Oakville, Ontario, Canada). Specific immune complexes were detected using a second goat anti-rabbit Ab (1/5000 dilution) coupled to peroxidase and were revealed by enhanced chemiluminescence (NEN). The intensity of the luminescent signal on Western blot was quantitated using a biological imaging system (BioImage, Ann Arbor, MI) and was standardized to the same signal obtained on each blot with the anti-actin Ab.

Immunoprecipitation. The TGC-elicited peritoneal macrophages were metabolically labeled with [^{35}S]methionine, as we have previously described (Vidal et al. 1996). Briefly, cells were incubated overnight in methionine-free medium (Life Technologies) containing 10% heat-inactivated dialyzed FBS, 5 mM L-glutamine, and [^{35}S]methionine (sp. act., 1000 Ci/mmol; DuPont, Wilmington, DE) at a final concentration of 50 μCi/ml. Labeled cells were washed in cold PBS and lysed in 0.2 ml of 1% SDS/50 mM Tris, pH 7.5, followed by addition of 0.8 ml of 1% Triton X-100, 160 mM NaCl, 0.2% SDS, and 50 mM Tris, pH 7.5. For immunoprecipitation, labeled cell extracts (5–10 x 10^6 incorporated counts) were incubated for 16 h at 4°C in a 500-μl volume with polyclonal anti-Naip 1.7 antiserum or a rabbit preimmune serum (1/200 dilutions). Immune complexes were recovered by incubation for 2 h at 4°C with 1/1 mixture of protein A-Sepharose:protein G-Sepharose beads (Pharmacia Biotech, Piscataway, NJ), followed by five consecutive washes in a buffer containing 0.1% Triton

X-100, 0.03% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 5 mg/ml BSA and two washes in 150 mM NaCl. The final pellet was incubated at room temperature in Laemmli sample buffer for 10 min. Immune complexes were then analyzed by SDS-PAGE on a 7.5% polyacrylamide gel. Fluorography was performed using a commercially available amplifier (Amplify, Amersham) as recommended by the manufacturer. The gel was dried and exposed for 2 wk at -80°C.

Infection of macrophages in vitro. L. pneumophila Philadelphia-1 strain (serogroup 1, ATCC 33152, American Type Culture Collection, Manassas, VA) was used and was obtained from the Centers for Disease Control (Atlanta, GA). The organism was passaged once i.p. in guinea pigs (Harley strain) before it was used in this study. Fresh isolates were obtained from the spleen on day 3 postinoculation and were grown on buffered charcoal yeast extract agar plates, which contained *Legionella* agar base (Difco, Detroit, MI) supplemented with L-cysteine (0.4 g/L) and ferric pyrophosphate (0.25g/L), followed by further incubation at 37°C for 72 h. The bacteria were harvested by scraping the surface of the agar, resuspended, and stored at -80°C in tryptic soy broth (Difco) supplemented with 20% (v/v) glycerol until use. In other experiments, an avirulent *dotA L. pneumophila* mutant was used (provided by Dr. H. A. Shuman, Columbia University, New York, NY). This mutant was propagated under conditions similar to those used for wild-type *L. pneumophila*. The TGC-elicited peritoneal macrophages were infected with either wild-type or *dotA* transposon mutant of *L. pneumophila* at a multiplicity of infection (MOI) of two bacteria per macrophage in antibiotic-free medium, according to the protocol described by (Yoshida et al. 1991b). At predetermined times after infection, infected macrophages were washed with PBS and scraped off the tissue culture flask. Macrophages were recovered by centrifugation and lysed, and protein extracts were analyzed by SDS-PAGE and immunoblotting.

For infection with *Salmonella typhimurium*, a temperature-sensitive, replication-defective *S. typhimurium* mutant TS27 was used (gift from Dr. A.D. O'Brien, Uniformed Services University of the Health Sciences, Bethesda, MD 29814 U.S.A.). The protocols for propagation of this strain, preparation of the infectious inoculum, and infection of macrophages were as recently described by our group (Govoni et al. 1999), with the

following modifications. The inoculum was from a *S. typhimurium* TS27 culture in TSB (OD600, 0.15), and the infection was with a MOI of 10. Phagocytosis was allowed to take place for 90 min at 37°C followed by extensive washing of extracellular bacteria.

For phagocytosis of inert particles, TGC-elicited peritoneal macrophages were fed a meal of latex beads (3 μm in diameter, diluted 1/50 in warm RPMI medium from stock suspension; Sigma, St. Louis MO) for 2 h at 37°C. Macrophages were then washed of nonphagocytosed beads and either harvested immediately or after a further 24 h incubation period as described above.

Acknowledgements

We thank Mr. G. Govoni and Dr. F. Canonne-Hergaux for their help in RNA and protein purification from tissues, respectively. We also thank Dr. D. Malo and L. Laroche for their assistance in performing *L. pneumophila* infections. The *dotA L. pneumophila* mutant used in this study was a kind gift from Dr. H. A. Shuman (Columbia University, New York, NY).

Chapter 4

Birc1e (*Naip5*) is the gene within the *Lgn1* locus associated with resistance to *Legionella pneumophila*

Expanded version of published manuscript, by permission from the publisher and Philippe Gros (corresponding author):

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4.1 Abstract

In inbred mouse strains, permissiveness to intracellular replication of *Legionella pneumophila* is controlled by a single locus (*Lgn1*), which maps to a region within distal Chromosome 13 that contains multiple copies of the gene baculoviral IAP repeat–containing 1 (*Birc1*, also called *Naip*) (Yoshida et al. 1991b; Diez et al. 1997; Growney and Dietrich 2000). Genomic BAC clones from the critical interval were transferred into transgenic mice to functionally complement the *Lgn1*-associated susceptibility of A/J mice to *L. pneumophila*. Here we report that two independent BAC clones that rescue susceptibility have an overlapping region of 56 kb in which the entire *Lgn1* transcript must lie. The only known full-length transcript coded in this region is *Birc1e* (also called *Naip5*).

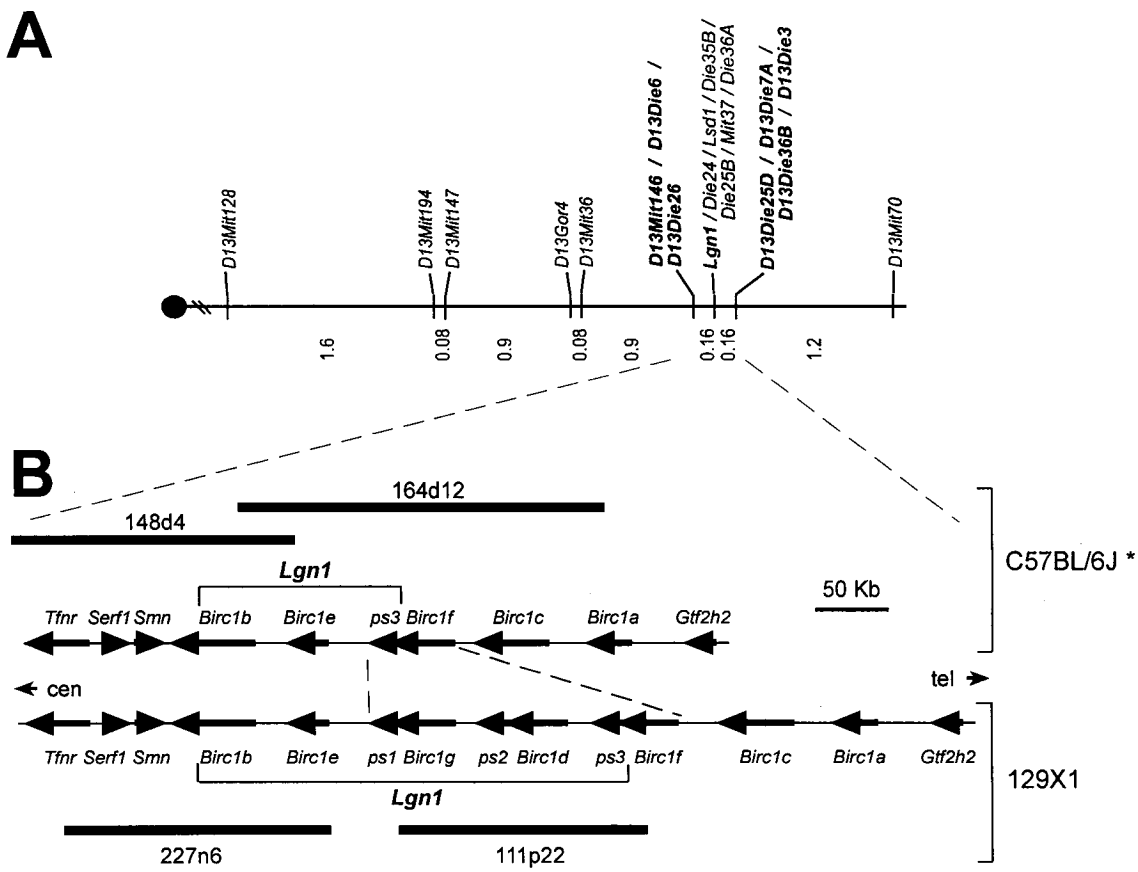
4.2 Introduction

The minimal genetic interval for *Lgn1* has been narrowed to 0.32 cM within distal mouse Chromosome (Chr) 13 with the marker order and inter-loci distances [*D13Mit146/D13Die6/D13Die26*] -0.16cM- *Lgn1* -0.16cM- [*D13Die25D/D13Die7A/D13Die36B/D13Die3*] (Beckers et al. 1995; Scharf et al. 1996; Diez et al. 1997; Growney and Dietrich 2000) (Fig. 4.1). The corresponding physical interval for *Lgn1* of 140 kb is within a direct-repeat genomic structure that includes multiple copies of *Birc1* (Scharf et al. 1996; Endrizzi et al. 2000) (Fig. 4.1). Only two *Birc1* copies (*Birc1b*, also called *Naip2*, and *Birc1e*) are included in the *Lgn1* interval (Growney and Dietrich 2000). *Birc1b* and *Birc1e* mRNA and Birc1 protein are present in macrophages, and their expression is upregulated after phagocytosis, consistent with *Birc1* being the gene of interest within the *Lgn1* locus (Diez et al. 2000). The structure of

FIGURE 4.1

Genetic and physical maps of the *Lgn1* region on mouse Chromosome 13.

A, The minimal *Lgn1* interval of 0.32 cM is delineated proximally by *D13Die26* and distally by *D13Die25D* (2 recombinations in 1270 (C57BL/6J ×A/J) F1 ×A/J backcross progeny each). The centromere is identified by a solid dot on the left.
B, The minimal *Lgn1* physical interval (bracketed) is approximately 140 kb (for the C57BL/6J strain) and contains *Birc1b* and *Birc1e*. All transcription units are shown as thick arrows in a 5' to 3' direction. The BAC clones used for transgenesis are drawn to scale beside the transcriptional map of their respective strains of origin (C57BL/6J, 129X1). The 129X1 mouse strain contains an extra repeat structure *(Birc1d* and *Birc1g*), increasing to seven the number of full-length *Birc1* copies known. Truncated, or pseudogene, *Birc1c-ps1*, *Birc1c-ps2* and *Birc1c-ps3* copies are shown as *ps1*, *ps2* and *ps3* respectively. The overall arrangement of the A/J *Lgn1* genomic region is similar to that depicted for the C57BL/6J strain (Growney and Dietrich 2000). This map is adapted from (Growney and Dietrich 2000).


A
D13Mit128
D13Mit194
D13Mit147
D13Gor4
D13Mit36
D13Mit146 / D13Die6 / D13Die26
Lgn1 / Die24 / Lsd1 / Die35B / Die25B / Mit37 / Die36A
D13Die25D / D13Die7A / D13Die36B / D13Die3
D13Mit70
1.6
0.08
0.9
0.08
0.9
0.16
0.16
1.2
B
164d12
148d4
Lgn1
50 Kb
C57BL/6J *
Tfnr Serf1 Smn Birc1b Birc1e ps3 Birc1f Birc1c Birc1a Gtf2h2
cen
tel
Tfnr Serf1 Smn Birc1b Birc1e ps1 Birc1g ps2 Birc1d ps3 Birc1f Birc1c Birc1a Gtf2h2
Lgn1
129X1
227n6
111p22

the *Birc1* cluster, including variable copy number among inbred strains (Growney and Dietrich 2000) and presence of truncated copies (*Birc1c-ps1*, *Birc1c-ps2* and *Birc1c-ps3*, also called *Naip1*, *Naip2* and *Naip3*, respectively; Fig. 4.1) (Growney et al. 2000), has made it difficult to pinpoint which *Birc1* copy underlies *Lgn1*. To circumvent these difficulties, we implemented a genetic complementation approach *in vivo* in transgenic mice bearing individual BAC clones overlapping discrete portions of the *Birc1* cluster.

4.3 Results

Susceptibility (S) or resistance (R, R/S) alleles at the *Lgn1* locus were inferred from the extent of intracellular replication of *L. pneumophila* (strain Philadelphia 1) in thioglycholate-elicited peritoneal macrophages *ex vivo*. Susceptible A/J macrophages supported an increase of up to 1,000-fold in the number of live bacteria (change in colony-forming units (CFU)) that could be recovered 72 h after infection, whereas macrophages from inbred strains C57BL/6J (B6), FVB and 129X1 (129) allowed little replication (twofold to eightfold increase; Table 4.1).

For functional complementation studies *in vivo*, bacterial artificial chromosomes (BAC clones) harboring large inserts overlapping portions of the *Birc1* cluster were obtained from libraries constructed with genomic DNA from resistant strains B6 and 129. Clones were individually injected in fertilized FVB eggs, and transgenic F0 pups were subsequently identified by PCR genotyping for the presence of vector-derived sequences. In addition, integrity of the transgenic BACs was monitored by genotyping with markers from the *Birc1* cluster, and that allow discrimination between B6 or 129 alleles (BAC-derived) from the endogenous A and FVB alleles (see Methods). FVB/BAC transgenics (F0) were crossed to the permissive A strain twice to generate the (F0 X A) X A backcross (Fig. 4.2). Such backcross mice were genotyped for presence of the transgene and either homozygosity (S/S; susceptible) or heterozygosity (FVB: A; R/S; resistant) for A alleles at the Chr. 13 *Lgn1* locus, and were used to assess functional complementation by the transgenic BAC (Fig. 4.3). In some instances, BAC-mediated complementation was validated by further backcrossing the transgenic BAC onto A background, followed

Table 4.1 *L. pneumophila* susceptibility of parental mouse strains used for BAC transgenesis

Strain	Replication, logΔCFU (mean ± s.d.)	Phenotype*
A/J	2.79 ±0.79	Permissive
C57BL/6J	0.76 ±0.52	Non-permissive
FVB	0.81 ±0.61	Non-permissive
129X1	0.97 ±0.07	Non-permissive

*Macrophages are arbitrarily defined as permissive if the average *L. pneumophila* replication during a 3-d infection period is above 20-fold.

FIGURE 4.2

BAC transgenic lines breeding scheme.

A, The BAC clone (gray diamond) was injected into *L. pneumophila*-resistant FVB mice (RR), and the FVB F0 transgenic founder (FVB + BAC) was subsequently backcrossed onto the *L. pneumophila*-susceptible A/J background (SS). The resulting F1 and subsequent backcross mice contain 50%, 25%, 12.5% and 6.3% of FVB donor DNA (proportionally shaded black) on A/J background (white).

B, Segregation of the transgenic BAC in mice was followed by genotyping for presence of BAC vector sequences (right) and polymorphic internal markers (shown in Fig. 4.4). Transgenic mice were further genotyped for the Chr 13 *Lgn1* region as either heterozygotes (RS) or homozygotes (SS) before crossing to A/J. Possible BAC-mediated complementation of *L. pneumophila* susceptibility was monitored in BAC transgenics homozygotes for Chr 13 susceptibility alleles (SS), and these mice are identified by a thick black outline (in **A**).

