

IL-1 Receptor antagonist as a positional candidate gene in a murine model of allergic asthma

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Received: 11 April 2006 / Accepted: 14 July 2006
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Abstract Interleukin-1 receptor antagonist (IL-1ra) is an inhibitor of the proinflammatory IL-1. The IL-1ra gene (*Il1rn*) maps near the allergen-induced bronchial hyper-responsiveness-1 locus, *Abhr1*, which we previously mapped to murine chromosome 2 using A/J (asthma susceptible) and C3H/HeJ (asthma resistant) mice. We evaluated the role of *Il1rn* in our mouse model by comparing its genomic sequence between A/J and C3H/

HeJ mice as well as assessing strain-specific RNA and protein production in response to allergen. We identified no functional sequence variations in the *Il1rn* gene between A/J and C3H/HeJ mice. *Il1rn* mRNA and protein were induced by ovalbumin (OVA) exposure in both strains, but to a greater extent in A/J mice at the earlier time points. We examined other IL-1 family members (*Il1a*, *Il1b*, *Il1f9*, and *Il1r2*) and found OVA-induced expression increases at 6 h, yet only *Il1b* and *Il1f9* had strain-specific differences. Of these, only *Il1f9* is located within *Abhr1*, and we found several non-coding polymorphisms in the *Il1f9* gene between A/J and C3H/HeJ mice. Our results exclude *Il1rn* as the gene for *Abhr1* and indicate that *Il1f9* warrants further investigation based on genetic and expression differences observed in our mouse model of allergic asthma.

Electronic supplementary material Supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s00251-006-0146-x> and is accessible for authorized users.

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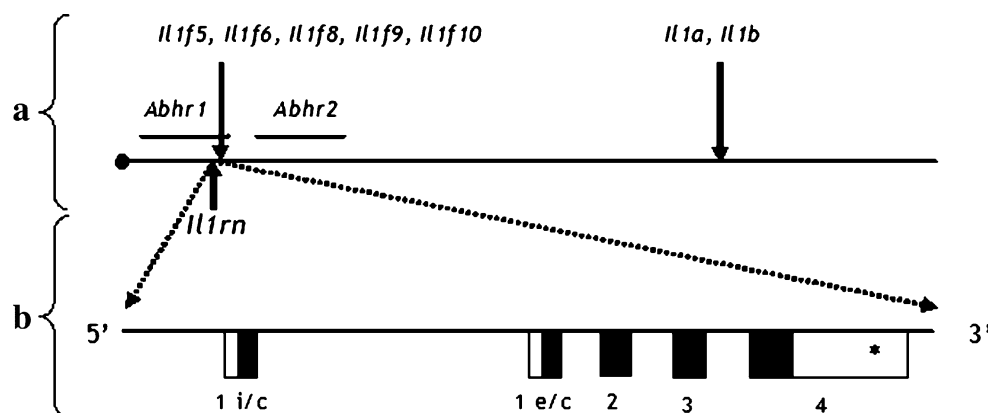
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Keywords IL-1 receptor antagonist · Asthma · Genetics · Airway inflammation · Mouse

Interleukin-1 receptor antagonist (IL-1ra) is an important anti-inflammatory cytokine that counter-regulates the pro-inflammatory effects of interleukin-1 (IL-1) in a variety of inflammatory disorders, including asthma (Arend et al. 1998; Arend and Guthridge 2000). The IL-1 complex consists of several agonists, antagonists, and receptors. IL-1 (IL-1 α or IL-1 β) binds to the IL-1 receptor (IL-1R1) and elicits a pro-inflammatory signal transduction cascade. This is counter-regulated by the IL-1ra, which competitively binds to IL-1R1 and prevents IL-1 binding to the IL-1R1. The decoy receptor IL-1R2, lacking the intracellular signal transduction domain, also counter-regulates IL-1-mediated inflammation by binding to IL-1 and preventing it from binding to the functional receptor IL-1R1. Several new genes (*IL1F5*–*IL1F10*) have been identified in the IL-1

Fig. 1 Sequencing of the murine *Il1rn* gene. **a** Many IL-1 family genes map to murine chromosome 2, several within the *Abhr1* locus. **b** A 16-kb region containing the four exons, including alternative exon 1 for intracellular (i/c) and extracellular (e/c) isoforms, splice sites and introns of *Il1rn*. □ Untranslated regions in exons; ▪ coding regions in exons. Asterisk indicates dinucleotide repeat polymorphism



gene family (Nicklin et al. 2002), whose functions remain to be fully elucidated.

