Forward genetic analysis of Toll-like receptor responses in wild-derived mice reveals a novel antiinflammatory role for IRAK1BP1

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Although inflammatory cytokines produced by activation of Toll-like receptors (TLRs) are essential for early host defense against infection, they also mediate a vast array of pathologies, including autoimmune disease, hypersensitivity reactions, and sepsis. Thus, numerous regulatory mechanisms exist in parallel with proinflammatory pathways to prevent excessive release of these potent effector molecules. We report elucidation of a novel regulatory function for interleukin receptor–associated kinase (IRAK)-1 binding protein 1 (IRAK1BP1, also known as SIMPL) through quantitative trait locus mapping of the TLR response in wild-derived mouse strains. This gene emerged as a negative regulator of TLR2-mediated interleukin (IL)-6 production in MOLF/Ei mice, which expressed IRAK1BP1 mRNA in an allele-specific manner when crossed with the C57BL/6J strain. Human peripheral blood mononuclear cells and primary macrophages from two other wild-derived mouse strains also induced IRAK1BP1 mRNA by 4 hours after stimulation with agonists of various TLRs. Examination of its effects on IL-6 and other cytokines demonstrated that IRAK1BP1 regulates transcription of a specific subset of TLR-responsive genes, producing an overall anti-inflammatory profile. Our results reveal that IRAK1BP1 is a critical factor in preventing dangerous overproduction of proinflammatory cytokines by the innate immune system and in influencing the specificity of TLR responses. Furthermore, these results show that the genetic diversity of wild-derived mouse strains makes them a valuable model of important human gene functions that have been lost in some laboratory-inbred strains.

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Recognition of pathogens by the innate immune system activates Toll-like receptor (TLR)-mediated pathways, resulting in NF-κB–induced transcription of inflammatory cytokines (1). These molecules subsequently direct the initiation of appropriate adaptive responses, leading ultimately to clearance or containment of the invading pathogen (2). Due to their potent biological activity, however, inappropriately prolonged or excessive release of proinflammatory mediators can result in deleterious effects for the host. This is exemplified by the acute-phase cytokine IL-6, whose role in the development of the potentially pathogenic Th17 subset of T cells has recently been described (3). Furthermore, therapies targeting the cytokines IL-1, IL-6, and TNF-α have all shown promising efficacy in the treatment of autoimmune disorders, implying a central role for these molecules in disease pathogenesis (4, 5).

Given the dual nature of proinflammatory cytokines—essential for host defense but potentially lethal in excessive quantities—the mammalian immune system has evolved numerous regulatory mechanisms to precisely control the magnitude and time course of inflammatory responses (6). Regulation occurs at various levels of TLR signaling, from receptor ligation to gene transcription (7). Known mechanisms include the release of soluble TLRs (8, 9), competition for adaptor molecules and signaling intermediates (10–13), direct inhibition of NF-κB function (14, 15), and transcriptionally nonpermissive chromatin structure (16). The multiple levels of regulation highlight the importance of these processes and allow for greater specificity in controlling individual components of the inflammatory response.

The critical importance of balancing host defense with protection from inflammatory disease suggests that mutations strongly affecting

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TLR responsiveness will be appropriately compensated through evolutionary selection. This scenario lends to the experimental use of evolutionarily distant mouse strains to elucidate novel regulatory gene functions. Among the ~450 established inbred mouse strains, most descended from a restricted number of founder animals derived from the *Mus musculus* group of subspecies, and thus have rather limited genetic diversity. There are, however, several *Mus musculus* wild-derived inbred strains available, which have been captured and bred in geographically and temporally distinct circumstances. Such strains have large genomic contributions from other subspecies and are reported to have greater than one million years of evolutionary distance from classical inbred mice (17, 18). This diversity has been successfully used to map traits such as flavivirus (19) and *Salmonella typhimurium* susceptibility (20), as well as resistance to lethal shock induced by TNF-α (21).

We have observed that several wild-derived mouse strains have increased TLR-stimulated cytokine production compared with classical inbred strains. In an ongoing quantitative trait locus (QTL) analysis to map the gene(s) underlying this phenotype, we have observed that the major locus conferring increased TLR-stimulated IL-6 production in the wild-derived strain MOLF/Ei is partially compensated for by another locus, which inhibits inflammatory cytokine synthesis. The compensatory allele confers on several wild-derived strains the ability to induce mRNA synthesis of IL-1 receptor–associated kinase (IRAK)-1 binding protein 1 (IRAK1BP1), a gene previously implicated in TNF receptor signaling (22, 23), after

