Published in final edited form as:

Genesis. 2013 October ; 51(10): . doi:10.1002/dvg.22424.

Genetic inactivation of the allograft inflammatory factor-1 locus

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Abstract

Allograft inflammatory factor-1 (Aif-1) is a 17 kDa EF hand motif-bearing protein expressed primarily in developing spermatids and cells of monocyte/macrophage lineage. Increased Aif-1 expression has been identified in clinically important conditions, including rheumatoid arthritis, systemic sclerosis, endometriosis, and transplant-associated arteriosclerosis. Largely similar gene products arising from the same locus are known as ionized Ca^{2+} binding adapter-1 (Iba1), microglial response factor-1 (MRF1), and daintain; Iba1 in particular has emerged as a histologic marker of microglia and their activation in pathologic CNS conditions, including the response to facial nerve axotomy and stroke, uveitis, and experimental autoimmune neuritis and encephalomyelitis. Nevertheless, how *aif-1* gene products affect cellular function is only partly understood, and the physiologic significance of these products for male fertility, immune system development, and inflammation has not been described. To permit such investigations, we generated a mouse line with targeted deletion of the coding regions of the *aif-1* gene. Here we report that mice lacking Aif-1 breed well and show normal post-natal growth, but show resistance to disease in a model of collagen-induced arthritis. We anticipate that these mice will be useful for studies of Aif-1 function in a variety of immune and inflammatory disease models.

Keywords

Aif-1; Iba1; Daintain; MRF-1; spermatid; macrophage

Introduction

Aif-1, a highly conserved, 17 kDa EF hand motif-bearing phosphoprotein (Utans *et al.*, 1995; Imai *et al.*, 1996), is expressed primarily by developing spermatids (Iida *et al.*, 2001; Kohler, 2007) and cells of monocyte/macrophage lineage. Increased Aif-1 expression has been identified in infiltrating macrophages in damaged or inflamed tissues, including allografted solid organs (*e.g.*, heart (Utans *et al.*, 1995) and liver (Nagakawa *et al.*, 2004)), autoimmune conditions (*e.g.*, rheumatoid arthritis), and drug-induced disease models (*e.g.*, kidney (Tsubata *et al.*, 2006)). In the nervous system, Aif-1 (often referred to as Iba1 in this literature) has emerged as a histologic marker of microglia and microglial activation (Ahmed *et al.*, 2007; Mittelbronn *et al.*, 2001) in several pathologic conditions, including the response to facial nerve axotomy (Graeber *et al.*, 1998), stroke (Postler *et al.*, 2000), uveitis (Fauser *et al.*, 2001), and experimental autoimmune encephalomyelitis (Schluesener *et al.*, 1998).

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How Aif-1 affects cellular activities is not well understood. When microglia are stimulated with colony stimulating factor-1, Aif-1 accumulates with F-actin in membrane ruffles, and overexpression studies *in vitro* suggest a role for this protein in cellular motility and phagocytosis (Imai and Kohsaka, 2002). It has also been suggested that Aif-1 expression in the cytoplasm of elongated spermatids contributes to the final stage of spermiogenesis (*i.e.*, elimination of residual cytoplasm) (Iida *et al.*, 2001), perhaps by reorganizing the actin cytoskeleton to promote residual body extrusion. Relevant intracellular signaling events may include Aif-1-mediated potentiation of a phospholipase C-gamma-dependent pathway that activates the Rac GTPase (Kanazawa *et al.*, 2002). Exogenous or overexpressed Aif-1 induces cytokine expression, including that of IL-6 by synovial fibroblasts and mononuclear cells (Kimura *et al.*, 2007) and IL-12 by monocyte/macrophages (Watano *et al.*, 2001), and promotes proliferation of cells derived from inflammatory lesions (Kimura *et al.*, 2007). Knockdown of Aif-1 with an siRNA decreased macrophage proliferation and migration, and limited activities of Akt and extracellular signal-regulated kinase 1/2 pathways, without affecting apoptosis as evaluated by caspase 3 activation (Tian *et al.*, 2006). Manipulation of Aif-1 expression *in vivo* affects neointima formation after mechanical vascular injury (Sommerville *et al.*, 2009), but the significance of Aif-1 expression in development and auto- and alloimmune inflammatory conditions is not known.

