Arterial Inflammation in Mice Lacking the Interleukin 1 Receptor Antagonist Gene

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Abstract

Branch points and flexures in the high pressure arterial system have long been recognized as
sites of unusually high turbulence and consequent stress in humans are foci for atherosclerotic
lesions. We show that mice that are homozygous for a null mutation in the gene encoding an
endogenous antiinflammatory cytokine, interleukin 1 receptor antagonist (IL-1ra), develop lethal
arterial inflammation involving branch points and flexures of the aorta and its primary and sec-
ondary branches. We observe massive transmural infiltration of neutrophils, macrophages, and
CD4+ T cells. Animals appear to die from vessel wall collapse, stenosis, and organ infarction or
from hemorrhage from ruptured aneurysms. Heterozygotes do not die from arteritis within a
year of birth but do develop small lesions, which suggests that a reduced level of IL-1ra is insuffi-
cient to fully control inflammation in arteries. Our results demonstrate a surprisingly specific
role for IL-1ra in the control of spontaneous inflammation in constitutively stressed artery
walls, suggesting that expression of IL-1 is likely to have a significant role in signaling artery
wall damage.

Key words: interleukin 1 • vasculitis • aorta • arteritis • chronic disease

Introduction

IL-1 is a primary inflammatory cytokine (for review see
reference 1). Its two forms, IL-1α and IL-1β, are products
of adjacent but highly divergent genes. Both forms bind
with high affinity and signal only through the type I IL-1
receptor (2, 3). Locally applied IL-1 causes all the features
of inflammation: it activates prostaglandin synthesis (4) and
induces nitric oxide synthase in smooth muscle (5), secre-
tion of proteolytic enzymes (6), and the release of further
inflammatory mediators, notably IL-6 (7) and chemokines
(8). IL-1 triggers increased surface availability of adhesion
molecules on endothelial cells and leukocytes (9). The
combination of chemokine secretion and induction of ad-
hesion molecules stimulates leukocyte extravasation and tis-
sue infiltration.

IL-1ra (receptor antagonist; mouse locus, *Il1rn*)[1] is a struc-
tural homologue of IL-1 (10) and occupies the type I IL-1
receptor with higher affinity and an association rate constant
similar to that of IL-1α and IL-1β (11) but appears to be un-
able to recruit the IL-1 receptor accessory protein, which in
turn is required to mediate intracellular signaling (12, 13).
IL-1ra thus antagonizes productive IL-1 binding. IL-1ra and
IL-1β are often coexpressed by activated monocytes. Though
the relative secreted concentrations of IL-1ra and IL-1β are
modulated (14, 15), IL-1ra is typically produced in much
greater abundance. To produce a large biological effect, it
appears that only a small proportion of IL-1 receptors need
be occupied by IL-1 (for review see reference 16). It follows
that the local ratio of available agonist/antagonist might de-
termine the extent of IL-1 receptor signaling rather than the
concentration of agonist alone, and it has been proposed that
this ratio is an important determinant in the course of in-
flammatory diseases (17).

Two forms of IL-1ra protein have been reported in both
mice and humans. One form is secreted (sIL-1ra; references
10 and 18), and one lacks the signal sequence and is retained
within the cytoplasm of intact cells (icIL-1ra; references 19
and 20). Other nonsecreted forms have been reported in
humans only (21, 22). The alternative forms result from the
activity of two promoters, differential splicing of the pri-

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[1]*Abbreviations used in this paper:* ES, embryonic stem; ra, receptor antag-
onist; SPF, specific pathogen–free.

mary transcripts, and the use of different translational initiation sites. With the exception of the recently described 16-kD form of IL-1ra, which has reduced affinity for the receptor (22), all forms of IL-1ra are biochemically equivalent. sIL-1ra is secreted by activated monocytes and macrophages (20). sIL-1ra in the circulation is probably maintained by constitutive secretion from hepatocytes, and its concentration is increased during the acute phase response (23). icIL-1ra mRNA is also present inducibly in monocytes (20) but is abundant in epithelial cells, from which it was originally identified (19).