It has been shown that IL-1ra knock-out mice have an increase in bronchial hyper-responsiveness (BHR), T-helper (Th) 2-cell proliferation, and increased IL-4 and IL-5 cytokines in response to allergic airway challenge (Nakae et al. 2003), confirming its protective role in asthma. IL-1ra also plays a protective role against ozone-induced BHR (Park et al. 2004), and the ratio between IL-1 and IL-1ra is also an important factor in asthma (Mao et al. 2000; Arend 2002).

We have previously performed a linkage study in our allergic asthma model using A/J (asthma hyper-responsive) and C3H/HeJ (asthma hypo-responsive) strains of mice, and found two quantitative trait loci (QTL) for allergen-induced BHR, termed *Abhr1* and *Abhr2*, on mouse chromosome 2 (Ewart et al. 2000). The IL-1 receptor antagonist gene (*Il1rn*) and the mouse homologues of the

newly identified IL-1 family member genes *Il1f5–Il1f10* (Nicklin et al. 2002) are located within the QTL *Abhr1*. Thus, in this study we examined several members of the IL-1 family in our model, with a focus on *Il1rn* as a positional candidate gene for *Abhr1* based on the genetic evidence from our model and its functional relevance in asthma. The following studies were approved by the All-University Committee on Animal Use and Care of Michigan State University.

To determine whether the increased allergen-induced BHR phenotype in A/J mice, compared to C3H/HeJ mice, was due to functional differences in the *Il1rn* gene, we examined *Il1rn* sequence in both strains. Our results (Fig. 1 and Genbank DQ383807 and DQ383808) confirmed the microsatellite *D2Mit60* (A/J, [TG]₂₁; C3H/HeJ, [TG]₂₀) in the *Il1rn* 3' UTR; however, no other polymorphisms between A/J and C3H/HeJ strains were observed in approximately 16 kb of sequence.

Table 1 Primers for quantifying mRNA expression of IL-1 related genes

Primers and probe ^a	Sequence (5'–3')	Assay based on
	<i>Il1rn</i>	GenBank M64404
Forward primer	AGTACTGCCGAGGCCTGTAATAA	
Reverse primer	TTGTTCTCAGGCCCAAT	
Probe	ACCAACTGCCTGATCACTCTGGCCAT	
	<i>Il1a</i>	GenBank NM_010554
Forward primer	CAGGGCAGAGAGGGAGTCAAC	
Reverse primer	CAGGAACCTTGCCATCTTGAT	
	<i>Il1f9</i>	GenBank NM_153511
Forward primer	CCCTTGTGACAGTTCCACGAA	
Reverse primer	GGTACTTGCATGGGAGGATAG	
	<i>Il1r1</i>	GenBank NM_008362
Forward primer	CGGCGCATGTGCAGTTAATA	
Reverse primer	TGTAGCCGTGAGGATGATAAAGC	
	<i>Il1r2</i>	GenBank NM_010555
Forward primer	AGTGCAGCAAGACTCTGGTACCTA	
Reverse primer	AGTTCCACAGACATTTGCTCACA	
	<i>18S rRNA</i>	
Forward primer	CGGCTACCACATCCAAGGAA	
Reverse primer	GCTGGAATTACCGCGGCT	

^a Primer Express software (Applied Biosystems, Foster City, CA, USA) was used for assay design.