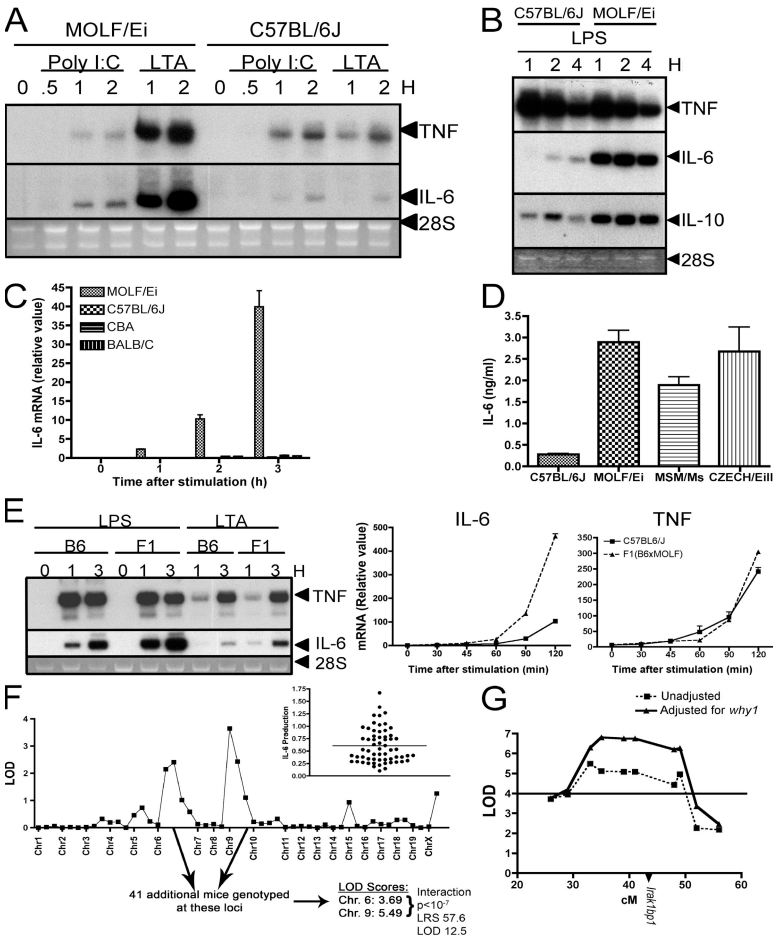

Figure 1. Phenotypic characterization and genetic mapping of TLR hyperresponsiveness in MOLF/Ei mice. (A and B) Peritoneal macrophages were elicited from MOLF/Ei and C57BL/6J (B6) strains and stimulated with the TLR agonists indicated. Northern blot analysis was performed using probes specific for TNF-α, IL-6 (A and B), and IL-10 (B). EtBr staining of 28s and 18s RNA was used as a loading control. (C) Macrophages from MOLF/Ei mice and the three classical strains shown were stimulated with 2 μg/ml LTA for the indicated length of time, and IL-6 mRNA was assessed by quantitative RT-PCR. (D) Macrophages from C57BL/6J and three wild-derived strains were stimulated with 2 μg/ml LTA for 6 h. Supernatants were assayed for IL-6 using ELISA. (E) F1 and C57BL/6J macrophages were stimulated with 2 μg/ml LTA for the indicated times. IL-6 and TNF-α mRNA was assayed using quantitative PCR and Northern blot analysis. All phenotypic assays represent at least three independent experiments. (F) 82 C57BL/6JxF1 backcross mice were analyzed by ELISA for IL-6 production 6 h after stimulation with 2 μg/ml LTA (insert). QTL analysis of genome-wide microsatellite marker coverage for 41 mice revealed a 10 cM, positively acting locus on chromosome 6 named *Why1* and a 23 cM, negatively acting locus on chromosome 9 named *Why2*. The remaining 41 mice were genotyped at these loci to achieve LOD scores of statistical significance. (G) Linkage map of the *Why2* interval. Markers at the indicated positions were used for QTL analysis and are shown both as raw values and adjusted using the contribution of genotype at *Why1* for background.

stimulation of TLR2. In contrast to its previously proposed role, we find that IRAK1BP1 inhibits the transcription of inflammatory cytokines from their endogenous promoters after TLR stimulation.

RESULTS AND DISCUSSION

TLR hyperresponsiveness in MOLF/Ei mice maps to two loci, one of which is inhibitory

Wild-derived strains of mice are separated from common laboratory strains by significant evolutionary distance, but are still able to breed with inbred strains and can thus be used in forward genetic analyses (17). Accordingly, we screened the wild-derived strain MOLF/Ei against the classical strain C57BL/6J for cytokine responsiveness to various TLR family agonists. Peritoneal macrophages from MOLF/Ei mice exhibited increased levels of cytokine mRNA after stimulation with the TLR2 agonist lipoteichoic acid (LTA), the TLR3 agonist polyI:C, and the TLR4 agonist LPS (Fig. 1, A and B, and Fig. S1, which is available at http://www.jem.org/cgi/content/full/jem.20071499/DC1). The largest effect was observed for LTA- and LPS-stimulated IL-6 production. To confirm that MOLF/Ei mice were hyperresponsive compared with other classical inbred strains, we examined LTA-induced IL-6 production in CBA and BALB/c mice. These strains responded similarly to C57BL/6J, suggesting that intersubspecific differences (18) rather than more recently introduced mutations underlie the phenotype (Fig. 1 C). The trait was also observed at the level of secreted IL-6 measured by ELISA and was present in two other wild-derived strains, MSM/Ms and Czech/EiII (Fig. 1 D), again consistent with an intersubspecific origin.

To examine the pattern of inheritance of TLR hyperresponsiveness, Northern blot and quantitative RT-PCR analyses were performed using F1(C57BL/6JxMOLF/Ei) peritoneal macrophages. IL-6, but not TNF-α, was elevated in F1 compared with C57BL/6J macrophages after LTA stimulation (Fig. 1 E). We thus chose LTA-stimulated IL-6 production as a marker of TLR hyperresponsiveness to use in our mapping studies. To map genetic loci underlying the observed phenotype, 82 C57BL/6JxF1(C57BL/6JxMOLF/Ei) backcross mice were analyzed by ELISA for IL-6 secretion 6 h after stimulation with LTA. Genome-wide microsatellite marker coverage for the initial 41 mice analyzed by QTL analysis revealed two loci on chromosomes 6 and 9, with suggestive linkage to the trait (Fig. 1 E). These loci were named *wild-derived hyperresponse* (*Why*)*1* and *Why2*, respectively. The remaining 41 mice were genotyped at these loci, and significant logarithm of the odds (LOD) scores of 3.69 (*Why1*) and 5.49 (*Why2*) were obtained. Heterozygosity at *Why1* (D6MIT328) was concordant with the observation in parental strains and conferred a positive effect on IL-6 secretion, whereas a partially compensating negative effect was linked to heterozygosity at *Why2* (D9MIT155). Furthermore, a significant epistatic interaction (LRS 48.1) was detected between the two loci. This interaction implies that both genes function in the same biological pathway, a feature commonly found between evolutionarily compensatory alleles (24).

