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A Hemidominant *Naip5* Allele in Mouse Strain MOLF/Ei-Derived Macrophages Restricts *Legionella pneumophila* Intracellular Growth▽

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Mouse-derived macrophages have the unique ability to restrict or permit *Legionella pneumophila* intracellular growth. The common inbred mouse strain C57BL/6J (B6) restricts *L. pneumophila* growth, whereas macrophages derived from A/J mice allow >10^3-fold bacterial growth within three days. This phenotypic difference was mapped to the mouse *Naip5* allele. The B6 restrictive *Naip5* allele is dominant, and six amino acid changes in its product were predicted to control permissiveness. By using the wild-derived mouse strain MOLF/Ei, we found that MOLF/Ei-derived macrophages also restrict *L. pneumophila* growth, yet the Naip5 protein is identical to the A/J Naip5 at the six-amino-acid signature. The MOLF/Ei restrictive trait, unlike that of B6-derived macrophages, was not dominant over the A/J trait. In spite of this phenotypic difference, the *L. pneumophila* growth restriction in MOLF/Ei macrophages was mapped to the *Naip5* region as well, indicating that the originally predicted change in the A/J *Naip5* allele may not be critical for restriction. In the product of the A/J *Naip5* permissive allele, there are four unique amino acid changes that map to a NACHT-like domain. Similar misregulating mutations have been identified in the NACHT domains of Nod-like receptor (NLR) proteins. Therefore, one of these mutations may be critical for restriction of *L. pneumophila* intracellular growth, and this parallels results found with human NLR variants with defects in the innate immune response.

Legionella pneumophila is a gram-negative intracellular bacterial pathogen. It is found ubiquitously in aquatic environments, where it parasitizes a variety of protozoan species (15). The aerosolization of *L. pneumophila* from contaminated public water supplies, such as steam baths, cooling towers, or large air conditioning systems, is thought to be the primary route for human infection (17). After it is inhaled, *L. pneumophila* is able to replicate within alveolar macrophages, resulting in a severe pneumonia known as Legionnaires' disease (17).

Dot/Icm, a chromosomally encoded type IV secretion system, is required for *L. pneumophila* to replicate within a membrane-bound vacuole in host cells (32, 39). The *Legionella*-containing vacuole avoids fusion with endosomes and lysosomes (24) and recruits endoplasmic reticulum-derived secretory vesicles that modify the *Legionella*-containing vacuole into a compartment in which endoplasmic reticulum-like material is imbedded (23, 35, 37). *L. pneumophila* replicates within macrophages for up to 24 h and then lyses out to repeat the infection cycle.

Macrophages derived from many inbred mouse strains have been shown to be restrictive or permissive of *L. pneumophila* intracellular growth, with the C57BL/6J (B6) strain being used as the canonical restrictive strain (9, 42, 43). A/J, a permissive mouse strain, supports 10^3- to 10^4-fold growth of *L. pneumophila* over a 3-day period, whereas B6 rarely supports <10-fold growth over this time period (9, 42, 43). Studies involving crosses of B6 and A/J showed that restriction of *L. pneumophila* growth is dominant and segregates in a Mendelian fashion via a single autosomal locus on mouse chromosome 13 named *Lgn1* (5, 9).

The *Lgn1* locus contains a variable number of *Naip* gene paralogs (~5 genes and pseudogenes) that share ~85% identity (19, 25). Positional cloning and complementation assays linked *L. pneumophila* restriction in mouse macrophages to a single gene called *Naip5* (also known as *Birc1e*) (10, 41). Naip5 is a nucleotide-binding domain-containing and leucine-rich Nod-like receptor (NLR) protein made up of three N-terminal baculoviral inhibitory repeat domains, a central NOD/NACHT domain, and C-terminal leucine-rich-repeat motifs (41). NLRs are cytosolic proteins that sense pathogen-associated molecular patterns, common microbial molecules that are released into the cytoplasm of the host cells, often as the result of microbial infection (33). Naip5 is believed to sense *L. pneumophila* flagellin, dependent on the presence of the type IV secretion system (29, 30). Additional regulation of *Legionella* infection via phagosome maturation is provided by another NLR protein, Ipaf (NLRC4) (3, 44). The cytoplasmic presentation of flagellin activates caspase-1 and restricts *L. pneumophila* intracellular growth (3, 16, 29, 30).

The genetic difference distinguishing *Naip5* in permissive A/J and restrictive B6 macrophages has been hypothesized to be linked to either the expression level or the amino acid sequence of Naip5, because both strains are predicted to encode an intact Naip5 protein (10, 41). In terms of expression, Diez et al., in 2000, investigated the mRNA expression level of the *Naip* homologous transcripts in B6 macrophages versus A/J macrophages by Northern blot analysis and showed there was enhanced expression of *Naip* transcripts in B6 macrophages (11). Wright et al., in 2003, looked at the expression level of Naip5 protein in B6 macrophages versus A/J macrophages and observed enhanced protein expression in B6 macrophages as

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well (41). However, in both studies, the detection methods did not rule out the possibility that other *Naip* paralogs could be contributing to these results. Therefore, it is unclear whether the enhanced expression level of *Naip* in B6-derived macrophages is specific to *Naip5*.

It was also observed that the Naip5 protein sequence is polymorphic. There are 14 differences in the amino acids encoded by the A/J and B6 *Naip5* alleles, suggesting that one of these variants could affect the function of Naip5 (41). A previous study narrowed down the putative amino acids regulating Naip5 activity by evaluating *L. pneumophila* intracellular growth in seven different inbred mouse strains (41). Through this analysis the authors found two strains (B6 and P/J) that restricted *L. pneumophila* intracellular growth, and five strains (A/J, C3H/HeJ [C3H], BALB/cJ [BALB], 129S1, and FvB/N) that were permissive (41). The *Naip5* gene was sequenced from each strain and six amino acid differences correlated with the permissive/restrictive phenotype (41). These six amino acid changes appeared to serve as a signature for a permissive or restrictive *L. pneumophila* mouse strain.