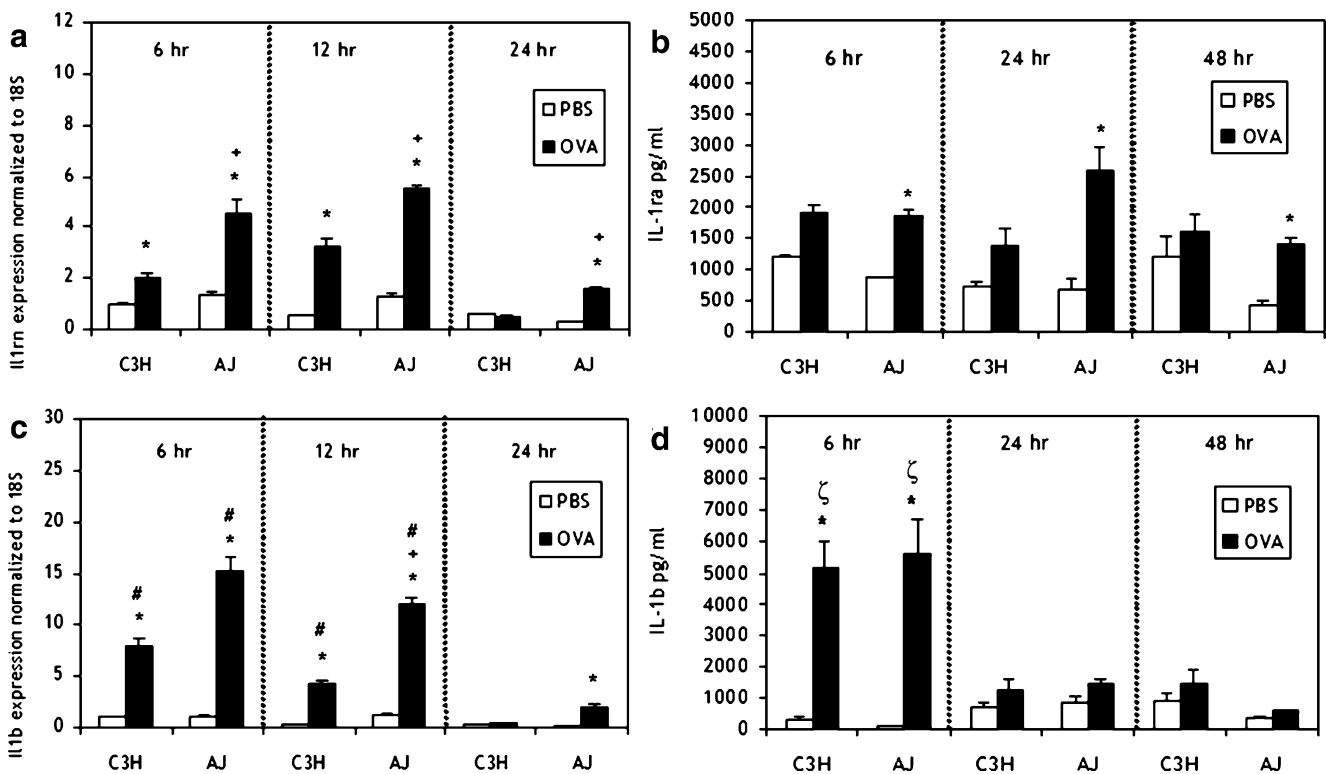


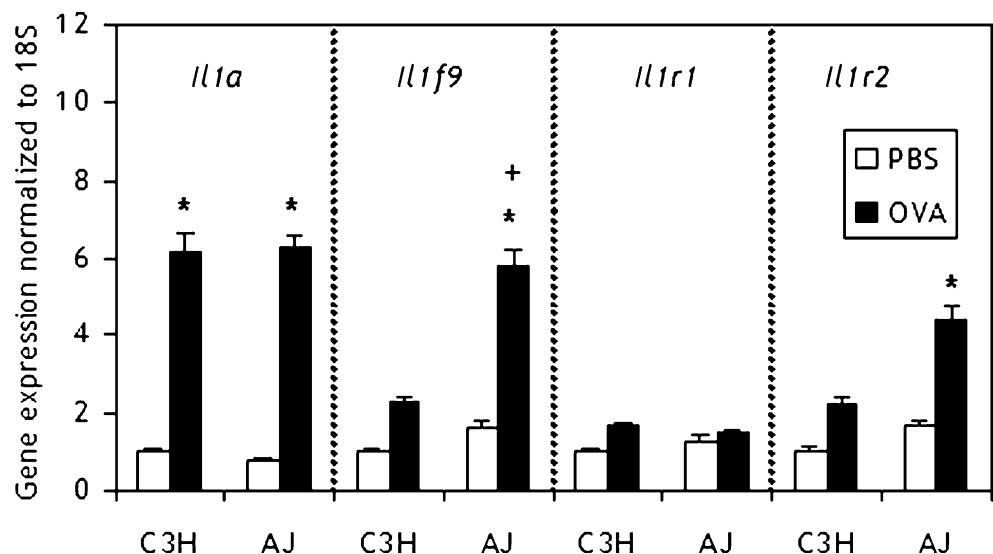
Fig. 2 Lung homogenates were assayed for **a** *Il1rn* mRNA, **b** IL-1ra protein, **c** *Il1b* mRNA, and **d** IL-1 β protein. The sensitivities of IL-1ra and IL-1 β ELISAs were 4–13 pg/ml and 25 pg/ml, respectively. Samples were assayed in duplicate and values reported as mean $\pm$ SEM.

Significance differences ($p < 0.05$) due to: asterisk treatment; plus sign strain; pound sign different from 24-h time point; and ζ different from 48-h time point

To test the hypothesis that reduced IL-1ra levels underlie allergen-induced bronchial hyper-responsive in A/J mice, we compared *Il1rn* mRNA and protein levels across strains and allergen-exposure treatments. A/J and C3H/HeJ male mice (JAX, Bar Harbor, ME) were sensitized i.p. with 10 μ g ovalbumin (OVA, Sigma, St. Louis, MO, USA) in 0.2 ml calcium and magnesium-free phosphate-buffered saline (PBS) or an equivalent amount of PBS alone on