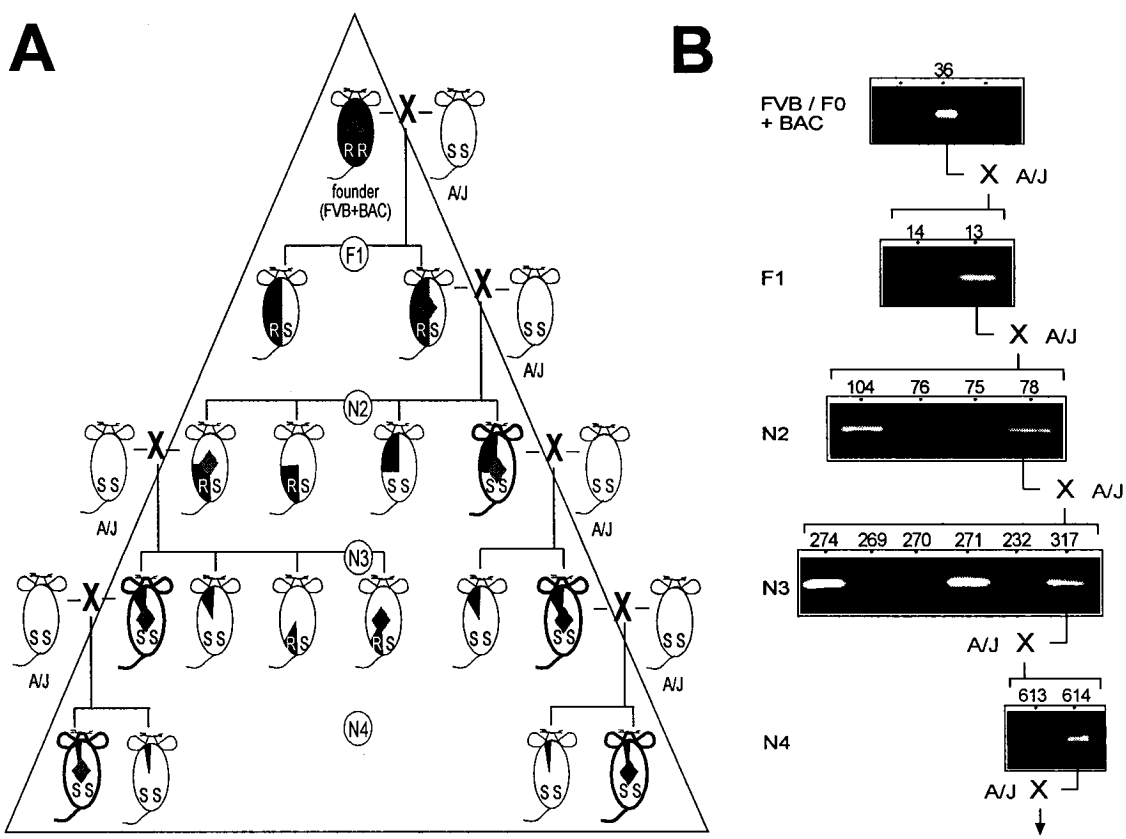

A
B
RR
SS
founder
(FVB+BAC)
A/J
F1
RS
N2
N3
N4
36
FVB / F0
+ BAC
X A/J
14 13
F1
104 76 75 78
N2
274 269 270 271 232 317
N3
613 614
N4

FIGURE 4.3

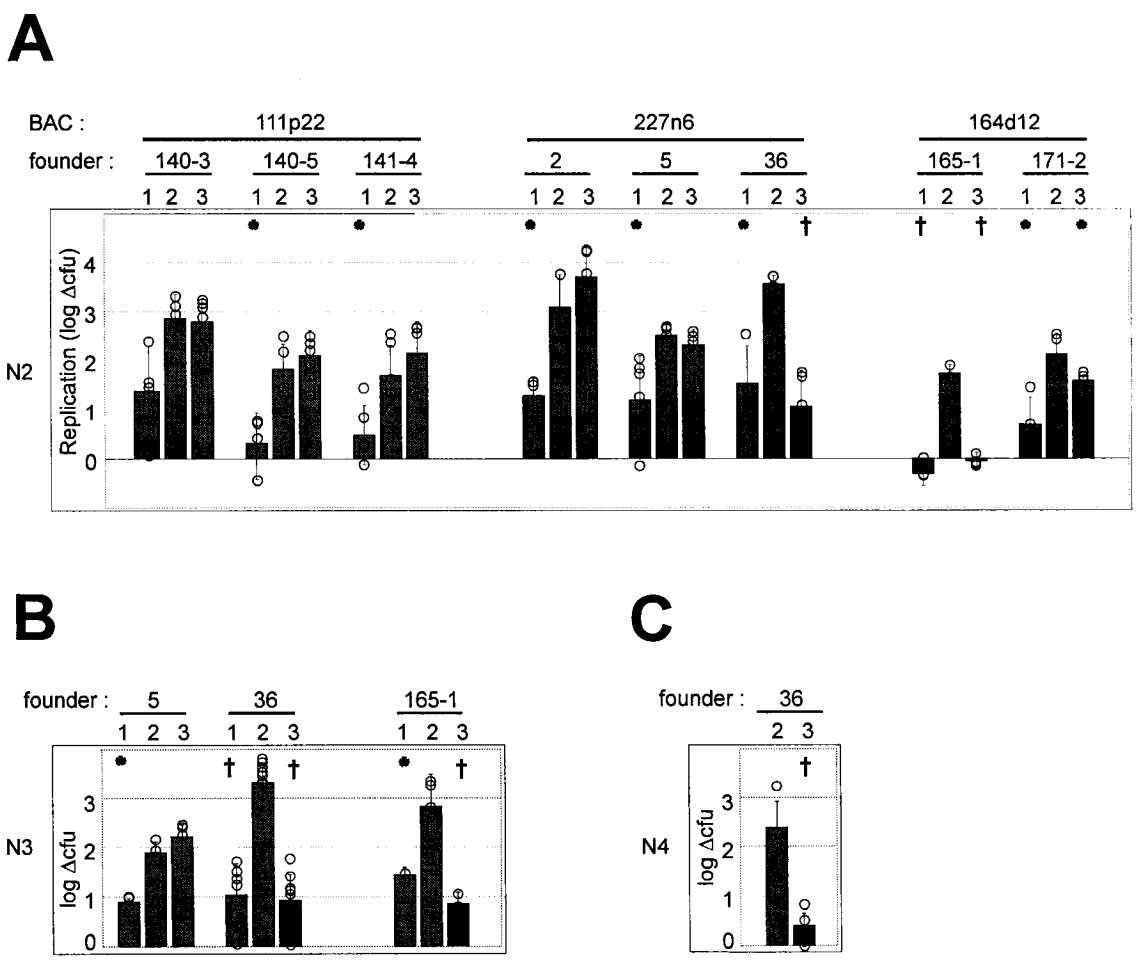
Correction of *L. pneumophila* susceptibility of A/J mice *in vivo* with transgenic BAC clones from the *Birc1* cluster.

A, The identity of the BAC clones used for transgenesis is indicated on top, with the identity of the FVB-BAC founders used for back-crossing to A/J immediately underneath. Backcross ((FVB ×A/J)F1 ×A/J) mice (N2) were genotyped for Chr 13 haplotypes and for the presence of the transgene and were grouped as heterozygotes with respect to the resistance allele at *Lgn1* inherited from FVB (column 1), control homozygotes with respect to the susceptible A/J allele at *Lgn1* lacking the transgene (column 2) or informative BAC transgenics homozygotes with respect to the susceptible A/J allele at *Lgn1* but harboring the transgene (column 3). Data are the mean ± s.d. (blue histogram) of log ΔCFU for peritoneal macrophages from individual mice (shown as single data points) measured 72 h after infection with *L. pneumophil*a. Significant reduction of *L. pneumophila* replication associated with the presence of the R allele at *Lgn1* (column 1) or with the presence of a complementing BAC (column 3, red histograms) was determined by one-tailed Student's t-test statistics (asterisk (*) indicates $P < 0.05$ and dagger (†) indicates $P < 0.001$). Continuing complementation of *Lgn1* susceptibility by the 227n6 and 164d12 BAC transgenes was monitored after subsequent backcrossing of the BAC transgene to A/J in **B**, the N3 and **C**, N4 generations and phenotyping of macrophages as indicated in **A**.

by testing for continuing complementation of susceptibility in successive N3 and N4 progeny (Fig. 4.3).

The consensus genetic, physical and transcription map of the *Lgn1* region (Fig. 4.1) shows the position of BAC clones (111p22, 227n6, 148d4, 164d12) that overlap different portions of the *Naip* cluster, and that were used in this study.

The BAC 111p22 180-kb insert contains the entire *Birc1g* (also called *Naip7*), *Birc1c-ps2*, *Birc1d* (also called *Naip4*) and *Birc1c-ps3* putative genes (Growney et al. 2000) from the 129X1 inbred strain (Fig. 4.1). Transgenic mice with germline transmission of an intact 111p22 BAC did not show correction of the susceptibility phenotype (Fig. 4.3).


A
BAC :
111p22
227n6
164d12
founder :
140-3
140-5
141-4
2
5
36
165-1
171-2
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
N2
Replication (log Δcfu)
0
1
2
3
4
B
founder :
5
36
165-1
1 2 3 1 2 3 1 2 3
N3
log Δcfu
0
1
2
3
C
founder :
36
2 3
N4
log Δcfu
0
1
2
3

The linearized BAC 227n6 insert (129X1 origin) spans approximately 140 kb from a *Not*I site proximal to *Serf1* to the cloning site; it thus contains *Serf1*, *Smn*, *Birc1b* and most of *Birc1e* (Figs. 4.1and 4.4), except for the non-coding exon 1. Of three transgenic lines showing germline transmission of intact BAC 227n6, two (2 and 5) did not show correction of the susceptibility phenotype (Fig. 4.3). But backcross mice that were derived from founder 36, homozygous with respect to A/J haplotypes at Chr 13 and positive for the BAC transgene were non-permissive to *L. pneumophila* replication. CFU counts in these mice were comparable to those observed in littermates heterozygous with respect to the A/J Chr 13 haplotype and were clearly distinct ($P < 0.001$) from A/J homozygous littermates lacking the transgene (Fig. 4.3*a*). Genotyping for presence of strain-specific polymorphic variants verified the presence of 129X1-derived *Birc1b* and *Birc1e* sequences in BAC 227n6 transgenics derived from founder 36 (Fig. 4.5). We confirmed the complementing activity of BAC 227n6 by backcrossing transgenic mice for one (N3) or two (N4) additional generations: we observed a complete correlation between presence of the transgene and resistance to *L. pneumophila* infection in macrophages from mice that were homozygous with respect to otherwise susceptible A/J alleles at *Lgn1* (Fig. 4.3*b*,*c*).

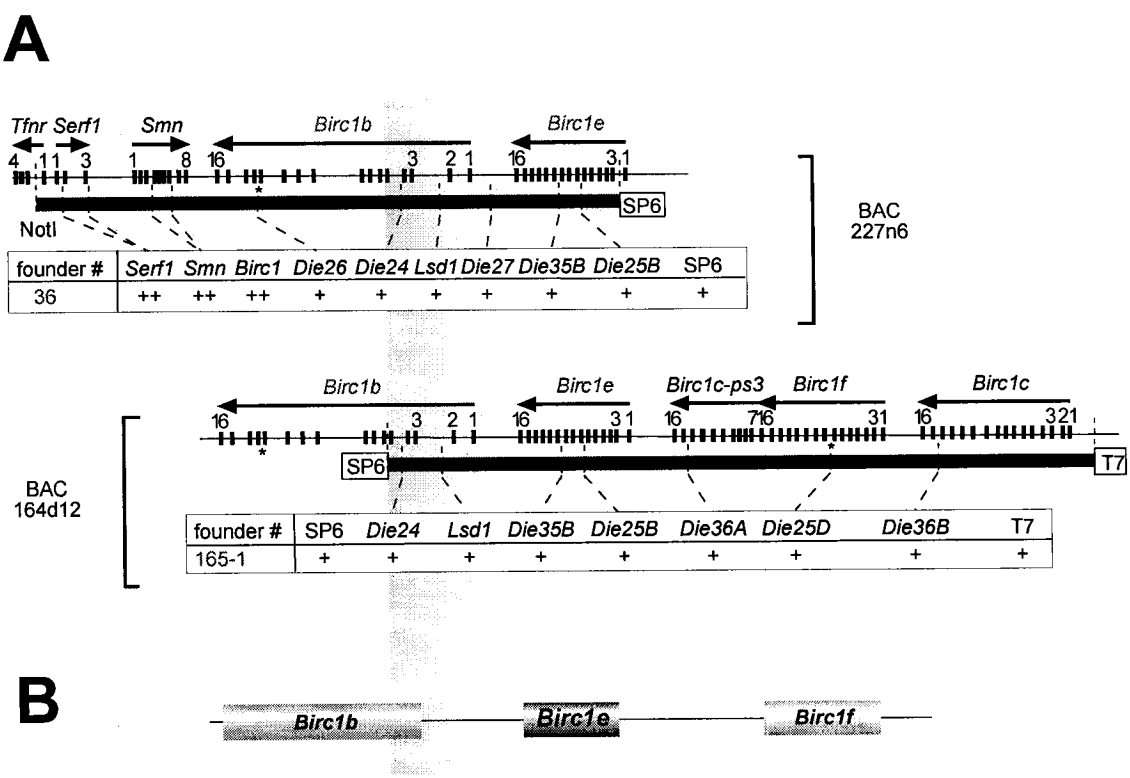
BAC 164d12 is derived from B6 and contains the entire *Birc1e*, *Birc1c-ps3*, *Birc1f* (also called *Naip6*) and *Birc1c* (also called *Naip3*) genes (Growney and Dietrich 2000)

within its 220-kb insert (Figs. 4.1 and 4.4). We successfully bred two transgenic founders (165-1, 171-2) showing germline transmission of 164d12 and then examined N2

FIGURE 4.4

Detailed organization of the transcript map of the genomic region corresponding to rescuing BAC clones 227n6 and 164d12.

A, The transcript maps are derived from the sequences of BAC 149m19 and BAC 26f17 available in GenBank as assembled in (Growney and Dietrich 2000). Predicted transcripts are identified by arrows (with name above) pointing in the direction of transcription (5' to 3'). The position of individual exons is also shown, with numbers identifying most 5' and 3' exons. The insert of BAC clones 227n6 and 164d12 is shown (blue line), along with the position and origin of attached vector sequences (SP6, T7). Immediately underneath each BAC insert, the nature and position of strain-specific, PCR-based polymorphic markers used to discriminate between C57Bl/6J or 129X1 (BAC DNA) and FVB or A/J (recipient strains) and to verify the integrity of the cloned genomic segment are indicated. Cloned DNA segments from *Serf1*, *Smn* and *Birc1* were used for Southern blotting and detection of over-represented and BAC-associated hybridizing restriction fragments in genomic DNA from transgenic mice carrying BAC clone 227n6. **B**, *Birc1e* is the only *Birc1* coding sequence that is contained entirely within the region of overlap (depicted in yellow) between complementing BAC clones 227n6 and 164d12.


A
Tfnr Serf1
Smn
Birc1b
Birc1e
SP6
NotI
BAC
227n6
founder #
Serf1 Smn Birc1 Die26 Die24 Lsd1 Die27 Die35B Die25B SP6
36
++ ++ ++ + + + + + + +
Birc1b
Birc1e
Birc1c-ps3
Birc1f
Birc1c
SP6
T7
BAC
164d12
founder #
SP6 Die24 Lsd1 Die35B Die25B Die36A Die25D Die36B T7
165-1
+ + + + + + + + +
B
Birc1b
Birc1e
Birc1f

progeny for susceptibility to *L. pneumophila*. We noted a small but significant effect ($P < 0.05$) of the transgene on *L. pneumophila* replication in N2 (Fig. 4.3*a*) and N3 (data not shown) mice derived from founder 171-2. Transgenic mice derived from founder 165-1 showed full rescue ($P < 0.001$) of susceptibility alleles at *Lgn1* (Fig. 4.3*a*). Genotyping with strain-specific markers suggested that the transgenic 164d12 BAC was intact only in the 165-1 line. We verified continued rescue of susceptibility phenotype by BAC 164d12 by additional backcrossing (N3) of transgenic founder 165-1 to A/J mice (Fig. 4.3*b*).

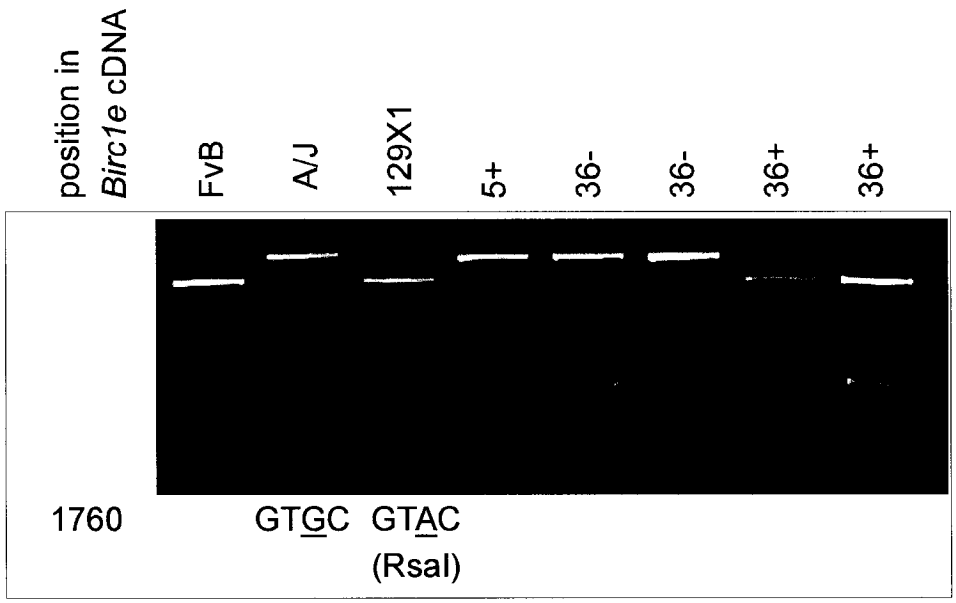
Alignment of the transcript map of rescuing BAC clones 227n6 and 164d12 (Fig. 4.4) showed that *Birc1e* is the only full-length coding sequence contained in the region of overlap between the two complementing clones.

We verified the presence of mRNA transcripts derived from the rescuing BAC clones in transgenic mice (Fig. 4.5) using a number of single-nucleotide polymorphisms (SNPs) that distinguish between transgenic 129 (BAC 227n6) or B6 (BAC 164d12) mRNA and host A/J mRNA, including the presence of strain-specific restriction enzyme sites. A polymorphism in *Birc1e* exon 11 (position 1,760) creates an *Rsa*I restriction site specific to the 129 strain, which diagnostically showed transgenic expression of *Birc1e* from rescuing BAC 227n6 in RT–PCR products of spleen RNA from transgenic mice of line 36 (Fig. 4.5). No FVB-specific expression of *Birc1e* was detected in any of the transgenic mice tested, consistent with homozygosity with respect to *Lgn1* Chr 13 A/J haplotypes in these mice. We obtained similar results using a polymorphism at position 1746 that abolishes an *Msc*I restriction site in 129 (data not shown). Likewise, we used a strain-specific *Msp*I restriction site (exon 12, position 3,545; Fig. 4.5) and *Bst*NI restriction site (position 3,566, data not shown) to indicate the presence of B6-derived *Birc1e* transcript in BAC 164d12 transgenic lines showing phenotypic rescue (165-1). Expression of *Birc1e* was undetectable in line 171-2 (Fig. 4.5). We genotyped the additional informative SNPs (positions 3,923, 4,025 and 4,342) by nucleotide sequencing of RT–PCR products after subcloning into plasmid vector (Fig. 4.5). Expression of BAC-derived B6 *Birc1e* sequences was associated with phenotypic rescue (in founder 165-1, 21 of 24 clones sequenced) but not with the non-rescued line (171-2, 1 of 14 clones sequenced). Thus, we found expression of the transgenic *Birc1e* mRNA in all lines in which we observed phenotypic rescue (*L. pneumophila* growth restriction).