To permit such investigations, we have generated a mouse line with targeted inactivation of the *aif-1* gene. Mice homozygous for the targeted allele appear grossly normal, show good viability and fertility, and have nearly normal peripheral blood cell profiles. In a collagen-induced arthritis model, they displayed significantly less paw swelling and disease activity compared to control mice. We anticipate that these mice will be useful for studies of Aif-1 function in a variety of inflammatory disease models.

Results and Discussion

Targeting the *aif-1* locus

The mouse *aif-1* locus lies in the class III major compatibility complex on chromosome 17. Transcripts described in Genbank entries NM019467.2 and AK006184.1 indicate that the locus has 8 exons. Two exons encode 5' untranslated sequences, while the other 6, which encompass the translation start and stop sites, span just 1.4 kb of the genome. To create a null allele with genetic reporter capability, we designed a targeting construct to substitute a modified *lacZ* gene encoding nuclear-localized β -galactosidase (Kupersmidt *et al.*, 1999) for all *aif-1* coding sequences (Figure 1a). This construct was electroporated into embryonic stem (ES) cells, and homologous recombinant clones were identified by PCR and evaluated by genomic Southern analysis using 5' and 3' external probes. Several clones with proper targeting were identified; in the example in Figure 1b, lane 2 shows the predicted change in Nde I restriction fragment size from 12.7 kb to 9.9 kb (5' probe) and to 6.6 kb (3' probe). This clone was injected into C57BL/6 blastocysts, and yielded highly chimeric founders. Germline transmission of the targeted, inactivated *aif-1* allele was confirmed, and these F1 mice were crossed with C57BL/6 mice to establish the colony. Genomic Southern analysis further confirmed expected genotypes (Figure 1c). Methods for routine genotyping by PCR were established (Figure 2a). Both *aif-1*^{+/-} and *aif-1*^{-/-} mice of both sexes appeared grossly normal and bred well; genotyping of 130 offspring showed no significant deviation from predicted Mendelian ratios of inheritance, indicating that loss of Aif-1 has little or no effect on fertility or survival in barrier-protected, C57BL/6 mice (Table 1).

Analysis of targeting efficacy

To confirm loss of Aif-1 expression through this strategy, we euthanized *wt*, *aif-1*^{+/-}, and *aif-1*^{-/-} mice and isolated total RNA from several organs. Samples were subjected to

Northern analysis using a cDNA probe encoding the entire Aif-1 open reading frame (Sibinga *et al.*, 2002). The filter was washed to high stringency and autoradiographed. As shown in Figure 2b, robust expression of *aif-1* mRNA was apparent in thymus, testis, and spleen samples from *wt* mice, with a faint signal also observed in liver. In the testis sample, higher molecular weight bands were observed, consistent with a previous report (Watano *et al.*, 2001), and these signals were notably more intense than those produced by other organs. All samples from *aif-1^{+/-}* mice showed decreased signal intensity relative to those from the corresponding *wt* tissue. Samples from *aif-1^{-/-}* mice were devoid of signal, as expected based on the targeting strategy. Immunoblotting also showed loss of Aif-1 signal, although in this case, faint bands of ~22 and 17 kDa were apparent in the testis and liver samples, respectively (Figure 2c). Because our targeting strategy removed all Aif-1 coding sequences, these bands likely result from non-specific cross-reaction of the antibody, but it is possible that the 17 kDa band represents a product of the Aif-1-like gene (ENSMUSG0000001864), which is 81% similar in sequence to Aif-1.