To expand upon these studies, we characterized *L. pneumophila* intracellular growth in the wild-derived mouse strain MOLF/Ei. Although inbred mouse strains have served as a genetic reservoir for pathogenesis, new emerging mouse models, such as wild-derived mice, have expanded the genetic repertoire, allowing novel genes and/or regulatory mechanisms that could play a role in determining host-microbe interactions to be identified (8, 31). We characterized *L. pneumophila* intracellular growth in MOLF/Ei-derived bone marrow macrophages (BM macrophages) to gain further insight into the genetic determinants regulating *L. pneumophila* restriction. Surprisingly, we found that MOLF/Ei-derived macrophages restrict *L. pneumophila* intracellular growth, even though the six-amino-acid signature found in the MOLF/Ei *Naip5* allele product is identical to the those encoded by alleles from the permissive FvB/NJ, BALB, and C3H strains. By mapping the *L. pneumophila* restriction phenotype, we found that it was also linked to *Naip5* in MOLF/Ei macrophages, suggesting that the originally proposed missense amino acids in the A/J protein are not critical for *L. pneumophila* intracellular growth. Instead, we propose that unique amino acid changes within the NACHT domain of A/J Naip5 are likely to be responsible for the permissiveness of this inbred mouse strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. pneumophila* philadelphia-1 strain Lp02 (*thyA*) is a derivative of Lp01 (*hsdR rpsL*) (6). The Lp02 *flaA*-negative strain (referred to as the *flaA* mutant strain) was generously provided by Tao Ren and William Dietrich (30). *L. pneumophila* strains were maintained on buffered charcoal-yeast extract solid medium and ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-buffered yeast extract (AYE) broth culture media (13, 18, 35). For all Lp02 derivatives, thymidine was included in the media at 100 μg/ml. For infections, *L. pneumophila* strains were patched from a single colony onto buffered charcoal-yeast extract containing 100 μg/ml of thymidine. After 2 days at 37°C, patches were used to inoculate AYE broth cultures. Cultures were grown overnight in AYE broth containing 100 μg/ml of thymidine to ensure that the bacteria were in postexponential phase (A_{600}, ~4.0) prior to infection.

Mice, phenotyping, and genotyping. A/J, B6, MOLF/Ei, and FvB/NJ were purchased from Jackson Laboratory. A/J and MOLF/Ei were crossed to generate F1 progeny. The F1 progeny were backcrossed to A/J, and N2 (F1 × A/J) progeny were phenotyped using the *L. pneumophila* growth curve assay (see below). *Legionella* growth was assessed at 2, 48, and 72 h postinfection (hpi), and mice were scored as either intermediate (10^2-fold growth) or permissive (10^3-fold growth) for *L. pneumophila* growth in comparison with an A/J or (MOLF/Ei × A/J)F1 control. Genotyping of N2 (F1 × A/J) progeny was performed with polymorphic microsatellite PCR using genomic DNA obtained from tail tissue (Qiagen).

TABLE 1. List of primers

Primer	Restriction site	Sequence[a]
Naip5F-myc	EcoRI	CC**GGAATTC**GGATGGCTGAG CATGGGGAG
Naip5FS1		GGTACCATGAAGAGGAGG
Naip5FS2		GTAGGAGTGAAGCCCAG
Naip5FS3		CTTCTATAATACTGTCTC
Naip5FS4		GTTTCAGTTTGTTAGAGG
Naip5FS5		CATGTCCAGGCTGGAGCT
Naip5FS6		CTGCAGCTTCCGTGCCTC
Naip5FS7		GAAGCTCTAGTCAGAGCAGG
Naip5RS1		CCTCCTCTTCATGGTACC
Naip5RS2		ACTGGGCTTCACTCCTAC
Naip5RS3		GAGACAGTATTATAGAAG
Naip5RS4		CCTCTAACAAACTGAAAC
Naip5RS5		AGCTCCAGCCTGGACATG
Naip5RS6		GAGGCACGGAAGCTGCAG
Naip5R	SalI	ACGC**GTCGAC**CCAGGAGGGC CCAACATAC

[a] Restriction sites are indicated in boldface.

Cell culture. BM macrophages were flushed from the femurs of 6-week-old to 3-month-old mice and differentiated in BM macrophage medium (BMM; RPMI, 1 mM glutamine, 10% fetal bovine serum, 30% L-cell supernatant) (35). Macrophages were differentiated for 7 to 8 days, collected, and frozen for use in multiple experiments, if needed, in media containing 20% serum and 10% dimethyl sulfoxide (DMSO).

Growth curves for *Legionella pneumophila* in BM macrophages. Macrophages were replated after 7 to 8 days of differentiation in fresh BMM plus 200 μg/ml of thymidine. BM macrophages were plated at 4×10^5 cells per well of a 24-well plate and allowed to settle overnight. *Legionella pneumophila* was grown in AYE broth to postexponential phase (A_{600}, ~4.0), when the bacteria are highly motile, and the cells were infected at a multiplicity of infection (MOI) of 0.05. After infection of the cells, culture plates were placed in a tabletop centrifuge and spun at 1,000 rpm for 5 min at room temperature to promote contact of bacteria with the macrophages. The macrophages were incubated at 37°C with 5% CO_2 for 2 h, after which the monolayers were washed three times in prewarmed BMM plus 200 μg/ml of thymidine. At 2, 24, 48, and 72 hpi, three independent wells at each time point were lysed with 0.2% saponin. Dilutions of each lysate were plated onto bacteriological media, and CFU were determined. For each time point, we determined the mean number of bacteria recovered from three independent wells ± the standard error.