It has been proposed that in atherosclerosis, IL-1 and other inflammatory cytokines are secreted by foam cells (macrophages) in atherosclerotic plaques, as well as by vascular endothelial and smooth muscle cells (for review see reference 24). IL-1 stimulates the local secretion of platelet-derived growth factor, which in turn acts as a mitogen for smooth muscle cells and fibroblasts (25) and thus may serve as a mediator in the invasion of the damaged vessel intima by hypertrophic smooth muscle. The superficial proliferation of matrix-depositing cell types appears to permit the accumulation of further atherogenic material into the growing plaque (24). IL-1ra mRNA and protein is present in human vascular cells within and in the vicinity of plaques (Francis, S., personal communication). A crucial role in arterial biology for IL-1ra or a closely linked gene is also suggested by the strong genetic association of single vessel coronary artery disease with the rarer allele of the human IL-1ra gene (26), though the functional difference between the two alleles in this context remains to be explored.

Two previous reports of IL-1ra gene disruption (27, 28) have shown that IL-1ra–deficient animals are developmentally normal but have a lower mean postnatal growth rate than their wild-type or heterozygous littermates. No other spontaneous pathology has so far been reported. $Il1m^{-/-}$ animals are hypersensitive to bacterial LPS and to fever induced by sterile local inflammation (28). Circulating levels of IL-1β were also reduced in LPS-stimulated mice compared with wild-type animals (27), implying some level of negative feedback in the IL-1 system. IL-1ra–deficient mice had an enhanced ability to clear the intracytoplasmic parasite *Listeria monocytogenes*, suggesting that the macrophages of mice deficient in IL-1ra are more readily activated by endogenously generated IL-1 in the absence of IL-1ra (27). It thus appears that in nature, IL-1ra is part of a balance that is achieved between the beneficial excitability of the primary immune system and its potentially devastating autotoxic effects.

Materials and Methods

Generation of the Il1rn-null Allele in Mice. A genomic λ clone spanning +0.1 to +14.6 kb (as defined from the first nucleotide of sequence available from EMBL/GenBank/DDBJ under accession no. L32838; reference 18) was identified in a strain 129 mouse library by hybridization with human IL-1ra cDNA and genomic clones. E14 TG2A embryonic stem (ES) cells were transfected as described previously (29) with a replacement construct that contained homologous arms consisting of nucleotides 916 to 3,530

and 4,791 to ~11,800 relative to L32838. The arms flanked a polyadenylation sequence, an inverted *aph* gene driven by the human β-actin promoter and a modified pBluescript vector (see Fig. 1 a; reference 30). The promoter was modified to remove an endogenous NotI site. Resistant colonies were selected with G418 and genomic DNA from expanded clones was screened for homologous recombination on Southern blots with the 5′ and 3′ flanking probes (see Fig. 1 a). ES cells were microinjected into C57BL/6 blastocysts, and chimeric mice were derived. Chimeras were bred with outbred MF1 albino females. With the exception of the confirmatory study, results presented are from the descendants of a single chimera and a single MF1 from the Edinburgh MF1 colony. The confirmatory colony was founded from a chimera derived from an independent G418-resistant ES colony. The chimera was backcrossed three times onto 129/Ola (Harlan UK) and mated with an MF1 female from the stock in Sheffield.

Genotype Screening. Initial screening of ES cell lines and test screens of DNA from founders (data not shown) and from selected progeny (see Fig. 1, b and c) were by Southern blot analysis with flanking probes containing nucleotides 46 to 567 and ~13,300 to ~14,600 (AccI–NotI). The carriage of wild-type and null alleles was subsequently followed by allele-specific PCR analysis of DNA extracted from ear clippings.

Detection of Arteritis. Live animals were killed either by overdosing with anesthetic and exsanguination from the heart or by cervical dislocation. Spontaneously dead or killed animals were dissected by a longitudinal cut into the peritoneum and thoracic cavity and were immersed in 4% formaldehyde for >48 h. After dehydration and embedding in paraffin, 5-μm sections were stained with hematoxylin and eosin. Blocks of tissue that were examined included (a) the aortic arch and the mediastinal arteries, (b) the abdominal aorta from the diaphragm to the iliac bifurcation, and (c) the arteries of the leg. All other tissues were sampled in a cohort of 53 animals, including 42 $Il1m^{-/-}$ and 11 $Il1m^{+/-}$ mice. For the final 30 animals, only the arteries were examined.