Effect of *aif-1* inactivation on spleen weight and hematologic indices

On necropsy, spleens from *aif-1^{-/-}* mice appeared smaller than those of *wt* littermates. On average, Aif-1-deficient spleens weighed less, while splenic cell density (cell number per gram of tissue) was similar to *wt* (Figure 3). We also evaluated peripheral blood components. While erythrocyte numbers, hemoglobin, and hematocrit concentrations from Aif-1-deficient mice were all slightly lower than those in *wt* mice, these individual differences did not reach statistical significance (Table 2). On the other hand, mice lacking Aif-1 had ~10% fewer circulating platelets compared to their *wt* counterparts. The cellular basis for these modest differences in hematopoietic cell populations is not known at present.

Effect of *aif-1* inactivation on autoimmune disease

Increased Aif-1 expression, infiltration of Aif-1-expressing cells, and single nucleotide polymorphisms in the *aif-1* locus have been linked to multiple autoimmune diseases, notably rheumatoid arthritis (Kimura *et al.*, 2007); (Pawlik *et al.*, 2012), but whether Aif-1 has an essential role in the pathogenesis of such processes is not clear. For initial evaluation of how Aif-1-deficiency affects inflammatory disease progression, we studied 12 week-old male *wt* control and *aif-1*-targeted mice, immunizing them by intradermal injection of chicken collagen type II (CII) emulsified in complete Freund's adjuvant (Inglis *et al.*, 2008). The animals were examined every few days to evaluate disease activity as reflected in measured paw thickness and overall clinical score, as described (Inglis *et al.*, 2008). We saw thickening and erythema in the paws, but not in knee or ankle joints, consistent with the milder level of disease seen in C57BL/6 compared with DBA/1 mice (Inglis *et al.*, 2007). In both genotypes, these indices increased during the period from 29 to 69 days after immunization, but in mice that lacked Aif-1, both paw thickening and disease scores were significantly attenuated relative to control mice (Figure 4).

In summary, these studies demonstrate the successful generation, by homologous recombination, of a null allele for the *aif-1* gene in mice. The effect of global loss of Aif-1 expression on mouse development has not been previously described. Although *aif-1* mRNA is normally expressed strongly in the testis (Figure 2b), mice lacking one or both copies of the gene proved to have good fertility and viability. Decreased spleen weight and lower peripheral blood platelet counts in *aif-1-null* animals indicate that loss of Aif-1 has modest effects on hematologic development; the precise cellular basis of these effects remains to be determined. Several prior reports have implicated Aif-1 in the pathogenesis of multiple inflammatory and immunologic conditions, but the functional significance of Aif-1 in these diseases and disease models has not been previously tested *in vivo*. Our initial studies using a model of autoimmune arthritis support the idea that Aif-1 promotes disease activity, and

demonstrate the utility of this new transgenic mouse line. We anticipate that the line will be useful in characterizing how Aif-1 contributes to a variety of other inflammatory diseases.

Materials and Methods

Gene targeting

The standing committee of the Albert Einstein Institute for Animal Studies approved these studies, and the mouse model will be shared with the research community after manuscript publication. A 4.2 kb EcoRI-EcoRI fragment containing the known coding sequences of the *aif-1* locus was obtained from a clone isolated from a 129SvJ mouse genomic library in the Lambda Fix II phage (Stratagene); this fragment was recloned into a PGK-tk/pBluescript targeting vector backbone. The Aif-1 coding sequences were excised and replaced by a modified *lacZ* gene encoding nuclear-localized β -galactosidase and a neomycin cassette (Kupersmidt *et al.*, 1999). The 5' arm of the construct was extended by ligating a 4.8 kb NsiI-NsiI *aif-1* genomic fragment into the Nsi I site 1.67 kb 5' to the Aif-1 translation start site. The final construct was confirmed by restriction digestion and nucleotide sequencing across all junctions. The construct was linearized and electroporated into 129SvEv mouse ES cells, which were cultured in the presence of neomycin for positive selection and ganciclovir for negative selection. Genomic DNA was prepared from ES cells using 100 mM Tris pH 8.5, 0.2 M NaCl, 5 mM EDTA, 0.2% SDS, and 0.1 mg/ml Proteinase K. Individual clones were screened for targeting of the *aif-1* locus by PCR with Redtaq polymerase (Sigma), forward primer PGK polyA (AACCAAATTAAGGGCCAGCT), and reverse primer *aif-1* 3' flanking (ATCCCTCGGAGAGAGAAAGG); cycling conditions were 94°C×30 s, 64°C×30 s, and 72°C×90 s for a total of 36 cycles.