Caspase-1 inhibition. The specific caspase-1 inhibitor Z-YVAD-FMK (YVAD; Calbiochem) was used. Macrophages were preincubated 1 h before infection with 40 μM of inhibitor dissolved in DMSO or with an equivalent volume of DMSO (control). Macrophages were infected as described above, and after the third wash, fresh inhibitor was added for the duration of growth.

Cloning and sequencing Naip5 from MOLF/Ei BMM. Total RNA was isolated from MOLF/Ei BM macrophages according to the instructions with the Qiagen RNeasy kit (Invitrogen). cDNA was amplified from 5 to 10 μg of total RNA by using oligo(dT) and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. *Naip5* was amplified from cDNA by using *Pfu* Ultra DNA polymerase (Stratagene) with primers Naip5F-myc and Naip5R and cloned into pMyc (Clontech) at EcoRI and SalI sites. pMyc-*Naip5* clones were screened by restriction digested and sequenced using primers Naip5F-myc and Naip5R (Table 1). The full-length sequence of *Naip5* was determined using the primers Naip5FS1-6 and Naip5RS1-6 (Table 1). HEK293T cells were transfected with MOLF Naip5 cDNA, and lysates were prepared for Western blotting to confirm expression of the full-length protein (data not shown).

RNA isolation and real-time PCR. Cells were lysed for RNA preparation using 1 ml of Trizol (Invitrogen) per 10^6 cells. The RNA pellet was reconstituted in 20 μl of water and treated with 20 U of DNase Q1 (Promega) for 1 h at 37C. RNA was extracted with phenol and phenol-chloroform, reprecipitated, and subse-

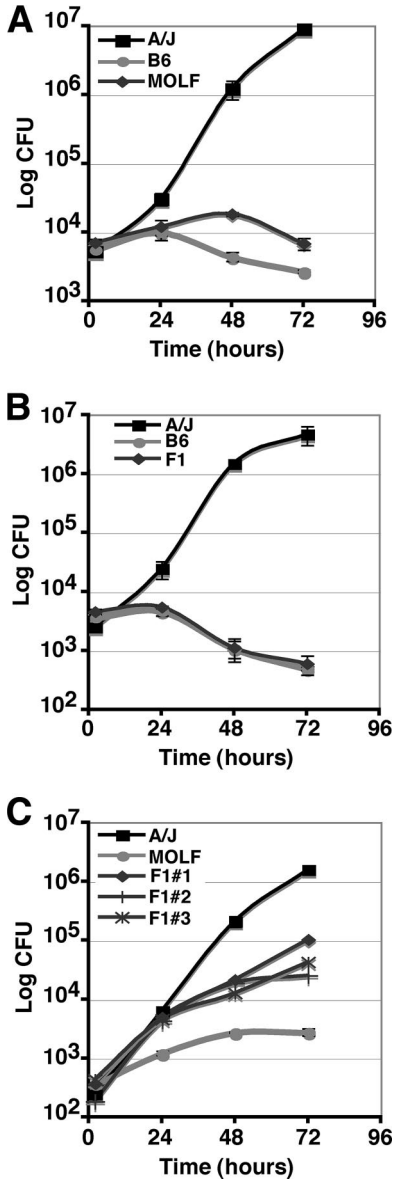

FIG. 1. MOLF/Ei-derived macrophages restrict *L. pneumophila* growth. Growth curves for *L. pneumophila* (Lp02) in A/J, MOLF/Ei, and B6 strains (A), A/J, B6, and (A/J × B6)F1 strains (B), and A/J, MOLF/Ei, and (MOLF/Ei × A/J)F1 strains (C). Three independent F1 mice were tested and labeled arbitrarily numbers 1 to 3. For each growth curve experiment, BM macrophages were infected with Lp02 at an MOI of 0.05. Cells were lysed at 2, 24, 48, and 72 hpi, and CFU were enumerated. Data represent the means and standard errors for triplicate samples.

quently used for cDNA synthesis using random primers of nine nucleotides, deoxynucleoside triphosphates, and Moloney murine leukemia virus reverse transcriptase (New England Biolabs). cDNA was analyzed by real-time PCR gene expression analysis using the Sybr green PCR master mix kit (Applied Biosystems) and the Naip5-specific primers Naip5F (5′-GTG CTG GTC ACC AAA CCT TTA TC-3′), Nip5R (5′-TCC TGT TGA CCT TGG TAT TGG AAG-3′), GAPDH_F (5′-CCA TGG AGA AGG CTG GGG), and GAPDH_R (5′-CAA AGT TGT CAT GGA TGA CC).

Data analysis. An enhanced version of Map Manager QT (quantitative traits) software, QTX, was used in linkage analysis of the growth permissiveness/restriction trait. Linkages with a logarithm of odds (LOD) greater than 3.0 were considered significant. All experiments were performed in triplicate unless stated otherwise. Statistical analysis of differences in expression of Naip5 was carried out using two-sample *t* test.

RESULTS

MOLF/Ei-derived BM macrophages restrict *L. pneumophila* growth. Wild-derived mice, such as MOLF/Ei strain mice, have many advantages over the commonly used inbred mouse strains, including novel allelic variants and greater density of single-nucleotide polymorphisms, that facilitate mapping strategies (20). Most of the commonly used inbred mouse strains have been established from the *Mus musculus domesticus* subspecies, representing a relatively small gene pool, making these strains limited in genetic variations. In contrast, wild-derived mice originated mainly from *M. m. musculus* and *M. m. castaneus* subspecies are genetically diverse compared to the classical inbred strains. Compared to one another, the genomes of the wild-derived and classical inbred strains show a polymorphism in every 100 to 200 bp (34), suggesting that screening wild-derived mice may uncover other host factors or novel alleles of previously characterized genes that influence *L. pneumophila* intracellular growth.