Immunohistology. Lesional sites were discovered by cutting 5-μm frozen sections from candidate tissue and staining every 20th section with hematoxylin and eosin. Sections were stored at −80°C and fixed with acetone after drying. To detect intracellular IL-1β, the sections were first treated with a solution of 0.1% saponin in PBS, a procedure that did not interfere with subsequent detection of either CD4 or the F4/80 antigen. Serial sections were stained with mAbs to F4/80 antigen, CD4, CD8, CD19 (PharMingen), IL-1β (R & D Systems, Inc.), and with control nonspecific mAbs. In Fig. 4, a–d, primary antibodies were detected with a two-layer biotinylated secondary antibody/peroxidase-conjugated avidin system (Vector Labs., Inc.) with final oxidation of diaminobenzidine to yield a brown pigment. In Fig. 4, e–h, IL-1β–containing cells were first detected as before but with nickel salt added to the substrate as described by the manufacturers, to yield a darker pigment. F4/80 antigen or CD4 was then also detected with a two-layer biotinylated secondary antibody/alkaline phosphatase–conjugated avidin system (Vector Labs., Inc.) and the substrate Vector Red (Vector Labs., Inc.) in the presence of the selective phosphatase inhibitor levamisole (Vector Labs., Inc.), as described by the manufacturer. Vascular tissue gave no significant cellular red staining in the absence of a primary antibody.

Pathogen Screening. Five $Il1m^{-/-}$ mice (four of five severely arteritic) were raised under specific pathogen–free (SPF) conditions. Serum was tested at the Diagnostic Service, Royal Veterinary College, London for evidence of infection with the following viruses: adeno-, cytomegalo-, ectromelia (mouse pox), hantaan, hepatitis,

K-, lymphocytic choriomeningitis, minute, parainfluenza-, pneumonia, polyoma-, Sendai, and Theiler's viruses; epizootic diarrhea of infant mice (mouse rotavirus), and reovirus 3, and for other agents, *Clostridium piliformis, Mycoplasma pulmonis,* and *Encephalitozoon cuniculi.* All results were negative. Serum from five *Il1m*−/− mice (four of five overtly diseased) were screened for mouse *Chlamydia* by Dr. B. Thomas (St. Mary's Hospital, London) and found negative. Fecal samples were screened by a PCR-based detection method (31) in our laboratories and the laboratory of Professor L. Riley (University of Missouri, Columbia, MO) for colonization by *Helicobacter* spp.

Results

Characterization of the IL-1ra-null Allele. The event leading to the inactivation of the IL-1ra gene was demonstrated by showing that the replacement vector was physically linked to uninvolved markers on both flanks (Fig. 1, a–c). Homologous recombination would result in the fusion of vector-derived sequence with the open reading frame in the middle of the single folding domain of IL-1ra (in exon 3), which is codon 77 of the mature sIL-1ra and codon 82 of icIL-1ra (18, 20). No functional IL-1ra protein would be expected from the engineered allele that we describe. To ensure that the phenotype that we observed did not result from modulation of the adjacent gene of the IL-1 gene family, *Il1l1* (Barton, J., and M. Nicklin, unpublished data; available from EMBL/GenBank/DDBJ under accession no. AJ250429), we performed reverse transcription and PCRs on total RNA from LPS-stimulated peritoneal macrophages of both wild-type and *Il1m*−/− mice. No transcript was found in either, although IL-1ra mRNA could be found in mRNA from the wild-type animals and IL-1–like protein 1 mRNA was detected in RAW 264.7 macrophages.

Il1m−/−, *Il1m*+/−, and *Il1m*+/+ offspring were produced in the expected Mendelian ratios. Activated macrophages from *Il1m*−/− mice did not contain detectable IL-1ra mRNA (Fig. 1 d) nor produce IL-1ra protein (Fig. 1 e). Both sexes of *Il1m*−/− mice were fertile, and *Il1m*−/− intercrosses were successful. We determined the mean body mass at between 27 and 33 d of age in 53 litters that contained *Il1m*−/− and *Il1m*+/− mice. In 37/53 litters, the mean mass of the homozygotes was less than the mean of the heterozygotes ($\chi^2 = 8.3$, $P < 0.005$), in agreement with previous reports (27, 28).