day 0. On day 14, mice were anesthetized (i.p. ketamine, 45 mg/kg and xylazine, 8 mg/kg), challenged by pharyngo-tracheal instillation of 1.5% OVA in 50 μ l PBS, or PBS alone. This protocol and dose of allergen were previously established as the lowest dose required to induce distinct asthmatic responses in our mouse model (Ewart et al. 2000). The mice were killed and tissues collected at various times post challenge.

Fig. 3 Lung homogenates from 6-h samples were examined by SYBR Green assays for transcript expression of *Il1a*, *Il1f9*, *Il1r1*, and *Il1r2*. Samples were assayed in duplicate and values are reported as mean $\pm$ SEM. Significant differences ($p < 0.05$) due to: asterisk treatment and plus sign strain



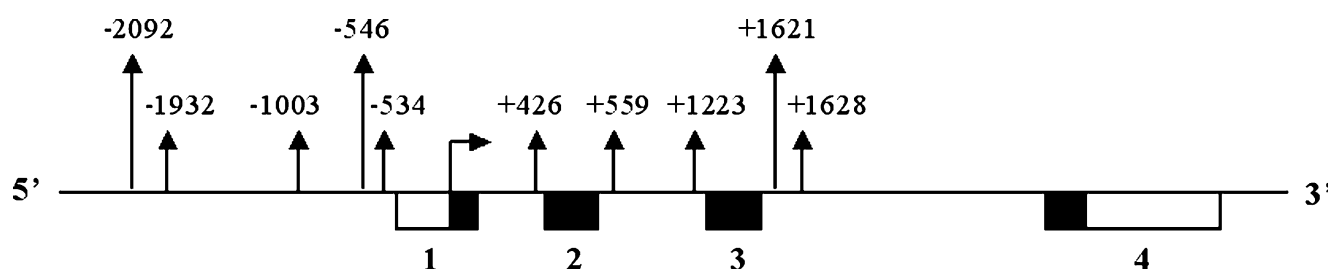


Fig. 4 Sequence differences in the murine *Il1f9* gene. Vertical arrows indicate the base-pair location of polymorphisms between A/J and C3H/HeJ strains. □ Untranslated regions in exons; ■ coding regions in exons. All coding exons and splice sites were completely sequenced