Genomic Southern analysis

Genomic DNA isolated from mouse tails was extracted with phenol-chloroform prior to overnight restriction enzyme digestion, electrophoresed through 0.8% agarose gels, and transferred to GeneScreen Plus (PerkinElmer) nylon filters. DNA probes were labeled with ³²P-dCTP using the Prime-It RmT Random Primer Labeling Kit (Stratagene), hybridized to filters, and processed as described (Sibinga *et al.*, 2002).

Mouse genotyping

Routine genotyping to identify the intact *aif-1* gene was performed by PCR of genomic DNA obtained from tail biopsies using primers (forward, TGGGCAAGAGATCTGCCATCTTGA, and reverse, AAGAGACACAGGCATCAGGGACAA) to amplify a 243 bp fragment spanning the 3' most exon-intron junction. Touchdown cycling conditions were used, decreasing the annealing temperature by 0.5° per cycle from 64–58°C over 12 cycles, followed by 25 cycles with an annealing temperature of 58°C. The targeted allele was identified by multiplex PCR using *lacZ*-specific (Jackson Labs IMR39 and IMR40) and PCR control (Jackson Labs IMR 15 and IMR16) primers; this reaction used standard PCR cycling with a 60°C annealing temperature.

Northern analysis

Total RNA was extracted from mouse organs using Trizol (Invitrogen). Samples (10 µg) were fractionated on 1.2% formaldehyde-agarose gels and transferred to BrightStar Plus (Ambion) nylon filters, which were then hybridized at 68°C for 2 h to the random-primed, ³²P-labeled mouse *aif-1* cDNA probe (10⁶ cpm/ml) in QuikHyb solution (Stratagene) (Sibinga *et al.*, 2002). The filters were washed in 30 mM sodium chloride, 3

mM sodium citrate, and 0.1% SDS at 55°C and autoradiographed with Kodak Biomax MS film for 8 h at –80°C.

Immunoblotting

Total proteins were extracted from *wt* and *aif-1*^{–/–} mice, separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membrane (Millipore), probed with a polyclonal antibody against Aif-1 (1:250, Wako), and evaluated by chemiluminescent detection (Pierce).

Collagen-induced arthritis model

The targeted *aif-1* allele was backcrossed at least 8 generations onto a C57BL/6 mouse genetic background. To induce arthritis, *wt* and *aif-1*^{–/–} mice (age 12–14 weeks, 3–6 per genotype) were immunized with CII using a standard protocol (Inglis *et al.*, 2008). Briefly, CII (4 mg/ml, MD Bioproducts) was dissolved overnight in 0.1 M acetic acid and mixed with equal parts of Complete Freund's Adjuvant containing 5 mg/ml of Mycobacterium Tuberculosis H37Ra (Difco). All mice were immunized with a total of 200 µg of this emulsion injected intradermally at the base of the tail and at a second site slightly anterior to the first. Mice were boosted with 100 µg CII in Incomplete Freund's Adjuvant 14 days after the primary immunization. Starting 20 days after initial immunization, paw diameters of the hind and fore limbs were measured using microcalipers (Kroeplin), and these measures were averaged. Scores for the two genotypes across 8 time points were evaluated using a two-way analysis of variance, with a Bonferroni correction and significance set at 0.05 (Prism 6 software, Graphpad). Disease activity was scored by evaluating each limb on a 0–3 scale, with 0 being normal and 3 referring to swelling of the entire paw with erythema and ankylosis, as described (Inglis *et al.*, 2008).