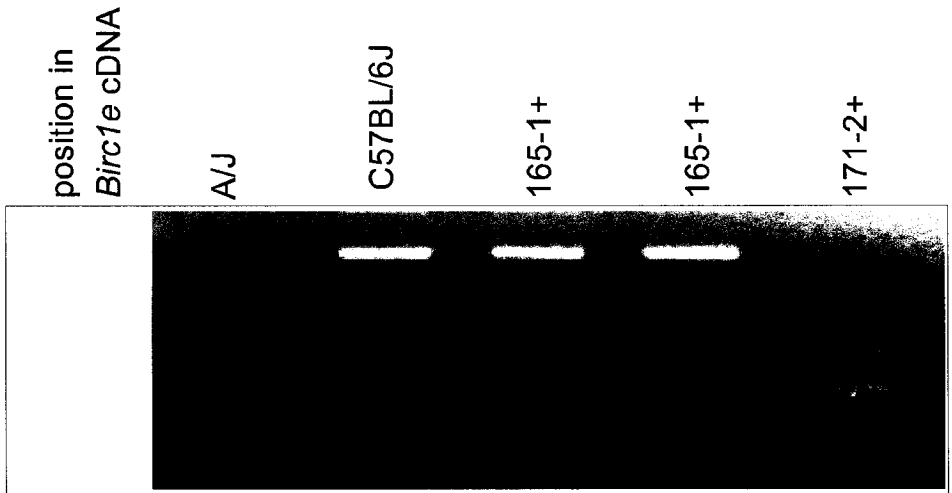
FIGURE 4.5

Transgenic *Birc1e* mRNA expression in lines in which complementation was observed.

Transgenic *Birc1e* expression was verified at the mRNA level in lines harboring the rescuing BAC 227n6 (top panel) and BAC 164d12 (lower panel). A number of strain-specific polymorphisms (SNPs) in coding and 3' untranslated regions (UTRs) of the *Birc1e* transcript were used to distinguish between transgenic 129 (BAC 227n6) or B6 (BAC 164d12) mRNA and host A/J *Birc1e* mRNA. Top panel (227n6), presence (GTAC) or absence (GTGC) of a diagnostic *Rsa*I restriction site (position 1,760) was monitored by agarose gel electrophoresis and ethidium-bromide staining of *Rsa*I-digested RT–PCR products from spleen RNA from controls (FVB, A/J, 129X1). Presence of 129X1-specific sequences in BAC 227n6 transgenic mice showing phenotypic rescue (36+) was observed, but no such transcripts were detected in transgenic mice not showing phenotypic rescue (5+) or non-transgenic mice (36–). The presence of *Birc1e* transcripts from BAC 227n6 in transgenic mice indicates that absence of exon 1 in this BAC does not preclude transgenic *Birc1e* mRNA expression. Bottom panel (164d12), presence (CCGG) or absence (CCGA) of a diagnostic *Msp*I restriction site (position 3,545) was monitored in controls (A/J, C57BL/6J) and in transgenic mice showing (165-1+) or not showing (171-2+) complete phenotypic rescue. Other SNPs (positions 3,923, 4,025 and 4,342) were genotyped after subcloning and sequencing of the PCR products, and the ratio of each sequence in independent clones is indicated. Independent mice derived from founders 36 and 165-1 were tested.



227n6



164d12

3923	A	G	0A/4G	1A/11G	5A/0G
4025	A	G	1A/7G	1A/14G	9A/1G
4342	C	T	1C/7T	2C/14T	11C/1T

4.4 Discussion

A functional complementation strategy *in vivo* was implemented to functionally dissect the *Birc1* cluster, and to identify the *Birc1* copy allelic with *Lgn1*. In this approach, firm conclusions could only be drawn from animals showing positive complementation. Indeed, rearrangements during BAC DNA isolation or microinjection, instability or lack of germ-line transmission of an intact clone, or stable integration into a transcriptionally silent chromosomal site are all likely to occur, and seriously complicate the analysis of animals showing a lack of complementation. Positive complementation was observed with BAC clones 227n6 and 164d12. The minimal region of overlap between these two clones (AF 131205; nucleotide position 109371 to 166081) contains the full-length coding sequence of *Birc1e* and a partial segment of *Birc1b* comprising the first 5 exons. These results suggest that a functional *Birc1e* polypeptide accounts for the complementing activity of both BAC clones. However, we cannot formally exclude the possibility that the severely truncated Birc1b protein encoded by BAC 164d12 may retain hypothetical complementing activity of the wild type Birc1b protein which is present in BAC 227n6. However, BAC 148d4 which contains a full *Birc1b* locus, the *Birc1b-Birc1e* intergenic region and a partial *Birc1e* gene (Fig. 4.1) has so far failed to show rescue in one intact transgenic line (data not published), arguing against but not excluding a role for *Birc1b*. Also, it is possible that splicing-competent exons encoding peptides biologically active in macrophages may have gone undetected in the region of overlap between the two BACs. Nevertheless, we believe that the above-mentioned scenarios are unlikely and that, by far, the most reasonable interpretation of our results is that a functional Birc1e polypeptide constitutes the complementing activity of both BAC clones.

Birc1e is intact in BAC clone 164d12, but lacks the non-coding exon 1 in complementing BAC 227n6. Expression studies in BAC transgenic 227n6 founder 36 and its descendants (semi-quantitative RT-PCR) identify increased *Birc1e* mRNA expression from multiple copies of the transgenic BAC compared to non-transgenic

controls (data not shown), suggesting that deletion of exon 1 does not affect *Birc1e* mRNA expression in these mice.

The transgene-mediated complementation at *Lgn1* may result from correction of a loss-of-function allele of *Birc1e* in A/J (structural or regulatory mutations). The apparent lack of functional redundancy in the *Birc1* family would be in agreement with this proposal. Indeed, the exclusion of *Birc1* copies from the minimal *Lgn1* interval and the observation that deletion of *Birc1a* (also called *Naip1*) has an effect on neuron survival (Holcik et al. 2000) but not on susceptibility to *L. pneumophila* infection (data not shown) support discrete functional roles for individual *Birc1* paralogs in the same or different tissues. This situation would be similar to that seen in the *Ly49* gene cluster on mouse Chr. 6 where the deletion of a single copy of *Ly49h* causes susceptibility to cytomegalovirus infection in BXD8 mice (Lee et al. 2001).

It is also possible that the BAC complementation represents a gain-of-function through overexpression of *Birc1e* on a haploid insufficient A/J background. The abundant transgenic *Birc1e* mRNA expression seen in all rescued lines would support this possibility (Fig. 4.5). The creation of a deletion mutant at *Birc1e* by homologous recombination should distinguish between the two possibilities. Very recently, partial rescue of the phenotype associated with *Lgn1 in vivo* with a BAC clone containing *Birc1e* has been reported (Wright et al. 2003), in agreement with results from this study.

These results indicate a role for *Birc1e* in macrophage resistance to *L. pneumophila* infection, but the mechanism of action is yet unknown. The *BIRC1* gene was initially identified through positional cloning of the gene causing spinal muscular atrophy in humans (Roy et al. 1995a). Although it is alteration of the neighboring *SMN* gene that triggers disease, *BIRC1* is deleted in severe cases of spinal muscular atrophy and is a potential modulator of disease severity (Somerville et al. 1997; Haider et al. 2001). BIRC1 proteins (150 kDa) are members of the inhibitor of apoptosis protein (IAP) family (Liston et al. 1996), structurally defined by baculovirus inhibitor of apoptosis repeat (BIR) domains implicated in protein–protein interactions (Hozak et al. 2000; Wu et al. 2000). BIRC1 also shows a putative nucleotide-binding site (Koonin and Aravind 2000). An anti-apoptotic effect of BIRC1 has been described *in vitro* (Liston et al. 1996) and *in vivo* (Holcik et al. 2000; Perrelet et al. 2000), probably through inhibition of

effector caspases by BIRC1 BIR domains (Maier et al. 2002). Notably, induction of apoptosis seems to be important for pathogenesis of *L. pneumophila* in human macrophages *in vitro* (Muller et al. 1996; Gao and Abu Kwaik 1999b). We have been unable to detect an association between apoptosis and *Lgn1*-regulated intracellular replication of *L. pneumophila* in mouse macrophages (data not shown). On the other hand, we have observed that BIRC1 protein expression is modulated in macrophages after phagocytosis (Diez et al. 2000). Thus, it is possible that BIRC1 may be part of a response pathway triggered during phagocytosis in these cells (Watarai et al. 2001). This mechanism may not be related to the known function of BIRC1 during apoptosis, but may be associated with the mechanism by which *L. pneumophila* inhibits maturation of the phagosome.

4.5 Materials and methods

Mice. Inbred mouse strains were initially purchased from The Jackson Laboratory and then maintained as breeding colonies in our laboratories. Maintenance and experimental manipulation of the mice were performed according to the guidelines and regulations of the Canadian Council on Animal Care.

Generation of transgenic mice. BAC clones 227n6 and 111p22 were isolated from a 129X1-derived BAC library (Genome Systems) and have been described (Diez et al. 1997; Yaraghi et al. 1998). BAC clones 164d12 and 148d4 have been mapped to the *Lgn1* interval (Growney and Dietrich 2000) and were from the Research Genetics RPCI-23 library (C57BL/6J). The BAC clones were linearized by digestion with *Not*I and the inserts were separated by pulsed-field gel electrophoresis, digestion with agarase and dialysis against microinjection buffer (phosphate-buffered saline). Each BAC insert was microinjected (< 1 g l^{-1}) into the pronuclei of an average of 500 FVB embryos and

implanted into pseudo-pregnant females to generate transgenic mice. Founder (F0) transgenic mice were identified by PCR amplification with primer pairs specific for the BAC vector as well as with polymorphic markers from the *Birc1* cluster that could discriminate between the BAC donor (B6 or 129 alleles) and recipient strains (A/J and FVB alleles). The *Not*I-excised inserts from BAC clones of the Genome Systems library retain about 350 bp of pBeloBAC11 sequence at the T7 promoter end within which the primer pair T7F 5'-TGGCTTAACTATGCGGCATC-3' / T7R 5'-ATTACAATTCACTGGCCGTC-3' were used to amplify a 268 bp. diagnostic PCR product. At the SP6 promoter end (about 290 bp of vector sequence), the primer pair SP6F 5'-TATCCGCTCACAATTCCACAC-3' / SP6R 5'-ATTCATTAATGCAGCTGGCAC-3' yielded a 152-bp vector-specific PCR product (see Fig. 4.2). The *Not*I-digested inserts from BAC clones of the RPCI-23 library retain less than 50 bp of pBACe3.6 vector sequence. Thus, identification of such BAC clones in transgenic mice required the alternate use of one of two primers within the vector (T7 arm "forward" 5'-TCACTATAGGGAGAGGATCCG-3' and SP6 arm "reverse" 5'-CCGTCGACATTTAGGTGACA-3') and a paired PCR primer within the cloned DNA itself and specific for each BAC (BAC164d12, T7-R 5'-AGTTCTTTCCAAGGGTGGTGA-3' within GenBank AF240508 / *Naip1* exon 16; BAC164d12, SP6-F 5'-GGAAACACAACCCAAACTGA-3' within GenBank AF240507 / *Naip2* intron 5 and BAC148d4-SP6-F 5'-TGCCTTTCTCCTTTATCCTCC-3' within GenBank AZ293791 / *Naip5* intron 12).

The polymorphic markers *D13Die26*, *D13Die24*, *D13Lsd1*, *D13Die27*, *D13Die35*, *D13Die25* and *D13Die36*, which have been described (Endrizzi et al. 1999; Growney and Dietrich 2000), were used as strain-specific polymorphisms to distinguish chromosomal gene copies from those of the BAC clones. FVB/BAC transgenics (F0) were backcrossed twice to the permissive A/J strain (Fig. 4.2), and then genotyped for the transgene and either homozygosity (S/S; susceptible) or heterozygosity (R/S; resistant) with respect to A/J alleles at the Chr 13 *Lgn1* locus with proximal (*D13Mit194*, *D13Mit36*) or distal (*D13Mit72*, *D13Mit70*) markers (Diez et al. 1997). These mice were then used to assess the biological effect of the transgenic BAC (Fig. 4.3).

L. pneumophila infection of thioglycholate-elicited peritoneal macrophages. Peritoneal macrophages were obtained 72 h after intraperitoneal injection of 1 ml of sterile 3% thioglycholate broth by washing the peritoneal cavity with 10 ml of serum-free RPMI 1640 medium supplemented with 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Life Technologies) as previously described (Yamamoto et al. 1988). The peritoneal exudate cells were plated in 24-well plates (0.5×10^6 per well) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and incubated for 16 h at 37 °C. Non-adherent cells were eliminated by washing with phosphate-buffered saline and the remaining cell monolayers were used for bacterial infection. *L. pneumophila* Philadelphia-1 strain (serogroup 1, ATCC 33152, American Type Culture Collection) was obtained from the Centers for Disease Control. Macrophages were infected with *L. pneumophila* at a multiplicity of infection of 2:1 in antibiotic-free RPMI 1640 medium with 10% fetal bovine serum using a modification of a published protocol (Yoshida et al. 1991b). Macrophages were incubated with the bacteria for 1 h at 37 °C under 5% carbon dioxide. The plates were then washed three times with warm RPMI 1640 medium to remove non-phagocytosed bacteria. At that point, the number of phagocytosed/macrophage-associated bacteria was determined by lysing one series of infected macrophages with distilled water (T0). The cell lysates were serially diluted in saline, plated on buffered-charcoal yeast-extract agar plates (OXOID) and CFU counts were determined. The duplicate wells were incubated for 3 d in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. The infected monolayers were then lysed and CFU counts determined. The extent of intracellular bacterial replication was determined by comparing CFU counts obtained at T0 and 72 h after infection.

Verification of transgenic Birc1e expression. RNA was prepared from the spleens of mice homozygous with respect to the A/J haplotype at Chr 13 *Lgn1* from N2 and N3 generations (backcross to A/J) either carrying or not carrying the transgenic BAC clone. The RNA was converted to cDNA using random hexamers and reverse transcriptase and the *Birc1e* sequences corresponding to positions 272–1,983 and 2,920–4,502 were PCR-amplified. A number of *Birc1e* SNPs, identified in the Celera Mouse Reference SNP

Database, distinguish between transgenic 129 (BAC 227n6; positions 1,746 and 1,760 within exon 11) or B6 (BAC 164d12; positions 3,545 and 3,566 within exon 12, positions 3,923 and 4,025 within exon 15 and position 4,342 in exon 16) mRNA and host A/J *Birc1e* mRNA. RT–PCR products were gel-purified, cloned with a TOPO TA Cloning Kit (Invitrogen) and transformed in *Escherichia coli* DH10. Colonies from each transformation were picked, their inserts sequenced by fluorescent dye terminator chemistry and analyzed on an ABI 3700 automated sequencer (Applied Biosystems). For SNPs that altered a restriction site, the RT–PCR products were purified by column chromatography (QIAquick PCR Purification Kit; QIAGEN) followed by enzymatic digestion and gel electrophoresis.

Accession numbers. Birc1e mRNA sequence, NM010870; genomic sequence within BAC 149m19, AF131205 (The BAC 227n6 insert spans nt 28,797–166,081 of this sequence and its overlap with BAC 164d12 corresponds to nt 109,371–166,081.); genomic sequence within BAC 26f17, AF242431. 4.6

4.6 Acknowledgements

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Chapter 5

Summary and Future Perspectives

Section 5.1

Complementary data and discussion

This section presents data that is important for a thorough understanding of the *Lgn1* cloning project but that was not included in the published chapters 2, 3 and 4. It also presents a retrospective look at the project up to this point with a special emphasis on how genome sequences and new technologies made available only in the last few years may have changed the steps involved in positional cloning.

5.1.1 Birc1 sequences

Having identified *Birc1e* as the gene responsible for the difference in permissiveness to intracellular *L. pneumophila* replication between A/J and C57BL/6J mice, it was imperative to compare the sequence of the *Birc1e* gene in the two strains. Not much sequencing was necessary in our laboratory. The Celera Mouse Reference SNP (single-nucleotide polymorphism) Database has had good coverage of the *Lgn1* region since 2002. The public domain SNP database (http://www.ncbi.nlm.nih.gov/SNP/) has also been improving its coverage and it was significantly enriched when the laboratory of W. Dietrich sequenced the *Birc1e* gene in several inbred mouse strains (Wright et al. 2003). Figure 5.1 displays the Birc1e protein sequence for the four mouse strains used in our BAC transgenesis experiments: the permissive A/J strain and the non-permissive FvB, 129X1 and C57BL/6J strains.