To address this point, *L. pneumophila* intracellular growth was assayed in BM macrophages over a 3-day period. BM macrophages from the A/J strain, the B6 strain, and the wild-derived mouse strain MOLF/Ei were infected with Lp02 at a low dose (MOI, 0.05), and *L. pneumophila* growth was monitored by enumeration of CFU at 2, 24, 48, and 72 hpi. We found that MOLF/Ei BM macrophages strongly restricted

TABLE 2. Naip5 amino acid polymorphisms in B6, MOLF, and A/J mice

Amino acid no.	Exon	Polymorphic residue[a]		
		B6	MOLF	A/J
92	3	R	R	R
144	3	R	R	R
234	5	E	K	E
368	9	T	M	T
472	11	**T**	**A**	**A**
496	11	Y	Y	N
512	11	D	D	G
514	11	G	G	E
517	11	N	N	K
533	11	**V**	**A**	**A**
538	11	**S**	**I**	**I**
647	11	A	A	T
692	11	S	S	P
755	11	V	V	M
855	11	S	T	S
952	11	S	T	S
1021	11	M	I	M
1092	12	**E**	**D**	**D**
1116	12	**N**	**D**	**D**
1123	12	**R**	**G**	**G**
1137	12	Q	R	Q
1140	12	T	R	T
1241	15	V	V	I
1275	15	D	D	N

[a] Naip5 amino acids in bold were originally predicted to correlate with permissiveness of mouse macrophages for *L. pneumophila* growth (41). Residues which are unique to A/J Naip5 compared to those of B6 or MOLF Naip5 are underlined.

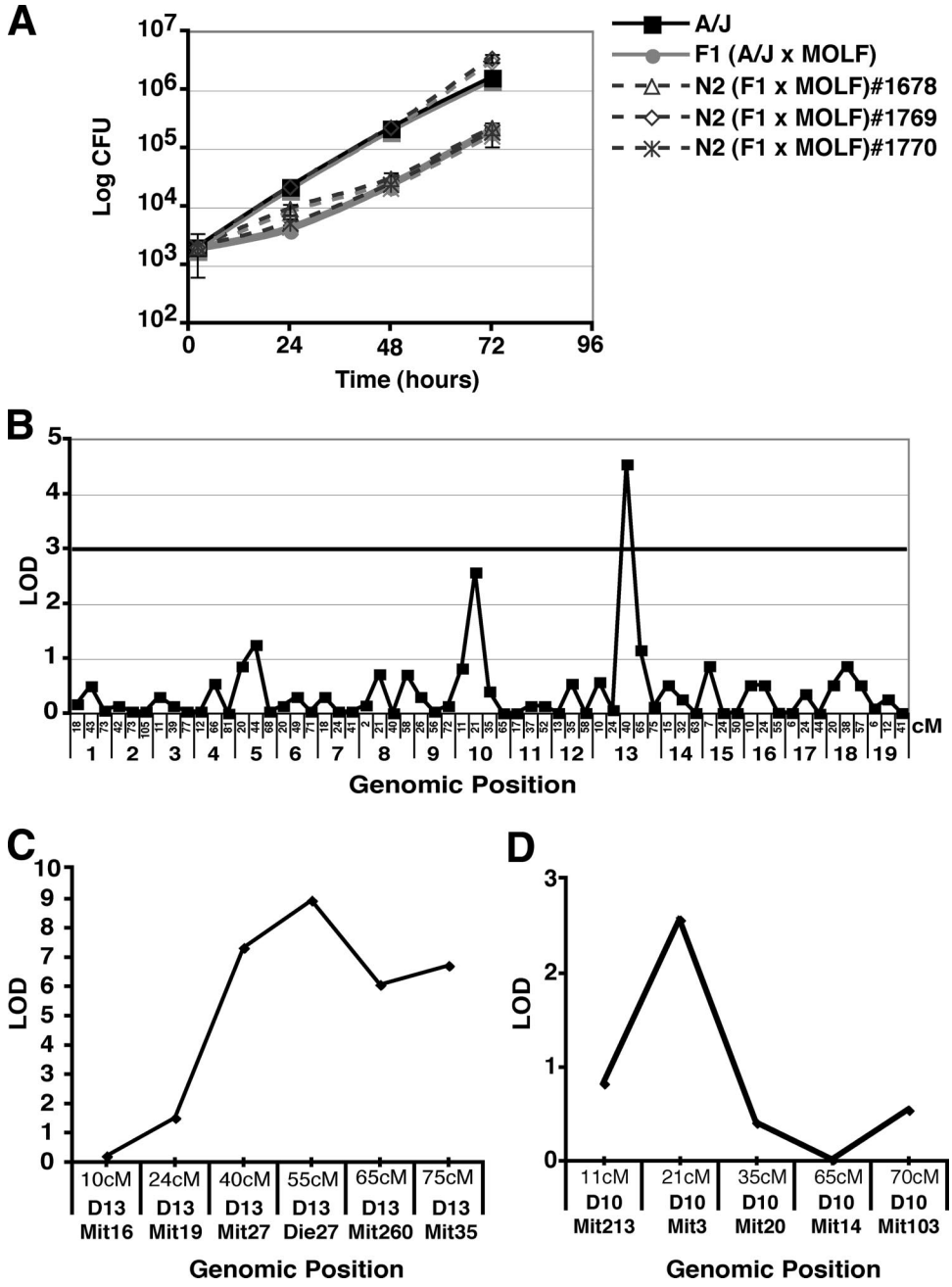

FIG. 2. *L. pneumophila* restriction in MOLF/Ei-derived macrophages maps to *Naip5*. (A) BM macrophages from N2 mice generated by mating strain (MOLF/Ei × A/J)F1 to strain A/J were tested for permissiveness to *L. pneumophila* growth. Macrophages from A/J, (MOLF/Ei × A/J)F1, and three representative N2 mice were infected at an MOI of 0.05, cells were lysed, and CFU were enumerated at 2, 48, and 72 hpi. Data represent the means and standard errors for triplicate samples. (B) Genome-wide scanning was performed according to standard procedures, using 62 polymorphic microsatellite markers throughout the genome, spaced at approximately 20- to 30-cM intervals per chromosome. The position of each genetic marker is indicated on the *x* axis in cM. Transgenomic log likelihood (LOD score) analysis was performed for 28 mice. The bold horizontal line indicates the cutoff for a significant LOD (≥3). (C) Forty-seven N2 mice were subjected to phenotypic analysis and genotyped for six chromosome 13 markers; names and positions (cM) are indicated on the *x* axis. D13Die27 markers the physical position of the *Naip5* allele (19). (D) Forty-seven N2 mice were subjected to phenotypic analysis and genotyped for five chromosome 10 markers; names and positions (cM) are indicated on the *x* axis.