Longevity of Il1m−/− *Mice and Incidence of Arteritis.* *Il1m*−/− and *Il1m*+/− mice were crossed to produce approximately equal numbers of *Il1m*−/− and *Il1m*+/− offspring. 18 litters containing *Il1m*+/− and *Il1m*−/− animals were monitored up to 550 d from birth (Fig. 2); 51/55 *Il1m*−/− mice died or were culled on humane grounds (93%), and the median age of death was 159 d, compared with 6/37 (16%) heterozygotes. Between 200 and 400 d from birth, the excess mortality exceeded 10. Autopsies on 46 *Il1m*−/− mice that had become ill (from this group and other litters) revealed arterial inflammation in 43 and ruptured aneurysms or indications of internal hemorrhage in 16. 2 of the 3 mice in which arteritis was not overt showed secondary signs of the disease, namely ischemic cardiomyopathy and interstitial
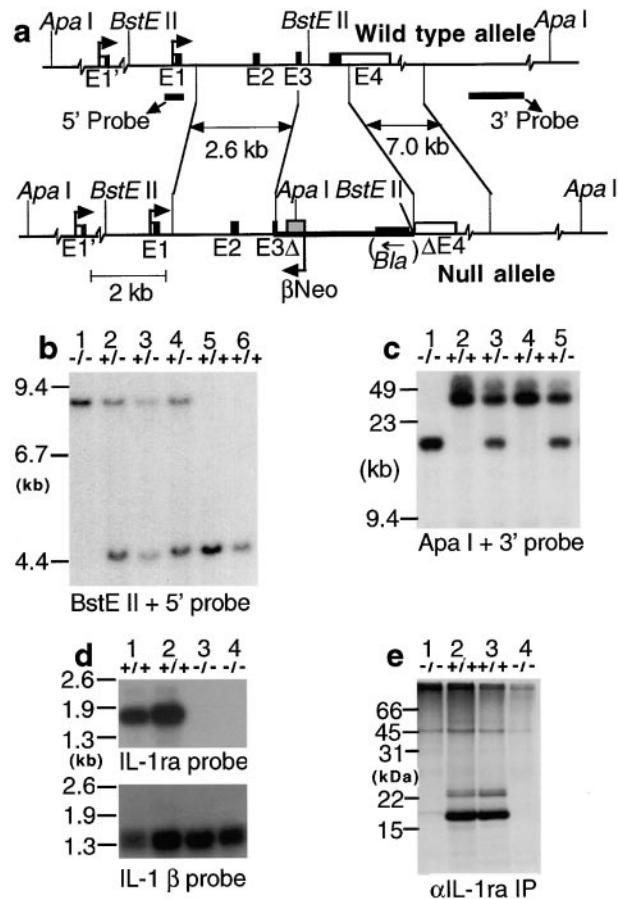
Figure 1. Molecular analysis of the *Il1m*-null allele. (a) Structure of the *Il1m* knockout construct. Top line: the genomic structure of the wild-type mouse *Il1m* allele (18). Positions of the two ApaI and two BstEII restriction sites within the region are indicated above the line. The exon structure is indicated by boxes, the coding region by filled areas. Exons are labeled below the line, and the two flanking probes are shown. The homologous arms used in preparing the knockout construct are indicated between the two lines. Bottom line: expected structure of the null allele generated by homologous recombination through both flanking arms. The thickened portion represents the knockout vector and contains the *aph* (aminoglycoside phosphotransferase) gene driven by a β-actin promoter (β-*Neo*). The position of the vector β-lactamase gene (*Bla*) is indicated. Restriction sites used in the screening of the genomic DNA are indicated above the line. (b) Southern blot hybridization of mouse liver DNA from littermates, digested with BstEII and probed with the 5′ flanking probe. Lane 1, *Il1m*−/−; lanes 2, 3, and 4, *Il1m*+/−; and lanes 5 and 6, *Il1m*+/+ animals. (c) Southern blot hybridization of high-molecular-mass mouse spleen DNA from littermates, digested with ApaI and probed with the 3′ flanking probe. Lane 1, *Il1m*−/−; lanes 2 and 4, *Il1m*+/+; and lanes 3 and 5, *Il1m*+/− animals. (d) Total RNA from ~10⁶ peritoneal macrophages, cultured for 4 h in the presence of 0.1 μg/ml *Escherichia coli* LPS (Sigma Chemical Co.), was sequentially hybridized with (top) a probe (nucleotides 50–726) spanning the coding sequence of the mouse IL-1ra cDNA (18) (the sequence is interrupted at nucleotide 400 in the null allele), and (bottom) a control cDNA probe for mouse IL-1β. Lanes 1 and 2 contain RNA from *Il1m*+/+ and lanes 3 and 4 are from *Il1m*−/− mice. (e) Immunoprecipitation with goat anti–mouse IL-1ra (R & D Systems, Inc.) of secreted IL-1ra from ~10⁶ peritoneal macrophages cultured for 7.5 h in the presence of 0.1 μg/ml *E. coli* LPS and labeled for 3 h in 4 ml serum-free RPMI with 67 μCi/ml [³⁵S]methionine. Immune complexes were precipitated with protein A–Sepharose. Cells are from *Il1m*−/− (lanes 1 and 4) and *Il1m*+/+ (lanes 2 and 3) mice.
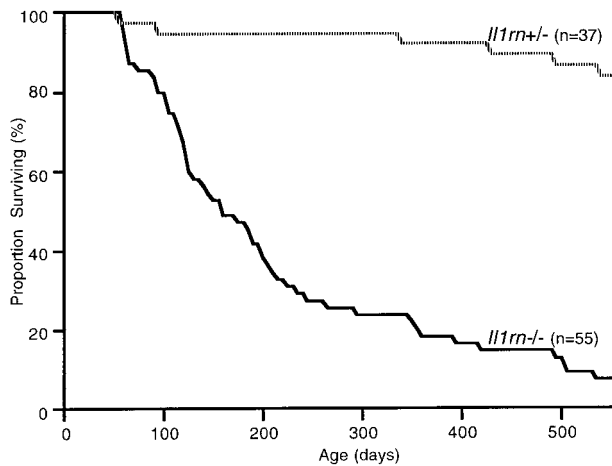
Figure 2. Mortality and disease in mice carrying the *Il1rn*-null allele. Survival curve during the first 550 d from birth of all *IL1rn$^{+/-}$* (broken line) and *Il1rn$^{-/-}$* (solid line) mice in the first 18 litters (excluding those culled as controls for other experiments). The mice either died spontaneously or were killed for humane reasons on veterinary advice.