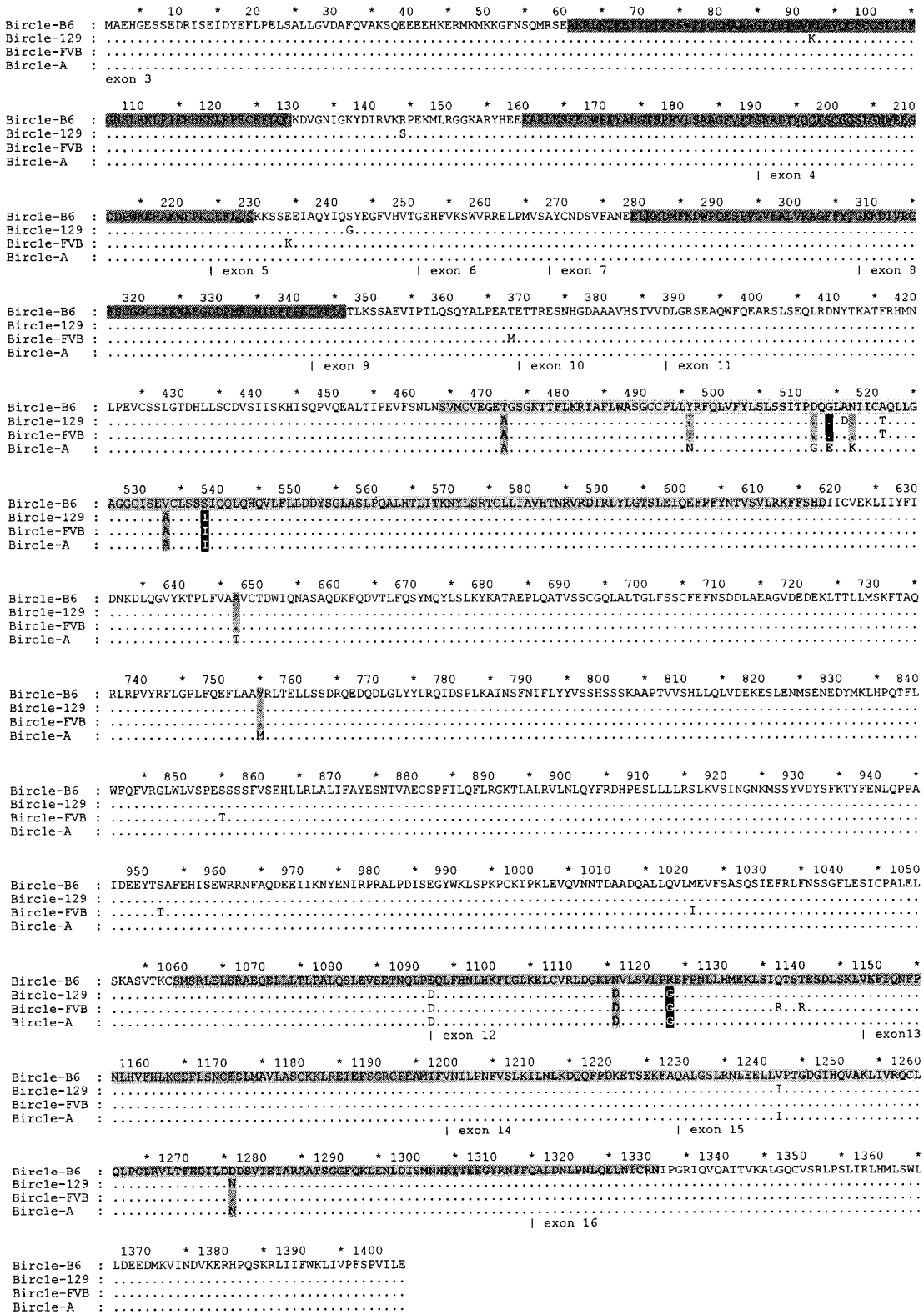
There are 14 polymorphisms distinguishing the Birc1e protein in the A/J and the C57BL/6J mouse strains. Those polymorphisms are concentrated mainly in the NACHT and LRR protein domains of Birc1e. Since none of the amino acid substitutions shown in figure 5.1 strike us as clear loss-of-function mutations, they will have to be reintroduced individually by site-directed mutagenesis into the C57BL/6J sequence and tested for their effect on Birc1e function. However, there is evidence of a resistance allele of the *Birc1e* gene in the 129X1 and FvB strains (see below). Therefore, priority should be given to testing the six polymorphisms shown in Figure 5.1 that are specific to the A/J strain.

Out of the six amino acid substitutions that are specific for the A/J strain, two are not part of a conserved protein domain and do not constitute dramatic changes in the physicochemical properties of the amino acid residue (aa positions 647 and 755). The four remaining A/J-specific amino acid substitutions are clustered within the NACHT protein domain of the Birc1e protein: Y496N, D512G, G514E and N517K. No particular function has been assigned to those four residue positions yet and they are not conserved in other NACHT domains. However, the glycine to glutamic acid substitution at position 514 is the most disruptive of the A/J-specific amino acid substitutions and none of the known NACHT domain-containing proteins contain a glutamic acid at that position.

FIGURE 5.1

Birc1e protein sequence in four different mouse strains.

Birc1e amino acid sequences are shown here for four mouse strains. The entire C57BL/6J-derived Birc1e sequence (Birc1e-B6; based on GenBank AAN60207) is shown in the first line. For the sequences in lines two, three and four only those residues that are different from the C57BL/6J sequence are shown. Line two represents the 129X1 mouse strain sequence (Birc1e-129; verified with the Celera SNP database to be identical to the strain 129S1 sequence, GenBank AAN60206). Line three represents the FvB mouse strain sequence (Birc1e-FVB; GenBank AAN60210) and line four represents the A/J mouse strain sequence (Birc1e-A; based on GenBank AAN77912). All of these Birc1e sequences were double-checked against the Celera SNP database as well as our own sequencing data. Only those amino acid substitutions that differentiate the C57BL/6J and A/J strain sequences and that result in a significant change of physicochemical properties are shaded; black shading was used on the most drastic changes. The six amino acid residues that are specific for the A/J Birc1e protein are highlighted in yellow. Red (BIR), blue (NACHT) and green (LRR) shading of the C57BL/6J sequence depict conserved protein domains. These protein domains were detected using the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The three Baculoviral Inhibition of apoptosis protein Repeat domains (BIR; smart00238) are found in inhibitor of apoptosis proteins (IAPs) and can act as a direct inhibitor of caspase enzymes. The NACHT domain (pfam05729) is named after some of the proteins in which it is found: NAIP, CIITA, HET-E and TP1 (Koonin and Aravind 2000). This putative NTPase domain is found in proteins with very diverse functions. The LRR domain found in Birc1 proteins is a leucine-rich repeat (LRR) of the ribonuclease inhibitor (RI)-like subfamily (cd00116). LRRs are present in many proteins that participate in protein-protein interactions. LRRs correspond to structural units consisting of a beta strand (LxxLxLxxN/CxL conserved pattern) and an alpha helix. Amino acid residue positions from the starting methionine are indicated above the sequences. As a reference to the genomic structure of the Birc1e gene, exon boundaries are indicated below the protein sequences.


Bircle-B6
Bircle-129
Bircle-FVB
Bircle-A
exon 3
exon 4
exon 5
exon 6
exon 7
exon 8
exon 9
exon 10
exon 11
exon 12
exon13
exon 14
exon 15
exon 16

Consideration of only the six polymorphisms that are specific to the A/J strain is only valid if the non-permissiveness of the FvB and 129X1 strains is due to a resistance allele of the *Birc1e* gene. Indeed, the 129X1 strain is non-permissive (see table 4.1) and its allele of the *Birc1e* gene confers resistance to L. pneumophila as exemplified by the rescuing BAC 227n6 shown in chapter 4. However, the FvB strain has been classified as "permissive" by Wright and colleagues (Wright et al. 2003). In our own work (table 4.1 and figure 4.3), presence of a FvB allele of the *Lgn1* locus was associated with non-permissiveness of transgenic and non-transgenic mice. Since the non-permissive BALB/cJ strain (Yoshida and Mizuguchi 1986; Yamamoto et al. 1988; Yamamoto et al. 1991) was also classified as permissive by Wright and colleagues (Wright et al. 2003), it is likely that differences in the experimental setup and/or the *L. pneumophila* strain used may have caused these differences. We did note, however, that some other region(s) of the FvB genome had the ability to modify the *Lgn1* phenotype. As shown in figure 4.3, a small number of N2-generation mice (25% FvB genome) homozygous for FvB *Lgn1* alleles were rather permissive to *Lgn1* replication. As expected from a modifier gene hypothesis, further backcrossing to the A/J strain to rid the mice from any residual FvB genome effectively gave back to the *Lgn1* locus full control of the *Legionella* replication phenotype (Figure 4.3). As a sidenote, the C3H/HeJ mouse strain was also shown by Wright and colleagues as a permissive strain and assumed to encode for a permissiveness allele of the *Birc1e* gene (Wright et al. 2003). In fact, there is experimental evidence to support that the *L. pneumophila*-permissiveness of the C3H/HeJ strain is due to its Tlr4 LPS signalling protein deficiency and not to its *Lgn1* locus (Yoshida et al. 1991a).

A puzzling observation: the clustered D512G, G514E and N517K substitutions provide A/J with a NACHT domain sequence that is close to the NACHT domain of Birc1b as found in C57BL/6J, FvB and 129X1 (556G, 558E, 561K; see figure 5.2 for the C57BL/6J-derived sequence). Interestingly, the Birc1b protein of the A/J strain reads 556D, 558G, 561N. A cloning artifact seems unlikely as different genomic and cDNA clones have been sequenced and placed in the GenBank database.

The four *Birc1* copies known to be transcriptionally active and to produce full-length transcripts in the genomes of A/J and C57BL/6J are *Birc1a*, *Birc1b*, *Birc1e* and *Birc1f*. The sequence of the four resulting proteins as encoded in the C57BL/6J

genome is shown in figure 5.2. Overall, the sequence of these four Birc1 proteins is extremely well conserved, with the most distant relatives, Birc1f and Birc1b, sharing 79% identity and 83% homology at the amino acid level. The two closest relatives, Birc1e and Birc1f, share 95% identity and 96% homology which is likely due to their recent duplication as supported by the conservation of their genomic structure and intronic sequences (see Figure 5.3). Having identified the *Birc1e* copy as the only *Birc1* transcript capable of controlling *L. pneumophila* replication within mouse macrophages points out that the function of the *Birc1* genes is not redundant despite their sequence similarity. As can be seen in Figure 5.2, there are non-conservative amino acid variations between the mouse Birc1 proteins that could explain their lack of functional redundancy. Which of the few differences between the Birc1e protein and the other mouse Birc1 proteins give it its unique function in bacteriostatic control of *L. pneumophila* infections? A lot of experimentation with chimeras, specific amino acid substitutions and deletions will have to be performed in the near future to thoroughly understand the *Birc1* cluster. Not surprisingly, most of the sequence variation between the different Birc1 proteins lies outside of the conserved protein motifs, where the functional implications of amino acid substitutions are the least obvious right now. A brief survey of the sequence variations that do lie within the conserved protein motifs of the C57BL/6J-derived Birc1 proteins yields the following observations. The first BIR domain is 100% conserved between Birc1e, Birc1f and Birc1a, and the second BIR domain is identical in the Birc1e and the Birc1a proteins. Thus, no BIR1 or BIR2-mediated protein-protein interactions are specific to Birc1e. There is a significant amount of sequence variation within the third BIR domain, however, only one non-conservative substitution sets the Birc1e and closely related Birc1f sequences apart. The Birc1e NACHT domain is 94- to 96% homologous to the NACHT domains of Birc1f, Birc1a and Birc1b. Residue L591 is specific to the NACHT domain of the Birc1e protein and results in a significant physicochemical change with respect to the residues found in the other three Birc1 copies. The LRR domains are riddled with non-conservative amino acid substitutions among the Birc1 copies. Reminiscent of sequence variation in other NBD_LRR proteins that display different specificities for bacterial or other pathogen antigens (Tanabe et al. 2004). There are five residues within the LRR that are specific to the Birc1e copy in the C57BL/6

genome and that result in significant physicochemical changes with respect to the residues found in other Birc1 copies (N1116, R1123, Q1137, T1269 and E1325). Not shown in Figure 5.2, the *Birc1b* copy splices in an extra 132 bp 5' of exon 11 and the rest of the transcript remains in-frame. Thus Birc1b encodes for an extra 44 amino acid residues starting at residue number 389. This Birc1b-specific protein sequence does not represent any known conserved motif.

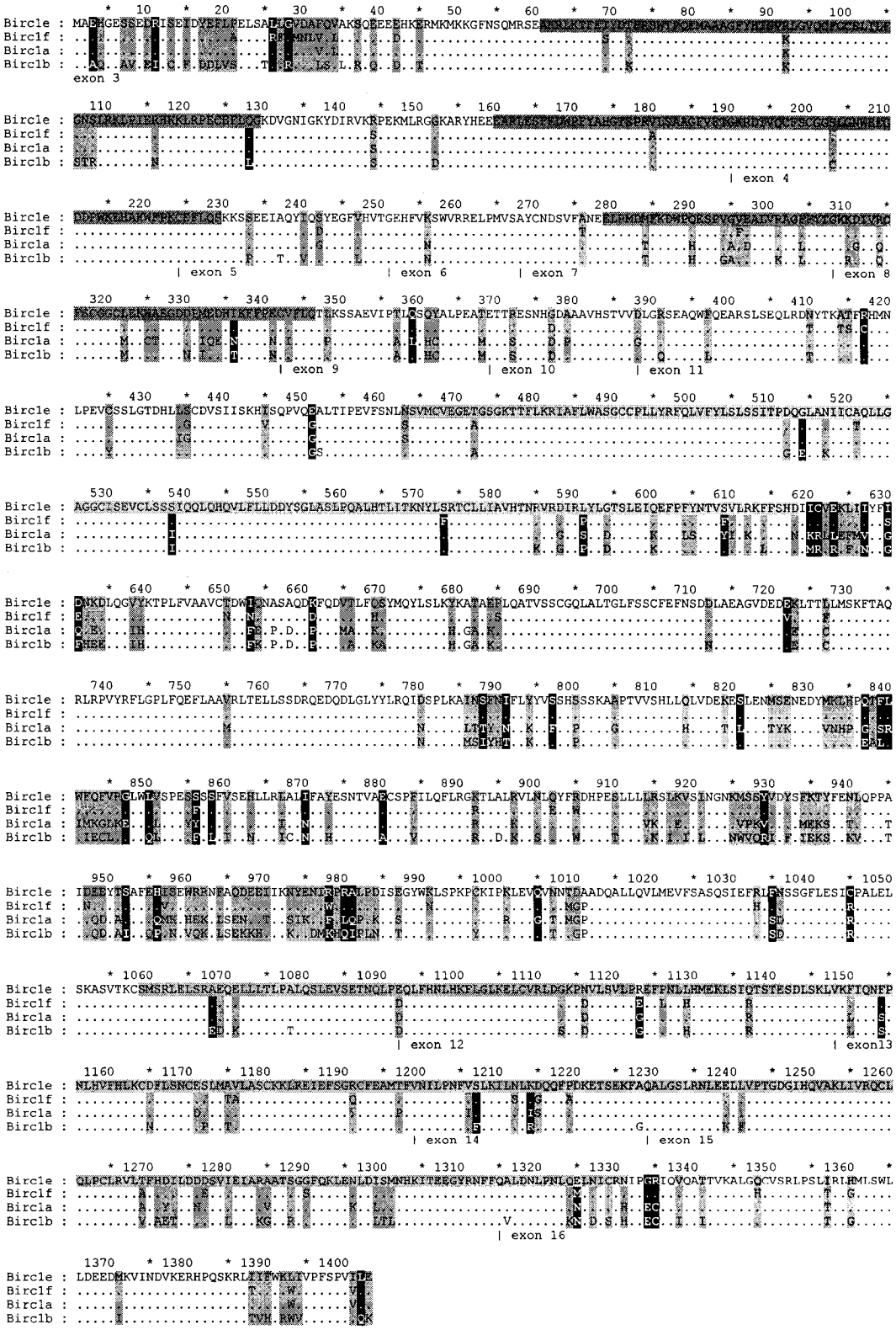
Another source of variation for the Birc1 proteins is the extensive alternative splicing events that have been detected. Alternative splicing in the promoter region of *Birc1b* was carefully documented at a time when *Birc1b* was favored as a candidate for the *Lgn1* locus (Yaraghi et al. 1999). Such alternative use of 5' untranslated sequences was thought to be important for tissue-specific and trigger-specific expression of the Birc1b proteins. Alternative splicing of *Birc1e* 5' sequences has also been detected and should be further documented in the near future to assess its relevance to the *Lgn1* phenotype.

Importantly, there is alternative splicing of coding exons within *Birc1e*. Indeed, although full-length *Birc1e* messages can be detected within mouse macrophages, there are transcripts that lack exon 11 plus a varying number of downstream exons. A Δexon11-12 *Birc1e* transcript sequence has been submitted to GenBank (AF135493). We have also detected *Birc1e* transcripts that lack exons 11 through 15. Interestingly, this observed failure to splice-in exon 11 (a partial failure as full-length transcripts are also produced) seems specific to the *Birc1e* locus. An explanation for this unique feature of the *Birc1e* gene can be observed when the genomic sequence of the *Birc1* genes is aligned (Figure 5.3). Duplication of the mouse *Birc1* genes, particularly between *Birc1e* and *Birc1f*, is believed to have occurred recently (Endrizzi et al. 2000) and is clearly reflected in the conservation of intronic sequences between these loci (Fig.5.3). One obvious *Birc1e*-specific feature (see Figure 5.3) is the integration, between exons 10 and 11, of a 3500 bp long terminal repeat (LTR) element belonging to an endogenous retrovirus (ERV-class I) as detected with the RepeatMasker software at http://repeatmasker.org. This large retroviral integration may cause the observed partial failure to splice-in exon 11. I tested whether the presence of such splice variants could be correlated with the *Lgn1* phenotype: however both the A/J and C57BL/6J mouse strains

FIGURE 5.2

Birc1 protein sequences in the C57BL/6J mouse strain.