Lp02 growth to an extent similar to that observed for the commonly used restrictive inbred B6 mouse strain (Fig. 1A).

Previous work identified six amino acid changes within Naip5 that were present in all strains of mice that were permissive of *L. pneumophila* intracellular growth (Table 2) (41). *Naip5* was cloned and sequenced from cDNA prepared from MOLF/Ei-derived macrophages to determine whether the *L. pneumophila* restriction in MOLF/Ei macrophages could be

due to mutations in *Naip5*. The signature six polymorphic amino acids in the MOLF/Ei Naip5 protein were identical to those in the A/J protein, yet MOLF/Ei-derived macrophages were restrictive for *L. pneumophila* growth (Table 2).

The B6 restrictive *Naip5* allele is known to be dominant over the A/J sensitive *Naip5* allele. Consistent with this observation, we found that BM macrophages from (B6 × A/J)F1 progeny restricted *L. pneumophila* growth (Fig. 1B). BM macrophages from (MOLF/Ei × A/J)F1 were tested to determine whether the MOLF/Ei *Naip5* allele was dominant or recessive. Surprisingly, all seven F1 crosses (MOLF/Ei × A/J) tested in our study were intermediate for *L. pneumophila* intracellular growth (Fig. 1C; data not shown). We consistently observed a 10-fold defect in *L. pneumophila* growth when the (MOLF/Ei × A/J)F1 strain was compared to the A/J strain. Altogether, the sequence similarity to a permissive *Naip5* allele and the difference in the (MOLF/Ei × A/J)F1 mode of inheritance of this trait indicated that another gene(s) beside *Naip5* may be influencing the restrictive MOLF/Ei phenotype. Alternatively, the restrictive B6 *Naip5* allele may behave differently than a restrictive MOLF/Ei allele.

***L. pneumophila* restriction in MOLF/Ei-derived macrophages is linked to *Naip5*.** In contrast to the B6 *Naip5* allele, the MOLF/Ei *Naip5* allele was not dominant over the A/J *Naip5* allele, so we hypothesized that another gene(s) within the MOLF/Ei strain background may be contributing to the restriction of *L. pneumophila* growth. To test this, the progeny of (MOLF/Ei × A/J)F1 mice were backcrossed to strain A/J to map the genetic difference influencing the *L. pneumophila* phenotype.

BM macrophages from the resulting N2 (F1 × A/J) mice were screened for phenotypic differences in *L. pneumophila* growth. A total of 75 N2 (F1 × A/J) mice were screened by assaying for *L. pneumophila* growth in BM macrophages at 2, 48, and 72 hpi. Of the 75 mice screened, 53% of N2 (F1 × A/J) mice were permissive (A/J-like) and 47% were found to be intermediate (F1-like) for *L. pneumophila* growth, suggesting a simple Mendelian segregation pattern and suggesting that a single gene may regulate the restriction phenotype (Fig. 2A; data not shown). Genome-wide scanning was performed on 28 N2 mice by using 62 polymorphic microsatellite markers spaced at 20- to 30-centimorgan (cM) intervals on each chromosome. The strongest linkage observed was to chromosome 13 at 40 cM, with a transgenomic log likelihood (LOD) of 4.54 (Fig. 2B). Further evaluation of linkage to chromosome 13 using 47 additional mice revealed that the strongest linkage was to marker D13Die27, located within the intergenic region between the *Naip2* and *Naip5* genes (Fig. 2C) (19). The LOD score peaked at D13Die27 (LOD = 8.9). Equivalent linkage was also observed with D13Die35 located within the *Naip5* intron (data not shown). We also observed a peak in LOD score at chromosome 10, but the LOD score was less than 3 and failed to increase when the additional 47 mice were included in the analysis, so the linkage was not considered significant (Fig. 2D). Our linkage analysis demonstrated that the primary restriction of *L. pneumophila* growth in MOLF/Ei macrophages is likely dependent on *Naip5*.

BM macrophages from FvB/N are not permissive of *L. pneumophila* intracellular growth. The mapping of *L. pneumophila* restriction to *Naip5* in the MOLF/Ei strain effectively argues that any common amino acid polymorphisms that control permissiveness for *L. pneumophila* intracellular growth have yet to be identified (Table 2). In fact, the MOLF/Ei Naip5 amino acid sequence is identical to those of the proteins from inbred FvB/NJ, BALB, and C3H mouse strains (data not shown), which were shown to be permissive (41). To evaluate this discrepancy, we performed growth curve analyses with FvB/NJ BM macrophages and found that, in contrast to previous published results, FvB/NJ BM macrophages were restrictive of *L. pneumophila* intracellular growth (Fig. 3). In support of our data, another study showed that peritoneal macrophages from FvB/NJ mice were also restrictive for *L. pneumophila* growth (10). Differences in our BM macrophage differentiation or growth curve analysis protocols may also have influenced the phenotype of FvB/NJ. If the identified residues in Naip5 are playing a role in controlling the restrictive/permissive phenotype, then we propose that the critical amino acids are likely the ones that are unique to the A/J *Naip5* allele product and are not found in any of the restrictive strains (Table 2).