Statistical methods

Paw measurements and disease scores are shown as mean ± standard error (SE). Statistical significance was determined by Student's *t* test (two-tailed), multiple analysis of variance (ANOVA) with Bonferroni correction, or Chi-square test, as appropriate, with significance accepted for *P* < .05. The analyses were conducted with GraphPad Prism.

Acknowledgments

We thank Jennifer Hadix for excellent technical assistance in early stages of this project.

Contract grant sponsor: US National Institutes of Health (NIH); Contract grant number: HL67944

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FIG. 1.

Generation of an *aif-1*-null allele in mice. (a) Gene targeting strategy. An arrow indicates the translation start site. *nlacZ*, modified β -galactosidase gene with nuclear localization signal; *neo*, neomycin positive selection cassette; ext, external; *tk*, thymidine kinase negative selection cassette. (b) Genomic Southern analysis of targeted ES cell clones. (c) Genotyping by Southern analysis of *wt* and *aif-1*-targeted mice.

FIG. 2.

Evaluation *aif-1* mRNA and protein levels in gene-targeted mice. (a) Routine genotyping, showing detection of targeted allele by PCR-based amplification of a 315 bp *lacZ* amplicon and loss of a 243 bp *aif-1* genomic amplicon. (b) Total RNA samples (10 µg) from mice with the indicated *aif-1* genotypes were assessed by Northern analysis using a radiolabeled *aif-1* cDNA probe. Filters were hybridized to the probe, washed to high stringency, and evaluated by autoradiography. (c) Total protein (25 µg) was extracted from organs and evaluated by immunoblotting for Aif-1 or GAPDH, as indicated. Abbreviations: thymus (Th), testis (Te), spleen (Sp), and liver (Li).

FIG. 3. Spleen weight and cell density. (a) Spleens from *wt* and Aif-1-deficient mice were weighed. *, $P < .05$. (b) cells per gram of spleen tissue, as measured by hemocytometer. ns, difference not significant.

FIG. 4.

Effect of *aif-1* inactivation in a collagen-induced arthritis model. *Wt* (n = 9) and *aif-1^{-/-}* (n = 6) mice were immunized with chicken collagen, as described in Methods. (a) Paw diameter was measured from 29 to 69 days after immunization using a microcaliper. (b) Disease activity was scored during the same time period, as described in methods. *, $P < .0001$ for genotype-dependent difference in paw thickness or disease activity.

Table 1genotype frequency in progeny of *aif-1*^{+/-} × *aif-1*^{-/-} crosses

Genotype	heterozygous (<i>aif-1</i> ^{+/-})	homozygous (<i>aif-1</i> ^{-/-})	Total
Number expected	65	65	130
Number observed ^a	60	70	130
Expected Ratio (%)	50	50	100
Observed Ratio (%) ^b	46.2	53.8	100

^a mice backcrossed to C57BL/6 strain and surviving to >8 weeks of age were genotyped^b difference from expected Mendelian ratio was not statistically significant (by Chi-square test, P = 0.5348)

Table 2

hematologic indices

	wt	aif-1 ^{-/-}	P value
WBC ^a	6.12±0.75	7.98±0.85	.132
%Neutrophil	20.64±1.13	21.69±1.08	.519
%Lymphocyte	75.80±1.27	74.81±1.33	.601
%Monocyte	3.35±0.24	3.12±0.33	.586
%Eosinophil	0.11±0.04	0.31±0.11	.121
%Basophil	0.09±0.03	0.08±0.02	.832
RBC ^b	10.11±0.20	9.67±0.17	.129
Hemoglobin ^c	15.05±0.29	14.30±0.23	.066
Hematocrit (%)	41.70±0.81	39.95±0.66	.126
Platelet ^d	0.794±0.017	0.715±0.025	.027*

^aWBC, white blood cell count in thousands per cubic millimeter

^bRBC, red blood count in millions per cubic millimeter

^cHemoglobin in g/100 milliliter blood

^dplatelet count in millions per cubic millimeter

n = 6 for each genotype