Amino acid sequences are shown here for the four Birc1 copies known to produce full-length transcripts in the C57BL/6J mouse strain. The entire C57BL/6J-derived Birc1e sequence (Birc1e; based on GenBank AAN60207) is shown in the first line. For the sequences in lines two, three and four only those residues that are different from the Birc1e sequence are shown. Line two represents the Birc1f sequence (Birc1f; GenBank AAN77617). Line three represents the Birc1a sequence (Birc1a; based on GenBank Q9QWK5) and line four represents the Birc1b sequence (Birc1b; GenBank AAN77585). All of these Birc1 sequences were double-checked against the Celera SNP database as well as our own sequencing data. Only those amino acid substitutions that result in a significant change of physicochemical properties with respect to the Birc1e sequence are shaded; black shading was used on the most drastic changes. The 20 non-conservative amino acid substitutions that are specific for the Birc1e protein are highlighted in yellow. Conserved protein domains are depicted exactly as in Figure 5.1. Amino acid residue positions from the starting methionine are indicated above the sequences. As a reference to the genomic structure of the Birc1e gene, exon boundaries are indicated below the protein sequences.


Bircle
Bircl1f
Bircla
Circlb
exon 3
exon 4
exon 5
exon 6
exon 7
exon 8
exon 9
exon 10
exon 11
exon 12
exon13
exon 14
exon 15
exon 16

express Δexon11 *Birc1e* transcripts (not published). Nevertheless, a thorough understanding of *Birc1e* function might not be achieved without exploring the role of these *Birc1e* proteins that lack both the NACHT conserved domain and parts of the leucine-rich repeats.

5.1.2 Retrospective look at the positional cloning of *Lgn1*.

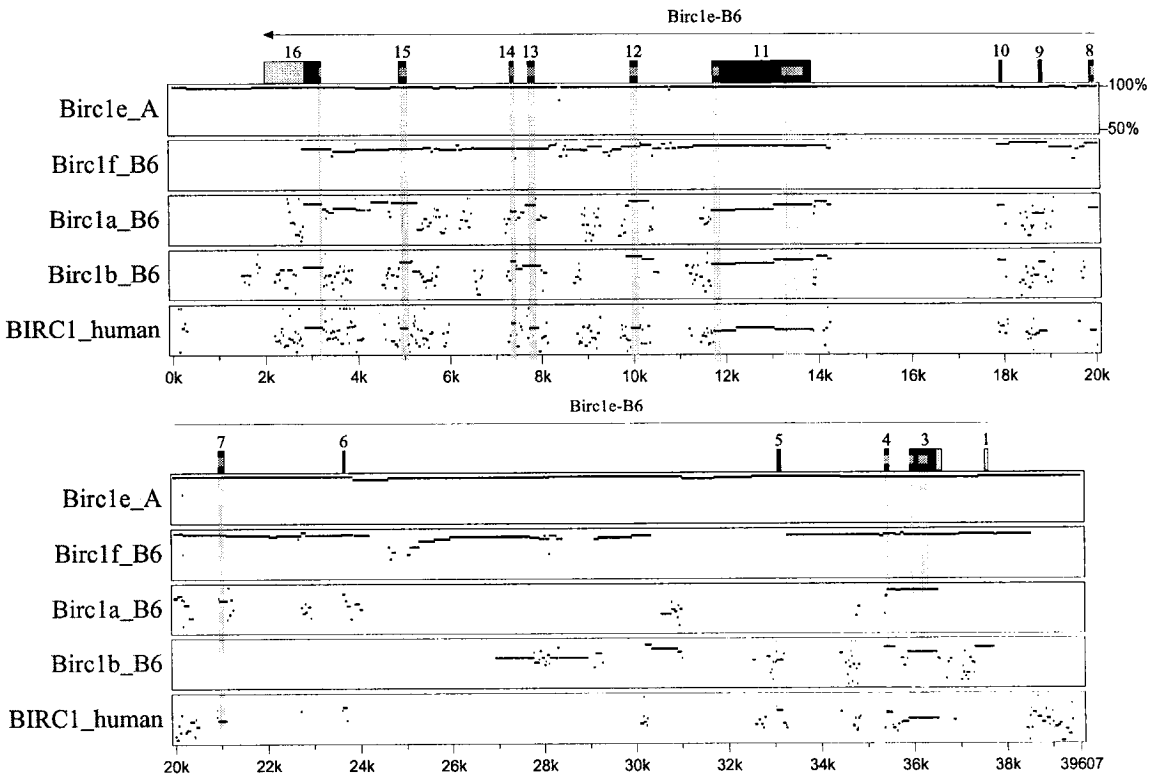
The (A×B) and (B×A) recombinant-inbred strains (RIS) were an effective tool for assigning a chromosomal position to the *Lgn1* locus. However, the effectiveness of such small sets of informative meioses for mapping genetic loci is highly dependent on the density of genetic markers for which a strain distribution pattern (SDP) has already been determined. Specifically, it is only after a substantial number of "Mit" genetic markers were mapped onto the A×B / B×A RIS that an unequivocal assignment of the *Lgn1* locus to distal mouse chromosome 13 was obtained (Beckers et al. 1995; Dietrich et al. 1995).

Also, generation of 1270 ABA backcross progeny proved highly effective for the high-resolution genetic mapping of the *Lgn1* locus (Beckers et al. 1997; Diez et al. 1997). At the time, genetic maps covering distal mouse chromosome 13 provided only a low resolution view of the region and were rather inaccurate. It is for that reason that backcross progeny were typed for many genetic markers (such as *D13Sel1*, *D13Mit108*, *D13Mit111*, *D13Mit110*, *D13Mit287*, *D13Mit112*, *D13Mit71*, *D13Mit47*, and the genes *Dhfr*, *Hexb*, *Gpcr18*, *Htr1*, *Ctla3*, *Itgal* and *Itga2*) that did not help to narrow down the previously identified *Lgn1* genetic interval between *D13Mit128* and *D13Mit70*. It is also for that reason that non-informative BSB and BSS backcross sets had to be used to order genetic markers with respect to each other when no polymorphism between A/J and C57BL/6J could be detected. Also, in an effort to identify a reasonable number of genetic markers within the *Lgn1* genetic interval to isolate genomic clones that would span the entire region, several B1 repeat-derived *Hun* markers were characterized. Unfortunately, both the repetitive nature of the *Hun* markers as well as the fact that most of them were specific to the *Spretus* species, made those markers inappropriate for screening YAC or BAC genomic clone libraries derived from either the C57BL/6J or 129 mouse strains. In

FIGURE 5.3

Percent-identity plots of *Birc1* genomic sequences.

The genomic region covered by mouse and human Birc1 genes is about 40 Kb in length. Alignment and graphical presentation of such large genomic sequences was achieved here with the PIPMAKER web-based software (Schwartz et al. 2000). The C57BL/6J-derived *Birc1e* genomic sequence (Mus musculus chromosome 13 genomic contig, strain C57BL/6J; GenBank NT_039590; bp. 8 398 772 to bp. 8 438 378) was used as the reference sequence to which other Birc1 sequences were aligned. Numbered boxes indicate the position of exon sequences within the C57BL/6J-derived *Birc1e* genomic sequence. Coding sequences are represented by black shading of the boxes. An arrow above the boxes indicates the direction of transcription for the *Birc1e* gene. The position of sequences encoding for conserved protein domains is shown with colored boxes (BIR in red, NACHT in blue, LRR in green). The five histograms indicate the percent identity between each of the indicated *Birc1* sequences and the C57BL/6J-derived *Birc1e* sequence. From top to bottom: A/J derived *Birc1e* genomic sequence (NT_039593 bp. 59 139 to bp. 98 271), C57BL/6J-derived *Birc1f* (NT_039590 bp. 8 470 214 to bp. 8 507 687), *Birc1a* (NT_039590 bp. 8 558 628 to bp. 8 601 378) and *Birc1b* (NT_039590 bp. 8 328 987 to bp. 8 393 114) sequences, and human BIRC1 sequence (NT_078018 bp. 152 843 to bp. 213 034). These histograms are called percent identity plots (PIP). A pip shows the position in one sequence of each aligning gap-free segment and plots its percent identity. Only percent identities above 50% are plotted. The scale of these graphic representations of the *Birc1* genomic sequences is indicated below the histograms in base pairs.


Birc1e-B6
16
15
14
13
12
11
10
9
8
100%
50%
Birc1e_A
Birc1f_B6
Birc1a_B6
Birc1b_B6
BIRC1_human
0k
2k
4k
6k
8k
10k
12k
14k
16k
18k
20k
Birc1e-B6
7
6
5
4
3
1
Birc1e_A
Birc1f_B6
Birc1a_B6
Birc1b_B6
BIRC1_human
20k
22k
24k
26k
28k
30k
32k
34k
36k
38k
39607

recent years, genomic clone libraries (mostly BAC clones) have been systematically screened for their STS content and ordered into contigs that can be browsed by any researcher through the web. It is therefore not necessary to screen genomic libraries directly for clones containing markers closely associated with the gene of interest. These BAC contigs have also resulted in accurate positioning of all *Mit* genetic markers with respect to each other.

Right now, with the mouse genome fully sequenced, finding genetic markers within most genomic regions is easier, their positioning is mostly unequivocal, and the possibility of using already identified SNPs as genetic markers has greatly increased marker density for high resolution genetic mapping. It should be noted also that the early availability of a full human genome sequence coupled to comparative mapping facilitated greatly the identification of transcripts within the minimal *Lgn1* genetic interval. Techniques such as exon trapping or cDNA selection that were helpful in identifying candidate genes for loci such as *Nramp* (Vidal et al. 1993) or *Lps* (Qureshi et al. 1999a) were therefore not performed in our positional cloning project. It is not an overstatement to say that recent access to complete mouse genomic sequences has completely revolutionized positional cloning efforts.

In the genetic and physical mapping work described in chapter 2 of this thesis (Fig. 2.2), the *Lgn1* physical interval, delimited proximally by *D13Die6* and distally by *D13Die3*, was determined to be between 125 Kb (size of strain 129X1-derived BAC clones 152p21 and 180i15 entirely comprised between markers *Die6* and *Die3*) and 350 Kb in size (size of C57BL/6J-derived YAC clone 188a1 that contains both markers *Die6* and *Die3*). Now, with genomic sequences available and detailed BAC contig information, those size predictions can be verified. In the C57BL/6J and the similar A/J genomes the distance between *D13Die6* and *D13Die3*, delimiting the *Birc1* array, is indeed of approximately 330 Kb (see Fig. 4.1). However, in the 129X1 genome, the size of the *Birc1* array is of approximately 520 Kb due to a recent gene duplication event that yielded two extra *Birc1* copies. Fortunately, in our 1997 publication (Diez et al. 1997), we did discuss the possibility that the organization of the *Lgn1* region, in particular the *Naip* repeated unit, could be different in the two strains. One more inaccuracy in the physical map depicted in Figure 2.2 can be pointed out now. Confirming a possibility

that was discussed in chapter 2, the two-allele system that was detected by SSLP for markers *D13Mit37* and *D13Die7* did not enable us to distinguish all of the marker copies that do exist within the *Birc1* array. We now know that BAC clones 227n6 and 26f17 do not overlap even though they contain *D13Mit37* and *D13Die7* copies that yield PCR products of the same length. In fact, there is a 210 Kb gap between those two BAC clones, corresponding to the distance between *Birc1e* exon 3 (BAC 227n6 SP6 end) and *Birc1f* exon 16 (BAC 26f17 T7 end, AF193024) in the 129X1 mouse strain genome. It is for this reason that the larger physical size of the *Lgn1* minimal interval in the 129X1 genome was not predicted from the map in Figure 2.2.

Figure 3.1 shows the mouse *Birc1* (*Naip*) array as consisting of 6 copies. Now we know with certainty that the C57BL/6J and A/J genomes contain 5 full-length *Birc1* copies (proximal to distal: *Birc1b*, *Birc1e*, *Birc1f*, *Birc1c* and *Birc1a*) and that the 129 mouse genome contains 7 copies (proximal to distal: *Birc1b*, *Birc1e*, *Birc1g*, *Birc1d*, *Birc1f*, *Birc1c* and *Birc1a*). It was mentioned in the figure legend to figure 3.1 that the only *Birc1* copies for which the genomic position was certain were *Birc1b* and *Birc1e*; *Birc1b* being the most closely linked to *Smn*, followed distally by *Birc1e*. Indeed, those were the only two copies that were shown in the correct order (see Figure 4.1).

In chapter 3, we were able to show that mouse *Birc1* genes are indeed expressed within macrophages. RT-PCR has been the most fruitful technique thus far to study copy-specific *Birc1* expression. When the analysis of RT-PCR product sequencing data was performed for the year 2000 publication, complete sequences were only available for the *Birc1a* and *Birc1b* copies. Moreover, a correspondence between exon 3 sequences (named *Naip* exon5-related sequences at the time) published by Scharf and colleagues (Scharf et al. 1996) and exon 1 and exon 5 (named exon 4 at the time) sequenced by Yaraghi and colleagues (Yaraghi et al. 1998) had not been determined with certainty. With the entire C57BL/6J and A/J genome sequences now available, the RT-PCR sequences collected in 1999 and additional RT-PCR reactions that were carried out after the manuscript was submitted for publication can be reanalyzed with ease. The results are shown in Fig. 5.4. As stated in the original manuscript (Chapter 3), about half of the *Birc1* mRNAs expressed in mouse macrophages are indeed derived from the *Birc1b* (*Naip2*) copy. However, some sequences that had few mismatches with the published

Birc1a sequence (96% to 97% sequence identity) were labelled as such when in fact they are clearly *Birc1f*-derived. This makes *Birc1f* a relatively abundant transcript in macrophages (about 20% of *Birc1* mRNAs) while only about 10% of *Birc1* mRNAs are derived from *Birc1a*. Additionally, clones originally labelled as *Birc1c*-derived (94% to 99% sequence identity) are in fact *Birc1e* sequences. The apparent lack of *Birc1c* expression in mouse macrophages is in agreement with other reports (Huang et al. 1999; Yaraghi et al. 1999). Three clones that did not match the available *Birc1a*, *Birc1b* and *Birc1c* sequences were labelled by default as *Birc1d*-, *Birc1e*- and *Birc1f*-derived copies in the original report. These clones are clearly derived from the *Birc1e* copy in this revision of the data. *Birc1e* is therefore the second most abundant *Birc1* transcript expressed in C57BL/6J inflammatory peritoneal macrophages. A comparison of the *Birc1* expression data obtained for C57BL/6J and A/J macrophages shows that the relative expression of *Birc1b*, *Birc1a* and *Birc1f* is equivalent in the two strains (only 5- to 8% higher relative expression in the A/J strain). However, *Birc1e* expression (1/20 clones in A/J, 6/24 clones in C57BL/6J) is significantly lower in the A/J strain (20% lower relative expression in the A/J strain).

Lower relative expression of the *Birc1e* gene in the A/J strain would automatically translate into the slightly higher relative expression of the other three *Birc1* genes that was observed. Therefore, further transformation of the data shown in Figure 5.4B is possible. Under the assumption that *Birc1b*, *Birc1a* and *Birc1f* absolute expression is equal in both mouse strains, the percent relative expression figures for those genes in the A/J strain can be homogeneously reduced by a factor (0.79) that makes their sum (95%) equal to the sum of the C57BL/6J percentages for the same three genes (75%). Such transformation of the percentages obtained for the A/J strain would yield the following values which can be thought of as a percentage of total *Birc1* mRNA expression in the C57BL/6J strain: 43% *Birc1b*, 12% *Birc1a*, 20% *Birc1f*, and 4% *Birc1e*. This transformation of the data arbitrarily brings closer the expression values for *Birc1b*, *Birc1a* and *Birc1f* in the two mouse strains (within a 3- to 7% difference). But, most importantly, it would indicate that A/J macrophages express 6 times less *Birc1e* transcripts than C57BL/6J macrophages, and that the total amount of *Birc1* transcripts present in A/J macrophages is 79% of that found in C57BL/6J macrophages.

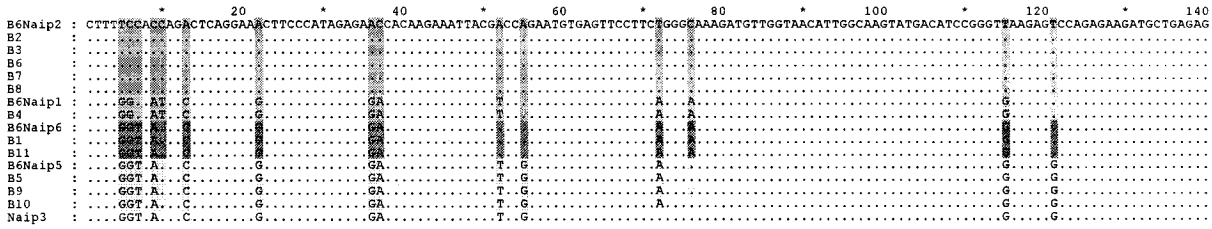
FIGURE 5.4

RT-PCR analysis of *Birc1* mRNA expression in mouse macrophages.