Total RNA was isolated from homogenized right lung lobes ($n=6/\text{group}$) and subjected to DNaseI treatment. Then 400 ng total RNA was reverse transcribed and used as the template for real-time PCR with 18 s rRNA as the internal control. An Assay-on-Demand Gene Expression kit (Applied Biosystems, Foster City, CA, USA) was used to measure expression of *Il1b*, we designed primers for all other assays (Table 1). Data were analyzed by two- or three-factor ANOVA mixed models using SAS v9.1 (SAS Institute, Cary, NC, USA), and the P values were adjusted for multiple comparisons using Tukey or Tukey–Kramer tests.

We observed both treatment-induced and strain-specific increases in *Il1rn* mRNA expression in the lungs of A/J mice at 6, 12, and 24 h subsequent to OVA challenge (Fig. 2a). The major active agonist, *Il1b*, showed a similar expression pattern as *Il1rn* with significant treatment-related increases in expression in the lungs of OVA-treated A/J mice at all time points (Fig. 2c) as well as a strain-specific increase (A/J>C3H/HeJ) at the 12-h time point.

To correlate protein with these RNA findings, we performed ELISAs for IL-1ra and IL-1 β on right lung lobes collected from A/J and C3H/HeJ mice ($n=5/\text{group}$) after OVA or PBS challenge. We developed an ELISA for IL-1 β using anti-mouse IL-1 β antibody, biotinylated anti-mouse IL-1 β antibody and recombinant mouse IL-1 β (R&D Systems, Minneapolis, MN, USA) and used the

Quantikine ELISA kit for IL-1ra/IL-1F3 (R&D Systems, Minneapolis, MN, USA). Treatment-specific increases in IL-1ra were present in A/J mice (Fig. 2b). There were no significant strain effects; however, within PBS-treated groups IL-1ra tended to be lower in A/J mice. IL-1 β protein production was significantly increased due to OVA in both strains of mice at the 6-h time point and declined to baseline at 24 and 48 h (Fig. 2d).

Along with *Il1rn* and its major agonist, we also wanted to examine other members of the IL-1 family. Transcript levels of *Il1a* showed significant treatment-induced increases at the 6-h time point, but without strain differences (Fig. 3). Both treatment- and strain-specific increases were observed in the new IL-1 family member, *Il1f9*, at 6 h (Fig. 3). The levels of these IL-1 agonists detected returned to or below baseline levels by 72 h (data not shown). We were unable to detect the expression of *Il1f5*, *Il1f6*, *Il1f8*, or *Il1f10* in OVA- or PBS-treated lung tissues indicating an essential absence of expression in murine lungs. The active receptor, *Il1r1*, was not induced by allergen exposure in either strain (Fig. 3). In contrast, the decoy receptor, *Il1r2*, showed an increased expression in the lungs of only the A/J strain due to OVA treatment at 6 h (Fig. 3). The expression levels of these two receptors returned to the baseline level at 72 h after OVA challenge (data not shown).

We predicted that the heightened allergen-induced BHR in A/J mice, compared to C3H/HeJ mice, was due to a