nephritis, probably due to undetected coronary and renal artery lesions, respectively.

We confirmed the existence of arteritis in a second line, derived from a different G418-resistant ES colony, where we found that two out of four homozygous animals had overt arteritis at 135 d and another animal had ischemic cardiomyopathy, which we have frequently found associated with aortic root inflammation in our main colony.

Effect of Lineage on Age of Onset in Il1rn$^{-/-}$ Mice. Our main colony, which was initiated by breeding a chimera with an outbred mother, could therefore contain up to three distinct haploid genotypes. The age of presentation of histologically confirmed arteritis across all litters has been broadly spread (31–493 d from birth). A number of lines have now been inbred by mating siblings. In the F2 litters, the age range over which animals became ill was 57–199 d. We have subsequently found that the age of onset of verified arteritis appears to be heritable. In one highly susceptible lineage of animals, all 38 *Il1rn$^{-/-}$* mice died at a mean age of 103 d (SD = 27 d). These animals were initially reared in quarantine rather than SPF conditions, but upon rederivation into SPF conditions the homozygotes of the first litter from this lineage also died very early. In a much less susceptible lineage, five of five *Il1rn$^{-/-}$* mice died at >350 d, but arteritis was seen in all of them. The presence of extensive fibrous, repaired lesions in aged survivors suggests that they were able to survive with arteritis rather than failed to develop the disease.

Mild Disease in Il1rn$^{+/-}$ Mice. Overt illness has been very rare in heterozygous mice, so to compare the incidence of arteritis in heterozygous and homozygous animals, we investigated outwardly healthy littermates of both genotypes. Arteritis was found in six of nine *Il1rn$^{-/-}$* animals (age range 112–130 d). Examination of a set of overtly healthy heterozygous animals has also revealed small lesions in 8/17 animals (age range, 105–158 d). To demonstrate that lesions were

not merely endemic and aggravated in IL-1ra–deficient mice, we raised wild-type animals from the most susceptible lineage for comparison with their *Il1rn$^{-/-}$* littermates. No lesions were found in 11 wild-type animals (age range, 127–190 d). All 11 *Il1rn$^{-/-}$* littermates (age range, 82–149 d) had arteritic lesions, and 7 had died before their wild-type littermates were culled.

Localization of Arteritic Lesions. Arteritis was found in the aorta and in its primary and secondary branches. Lesions have not been found in veins or capillaries or in smaller arteries, such as those of the kidney, that are typically affected by polyarteritis nodosa. Only one lesion in a pulmonary artery has recently been discovered. Full arterial examination of *Il1rn$^{-/-}$* animals (regardless of the cause of death) revealed lesions in 49/55 mice, and we frequently observed thoracic lesions (33/55) at the aortic root (12/55), in one or more coronary arteries (18/55), or affecting other vessels of the mediastinum (12/55). The abdominal aorta was affected in 30/55 cases. The iliac bifurcation (4/55) and the highly flexed popliteal arteries (8/55) were also affected. Sudden behavioral disorders alerted us to discover cerebral lesions in two cases. *Il1rn$^{-/-}$* mice often had multiple lesions. By comparison, only one lesion was discovered above the diaphragm in a heterozygote and only 2/17 mice examined had two lesions.