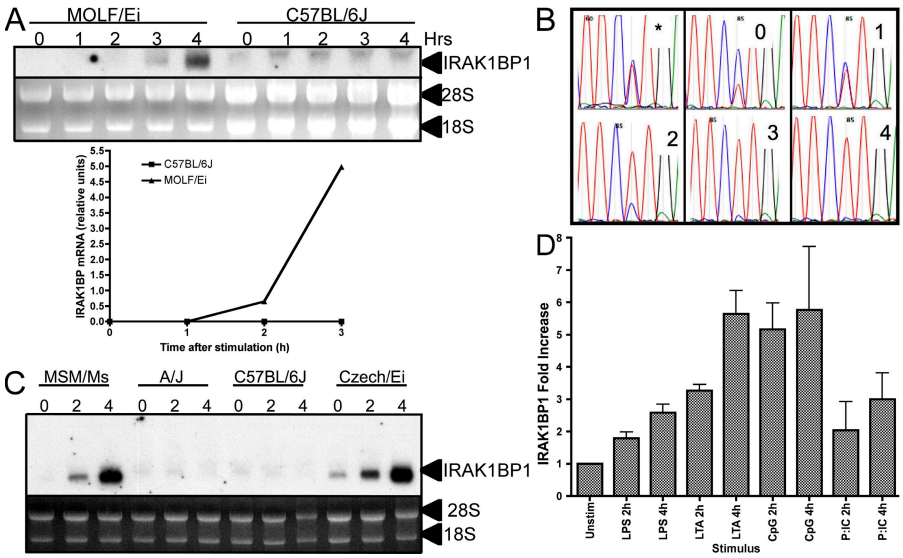

Figure 2. ***Cis*-acting regulatory elements confer TLR-mediated IRAK1BP1 inducibility in MOLF/Ei, but not C57BL/6J, macrophages.** (A) Peritoneal macrophages were stimulated with 2 μg/ml LTA for the indicated length of time. IRAK1BP1 mRNA was detected by Northern hybridization and quantitative PCR (each from independent experiments). (B) Macrophages elicited from F1(B6xMOLF) mice were stimulated with 2 μg/ml LTA for the number of hours indicated in each box. cDNA was prepared and IRAK1BP1-specific primers were used to amplify a 300-bp fragment containing a silent c→t mutation. Gel-purified products were sequenced to compare the amount of cDNA present from the MOLF/Ei (red peak) to C57BL/6J (blue peak) alleles. *, F1 genomic DNA. (C) Peritoneal macrophages from the indicated mouse strains were stimulated with 2 μg/ml LTA for 2 or 4 h, and IRAK1BP1 mRNA was assessed by Northern blot hybridization. (D) Human PBMCs were stimulated with the indicated TLR agonists for 2 or 4 h. IRAK1BP1 mRNA was assayed by quantitative RT-PCR. Data shown are mean ± range from duplicate wells, representative of two independent experiments.

Table I. Strain-specific variation of gene expression in the vicinity of the *Why2* locus

Transcript	FC	Gene name	Genbank	position (Mb)	Annotation
1452738_at	0.33	SLP-1; STORP; WPB72	AK007508	58051328	GO:5498(sterol carrier activity)
1443037_at	4.304	Sdfr1; AW554172, Nptn	BB183534	58380545	GO:50839(cell adhesion molecule binding)
1458512_at	23.19	ESG; Grg3a; Tle3; mKIAA1547	AW490470	61171677	GO:16055(Wnt receptor signaling pathway); GO:7283(spermatogenesis); GO:6350(transcription)
1421918_at	0.462	LANP; pp32; Anp32; PHAP1	AF022957	62139433	GO:5515(protein binding)
1442735_at	9.069	AZ-2; Sez15, Oaz2	BM941420	65474595	GO:5509(calcium ion binding)
1438246_at	3.178	9130020E21Rik	BM201663	65707015	GO:16055(Wnt receptor signaling pathway)
1420583_a_at	0.498	sg; ROR1; ROR2; ROR3; Nr1f1	NM_013646	68452965	GO:46068(cGMP metabolic process); GO:43030(regulation of macrophage activation); GO:6350(transcription)
1460083_at	3.027	kuz; MADM; Adam10 1700031C13Rik	AW552781	70478342	GO:7220(Notch receptor processing); GO:7267(cell-cell signaling)
1421955_a_at	0.413	Nedd4a; Nedd4-1; AA959633	NM_010890	72460913	GO:6464(protein modification process); GO:6512(ubiquitin cycle)
1456329_at	2.419	A230098A12Rik, Prtg	BB130087	72605779	GO:7155(cell adhesion)
1444411_at	5.932	d; Dbv; MVa; flr; Myo5; MyoVA; Sev-1	BB309795	74857810	GO:51643(ER localization); GO:7268(synaptic transmission); GO:7601(visual perception)
1436346_at	0.399	GARP; AI480638; CD109	AV246882	78401460	GO:48503(GPI anchor binding);GO:17114(wide-spectrum protease inhibitor activity)
1445387_at	4.754	Susp1; Senp6 mKIAA0797; 2810017C20Rik	BM118663	79852586	GO:6508(proteolysis); GO:6512(ubiquitin cycle)
1447762_x_at	3.749	AI314958; 2310047E01Rik, Car12	BB503164	66512885	GO:6730(one-carbon compound metabolic process)
1431771_a_at	0.0454	AIP70; Aabp3; SIMPL; AI851240; IRAK1BP1	AK014712	82626716	GO:7249(I-κB kinase/NF-κB cascade)
1427153_at	0.358	Bckdhb	AW047304	83745498	GO:9063(amino acid catabolic process)
1428547_at	0.409	NT; Nt5; eNT; CD73; AI447961	AV273591	88125532	GO:9166(nucleotide catabolic process)

Of the ∼560 genes present between 50 and 96 Mb on chromosome 9, 46 genes with a greater than twofold difference in expression levels between C57BL/6J and MOLF/Ei were identified. Genes that did not pass a standard expression threshold for either strain were discarded. The remaining 20 genes are listed in the order of their physical location on the chromosome. Average fold change (FC) indicates the ratio of expression levels in C57BL/6 relative to MOLF/Ei; the left column indicates Affymetrix accession number. Microarray data were deposited in the GEO database under accession numbers GSM259766 and GSM259767.