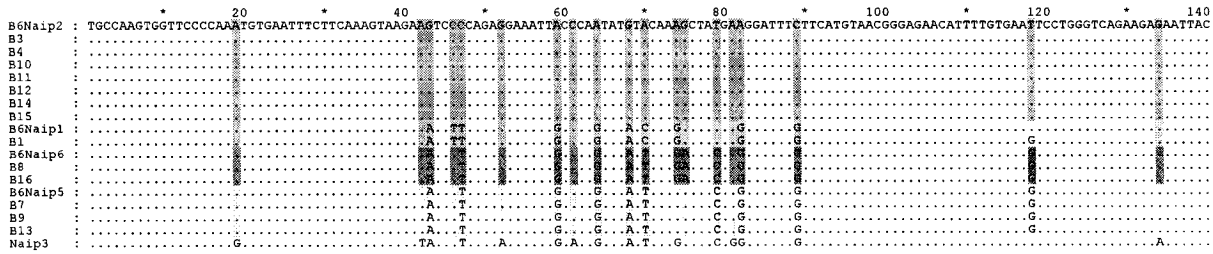
A, Alignment of sequenced clones from *Birc1* exon 3 and exon 5 RT-PCR amplification products. cDNA synthesis and PCR amplification conditions were as previously described (Chapter 3). Exon 3 sequences were PCR-amplified using the primer pair (exon 3F, 5'-GCTCTAGATCATGGACGCCACAGGAGATG-3'; exon 3R, 5'-CCGCTCGAGATGTCCCATGGGCATAAAATGGC-3'). Exon 5 sequences were PCR-amplified using the primer pair (forward primer on exon 4, 5'-GCTCTAGAGTAAAAGGGACACTGTGCAG-3' and a reverse primer on exon 6, 5'-CCGCTCGAGTAATTCTCTTCTGACCCAGG-3'). These primer pairs have been verified to correspond to *Birc1* sequences that are neither copy- nor strain-specific. Amplification products were gel-purified and subcloned in plasmid vector pBluescript, using restriction enzyme sites included in the oligonucleotide primers (underlined). The nucleotide sequence of independent clones from each PCR amplification (named on the left of the alignment) was determined and used to identify the *Birc1* transcripts expressed in macrophages according to diagnostic sequence polymorphisms in exons 3 and 5 unique to each *Birc1* copy (highlighted with distinct colors). Residues identical to the sequence shown at the top of the list (*Birc1b/Naip2*) have been represented with a dot.
B, Analysis of RT-PCR product sequences in terms of relative *Birc1* copy expression. The fraction of all sequenced clones matched to a specific *Birc1* copy is indicated for each of the exon 3 and exon 5 RT-PCRs that were performed. In the rightmost two columns, results from both exons were compiled and the resulting fractions are also displayed as percentages (the percentage of all sequenced RT-PCR-derived clones that were derived from a specific *Birc1* copy).

A

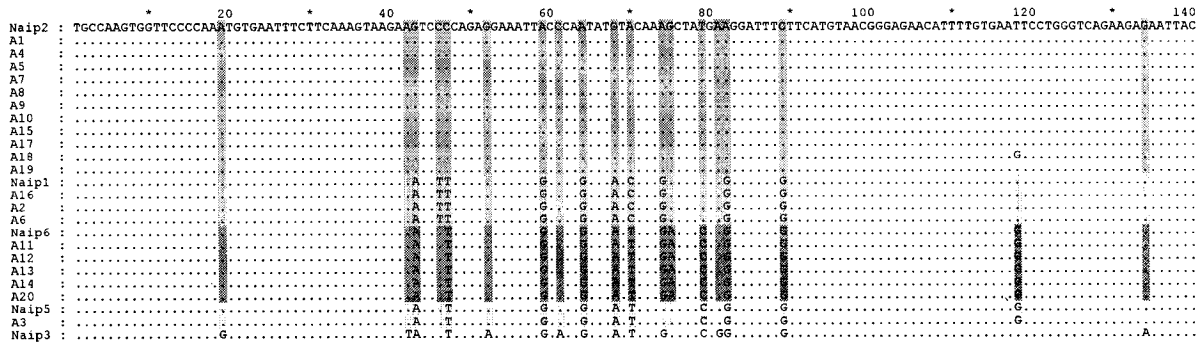
Exon3 C57BL/6J



Exon5 C57BL/6J



Exon5 A/J



B

	Exon 3	Exon 5		Total	
	B6	B6	A/J	**B6**	**A/J**
Birc1b	5/11	7/13	11/20	12/24 = **50%**	11/20 = **55%**
Birc1a	1/11	1/13	3/20	2/24 = **8%**	3/20 = **15%**
Birc1f	2/11	2/13	5/20	4/24 = **17%**	5/20 = **25%**
Birc1e	3/11	3/13	1/20	6/24 = **25%**	1/20 = **5%**

Unfortunately, the assumption of equal absolute expression of the *Birc1b*, *Birc1a* and *Birc1f* genes in both mouse strains might be wrong: our own Northern blot results shown in Figure 3.2 indicate total *Birc1* mRNA levels in A/J macrophages to be approximately 2.5 times lower than in C57BL/6J macrophages. This raises the possibility that a comparison of absolute expression levels might be beyond the scope of our relative expression data. We have not produced any additional quantitative data pertaining to the difference in *Birc1e* mRNA expression between A/J and C57BL/6J macrophages. Our Western blotting data using an antibody that recognizes all Birc1 copies showed a four-fold lower protein expression level in A/J macrophages relative to C57BL/6J macrophages. It was impossible to know, however, which of the Birc1 copies is/are differentially expressed. Wright and colleagues have attempted to produce a Birc1e-specific antibody. Although their α-Birc1e antibody crossreacts with Birc1f, it is worth mentioning that a significantly reduced immunoreactive signal was observed on A/J macrophage-derived protein preparations compared to C57BL/6J (Wright et al. 2003). A Birc1b-specific antibody produced equivalent signals in both protein samples (Wright et al. 2003). Therefore, considering this protein expression data as well as our RT-PCR expression data, it cannot be excluded that a mutation in a regulatory region of the *Birc1e* gene (usually within the 5' untranslated region of the mRNA) rather than, or in addition to, a missense mutation amongst the ones described in section 5.1.1 might be responsible for A/J macrophage permissiveness to intracellular replication of *L. pneumophila*.

Section 5.2

Future experiments

A lot of further research will be necessary to fully understand the mechanism by which mouse macrophages control intracellular *L. pneumophila* replication. Identification of the *Birc1e* gene as a candidate for the *Lgn1* locus was one important step in the process. This section will propose some experiments that could be performed in the near future as a continuation of the *Lgn1* research project. The experiments in the first subsection are aimed at testing further the candidacy of the *Birc1e* gene for the *Lgn1* locus. The second subsection proposes experiments to elucidate the molecular function of the cloned *Lgn1* gene.

5.2.1 Future confirmation of *Bircle* candidacy

The candidacy of the *Bircle* gene for the *Lgn1* locus has been strongly supported by our BAC transgenesis experiments. This section will propose some experiments that would test further the candidacy of *Bircle*. Gene-targeting and cDNA transgenesis represent some of the elegant experiments available, although at a high price in time and resources. Combining more than one of the methods described will identify the *Lgn1* gene firmly and beyond any reasonable doubt.

5.2.1.1 Study of K.O. mice

One important method available for verifying that the *Bircle* gene is indeed responsible for controlling intracellular replication of *L. pneumophila* is to disrupt the gene so that no functional Bircle protein is produced in an otherwise non-permissive mouse strain. This procedure is known as gene knockout, and would result in a *Bircle* knockout (KO) mouse. Functional studies of the *Bircle* gene would greatly benefit from the creation of such a mutant animal, especially true as no obvious loss-of-function mutations have been found to differentiate the A/J-derived allele of the *Bircle* gene from that of non-permissive mouse strains.

The technique of gene targeting takes advantage of the phenomenon known as homologous recombination. Cloned copies of the target gene are altered to make them nonfunctional and are then transfected into continuously growing lines of embryonic stem cells (ES cells) where they recombine with the homologous gene in the cell's genome, replacing the normal gene with a nonfunctional copy.

It should be noted that to knock out a gene *in vivo*, it is only necessary to disrupt one copy of the cellular gene in an ES cell. Once ES cells heterozygous for a knockout mutation in the gene of interest are obtained, they are injected into a recipient mouse blastocyst (early mouse embryo), which subsequently is transferred into the uterus of a surrogate pseudopregnant mouse. The cells carrying the disrupted gene become incorporated into the developing embryo, which results in chimeric offspring. These

chimeras contain tissues, including those of the germline, derived from both the transplanted ES cells and the host cells. The mutated gene can therefore be transmitted to some of the offspring of the original chimera. Finally, mating mice, each heterozygous for the knockout allele, will produce progeny homozygous for the knockout mutation and thus lacking expression of that particular gene product. The effects of the absence of the gene's function can then be studied.

Because the most commonly used ES cells are derived from the 129 strain of mice, should it be preferred to perform the analysis of the function of the disrupted *Bircle* gene in another mouse strain (say C57BL/6J), this would require extensive back-crossing to the alternative strain. After sufficient backcrossing, the mice would be intercrossed to produce homozygous mutants on a stable genetic background.

A problem with gene knockouts arises when the function of the gene is essential for the survival of the animal; in such cases the gene is termed a recessive lethal gene and homozygous animals cannot be produced. To circumvent this problem, there is a powerful technique that achieves tissue-specific or developmentally regulated gene deletion. This is achieved by employing the DNA sequences and enzymes used by bacteriophage P1 to excise itself from a host cell's genome. Integrated bacteriophage P1 DNA is flanked by recombination signal sequences called loxP sites. A recombinase, Cre, recognizes these sites, cuts the DNA and joins the two ends, thus excising the intervening DNA in the form of a circle. This mechanism can be adapted to allow the deletion of specific genes in a transgenic animal only in certain tissues or at certain times in development. First, loxP sites flanking a gene, or perhaps just a single exon, are introduced by homologous recombination. Mice containing such loxP mutant genes are then mated with mice made transgenic for the Cre recombinase, under the control of a tissue-specific or inducible promoter. Mating of these two mice will yield progeny that carry the gene of interest modified by insertion of flanking lox P sites and the *cre* gene controlled by a cell-type-specific promoter. In these mice, recombination between the loxP sites, which disrupts the gene of interest, will occur only in those cells in which the Cre promoter is active. Thus, for example, using a macrophage-specific promoter to drive expression of the Cre recombinase, a gene could be deleted only in macrophages, while remaining functional in all other cells of the animal. There are several macrophage-

specific promoters known; the macrophage colony-stimulating factor 1 (CSF-1) (Hume et al. 1997; Himes et al. 2001) and the scavenger receptor A (Wilson et al. 2001) promoters are examples of genes whose promoters have been successfully used to confer macrophage-specificity to the expression of other genes. Although the A/J strain can be putatively thought of as a viable *Birc1e* KO, systematic adoption of the Cre-lox strategy to disrupt the *Birc1e* gene would prevent an unforeseen problem of recessive lethality from jeopardizing the chances of success of this long and expensive process.

Having obtained mutant mouse macrophages with a functional defect, the defect can be ascribed definitively to the mutated gene if the mutant phenotype can be reverted with a copy of the normal gene transfected into the mutant mouse. Restoration of function means that the defect in the mutant gene has been complemented by the normal gene's function. In addition, the parts of the gene that are essential for its function can be identified by determining whether function can be restored by introducing different mutated copies of the gene back into the genome by transgenesis. The general unsuitability of the only known *Lgn1*-defective mouse strain (A/J) to genetic manipulation and direct transgenesis significantly increases the motivation to produce a *Birc1e* KO mouse.

A *Birc1a* (*Naip1*) KO mouse has been created (Holcik et al. 2000). Although we have shown *Naip1* mRNA expression to be primarily directed at the central nervous system (Yaraghi et al. 1999), *Naip1* transcripts can also be found within mouse macrophages (Huang et al. 1999; Diez et al. 2000). Therefore, not having formally ruled out the candidacy of the *Naip1* gene for *Lgn1*, I was allowed to phenotype macrophages derived from these KO mice with respect to *L. pneumophila* permissiveness (special thanks to Dr. A. MacKenzie, Dr. R. Korneluk and N. Gendron for their help). *Naip1* knockout mice turned out to be just as resistant to intracellular *L. pneumophila* replication as the parental mouse strain 129X1 (results not published). Briefly, elicited peritoneal macrophages from two *Naip1* KO mice and three parental strain 129 controls were infected *ex vivo* with *L. pneumophila* at an MOI of 2. The difference in CFUs collected from the infected monolayers after one hour of phagocytosis and a further 72 hours of intracellular replication was of 0.65 ± 0.34 log on average for the *Naip1* KOs, similar to the 0.34 ± 0.31 log obtained for the control mice. As published by our

collaborators, *Naip1*-deleted mice develop normally; however, the survival of pyramidal neurons in the hippocampus after kainic acid-induced limbic seizures is greatly reduced in these animals (Holcik et al. 2000). These studies were obviously performed in the mind frame of a putative role for the Naip proteins in modifying the severity of the spinal muscular atrophy phenotype. Thus, although Naip1 was shown not to be necessary for normal development of the murine central nervous system, endogenous Naip1 can be deemed to be required for neuronal survival in pathological conditions. At about the same time, detailed genetic analysis of the mouse *Naip* cluster formally segregated *Naip1* from the *Lgn1* critical interval (Growney and Dietrich 2000). By leaving only *Naip2* and *Naip5* as candidates for *Lgn1*, Growney and colleagues severely limited the possibility that the mouse *Naip* copies, although very closely related at the sequence level, might have redundant functions.

5.2.1.2 Post-transcriptional gene silencing studies

In addition to the possibility of knocking out gene function at the DNA level, transient inactivation of expression can be achieved for specific genes. Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene. Such effects have been proposed to result from an antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. Antisense inhibition of *Birc1e* has already been performed by a group of researchers with reasonable success (Wright et al. 2003). A 25-nucleotide long antisense morpholino oligo was designed to anneal to the 5' UTR of *Birc1e*, just prior to the initiating methionine. Morpholino oligos have substitutions of the riboside moieties with nitrogen-containing morpholine moieties and are phosphorodiamidate linked (Summerton and Weller 1997). These oligos have become popular for performing antisense experiments. One of the reasons for their popularity is that the nuclease-resistant morpholino backbone increases significantly the half-life of the oligo (Summerton and Weller 1997; Heasman et al. 2000). One problem that was encountered by Wright and colleagues remains difficult to avoid: their antisense oligo

can potentially inhibit the translation of *Birc1f* which is very closely related to *Birc1e* (Growney et al. 2000). For their assays, differentiated primary macrophages were treated with the morpholino oligos and the cells were then allowed to recover overnight before the *Legionella* infections were performed (Wright et al. 2003). Since their own BAC transgenic lines exhibited partial rescue of the permissiveness phenotype, it was reasoned that it would be a sensitized background in which an effect of the morpholino treatment could be observed, even if the translational inhibition was incomplete (no expression data was shown). Macrophages from their FvB-N5BAC line that were pretreated with morpholino had higher bacterial yields than untreated control macrophages at 72 and 120 hr post-infection. This relative increase in permissiveness was somewhat diminished by 144 hr post-infection, suggesting that the inhibitory effect of the morpholinos is transient. More importantly, supplemental data with C57BL/6J macrophages suggested that treatment with this morpholino can also modestly increase permissiveness in this nonpermissive background (Wright et al. 2003). In conclusion, their *Birc1e* antisense data did reinforce *Birc1e* as being the gene within their BAC transgenics that is able to provide resistance to *L. pneumophila*. However, their data showed only partial rescue of resistance phenotypes at best. The partial rescues observed could be accounted by a partial and transient inhibition of *Birc1e* translation by their morpholino reagent.

Recently, it has been demonstrated that dsRNA can be a substantially more efficient inducer of post-transcriptional gene silencing (PTGS) than sense or antisense RNA alone (Fire et al. 1998). Microinjection of dsRNA into the gut of *C. elegans* triggered highly specific silencing of the targeted endogenous genes. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stoichiometric interference with endogenous mRNA and suggesting that there could be a catalytic or amplification component in the interference process (Fire et al. 1998). Later studies determined that dsRNA-induced silencing occurs post-transcriptionally, through degradation of homologous mRNA (Montgomery et al. 1998). Interestingly, the effects of this interference were evident in both the injected animals and their progeny (Fire et al. 1998). A highly efficient induction of PTGS by direct production of dsRNA, termed RNAi, was subsequently demonstrated in plants through expression of inverted-repeat transgenes and through simultaneous expression of sense

and antisense transgenes (Waterhouse et al. 1998). Several groups have now reported near-absolute suppression of various endogenous and transgene mRNA species in >90% of transformants by the expression of complementary gene sequences separated by a nonhomologous linker region or a spliceable intron (Chuang and Meyerowitz 2000; Smith et al. 2000). The stem-loop or hairpin RNAs resulting from transcription of these constructs must possess a dsRNA region of at least 100 nt to efficiently induce PTGS in plants, which likely explains why the limited secondary structure of endogenous mRNAs does not trigger silencing (Wesley et al. 2001). The exceptional potency and efficiency of dsRNA as an inducer of PTGS was recently exemplified by the systematic functional analysis of *C. elegans* chromosomes I and III through feeding studies with dsRNA-expressing *E. coli* bacteria (Fraser et al. 2000; Gonczy et al. 2000).

These recent advances in post-transcriptional gene silencing could lead the way to more convincing *Bircle*-silencing data. For the moment, the only way to deliver dsRNA into cells seems to be through microinjection. Therefore, it would not seem practical to introduce interfering dsRNA into large numbers of macrophages just before a *L. pneumophila* infection as was done with the morpholino oligos. Should proper transfection protocols not become available soon, two choices come to mind. First, interfering dsRNA could be directly injected into a mouse oocyte thus creating a mouse that can provide the researcher with many macrophages lacking expression of *Bircle*. According to the studies in *C. elegans* and in plants, the progeny from such a mouse would also lack *Bircle* expression, thus creating an unlimited supply of macrophages for infection studies. Second, a construct that encodes for an interfering dsRNA could be either transfected into macrophage cultures or microinjected into a mouse oocyte thus effectively creating a transgenic mouse lacking expression of *Bircle*. Although these alternatives make for longer experimental setups than morpholino oligo treatments, they do have interesting advantages. Unlike morpholino treatment, gene silencing by dsRNA seems to be permanent and complete.