Nature of the Arteritic Lesions. Fig. 3 illustrates some typical large lesions from *Il1rn$^{-/-}$* mice. An inflamed right coronary artery (Fig. 3 a, R) was found to be surrounded by a massive cellular infiltrate (I) that tracked through the swollen free wall of the right ventricle, following the expected path of the vessel. A branch of the left coronary artery (L) and a nearby vein (V) were unaffected. Higher magnification (Fig. 3 b) revealed the characteristic whorled structure of chronic inflammation; the arrow indicates leukocytes apparently adhering to endothelium (see also L and E, respectively, in Fig. 3 c). The morphology of the medial cells (Fig. 3 c, M) suggests that smooth muscle was replaced by or had dedifferentiated into fibroblast-like (synthetic, noncontractile) cells (24). The chronic nature of the disease is illustrated by the presence of myocardial scarring (Fig. 3 d), indicating previous infarctions, which are uncommon in mice.

The collapse and stenosis or ballooning and rupture of affected vessels probably results from the destruction of the elastic laminae. Fig. 3, e and f, shows consecutive glancing sections of a mesenteric artery stained for cells (Fig. 3 e) and for elastin and connective tissue (Fig. 3 f). Full-thickness sections of three adjacent loops of artery are shown. The leftmost portion of the vessel was unaffected, with compact adventitia and intact elastic layers. The right portion of the vessel was severely inflamed, with a massive transmural infiltrate of leukocytes (Fig. 3 e), and the elastin layers were destroyed (Fig. 3 f). A longitudinal section of the adjacent portion of the aorta (Fig. 3, g and h) shows similar focused destruction of the elastic lamina. Repaired lesions were also found in both *Il1rn$^{-/-}$* and *Il1rn$^{+/-}$* mice and were characterized by damaged elastic layers with fibrous scarring but an absence of infiltrate (data not shown).