These quantitative trait-mapping results indicate that the major underlying genetic basis of TLR hyperresponsiveness in MOLF/Ei mice is contained within the *Why1* interval. *Irak-2* is the only gene in this region with a known role in TLR signaling. Despite the presence of several polymorphisms between the MOLF/Ei and C57BL/6J *Irak-2* alleles, we have thus far been unable to link any of these differences to the phenotype, though a serial outcross strategy has narrowed the interval even farther on either side of *Irak-2* (unpublished data). In addition to revealing the *Why1* locus, our mapping strategy also unveiled genetic mechanisms that wild-derived mouse strains have evolved to compensate for this trait and uncovered *Why2* as a major negatively acting locus in MOLF/Ei mice. Although *Why2* clearly does not act strongly enough to mask the hyperresponsive phenotype observed in MOLF/Ei mice, we reasoned that a regulatory allele was likely preserved at this locus under selective pressure from the overall proinflammatory bias conferred by *Why1*.

Cis-acting regulatory elements on *Why2* confer TLR inducibility on IRAK1BP1

Although the genetically determined interval containing *Why2* was relatively large (23 cM; Fig. 1 G), fine mapping of quantitative traits is often extremely difficult due to the effects of other loci with minor contributions to the phenotype. These undetected loci provide variability that prevents the use of single crossover events for defining interval boundaries. Thus, we combined a gene microarray approach (Fig. S2 and Table S1, available at http://www.jem.org/cgi/content/full/jem.20071499/DC1) with our mapping studies to identify potential candidate genes. Among genes differentially expressed in TLR-activated MOLF/Ei and C57BL/6J splenocytes from the *Why2* locus (Table I), *Irak1bp1* stood out both for the magnitude of its expression difference (∼20-fold) and for its known role in inflammatory signaling. Furthermore, this gene's previously defined ability to physically bind another IRAK family member, IRAK-1 (23), made

it an even more attractive candidate given the detected epistasis with the locus containing *Irak-2*.

To verify the microarray results in the conditions used during the mapping analysis, MOLF/Ei and C57BL/6J peritoneal macrophages were stimulated with LTA, and IRAK1BP1 mRNA was measured. Northern blotting revealed a significant increase in transcript level after LTA stimulation in MOLF/Ei, but not C57BL/6J, macrophages (Fig. 2 A). To determine whether the observed TLR inducibility of IRAK1BP1 mRNA in MOLF/Ei macrophages was linked to the detected locus on chromosome 9, allelic imbalance tests were performed. In F1(C57BL/6JxMOLF/Ei) macrophages, IRAK1BP1 mRNA was present almost exclusively from the MOLF/Ei allele 3 h after LTA stimulation, demonstrating the presence of cis-acting polymorphisms that make the gene transcriptionally responsive in MOLF/Ei mice (Fig. 2 B). Thus, the ability to up-regulate IRAK1BP1 mRNA after TLR stimulation is genetically linked to the inhibitory *Why2* locus detected in the analysis of IL-6 hyperresponse.

Why2 was found in our mapping analysis as a compensating mutation in the presence of an overall hyperresponsive phenotype. We therefore reasoned that if IRAK1BP1 was responsible for the linkage to this region, two other wild-derived strains with the same phenotype, Czech/EiII and MSM/Ms (Fig. 1 D), would also be under selective pressure to induce expression of IRAK1BP1 in response to TLR stimulation. Northern blot analysis revealed that these strains up-regulate IRAK1BP1 mRNA after stimulation with LTA, whereas C57BL/6J and another classical inbred strain, A/J, were transcriptionally unresponsive (Fig. 2 C). Macrophages in the quiescent state did not show high levels of IRAK1BP1 mRNA in any of the five strains analyzed in Fig. 2 (A and C), although previous data have shown basal expression levels in other tissues (23). We further extended our transcriptional analysis of IRAK1BP1 to human PBMCs and showed that it was transcriptionally responsive to a variety of TLR agonists in these cells as well (Fig. 2 D). Thus, IRAK1BP1 may also function in the context of human TLR signaling biology, and this trait is well modeled in three wild-derived mouse strains.

IRAK1BP1 contributes to the effect of *Why2* on LTA-induced IL-6 production

We next assessed the functional implication of *Irak1bp1* inducibility from the *Why2* locus in the context of our genetic mapping data. Because the MOLF/Ei allele at the *Why2* locus had an inhibitory effect on IL-6 production, and IRAK1BP1 was up-regulated in MOLF/Ei but not C57BL/6J mice, we predicted that IRAK1BP1 would down-regulate TLR-stimulated IL-6 production in macrophages. Previous studies have implied that IRAK1BP1 functions in the context of TNF-α signaling as a co-activator of RelA-mediated transcription (22, 23). However, these studies have been conducted using reporter plasmids, whereas our phenotype relied on measuring endogenously produced IL-6. Although ectopically introduced reporter assays are often used to assess the general function of inflammatory regulators, the intricate nature of site-specific chromatin remodeling and individual cytokine expression patterns is increasingly gaining appreciation (25, 26). Furthermore, reporter assays are not able to reveal all trans-acting regulatory effects that may be influencing the actual amount of a specific cytokine that is produced.