5.2.1.3 *Bircle*-transgenic mice

Birc1e is the only full-length gene encoded by both of the BAC clones that showed rescue of the *L. pneumophila* resistance phenotype in our experiments. This, however, did not provide irrefutable proof that the predicted full-length *Birc1e* mRNA is indeed responsible for controlling the *Lgn1* phenotype. Three alternative scenarios should be considered, arguing that further experiments are needed to test if *Birc1e* is indeed *Lgn1*. The first five exons of *Birc1b* that were also present in both rescuing BAC clones, although coding only for its BIR domains, could still code for a functional *Lgn1* gene. Also, intergenic and intronic regions common to both BAC clones could encode for functional messages that are not detected by current gene prediction software. Another possibility is that alternative splice forms of *Birc1e*, several of which have been detected, could be the ones responsible for controlling the *Lgn1* phenotype instead of the predicted full-length message.

One method for testing the candidacy of *Birc1e* for *Lgn1*, besides the loss-of-function experiments described above, resides in the creation of *Birc1e* cDNA transgenic mice. For this, it is first necessary to clone the *Birc1e* gene from a mouse strain that is known to possess the resistance allele of the *Lgn1* gene: the C57BL/6J strain would be a good choice. Messenger RNA (mRNA) from such a mouse should be isolated and reverse-transcribed into coding DNA (cDNA). The cloned gene can then be microinjected into the oocyte of an appropriate recipient mouse strain. Just as explained for the creation of BAC transgenics in our own work and for the creation of knockout animals, it would not be technically efficient to try to introduce the resistance *Birc1e* cDNA directly into the permissive A/J strain. A strain such as FvB would have to be used again, as it is the most fertile and amenable to genetic manipulation. The FvB strain being relatively resistant to *L. pneumophila* replication, any resulting transgenic progeny would have to be crossed to the A/J strain a minimum of two generations. The resulting transgenic mice would express the C57BL/6J-derived resistance allele of the *Birc1e* gene while being homozygous for endogenous *Birc1e* copies derived from the permissive A/J strain. In this case, the transgene would not encode for any other gene than *Birc1e* and would not include any intergenic or intronic sequences. Specific splice forms of the *Birc1e* gene could also be cloned to answer the third alternative explanation for the BAC transgenesis results that were mentioned above.

It should be noted that following our BAC transgenesis experiments it was not possible to dismiss the possibility that overexpression of any functionally redundant *Birc1/Naip* copy could result in the rescue of a haploid insufficiency phenotype. This possibility has urged us to recognize that although it is clear that *Birc1e* overexpression can render A/J mice non-permissive to *L. pneumophila* replication, *Birc1e* might not be the *Birc1* copy that is mutated in the A/J strain. Such criticism would be encountered again after creating a mouse that overexpresses the *Birc1e* gene specifically. One way to address that issue would be to clone the *Birc1e* allele derived from the permissive A/J mouse strain in parallel with that of the C57BL/6J strain and to take it through the same transgenesis process. The resulting transgenic A/J mouse, overexpressing its own version of the *Birc1e* gene, would clearly define whether the A/J *Birc1e* allele is functional against intracellular *L. pneumophila* replication.

5.2.1.4 *Birc1e*-transfected cell-lines

The process of making *Birc1e* cDNA transgenic mice can be expensive and time-consuming just as was the generation of BAC transgenics. Specially true if, as explained before, A/J-derived *Birc1e* transgenics were made in parallel and that several splice variants needed to be tested. Luckily, the *L. pneumophila*-resistance phenotype that is controlled by *Lgn1* is studied through *in vitro* infection of explanted mouse cells. This fact opens up the possibility of performing *Birc1e* cDNA transfections of cultured cells and to test directly the effect of the introduced cDNA on their *L. pneumophila*-permissiveness phenotype. Such experiments rely on the availability of *L. pneumophila*-permissive cells that would be amenable to transfection. Since primary macrophages have a limited life span in culture (less than one week) only transient transfections should be considered; unfortunately, such cells readily degrade any introduced DNA that does not get incorporated in their genome. Another impediment is that A/J is the only mouse strain that is clearly permissive to *L. pneumophila* replication and there are no commercially available or trustworthy monocyte/macrophage-like cell lines derived from this strain. An alternative *L. pneumophila*-permissive host is the human macrophage, and

there are many human monocytic or macrophage-like cell lines available. HL-60 and U937 are two such cell lines, widely used and known to be good permissive models for the study of *L. pneumophila* replication (see Chap1, section 1.2.2). Immortal HL-60 or U937 cells could be stably transfected and differentiated into macrophages with the phorbol ester PMA just prior to infection with *L. pneumophila*. Alternatively, a human lung epithelial cell line such as WI-26, also accepted as a permissive host for *L. pneumophila* replication (Chap1, section 1.2.2), would be a much easier cell to transfect. In fact, we have successfully overexpressed *Birc1b* protein within stably transfected WI-26 cells using a *Birc1b* cDNA construct under transcriptional control from a CMV promoter (unpublished data). Therefore, *Birc1e* transfections could be performed in this system to investigate their effect on *Legionella* replication.

5.2.1.5 Additional BAC transgenics

Supplemental BAC transgenesis experiments to the ones described in Chapter 4 could strengthen the conclusions that were drawn at the time. For example, only one founder from BAC 164d12 (named 165-1) showed a clear rescue of the *L. pneumophila*-resistance phenotype. That single rescue event was deemed sufficient to demonstrate that the genomic region covered by BAC 164d12 contained a sequence capable of controlling *L. pneumophila* replication. However, two additional founders had been obtained from a second round of microinjections, and the phenotypic data for their backcross progeny was only finalized after the manuscript had been accepted. The two new founders for BAC164d12 were named 254-4 and 254-6. Fig. 5.5A shows the phenotypes of their backcross progeny. Both founders showed clearly that the presence of the C57BL/6J-derived genomic region covered by BAC 164d12 can convert mice that are homozygous for the susceptibility allele of *Lgn1* into non-permissive hosts. Founder 171-2 from the same 164d12 BAC clone was published as showing only a partial rescue (statistically significant) of the *L. pneumophila* non-permissive phenotype. Phenotyping of the progeny from one additional backcrossing of these transgenic mice to the A/J strain to further remove any FvB-derived genetic background was also finalized after the

acceptance of the manuscript. The phenotyping results can be seen in Fig. 5.5B. Again, there was a statistically significant reduction of the permissiveness of mice harboring the BAC clone within their genome, but it remained an intermediate rescue unlike the one seen with founders 165-1, 254-4 and 254-6. This supplemental data further emphasizes that the partial rescue achieved for founder 171-2 was likely due to the low expression of the BAC-encoded transcripts (see Fig. 4.5) rather than the effect of any residual FvB genetic background (a theoretical 25% in N2 mice and only 12.5% in N3 mice).

One more C57BL/6J-derived BAC clone was microinjected and the phenotypic results were only obtained in 2003. The clone was called 148d4 and a graphic representation of the genomic region it covers can be seen in Fig. 4.1. Only one founder was obtained from these microinjections and was named 122-3. Analysis of the STS-content of C57BL6/J origin within the transgenic progeny showed that these mice contained at least one intact BAC clone within their genome. The introduced clones span without apparent internal deletions between *Birc1e* intron 12 to the same NotI restriction site within the *Tfnr* gene that formed the proximal boundary of the BAC 227n6 fragment that had been studied earlier. The interest in using BAC 148d4 for transgenesis resided in that it contained the entire *Serf1*, *Smn* and *Birc1b* genes just like BAC 227n6 did, but it only contained the last four exons of *Birc1e*, which should not produce a functional transcript. As shown in Fig. 5.5A, no rescue of the *L. pneumophila* resistance phenotype was observed in N2 progeny from BAC 148d4 founder 122-3. Lack of rescue from BAC transgenics should not be interpreted as meaning that the genomic region covered by the BAC clone does not contain the gene of interest. Indeed, there are many factors that can prevent a transgenic mouse that contains DNA encoding for a resistance gene allele from showing the resistance phenotype. One of these factors is that randomly integrated BAC DNA can end up within silent genomic regions, thus failing to express the transcripts it encodes. One alternative possibility is that small internal deletions or mutations within the integrated BAC clone, anomalies that STS-content determination would not detect, would abolish expression of a functional "resistance" transcript. Therefore, although this supplemental data does not formally rule out the presence of a *Lgn1* "resistance" transcript corresponding to *Birc1b* or somewhere in the *Birc1b-Birc1e* intergenic region, it does support the candidacy of the *Birc1e* gene for the *L. pneumophila* resistance locus.

It would be possible to perform additional BAC transgenesis experiments to further refine the genomic region capable of transferring a *L. pneumophila* resistance phenotype. Namely, rare-cutter digestion of BAC clones from the *Lgn1* region could yield fragments that would only contain the *Bircle* gene and not any other flanking genes. A rescue event from such transgenic genomic fragment, rather than the intersection of two long rescuing BACs as was the case in our studies, would directly point out to the *Bircle* gene being *Lgn1*. My opinion though, is that resources would be best invested in any of the aforementioned experiments (*Bircle* knockout, post-transcriptional silencing, cDNA transgenesis or transfection for *in vitro* studies).

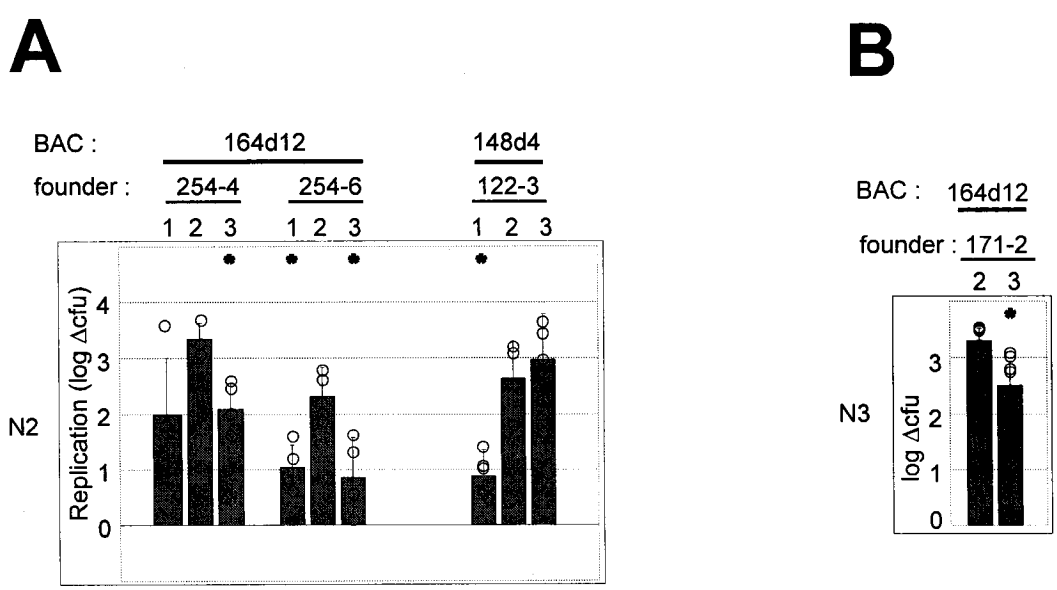
5.2.2 Future *Bircle* functional studies

Although *Bircle* has been proposed in our studies as being responsible for the control of *L. pneumophila* replication within mouse macrophages, the molecular mechanism through which such bacteriostatic control takes place remains unknown. This section thus describes some key experiments that should be conducted to elucidate the Bircle protein mode of action. First, it is appropriate to observe objectively the mechanistic/morphological differences in nascent phagosome maturation between macrophages expressing or not the resistance allele of *Bircle*. It is also possible to test directly the involvement of the Bircle protein in signalling pathways in which it may play a role, based on sequence homology to proteins with known functions.

FIGURE 5.5

Additional transgenesis experiments with BAC clones from the *Birc1* cluster.

A, The identity of the BAC clones used for transgenesis is indicated on top, with the identity of the FVB-BAC founders used for back-crossing to A/J immediately underneath. Backcross ((FVB ×A/J)F1 ×A/J) mice (N2) were genotyped for Chr 13 haplotypes and for the presence of the transgene and were grouped as heterozygotes with respect to the resistance allele at *Lgn1* inherited from FVB (column 1), control homozygotes with respect to the susceptible A/J allele at *Lgn1* lacking the transgene (column 2) or informative BAC transgenics homozygotes with respect to the susceptibility A/J allele at *Lgn1* but harboring the transgene (column 3). Data are the mean ± s.d. (blue histogram) of log ΔCFU for peritoneal macrophages from individual mice (shown as single data points) measured 72 h after infection with *L. pneumophil*a. Significant reduction of *L. pneumophila* replication associated with the presence of the R allele at *Lgn1* (column 1) or with the presence of a complementing BAC (column 3) was determined by one-tailed Student's t-test statistics (asterisk (*) indicates $P < 0.05$).
B, Complementation of *Lgn1* susceptibility by the 164d12 BAC transgene, founder 171-2, was monitored after one additional backcrossing to A/J (N3 generation) and phenotyping of macrophages as indicated in **A**.


A
BAC :
164d12
148d4
founder :
254-4
254-6
122-3
1 2 3 1 2 3 1 2 3
N2
Replication (log Δcfu)
0
1
2
3
4
B
BAC :
164d12
founder : 171-2
2 3
N3
log Δcfu
0
1
2
3

5.2.2.1 Electron microscopy using transgenic macrophages

Electron microscopy (EM) offers unparalleled resolution for the observation of subcellular compartments. Based mainly on their characteristic morphology, organelle interactions can be readily observed without resorting to antigenic marker colocalization studies. Also, bacterial cell morphology, indicative of their growth phase and overall health (Rodgers 1979; Chandler et al. 1980; Rodgers et al. 1980; Rodgers and Davey 1982; Katz et al. 1984; Surgot et al. 1988; Harley et al. 1997), can be viewed best at the great magnifications achieved by EM. It is for these reasons that a good part of the knowledge available on the *L. pneumophila* intracellular life cycle, including the specific steps required for formation of the *L. pneumophila* replicative phagosome, have been obtained through EM studies (Horwitz and Silverstein 1983; Horwitz 1984; Nash et al. 1984; Rechnitzer and Blom 1989; Tilney et al. 2001). Understandably, given the clinical importance of *L. pneumophila* infections, a lot of observations have been done on the replication of the bacterium within human macrophages (Horwitz and Silverstein 1983; Nash et al. 1984; Rechnitzer and Blom 1989). However, for comparative studies it is important to use macrophages that are very closely related at a genetic level yet displaying differential *L. pneumophila* permissiveness. Until now, the most closely related macrophages whose morphology has been studied in parallel during a *L. pneumophila* infection have been permissive, A/J-derived mouse macrophages and non-permissive, BDF_1-derived mouse macrophages (Yamamoto et al. 1992a). There is one important comparative study that has not yet been done: two mouse strains for which it is specifically known that only one gene controls the differential *L. pneumophila* replication observed, namely the permissive, *Lgn1*^{s}, A/J-derived macrophages and non-permissive, *Lgn1*^{r}, C57BL6/J-derived macrophages. It could be argued, however, that there are many genetic differences between the mentioned inbred mouse strains to attribute any of the observed morphological differences to the *Lgn1* gene specifically. The BAC transgenic animals produced in our own studies could prove very useful for performing comparative EM studies. Indeed, these mice represent a very limited number of genes of either C57BL6/J or 129X1 origin within a genetic background that is essentially identical to A/J

(<1% residual FvB-derived genetic background at the current N7 backcrossed generation). Additionally, the availability of different rescuing (non-permissive) founders limits considerably the possibility that any morphological differences observed between A/J macrophages and *Birc1e*-containing BAC transgenics could be due to any other gene than the ones contained in the BAC clones. Undeniably, *Birc1e* cDNA transgenic animals would constitute the best tool possible to describe a gene/phenotype correspondence via comparative EM studies; one more incentive for the creation of such transgenics.

The mechanism through which Birc1e may control intracellular *L. pneumophila* replication is not known. Careful observation of the process of bacterial uptake and of nascent phagosome maturation in macrophages differing only with respect to the *Birc1e* allele that they express, may give some hints as to the mode of action of the Birc1e gene product.

As described in detail in section 1.1.4.2, the most relevant morphological observation with respect to *L. pneumophila* replicative phagosome formation was due to the possibility of distinguishing endoplasmic reticulum lipid bilayer membranes (60±2 Å thick) from most other membranes (72±2 Å thick) on EM micrographs (Tilney et al. 2001). Briefly, it was shown that ER-derived vesicles attach to the nascent *L. pneumophila* phagosome within 5 min. of uptake and that within a further 25-30 min., the thickness of the phagosomal membrane becomes similar to ER (Tilney et al. 2001). It would be important to ascertain that such is the first identifiable difference that can be observed between $Lgn1^r$ and $Lgn1^s$ macrophages.

5.2.2.2 Immunofluorescence microscopy studies using transgenic macrophages

Birc1e cDNA and *Birc1e*-containing BAC transgenic mice can be important tools to perform comparative microscopy studies in addition to the electron microscopy studies described in the previous section. Fluorescence microscopy can be used to view subcellular structures or prokaryotic cells that have been labelled with specific antibodies

or soluble markers coupled to a fluorescent peptide. Although the resolutions obtained with the best confocal fluorescence microscopes cannot match the resolution of electron microscopy micrographs, it is a technique that will remain popular as sample preparation times are considerably shorter and the fact that fluorescence microscopes are more readily available to most laboratories.