The margins of the arteritic lesions appeared to be sharply
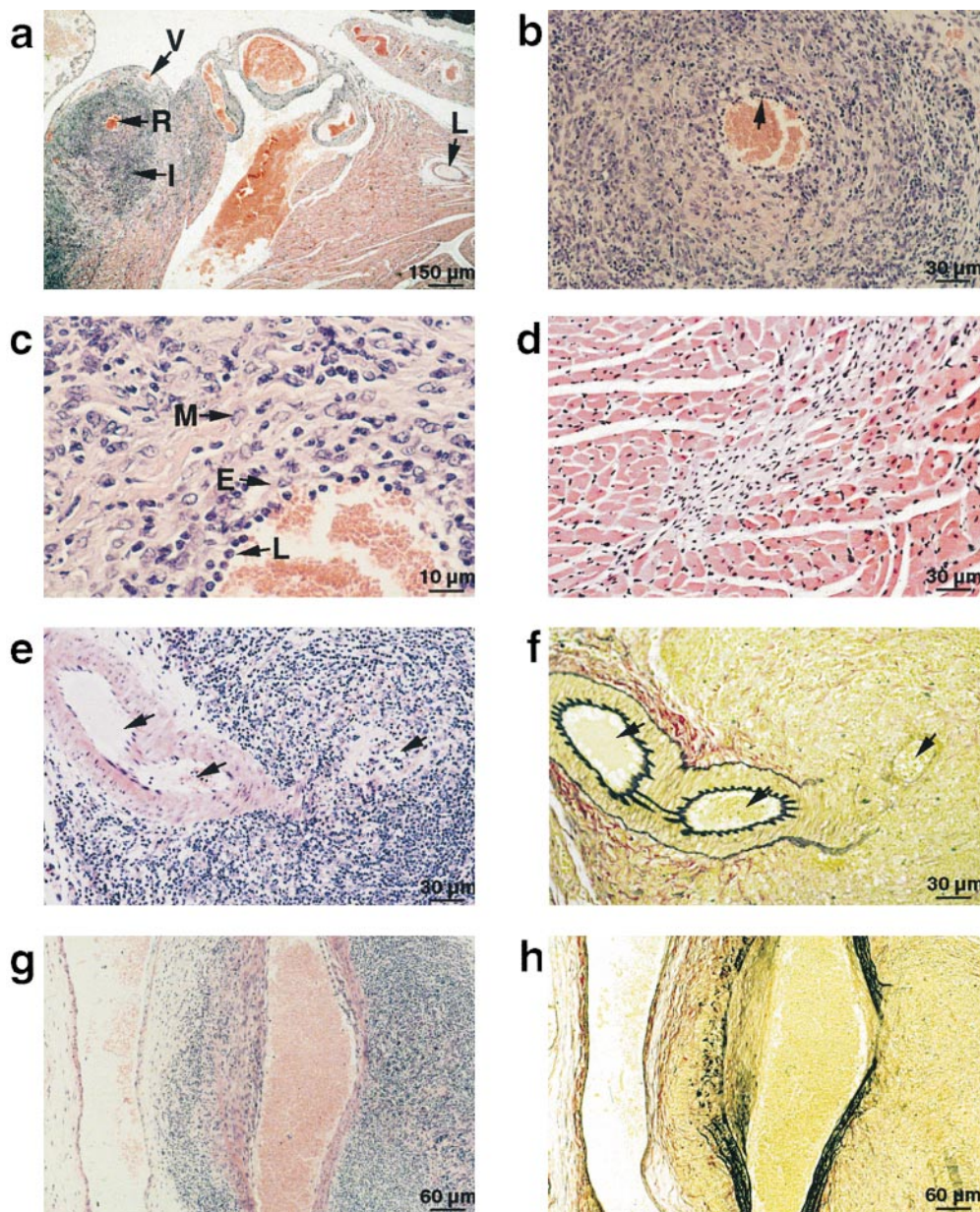
Figure 3. Arterial inflammation in $Il1m^{-/-}$ mice. (a–d) Localized severe inflammation of a coronary artery. A section has been cut through the pulmonary outflow of an affected 182-d-old $Il1m^{-/-}$ mouse and stained with hematoxylin and eosin. (a) Low magnification. Arrows indicate a grossly inflamed right coronary artery (R), inflammatory infiltrate surrounding the track of the artery out of the plane of the section (I), unaffected left coronary artery (L), and an unaffected vein (V). (b and c) Higher magnifications of the coronary lesion showing whorled inflammatory infiltrate and leukocytes (L, arrow) adhering to the endothelium (E) and relatively undifferentiated mesenchymal cells (M). (d) Myocardial scar from the same section. (e–h) Typical lesions, showing localized transmural destruction of arterial walls. (e and f) Sequential glancing sections were cut through the wall of an affected mesenteric artery from a 72-d-old $Il1m^{-/-}$ mouse and stained for cells with hematoxylin and eosin (e) and for elastin and connective tissue fibrils with elastin van Gieson's stain (f). Three increasingly affected loops of the artery are visible. In panel e, note that the smooth muscle layer becomes expanded and progressively disorganized (left to right). Inflammatory infiltrate, filling much of the field, surrounds the last loop. In panel f, note that the two layers of elastic tissue that surround the first and second loops are completely absent from the third. Also, loose connective tissue of the tunica adventitia (which stains red) surrounds the first loop closely but is dispersed by the infiltration around the third loop. (g and h) Corresponding staining of a longitudinal section of an aortic lesion from the same mouse, showing localized damage and dilation of the vessel.

defined and centered upon flexures, branches, and bifurcations, which are known sites of turbulent blood flow and have long been recognized as focal sites for atheroma.

Identification of Infiltrating Cells and the Presence of IL-1β. Immunohistochemistry experiments were done with several sections each from four fresh-frozen lesions (taken from an aortic root, a small subclavian aneurysm, and a large carotid aneurysm, which is shown in Fig. 4). We found abundant macrophages (Fig. 4 c) and CD4^{+} T cells (Fig. 4 d) in the media of the vessel wall. CD19^{+} B cells and CD8^{+} T cells were rarely found (data not shown). Neutrophils, detected by chloroacetate esterase activity, were also abundant (data not shown).