Given the complexity of cytokine gene regulation that is not incorporated into basic reporter assays, we sought to first characterize the effect of IRAK1BP1 in a more functional model of TLR signaling. The RAW264.7 mouse macrophage cell line is responsive to TLR activation and synthesizes cytokines from their endogenous genomic loci (27). To examine the role of IRAK1BP1 in a fully functional TLR pathway, we generated stably overexpressing RAW264.7 cells through retroviral transduction. IRAK1BP1-transduced cells produced significantly less IL-6 mRNA after LTA stimulation compared with empty vector–transduced cells. Furthermore, secreted IL-6 levels were decreased by ∼75% in IRAK1BP1-transduced cells (Fig. 3 A). Unexpectedly, in analyzing the levels of IRAK1BP1 mRNA in these cells, it was observed that RAW264.7 macrophages constitutively express high levels of transcript (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20071499/DC1). Therefore, we used a lentiviral-based short-hairpin RNA (shRNA)

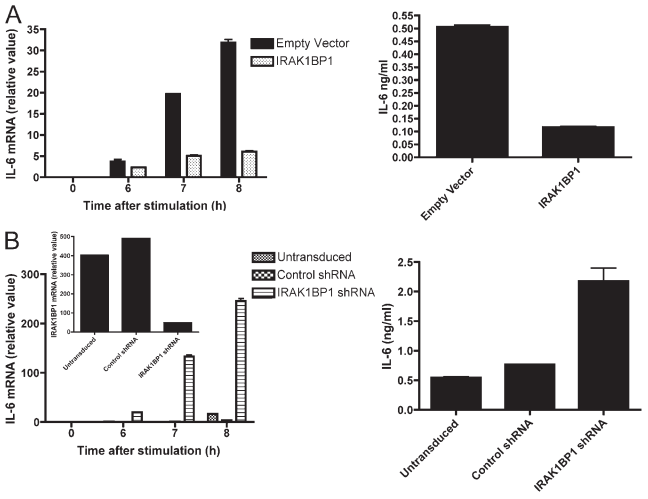

Figure 3. IRAK1BP1 inhibits LTA-stimulated IL-6 production. (A) IRAK1BP1 was stably introduced into RAW264.7 macrophages using retroviral transduction. IRAK1BP1-overexpressing and empty vector–transduced cells were stimulated with 2 μg/ml LTA and IL-6 mRNA was quantified using RT-PCR at the indicated time. Secreted IL-6 was quantified 16 h after stimulation using ELISA. (B) Stably transduced IRAK1BP1 or control (nonhomologous) shRNA-expressing cells were assessed by quantitative PCR for levels of IRAK1BP1 mRNA (inset) along with untransduced RAW264.7 macrophages. All cell types were assayed for LTA-stimulated IL-6 production as in A. The small variation between control shRNA and untransduced cell lines may represent residual effects of viral infection. Mean ± range from duplicate (RT-PCR) or ± SEM from triplicate (ELISA) wells are shown. RT-PCR experiments are representative of at least $n = 3$ independent experiments.

gene knockdown approach to confirm IRAK1BP1's inhibitory effect on IL-6 production. Expression of IRAK1BP1 was reduced >95% in knockdown cells compared with untransduced RAW264.7 cells and those receiving a control shRNA construct with no homologous sequence in the mouse genome. Consistent with its inhibitory role, IRAK1BP1 knockdown potently increased both LTA-induced IL-6 mRNA and protein expression compared with control and untransduced cell lines (Fig. 3 B).

We next sought to confirm the role of IRAK1BP1 in the observed linkage to the *Why2* interval (Fig. 1, F and G) by examining its role in primary macrophages from the strains used in the mapping study. We used the ability of the lentiviral shRNA system to infect primary mammalian cells as a means to inhibit LTA-inducible expression of IRAK1BP1 from the MOLF/Ei allele. Transduction and selection for stable integration of the shRNA construct in bone marrow–derived macrophages (BMDMs) from MOLF/Ei mice decreased the amount of IRAK1BP1 mRNA in these cells after LTA stimulation. Consistent with its effect in RAW264.7 cells and the mapping data, inhibition of IRAK1BP1 in MOLF/Ei BMDMs reduced IL-6 mRNA and protein levels after TLR activation (Fig. 4 A).

To assess the allelic specificity of IRAK1BP1's effect directly on the phenotype used in the mapping of *Why2*, we introduced the shRNA construct into both MOLF/Ei and C57BL/6J peritoneal macrophages. Although these cells are terminally differentiated and nondividing, thus decreasing efficiency of infection and preventing selection of a pure population of transductants, we were still able to significantly impair the ability of MOLF/Ei and F1(C57BL/6JxMOLF/Ei) peritoneal macrophages to induce IRAK1BP1. Furthermore, this inhibition directly affected the phenotype of LTA-stimulated IL-6 secretion in MOLF/Ei and F1, but not C57BL/6J, macrophages, implying a strain-specific effect on the phenotype (Fig. 4 B). Finally, to directly demonstrate that IRAK1BP1 was contributing to the linkage of *Why2* to the trait, we chose a small panel of 21 N2 mice from the mapping experiments to receive IRAK1BP1 shRNA. Introduction of this construct into peritoneal macrophages decreased the LOD score for this group of mice by 0.67 compared with introduction of control shRNA into cells from the same mice (Fig. 4 C). Our inability to completely abolish linkage to *Why2* in the presence of IRAK1BP1 shRNA may be due to a low efficiency of transduction or incomplete knockdown, allowing residual contributions to persist. Nonetheless, these data demonstrate that inhibition of LTA-induced IRAK1BP1 expression decreases linkage to the *Why2* locus and implies a role for expression of this gene in the context of the overall phenotype.