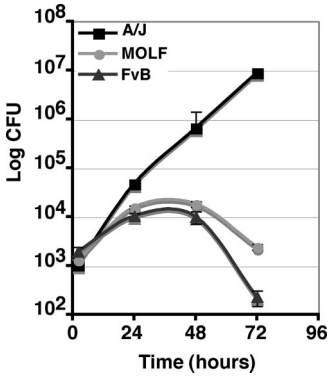

FIG. 3. FvB/NJ-derived macrophages restrict *L. pneumophila* intracellular growth. Growth curve for Lp02 in A/J, MOLF/Ei, and FvB/NJ strains. BM macrophages were infected at an MOI of 0.05, and then cells were lysed at 2, 24, 48, and 72 hpi. Data represent the means and standard errors for triplicate samples.

Unique residue variants in the A/J Naip5 allele product map to the NACHT domain. There are nine residues unique to the A/J Naip5 in comparison to the amino acid sequences of the B6 and MOLF/Ei Naip5 proteins (Table 2). Interestingly, four of the unique A/J residues cluster within exon 11, which contains the NACHT nucleoside triphosphatase (NTPase) domain between amino acids 464 and 618. NACHT-containing proteins in mammalian cells are thought to be involved in the innate immune response and the sensing of microbial ligands (40). Using PSI-BLAST and CLUSTALW, we aligned the Naip5 NACHT domains from B6, MOLF/Ei, and A/J Naip5 with the NACHT domains from the human Nalp3, Nod1, Nod2, CTIIA, Naip, and Ipaf proteins (Fig. 4). There are five conserved motifs that are found in these NACHT NTPase proteins (4). Motifs I and III are found in nearly all NTPases, and these motifs contain Walker A and B nucleotide-binding signatures. The Walker A box in motif I has the phosphate-binding site (P-loop), and the Walker B box in motif III has one to three aspartate or glutamate residues, which act to coordinate the Mg^{2+}-water molecule and provide the catalytic carboxylate for NTP hydrolysis (21). Motifs II, IV, and V are