It was mentioned in section 1.1.4.1 that a group of researchers recently described permissive mouse macrophages as engulfing *L. pneumophila* through a process of macropinocytosis (Watarai et al. 2001). In their study, A/J macrophages were compared directly to C57BL6/J macrophages, and a set of recombinant inbred progeny derived from these two strains was tested for a correlation between *L. pneumophila* replication, *Lgn1* genotype, and macropinosome formation. Their conclusion was that the $Lgn1^{s}$ allele found in A/J mice is somehow responsible for the formation of macropinosomes around *L. pneumophila*, a process that is invariably followed by successful bacterial replication (Watarai et al. 2001). This was the first report to suggest that the *Lgn1* gene product controls *L. pneumophila* replication by a mechanism that can be viewed right from the time of phagocytosis. As this has been the only report ever of this mode of uptake for *L. pneumophila*, and as we do not use the same bacterial strain for our own studies (they used a GFP-expressing Lp02 strain which is a streptomycin-resistant, thymine-auxotrophic derivative of the Philadelphia-1 strain that we use), it would be highly advisable to study in detail the mode of uptake of *L. pneumophila* under our own experimental conditions. First, A/J and C57BL6/J mice could be tested to try and reproduce their observations, then, BAC 227n6 and 164d12 transgenics, as well as any future *Birc1e* cDNA transgenic animal should be carefully inspected at the time of phagocytosis for an indication that the *Birc1e* gene might indeed play a role in the formation of macropinosomes around *L. pneumophila*. Although phase contrast imaging was deemed sufficient to distinguish the diffuse and phase-transparent ruffles that form around the bacteria being engulfed, the clearest results were obtained with a fluorescent fluid-phase marker. For that purpose, GFP-expressing *L. pneumophila* were incubated with macrophages in the presence of TRITC-Dextran, MW=155 KDa (Rh-Dx155); only permissive macrophages engulfing virulent *L. pneumophila* accumulated the marker in large vacuoles containing bacteria (Watarai et al. 2001). One potential problem is that

GFP expression by *L. pneumophila* might modify its interactions with host cells. Future experiments should therefore verify that the same macropinocytic uptake could be observed when wild-type bacteria are engulfed. The bacteria could be visualized by labeling with a specific antibody after fixation of the cells. It was proposed by Watari and colleagues that creation of macropinosomes could delay the interaction of the nascent "spacious" phagosome with the early endosomal compartments. This would give the bacterium enough time to convert to a form that can survive within, and exploit to its own advantage, the hostile environment of the phagolysosome (Watarai et al. 2001).

Interestingly, *L. pneumophila*-containing macropinosomes were shown to colocalize with components known to be associated with cholesterol-rich membrane rafts such as GM1 gangliosides and GPI-linked proteins. *Bircle*-transgenic macrophages should help to determine clearly if the Bircle gene controls an association between L. pneumophila and lipid rafts specifically. If such should be the case, a set of supplemental microscopy experiments could investigate whether there exists any difference in lipid raft organization or composition between macrophages harboring or not a resistance allele of the *Bircle* gene. Infected as well as non-infected macrophages should be analyzed in such studies. A thorough proteomics approach could also be applied to the determination of lipid raft composition in the two macrophage populations.

Later events in the *L. pneumophila* phagosome maturation process that can be readily observed using a fluorescence microscope include the recently described interaction with early secretory vesicles derived from the ER (Kagan and Roy 2002). *Bircle*-transgenic and non-transgenic macrophages could be compared with respect to recruitment of the early secretory vesicle and ER lumen marker YFP-KDEL to the nascent *Legionella*-containing phagosomes as in the work described in section 1.1.4.2 (Kagan and Roy 2002).

5.2.2.3 Birc1e protein interactions

A systematic search for proteins that interact with the Birc1e/Naip5 protein could reveal the mechanism through which it controls intracellular *L. pneumophila* replication. This has not been done before for the Birc1e protein or for any of the mouse Birc1 proteins. Only the baculovirus inhibitor of apoptosis repeat (BIR) of human BIRC1/NAIP has ever been used in a search for interacting partners, as presented in sections 1.4.2 and 1.4.4. As a brief reminder, the only confirmed protein-protein interactions involving the BIRC1 BIR domains were with the neuron-restricted, calcium-binding Hippocalcin protein (Mercer et al. 2000; Lindholm et al. 2002) and with the proteins TAK1 and TAB1, both of which mediate a functional interaction between BIRC1 and JNK1 (Sanna et al. 2002). Given the high degree of conservation of the BIR domain sequences between BIRC1 and Birc1e (76% identical, 82% similar using a PAM40 matrix across the three BIR domains), and since a functional interaction between Birc1e and the JNK1 signalling pathway can be easily envisioned as a mechanism through which Birc1e achieves bacteriostatic control (see next section), it would be advisable to test directly the possibility that Birc1e also interacts with mouse Jak1 and Tab1. These candidate protein interactions could be verified with co-immunoprecipitation or with affinity chromatography assays. Despite the potential interest in testing these candidate protein interactions, a thorough and systematic approach would be welcome as a means of discovering unsuspected and revealing interactions. A popular yeast two-hybrid system could be set up. Although protein microarray chips are becoming accessible and can provide speed and convenience to the task of detecting protein interactions (MacBeath and Schreiber 2000; MacBeath 2002; Lee et al. 2003; Sydor et al. 2003; Zhu and Snyder 2003), they can lead to a loss of thoroughness should the interacting proteins not be represented on the chip surface. Previous studies that looked for BIRC1-interacting proteins made use of bait constructs expressing only the BIR domains. Indeed, the zinc coordination structures encoded by the BIR domains are the only ones within the Birc1 sequences predicted *in silico* to be implicated in protein-protein interactions. However, the use of a full-length Birc1e protein as bait in yeast two-hybrid or coimmunoprecipitation assays would be most

advisable to address the possibility that important protein-protein interactions might only occur in the context of a full-length protein. According to previous experience with the closely related Birc1b protein (80% identical, 84% similar using a PAM40 matrix) it should not be difficult to express full-length Birc1e protein within transfected cells. It should also be kept in mind that some protein-protein interactions might require the presence of ATP in the reaction mixtures as recently described for the ATP-dependent binding of human BIRC1 to caspase-9 (Davoodi et al. 2004).

One more possibility that could be addressed in the future is that Birc1e interacts directly with *L. pneumophila* macromolecules. The use of *L. pneumophila* cell lysates or cDNA collections for the detection of such putative interactions could prove essential to fully understand the Birc1e mechanism of action. Such an interaction could also provide a rationale for the apparent specificity of the *Lgn1* gene for resistance against the *L. pneumophila* species (Miyamoto et al. 1996). In particular, the leucine-rich repeats (LRR domains) of Birc1e are good candidates for binding bacterial ligands, as they do in the related NBS-LRR pattern recognition proteins Nod1 and Nod2 (Chamaillard et al. 2003).

5.2.2.4 Exploring the JNK signalling pathway during infection in resistant versus susceptible macrophages

The MAP-kinase (mitogen-activated protein kinase) signalling pathways regulate programmed cell death (Shibuya 1999; Yamaguchi et al. 1999), are involved in both myeloid and neuronal cell differentiation (Wooten et al. 1999; Mielke and Herdegen 2002; Waetzig and Herdegen 2003) and play a central role in immunity, including LPS signalling, regulation of interleukins/cytokines and EEA1 endocytic vesicle fusion with nascent phagosomes (Imai et al. 1999; Cheung et al. 2003; Fratti et al. 2003). It is conceivable that pathways so versatile and complex still conceal secrets and unknown players. The fact that BIRC1 BIR domains have been shown to interact with the MAP-kinase kinases TAK1 and TAB1, known to mediate JNK (c-Jun-NH(2)-terminal kinase; a MAP-kinase) activation (Sanna et al. 2002), points out that the Birc1e protein could

indeed be a novel mediator of this signalling pathway. Therefore, high priority should be given to the verification of Birc1e's physical and functional interactions within the JNK pathway. Should Birc1e interact indeed with Tak1 and Tab1, its function within the JNK signalling pathway should be further defined in the context of a *L. pneumophila* infection. In recent literature, *L. pneumophila* infection has been shown to be followed by activation (phosphorylation) of JNK1 and JNK2 within permissive monocyte-derived macrophages (Welsh et al. 2004). Addition of a JNK inhibitor during the *L. pneumophila* infection resulted in reduced bacterial replication (Welsh et al. 2004). Given that both overexpression of BIRC1 (Sanna et al. 2002) and *L. pneumophila* infection (Welsh et al. 2004) result in JNK activation, it is tempting to suggest that the *L. pneumophila* bacterium uses the Birc1 proteins to trigger a JNK signalling cascade. Although this hypothesis is at this point based on many assumptions, it is very easy to test: permissive (A/J-derived) and non permissive (C57BL6/J-derived) macrophages could be infected with *L. pneumophila*, followed by detection of activated, phospho-JNK within the host macrophages. One improvement on that experiment would be to use *Birc1e* cDNA or *Birc1e*-containing BAC transgenic macrophages as the non-permissive host to confirm any correlation between *Birc1e* allele and JNK activation by *L. pneumophila*.

Why would induction of the mitogen-activated signalling cascade be essential for *L. pneumophila* replication within a host macrophage? IL-12 is an important trigger of inflammatory responses in macrophages and can be secreted in response to bacterial lipopolysaccharide (LPS) (Matsunaga et al. 2001; Matsunaga et al. 2003; Utsugi et al. 2003). *L. pneumophila*, although being a Gram-negative bacterium and thus expressing LPS on its surface, has been shown to suppress IL-12 production by permissive macrophages (Matsunaga et al. 2001). Curiously, the IL-12 suppression was later found to be dependent on the activation of a MAP-kinase signalling cascade by *L. pneumophila* (Matsunaga et al. 2003). Recent reports have determined that JNK activation negatively regulates LPS-induced IL-12 production in macrophages (Hidding et al. 2002; Utsugi et al. 2003). It must be critical for *L. pneumophila* to suppress IL-12 production by the host cell as it has been shown that treatment of a permissive host with IL-12 prevents bacterial replication (Brieland et al. 1998; Brieland et al. 2000). Moreover, nonpermissive A/J-

derived dendritic cells have recently been shown to produce IL-12 in response to *L. pneumophila* infection (Neild and Roy 2003).

I propose the following testable model for Birc1e function. The ability of *L. pneumophila* to repress activation of A/J macrophages would be essential for successful formation of a replicative vacuole. This repression of macrophage activation would occur through triggering of the Jnk signalling pathway. *L. pneumophila* activation of the Jnk signalling cascade is likely to occur through binding of *Legionella* antigens by BIRC1 in human macrophages and either the *Birc1e*^{s} allele or any of the other Birc1 proteins within mouse macrophages. The function of the resistance allele of the *Birc1e* gene would be therefore to prevent Jnk activation by *L. pneumophila*. I therefore propose that the *Birc1e*^{r} allele behaves as a dominant-negative form of either the *Birc1e*^{s} allele or of other *Birc1* genes.

The Δexon11 *Birc1e* transcripts presented in section 5.1.1 (lacking the nucleotide-binding site) are good candidates for dominant-negative behavior. Either the A/J-encoded Birc1e truncated protein lacks the putative dominant-negative function due to missense mutations within exons 12 and 15 (Fig. 5.1), or it is a regulatory mutation (low expression) that prevents it from shutting down Jnk activation effectively.

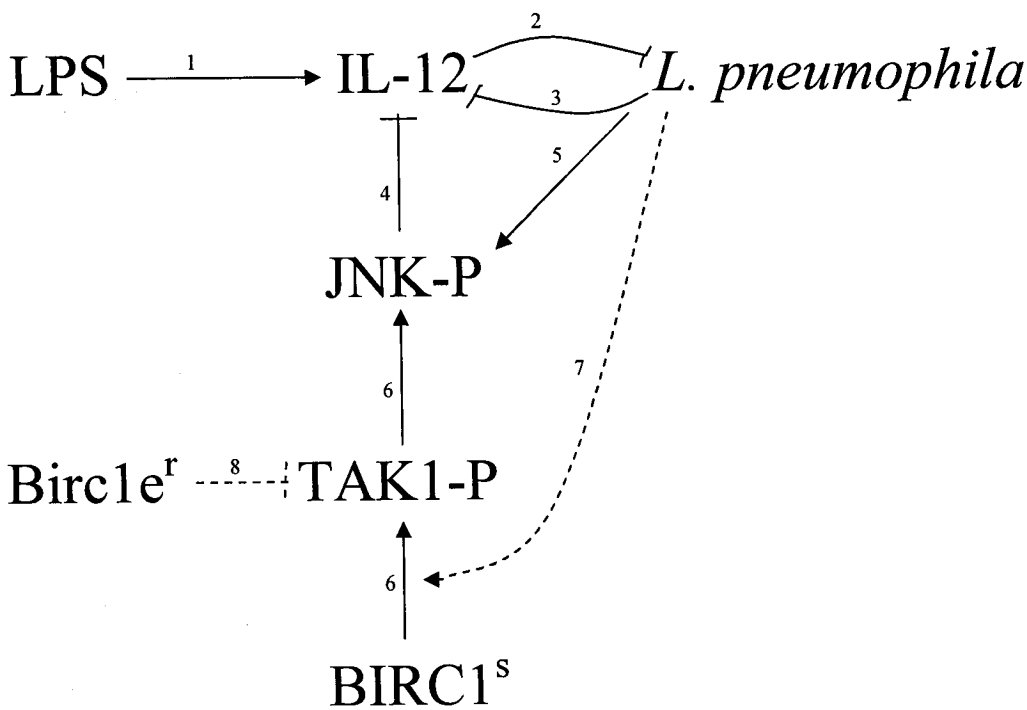

LPS
1
IL-12
2
3
L. pneumophila
4
5
JNK-P
6
7
Birc1e^r
8
TAK1-P
6
BIRC1^s

FIGURE 5.6

Schematic representation of proposed Birc1e function.

Known functional interactions (solid lines):

1- Lipopolysaccharide (LPS) induces IL-12 production in macrophages (Matsunaga et al. 2001; Matsunaga et al. 2003; Utsugi et al. 2003).

2- IL-12 represses *L. pneumophila* replication within its host (Brieland et al. 1998; Brieland et al. 2000).

3- *L. pneumophila* represses IL-12 production in permissive host macrophages (Matsunaga et al. 2001).

4- JNK (c-Jun-NH(2)-terminal kinase; a MAP-kinase) activation results in reduced IL-12 production by macrophages (Hidding et al. 2002; Utsugi et al. 2003).

5- *L. pneumophila* infection results in JNK activation (phosphorylation) within permissive host macrophages (Welsh et al. 2004).

6- BIRC1 (human NAIP) overexpression results in activation of the MAP-kinase kinase TAK1 which then results in JNK activation (Sanna et al. 2002).

Proposed functional interactions (dashed lines):

7- Within permissive macrophages, *L. pneumophila* antigens interact with Birc1 protein(s), resulting in TAK1 activation and subsequent inhibition of IL-12 production by the host macrophage.

8- Within nonpermissive macrophages, the resistance allele of Birc1e inhibits TAK1 activation by *L. pneumophila*.

Section 5.3

Final conclusion

"*Birc1e* is a host-resistance gene that controls *Legionella pneumophila* replication within mouse inflammatory macrophages". One phrase is all that is needed to describe the achievement of the many years of research described in this Ph.D. thesis (including the eight years of my own graduate work).

Essentially, we have been able to transform the progeny of mice that were permissive for *Legionella pneumophila* replication into nonpermissive mice. This was achieved by transferring a genomic segment encompassing the *Birc1e* gene from nonpermissive mouse strains to the permissive animals. We have therefore applied gene therapy to the prevention of legionellosis in A/J mice. However, the motivation behind this research is the possibility of identifying novel therapeutic strategies effective against *Legionella pneumophila* infection in humans.

Having shown that *Birc1e* controls *L. pneumophila* replication within mouse macrophages, BIRC1 may be a determinant of legionellosis susceptibility in humans. *Birc1e*-based gene therapy in humans does not represent a practical application of our research. Instead, development of applicable therapies is dependent on the identification of the mechanism(s) by which *Birc1e*^{r} prevents *L. pneumophila* replication within macrophages. Small molecule drug therapies targeting such *L. pneumophila* resistance pathways might eventually represent effective and specific alternatives to the antibiotic treatments of today.

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Original Contributions to Knowledge

1- Identification of the smallest *Lgn1* genetic interval thus far (0.32 cM).

2- Identification of genomic clones (YAC and BAC) covering the *Lgn1* genetic interval; useful for physical mapping of the region and subsequent functional complementation analyses.

3- First estimation of the physical size of the *Lgn1* critical interval (125-350 Kb).

4- Characterization of *Naip/Birc1* tissue-specific mRNA expression.

5- Detection of Birc1 protein expression within selected mouse tissues and inflammatory peritoneal macrophages.

6- Detection and quantification of a difference in Birc1 mRNA and protein expression within A/J and C57BL/6J macrophages (4 times lower expression in A/J).

7- Characterization of Birc1 protein expression within A/J and C57BL/6J macrophages during a *L. pneumophila* infection.

8- Characterization of Birc1 protein expression within A/J and C57BL/6J macrophages during different phagocytosis events.

9- Creation of transgenic mouse lines carrying cloned genomic segments from the *Lgn1* region.

10- Identification of a discrete 56 Kb genomic interval that encodes for the *L. pneumophila*-resistance gene *Lgn1*.

11- Identification of *Birc1e* as a *L. pneumophila*-resistance gene in mice.