IRAK1BP1 exhibits an antiinflammatory regulatory profile at the transcriptional level

The unique transcriptional requirements of individual cytokines are key mechanisms by which immune cells direct the nature of an inflammatory response. To characterize the breadth of cytokines affected by IRAK1BP1, we used the ability of

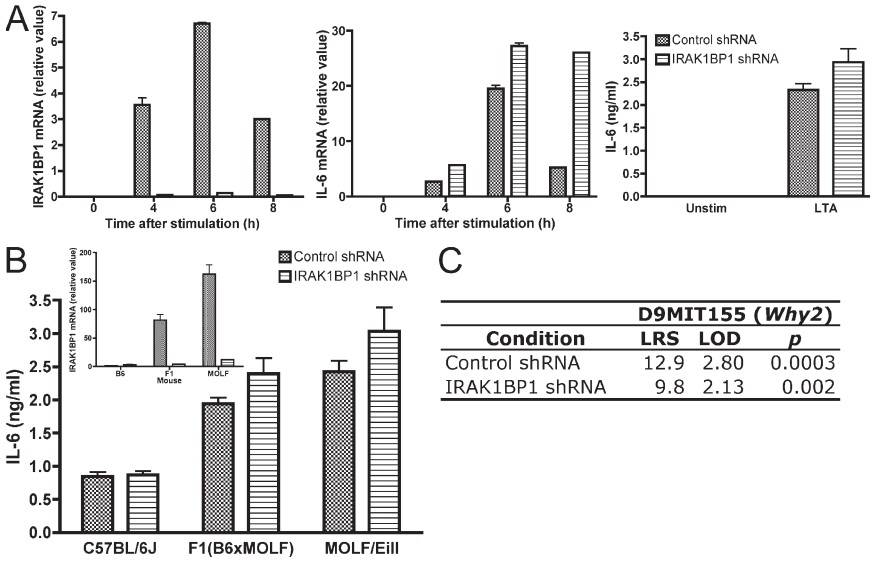

Figure 4. IRAK1BP1 regulates the LTA-stimulated IL-6 phenotype in primary MOLF/Ei macrophages and contributes to the linkage of the *Why2* locus. (A) BMDMs were infected with IRAK1BP1 or nonhomologous control shRNA constructs during in vitro differentiation. Stable transductants were selected and assessed for IRAK1BP1 mRNA, IL-6 mRNA, and protein after stimulation with LTA. IL-6 protein levels were assessed at 6 h after stimulation. (B) Peritoneal macrophages from the indicated strains were infected with control or IRAK1BP1 shRNA and assayed for LTA-induced IRAK1BP1 (inset) or IL-6 production 6 h after stimulation. Data representative of two (A) or three (B) independent experiments. (C) Macrophages from 21 N2(C57BL/6JxF1) mice were transduced with IRAK1BP1 and control shRNA (three wells each construct per mouse) and assessed for LTA-stimulated IL-6 secretion. Linkage to D9MIT155 (*Why2*) was calculated for each condition using a QTL analysis.