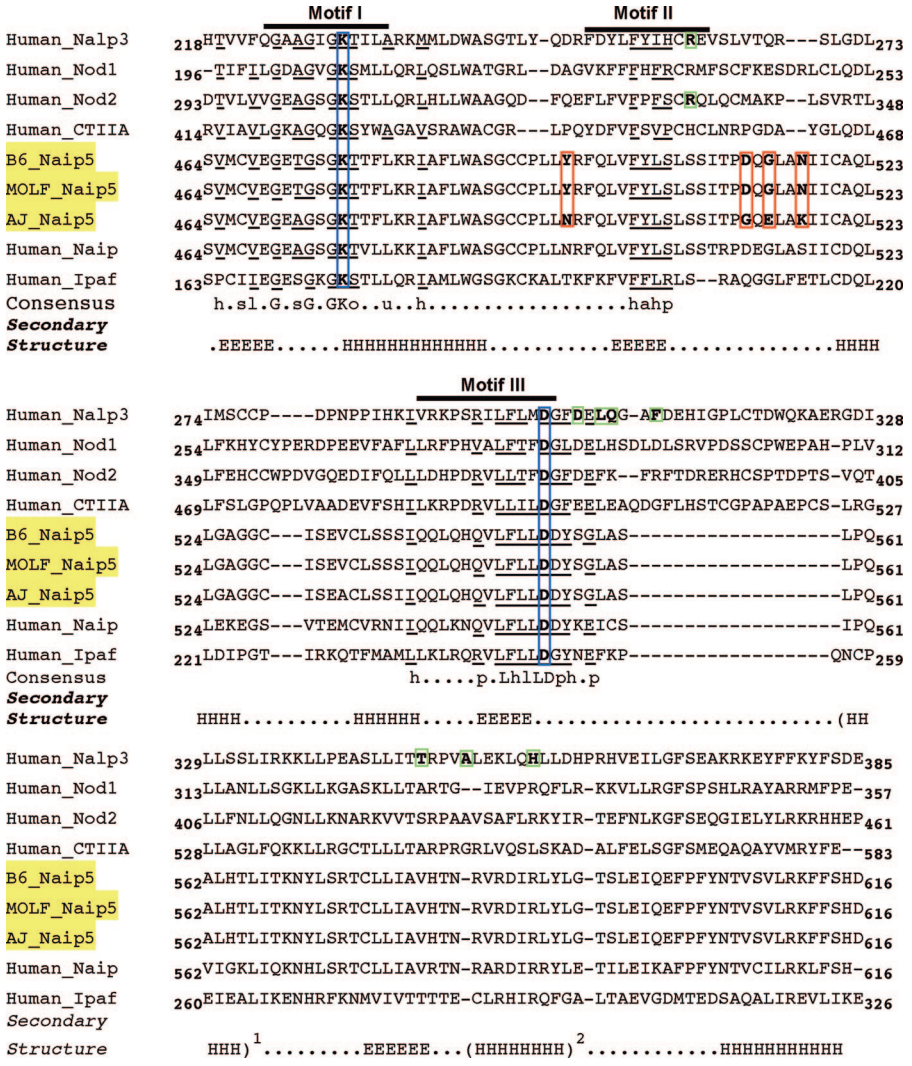

FIG. 4. Alignment of Naip5 to other NACHT domain-containing proteins. Three classic motifs are found in NACHT domain-containing proteins (4). Motif I contains the phosphate-binding lysine (K) of the Walker A box. Motif II contains hydrophobic and conserved polar residues. Motif III contains an aspartate (D) residue to coordinate the Mg^{2+} of the Walker B motif. NACHT domains from Naip5 of B6, MOLF/Ei, and A/J mouse strains were compared to human Nalp3, CIITA, Nod1, Nod2, Naip, and Ipaf proteins. The consensus amino sequence is present in 90% of NTPases, as described by Aravind et al. in 1999 (4). The amino acid residues within these motifs are underlined and marked as aromatic (a), hydrophobic (h), aliphatic (l), small (s), tiny (u), hydroxyl (o), and polar (p). The conserved Walker box residues are boxed in blue. The sites of the Nalp3 (R260W, D303N, L305P, Q306L, F309S, T348M, A352V, and H358R) and Nod2 (R334W/Q) gain-of-function mutations are shown boxed in green (38). The Naip5 allelic variants Y496N, D512G, G514E, and N517K are shown boxed in red. Marked below each region is the predicted secondary structure from PSIPRED (27). Residues with a score of ≥7 are marked as H for α-helices and E for β-sheets. A superscript 1 indicates that the secondary structure was present only in mouse Naip5s and human Naip and Ipaf. A superscript 2 indicates that the secondary structure was present only in human Naip and Ipaf.

present in some, but not all, NACHT NTPases (4). Only motifs I, II, and III were easily discernible in Naip5 (Fig. 4).

Of particular interest were four amino acids that differ between restrictive B6 and MOLF/Ei Naip5 proteins and the permissive A/J Naip5 protein that cluster near motif II (Fig. 4). This is where other NACHT domain mutations in Nod2 and Nalp3 which result in a gain of function leading to autoinflammatory diseases have been identified (Fig. 4) (1, 2, 12, 7, 14, 22, 38). Protein modeling has mapped these mutations to a loop region near the NTPase active site (2). These mutations could disturb NTP binding and hydrolysis or interfere with protein domain interactions (2). Three of the four amino acid variants in A/J Naip5 are dramatic. The B6 and MOLF/Ei Y_{496} residue changes from an aromatic residue into a polar residue in the A/J protein. The B6 and MOLF/Ei D_{512} changes from a negatively charged amino acid into a nonpolar residue and G_{514} changes from a nonpolar residue into a negatively charged residue in the A/J Naip5 protein (Fig. 4). Based on this model, we propose that the missense amino acids surrounding motif II may be critical for either Naip5 activation or function and may explain why the A/J *Naip5* is a permissive allele.

Expression analysis of Naip5 in different mouse strains. To investigate whether the differences in expression of Naip5 may still explain the phenotypes associated with the different alleles

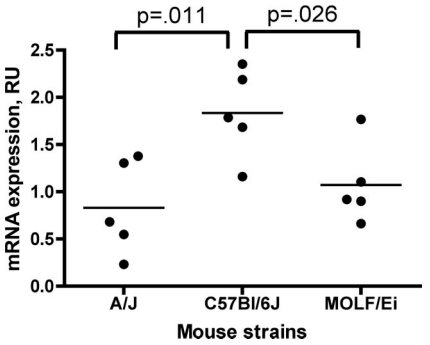

FIG. 5. Differences in *Naip5* expression in inbred strain macrophages. Isolated from the BM macrophages of A/J, B6, and MOLF/Ei mice, total RNA was analyzed by means of a quantitative PCR that specifically amplifies Naip5. Five mice per strain were used. Significance in variances was evaluated with a one-way analysis of variance test with a 95% confidence interval. RU, relative units.

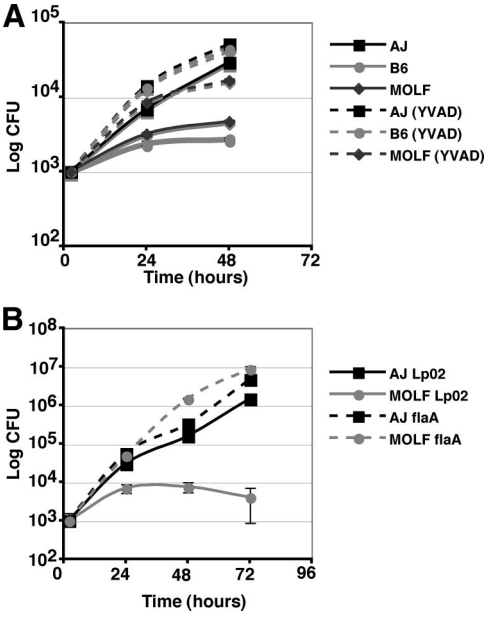

FIG. 6. The absence of flagellin or inhibition of caspase-1 restores *L. pneumophila* growth within MOLF/Ei-derived macrophages. (A) BM macrophages were pretreated for 30 min with DMSO (control) or the caspase-1 inhibitor YVAD. Macrophages from the A/J, B6, and MOLF/Ei strains were infected with Lp02 and assayed for *L. pneumophila* intracellular growth at 2, 24, and 48 hpi. (B) Growth curve for BM macrophages from A/J or MOLF/Ei mice that were infected with the *L. pneumophila* Lp02 strain or the *flaA* mutant strain. Data represent the means and standard errors for triplicate samples.

of the gene, we compared the expression levels of *Naip5* in B6, A/J, and MOLF/Ei BM macrophages by using a set of primers designed for a region within the 3′ untranscribed region that was specific to *Naip5* (Fig. 5). No significant difference was observed between the *Naip5* expression levels of A/J and MOLF/Ei macrophages, further supporting the model in which phenotypic differences between these strains are unlikely due to differences in expression levels. In contrast, expression of *Naip5* in B6 mice was found to be significantly higher than that in A/J and MOLF/Ei strains (Fig. 5). Therefore, restriction of *L. pneumophila* growth by mice bearing the B6 allele could be due to high levels of Naip5 in this strain compared to those in the A/J strain.