Table 2 Polymorphisms between A/J and C3H/HeJ mice in the *Il1f9* gene

Location ^a	AJ	C3H	Forward primer	Reverse primer	Temp
-2092	–	C	CCAAAAGCTGAACTTAAAGACACAT	GGCTTGGGGATACTAAAGAATAAAA	60
-1932	T	A	CCAAAAGCTGAACTTAAAGACACAT	GGCTTGGGGATACTAAAGAATAAAA	60
-1003	C	T	TACACTTTTTCTAAGGCAACACA	CAGATCTGTCCATTCACTGAACC	60
-546	C	T	ACTCATGCTGACTCTGAGATTCTT	CTCCCTACAAGTTTCAGACCATAAA	60
-534	CA	TG	ACTCATGCTGACTCTGAGATTCTT	CTCCCTACAAGTTTCAGACCATAAA	60
+426	C	T	TTTGTTTCCCAGAGATTTCTACA	AATGATGAAACCAGGGTAATCAGTA	60
+559	A	G	TTTGTTTCCCAGAGATTTCTACA	AATGATGAAACCAGGGTAATCAGTA	60
+1223	C	T	TTCAAACATGGGTATGAGAATGTAG	GAGCTGAGGTGTGGAGAATCTATTA	60
+1621	(TTTA) ₆	(TTTA) ₇	TTCAAACATGGGTATGAGAATGTAG	GAGCTGAGGTGTGGAGAATCTATTA	60
+1628	T	G	TTCAAACATGGGTATGAGAATGTAG	TTCAAACATGGGTATGAGAATGTAG	60

Temp annealing temperature in the PCR reactions

^aLocation in relation to start codon in Ensembl *Il1f9* genomic sequence (Ensembl ID: ENSMUSG00000044103)

sequence difference in the *Il1rn* gene between these strains with a resulting insufficient production of *Il1rn* mRNA and protein in the A/J strain. In the present study, we did not find functional *Il1rn* gene polymorphisms between A/J and C3H/HeJ mice. Furthermore, while we found treatment-induced and strain-specific differences in the endogenous mRNA and protein production profiles of *Il1rn*, contrary to our expectations, the A/J strain had increased *Il1rn* mRNA and protein expression due to allergen challenge compared to C3H/HeJ mice (Fig. 2a,b). We suggest that these increased *Il1rn* levels in the asthma-susceptible A/J mice are in response to allergic inflammation, but not the cause of the differential allergen-induced BHR phenotype in our model. We did not conduct additional functional studies on *Il1rn*, as our results do not support a causative role in allergic asthma in this mouse model.

Interestingly, we found that the expression of *Il1f9* increased in A/J mice, compared to C3H/HeJ mice, at 6 h after allergen challenge (Fig. 3). This gene has been shown to increase the production of nuclear factor kappa B (NF- κ B) through an orphan receptor, IL-1 receptor (IL-1R)-related protein 2 (IL-1Rrp2) (Debets et al. 2001; Towne et al. 2004). IL-1 has also been shown to induce the production of NF- κ B (Li and Verma 2002), which is critical for the expression of the Th2-cell-specific transcription factor GATA-3 (Das et al. 2001), which in turn is necessary for the expression of pro-asthmatic cytokines IL-4, IL-5, and IL-13. Moreover, human IL-1F9 has also been shown to exhibit inducible expression in bronchial epithelial cells similar to IL-1 β in response to *P. aeruginosa* (Vos et al. 2005). Our findings of *Il1f9* strain- and treatment-specific expression differences, its location within *Abhr1*, and its potential for functional relevance in asthma led us to examine *Il1f9* in more detail.

Thus, we sequenced important regions of the murine *Il1f9* gene in A/J and C3H/HeJ mice to determine if the expression difference we observed could be due to polymorphisms within the *Il1f9* gene. We designed primers, amplified and sequenced the 5' upstream sequences and all the exons in the gene, using genomic DNA sequence of mouse *Il1f9* (Ensembl Gene ID: ENSMUSG00000044103) as a reference. We found several polymorphisms in the 5' upstream region of *Il1f9* and in intronic regions, but did not find polymorphisms in splice sites or exons (Fig. 4 and Table 2). These results indicate that further investigation focused on *Il1f9* is warranted to determine the importance

of these polymorphisms with the expression of the gene and its influence on asthma.

In conclusion, our results do not support *Il1rn* as the susceptibility gene for the QTL *Abhr1*, but mRNA and protein expression profiles indicate that several IL-1 complex genes play an important role in the early stages of allergic inflammation. Of these, *Il1f9* should be pursued further because of increasing evidence of its IL-1-like activity and the genetic and mRNA expression differences presented in our study that suggest its role in early stages of inflammation.

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