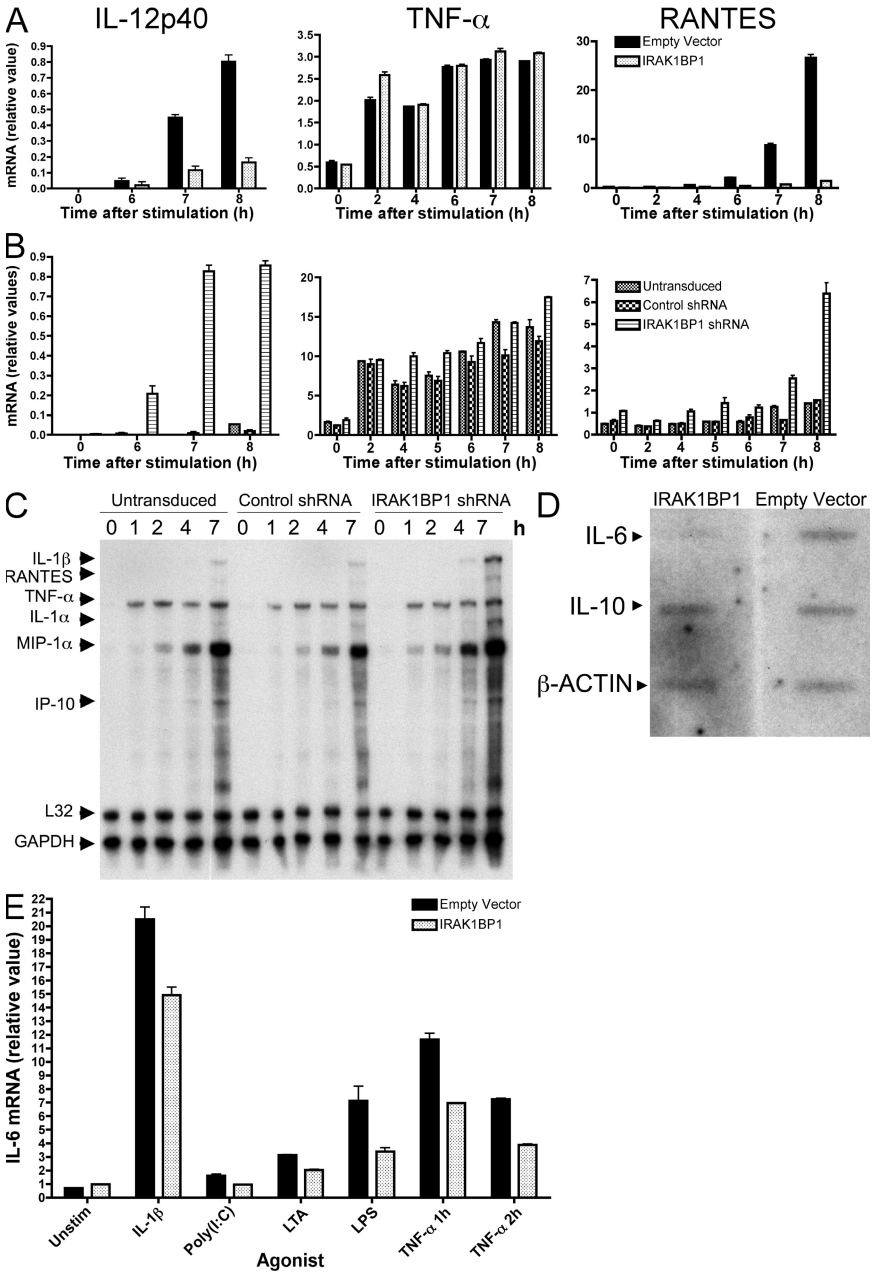

Figure 5. IRAK1BP1 regulates a subset of TLR-responsive genes and functions at the level of transcription. (A and B) IRAK1BP1-overexpressing or knockdown RAW264.7 macrophages were stimulated with 2 μg/ml LTA and assayed by quantitative RT-PCR for IL-12p40, TNF-α, and RANTES at the indicated times. Mean ± range for duplicate wells is shown, representative of at least two independent experiments. (C) Untransduced or shRNA-expressing cells were stimulated for the indicated lengths of time with 2 μg/ml LTA. RNA was hybridized with the indicated cytokines in an RNase protection assay, using L32 and GAPDH as loading controls. (D) Nuclei were extracted from empty vector–transduced or IRAK1BP1-overexpressing cells, and IL-6, IL-10, and β-actin–specific plasmids were used for nuclear runoff assays. (E) C57BL/6J MEFs were lentivirally transduced with IRAK1BP1 overexpressing of empty vector (pLEX) control constructs and stimulated with the indicated agonists for 1 (all agonists) or 2 (TNF-α, as indicated) h. IL-6 mRNA was assessed by quantitative RT-PCR.

RAW264.7 macrophages to activate transcription of endogenous TLR-responsive genes, using all of the subtle modifications required at each regulatory region. As shown in Fig. 5 (A and B), shRNA and overexpression studies demonstrated that IRAK1BP1 inhibits the ability of LTA-stimulated macrophages to transcribe the proinflammatory cytokines IL-12p40 and RANTES (CCL5). However, no effect was observed on the ability to transcribe TNF-α. As TNF-α is known to be an "early response" cytokine (25), we confirmed that IRAK1BP1 was not mediating effects before the time points used in our

assay by assessing mRNA levels at 30, 60, and 90 min after stimulation with LTA and still observed no significant difference in mRNA levels between control and IRAK1BP1-overexpressing or knockdown cell lines (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20071499/DC1). To further expand the panel of proinflammatory cytokines affected by IRAK1BP1, we conducted RNase protection assays using untransduced and stably expressing control shRNA and IRAK1BP1 shRNA RAW264.7 cells after stimulation with LTA. In addition to confirming the presence of an effect for RANTES and the lack of an effect for TNF-α, these results showed that IRAK1BP1 also inhibits the mRNA accumulation of IL-1α, IL-1β, and to a lesser degree MIP-1α, whereas exerting only a minimal effect on IP-10 (Fig. 5 C).

The observed differences in mRNA levels for the cytokines described could be due to transcriptional inhibition or to an increase in the rate of mRNA degradation mediated by IRAK1BP1. To discriminate between these scenarios, a nuclear runoff assay was performed for IL-6 in IRAK1BP1-overexpressing or empty vector–transduced cell lines. At 6 h after stimulation with LTA, IRAK1BP1-overexpressing RAW264.7 cells had noticeably less IL-6–nascent transcript, indicating that IRAK1BP1 exerts its effect at a stage preceding the transcription of inflammatory cytokines rather than through posttranscriptional regulatory mechanisms (Fig. 5 D). Finally, we sought to determine whether IRAK1BP1 acts at the level of proximal signaling events specific for TLR pathways or has a more general inhibitory role in inflammatory gene transcription. To address this question, mouse embryonic fibroblasts (MEFs) from C57BL/6J mice were transduced with IRAK1BP1 or empty vector controls and stimulated with IL-1β, TNF-α, or various TLR agonists. Although varying degrees of inhibition were observed, all agonists consistently exhibited decreased IL-6 production in the presence of overexpressed IRAK1BP1 (Fig. 5 E). Collectively, these studies show that the presence of IRAK1BP1 has an overall transcriptionally inhibitory effect on a subset of proinflammatory cytokines that is exerted in the presence of several receptor signaling pathways.

Conclusion

We have used QTL analysis of LTA-stimulated IL-6 production in wild-derived mice combined with microarray studies to elucidate a novel antiinflammatory function of IRAK1BP1. The ability of IRAK1BP1 to inhibit inflammatory cytokine production was demonstrated using ectopic expression as well as shRNA knockdown of IRAK1BP1 in a mouse macrophage cell line, two types of primary mouse macrophages, and MEFs. Although previous studies have examined this gene in the context of TNF-α–stimulated RelA co-activation, the functional data presented here are the first to show its effects on specific cytokines transcribed from their endogenous loci. IRAK1BP1 could be mediating its effects in a trans fashion by preferentially activating antiinflammatory NF-κB–dependent genes, which subsequently repress inflammatory cytokines. Alternatively, it could function as a "molecular switch," activating gene transcription from some promoters while inhibiting others. The importance of a balanced and precisely coordinated inflammatory response is increasingly recognized for its contribution to immune-mediated disease. As greater numbers of diagnostic and therapeutic techniques rely on specific biological activities, the regulatory function of IRAK1BP1 in TLR-mediated inflammation described here extends our understanding of how these processes are controlled.

MATERIALS AND METHODS

Genetic analysis. Parental mouse strains were obtained from The Jackson Laboratory. Peritoneal macrophages were elicited from N2 mice and phenotyped as described below, and genome-wide scanning was performed according to standard procedures, using two to three known polymorphic microsatellite markers per chromosome. Both genotypic and phenotypic data were analyzed using QTX software for genetic mapping of quantitative trait loci. 21 of the N2 mice used in the screen were also phenotyped after transduction with the pLKO.1 knockdown construct (described below), and linkage was assessed using the same methods. All experiments with mice were performed in accordance with the regulations and with the approval of Tufts/New England Medical Center Institutional Animal Care and Use Committee. All mice were maintained in a pathogen-free mouse facility at Tufts University School of Medicine.