MOLF/Ei Naip5 restricts *L. pneumophila* growth by recognition of flagellin and activation of caspase-1. Naip5 has been shown to be present in a signaling pathway that involves caspase-1 and Ipaf, two members of the inflammasome complex (3, 30, 44). The inflammosome regulates a pyroptosis pathway, which can result in caspase-1-dependent cell death (26). Since knockout of caspase-1 in B6 macrophages can rescue *L. pneumophila* growth, we wanted to determine whether caspase-1 was critical for MOLF/Ei restriction of *L. pneumophila* as well. A/J, B6, or MOLF/Ei BM macrophages were treated with or without the membrane-permeable caspase-1 inhibitor YVAD. Consistent with previous findings, wild-type *L. pneumophila* (Lp02) growth was enhanced ~10-fold in the B6 strain at 48 hpi (44). Similarly, treatment of MOLF/Ei macrophages with YVAD restored intracellular *L. pneumophila* growth to the level of that in untreated A/J macrophages at 48 hpi (Fig. 6A). Therefore, restriction of *L. pneumophila* intracellular growth by macrophages bearing the MOLF/Ei *Naip5* allele appeared to occur via the same mechanism as occurs in B6 macrophages, as caspase-1 activation is a critical component in the restriction phenotype observed in both mouse strains.

L. pneumophila flaA mutants, lacking flagellin, fail to activate the Naip5/Ipaf-dependent response that restricts *L. pneumophila* growth in B6 macrophages (3, 29, 30). It was predicted that flagellin is delivered into the cytosol of mammalian cells, dependent on the presence of Dot/Icm, and is recognized by Naip5, resulting in caspase-1-dependent restriction. Growth curve analyses were conducted using the wild type (Lp02) and a *flaA* mutant strain, to determine if delivery of *L. pneumophila* flagellin was contributing to MOLF/Ei restriction. The *L. pneumophila flaA* mutant strain was able to grow efficiently in B6 (data not shown) as well as MOLF/Ei BM (Fig. 6B) macrophages. We also found that the absence of flagellin was able to fully restore *L. pneumophila* growth in FvB/NJ BM macrophages (data not shown). This result further supports the model in which *L. pneumophila* restriction in MOLF/Ei-derived macrophages is due to a restrictive *Naip5* allele. The fact that FvB/NJ mouse strain has a *Naip5* allele identical to that of MOLF/Ei indicates that several restrictive alleles of *Naip5* may operate via recognition of flagellin and activation of caspase-1.

DISCUSSION

Here, we showed that a *Naip5*-linked determinant controls *L. pneumophila* growth in the wild-derived mouse strain MOLF/Ei in addition to the commonly used inbred B6 mouse strain. In contrast to previous studies using inbred mice, intriguingly, we observed that the restrictive MOLF/Ei *Naip5* allele is not dominant over A/J *Naip5*. The reason for the different behaviors of the (MOLF/Ei × A/J)F1 and (B6 × A/J)F1 mice is not clear, although the relative expression levels of *Naip5* in the MOLF/Ei and B6 strains offer a possible explanation. *Naip5* is more highly expressed in the B6 strain than in the MOLF/Ei strain, consistent with the dominant phenotype observed in mice bearing the B6 allele.

One question that still remains is whether the amino acid

polymorphisms are critical to the function of Naip5. A study by Zamboni et al. used an HEK293 ectopic expression system to evaluate whether Naip5 was sufficient to induce caspase-1-dependent cell death (44). In that study, HEK293 cells were cotransfected with Fc receptor to increase *L. pneumophila* uptake and red fluorescent protein to monitor cell morphological changes associated with cell death, caspase-1, and Naip5 from either B6 or A/J mice. The authors showed that upon *L. pneumophila* infection, only cells expressing the B6 *Naip5* allele were sufficient to induce cell death, and there was little cell death with cells expressing the A/J *Naip5* allele (44). This result supports a model in which the A/J Naip5 protein is deficient for signaling. We have attempted to determine if the MOLF/Ei allele of *Naip5* is able to activate caspase-1 signaling in response to *L. pneumophila* challenge in HEK293 cells. However, we found that it is difficult to produce a robust caspase-1 response even in the presence of the B6 allele, so we have been unable to test this model directly (data not shown).

To further support a role of the amino acid variants identified in controlling the response to *L. pneumophila*, similar changes have been identified in members of the NLR family that function in the innate immune response to microbial infection in mammalian cells. We propose that the key residues in A/J Naip5 that lead to inactivation of protein function are clustered near motif II in the NACHT domain (Fig. 4), since other misregulating mutations have been identified in this region. In particular, mutations that contribute to autoinflammatory human diseases have been identified in the NACHT domain of NLR proteins. For instance, several mutations in the NACHT domain of NALP3 (also known as CIAS1, PYPAF1, or cryoprin) have been identified (1, 12, 14, 22). Missense mutations in NALP3 in this region are believed to result in autoactivation leading to the Muckle-Wells syndrome and familial cold urticaria (38), which are associated with an excess of interleukin-1β production. Three mutations in the NACHT domain of Nod2 (R334Q/W and L469F) are associated with Blau syndrome, an autosomal dominant trait leading to granulomatous arthritis, iritis, and skin rash (28), which may result in enhanced activation of transcription factor NF-κB (36). In addition, mutagenesis on NACHT family members has revealed that subtle amino acid changes in this region can affect protein function (38). Transgenic mice carrying mutations affecting these residues in the B6 *Naip5* allele product should help to determine if these residues are critical to Naip5 function as well.

In conclusion, the results of our study is consistent with the model in which *Naip5* controls *L. pneumophila* restriction even in the more genetically diverse wild mouse strain, MOLF/Ei. Naip5 restriction is dependent on caspase-1 activation and expression of *L. pneumophila* flagellin, similar to the more commonly used restrictive B6 strain. However, our study revises the critical amino acid residues in Naip5 function and suggests further examination of the polymorphisms within the NACHT domain. Lastly, the MOLF/Ei *Naip5* allele is hemidominant over the A/J *Naip5* allele, suggesting that there should be a more complex level of Naip5 regulation in murine macrophages than previously described.

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