Cell culture. Primary mouse macrophages were elicited by intraperitoneal injection of 3% thioglycollate in 6–8-wk-old mice. 3 d later, the peritoneal cavity was washed with PBS, cells were resuspended in DMEM, 10% FBS, and plated. Assays were performed 6–16 h after plating or cells were transduced for 24 h as described below. Bone marrow macrophages were isolated from femurs of 6–8-wk-old MOLF/Ei mice as described previously (28). MEFs were isolated from 14-d embryos by trypsinization after dissection of the head and fetal liver. All experiments with MEFs were performed between passages two and five. Human PBMCs were obtained from healthy anonymous donors and were used with approval of the Institutional Review Board at Tufts University School of Medicine. PBMCs were prepared by Ficoll-Hypaque centrifugation on a Lymphoprep gradient. Cells were plated and stimulated with TLR agonists 6 h after plating. All cell lines were obtained from American Type Culture Collection (ATCC) and grown in DMEM, 10% FBS.

Plasmids. IRAK1BP1 cDNA was reverse transcribed and amplified from RAW264.7 RNA and cloned into the BamHI restriction site of the PINCO vector (provided by G. Nolan, Stanford University, Stanford, CA) (29) for transduction of RAW264.7 or pLEX (Open Biosystems) for transduction of MEFs. Orientation of the insert was verified by sequencing. All shRNAs were expressed in the pKLO.1 vector and obtained from Open Biosystems. Plasmid DNA for psPAX2 and pMD2.G vectors was from Addgene.

Transfections and transductions. The PINCO retroviral system was used as described previously (29). Viral supernatants were harvested from PHOENIX-AMPHO HEK293 cells (ATCC) 3 d after transfection (Fugene; Roche) and added to dividing RAW264.7 cells for 24 h. 3 d later, transductants were selected by FACS for GFP expression. shRNA was expressed in the pLKO.1 plasmid (Open Biosystems). Viral supernatants were generated by transfecting 293-T cells with the shRNA construct and packaging vectors pSPAX2 and pMD2.G (Addgene). Stably expressing RAW264.7 knockdown clones were selected by growth in puromycin for 2 wk. BMDMs were infected with the pLKO.1 shRNA construct for 24 h on day 4 of maturation. Cells were washed twice with RPMI, 10% FBS, and were further matured in 3 μg/ml puromycin for 3 d. Peritoneal macrophages were infected 3 h after elicitation for a 24-h period. Cells were washed twice with DMEM, 10% FBS, and recovered for 48 h before assays were performed. MEFs were transduced with IRAK1BP1 using the pLEX retroviral vector packaged using pSPAX2

and pMD2.G as described above, and stable transductants were selected in 5 μg/ml puromycin.

Phenotypic analyses. Salmonella minnesota Re595 LPS was purchased from Qbiogene. Poly I:C was obtained from GE Healthcare. All other agonists were obtained from InvivoGen. After stimulation, supernatants were analyzed using IL-6 ELISA (R&D Biosystems), and mRNA was prepared with Trizol (Invitrogen). mRNA was subsequently analyzed by Northern blot hybridization, RNase protection (BD Biosciences), or quantitative RT-PCR using Taqman gene-specific probes (Applied Biosystems).

DNA sequencing and cDNA synthesis. PCR products were amplified from genomic DNA or cDNA. Fragments were sequenced bidirectionally with internal primers. cDNA was synthesized from purified RNA (Trizol) using either random nanomer or oligo-dT primers and M-MLV reverse transcription (New England Biolabs, Inc.).

Nuclear runoff assay. Nuclei were isolated in Tween lysis buffer after 6 h of stimulation of cells with LTA. If not used immediately for transcription, nuclei were frozen in liquid nitrogen. 200 μl of nuclei suspension in 1× runoff buffer (25 mM Tris-HCl, pH 8, 12.5 mM MgCl2, 750 mM KCl, and 1.25 mM each of ATP, CTP, and GTP), 2 μl of RNase inihibitors (1 U/ml; New England Biolabs, Inc.), 200 mCi of a-[^{32}P]UTP, and 3,000 Ci/mmol (PerkinElmer) were added to 220 liters of nuclei suspension and incubated at 30°C for 30 min. Elongated transcripts were then isolated with Trizol, denaturated at 65°C for 5 min, and hybridized at 42°C for 48 h to 5 μg of denatured DNA immobilized on nylon filters in a few milliliters of hybridization solution (200 mM NaHPO4, pH 7.2, 1 mM EDTA, pH 8, SDS 7%, deionized formamide 45%, 250 mg/ml *Escherichia coli* tRNA). Filters were then washed once or twice at 37°C for 20–30 min in 40 mM NaHPO4-SDS 1% and exposed for autoradiography.

RNase protection assay. Total cellular RNA from individual samples was isolated with Trizol and hybridized for 16 h with custom-designed probes (BD Biosciences) according to the manufacturer's instructions. Nonhybridized RNA was digested with RNase, and remaining hybrids were run on a 5% PAAG gel together with probe. The gel was dried and exposed for 30 min to x-ray film.

Online supplemental material. Fig. S1 shows an extended panel of cytokine differences in C57BL/6J and MOLF/Ei. Fig. S2 depicts the microarray data discussed. Fig. S3 shows constitutive expression of IRAK1BP1 mRNA in RAW264.7 cells. Fig. S4 shows no effect of IRAK1BP1 on TNF-α production at early time points after LTA stimulation. Table S1 shows genome-wide results from the microarray experiment. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20071499/DC1.

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