We have proposed that the formation of arterial lesions is mediated by the presence of unopposed IL-1. To demonstrate its presence at lesional sites, we performed immunohistochemistry on saponin-treated frozen sections of three arterial lesions from $Il1m^{-/-}$ animals with antibodies specific to mouse IL-1β (Fig. 4 b). Simultaneous detection of the macrophage-specific antigen F4/80 (red staining) and IL-1β (dark brown staining) suggested that many, but not necessarily all, of the cells that were strongly positive for IL-1β were macrophages (Fig. 4, e and g). This population was generally found deep within the vessel wall (examples are labeled M2 in Fig. 4 g). Macrophages were clearly very abundant, but not all of them are activated to express IL-1β;
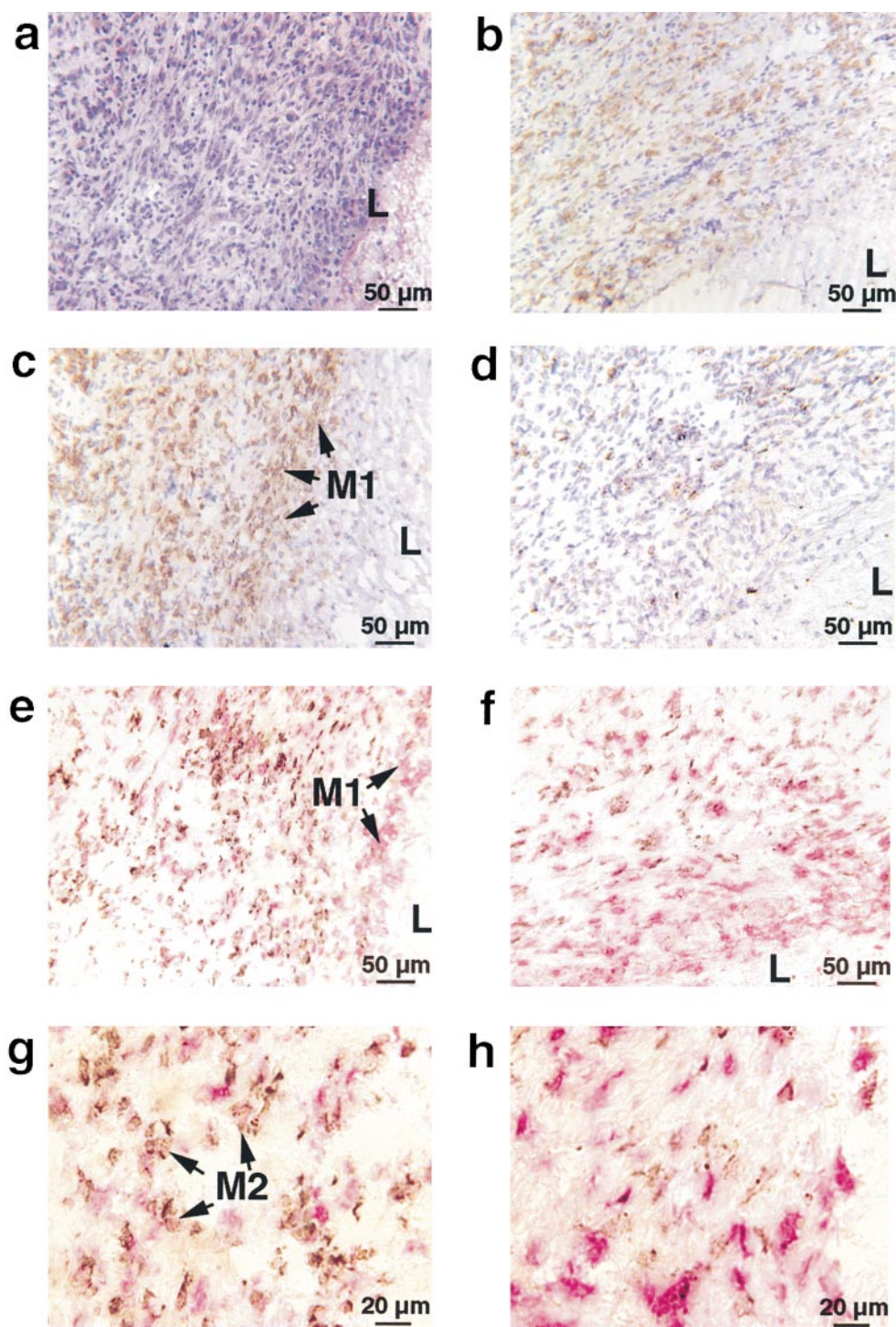
Figure 4. Immunohistochemical analysis of an aneurysm from a 95-d-old *Il1rn$^{-/-}$* mouse. Serial frozen sections were probed with specific antibodies to detect IL-1β, CD4, and the macrophage-specific marker F4/80 antigen. Comparable areas of the sections are shown. The lumenal face of the lesion is marked L. (a) Hematoxylin/eosin-stained section, showing the densely cellular vessel wall. (b) A section stained for IL-1β expression (brown), counterstained with hematoxylin. (c) A section stained for F4/80 antigen expression (brown) and counterstained with hematoxylin. Note the dense band of macrophages (M1) flanking the lumen and dispersed macrophages throughout the lesion. (d) A section stained for CD4 antigen (brown) and counterstained with hematoxylin. CD4$^+$ cells are dispersed through the wall of the lesion. (e) Dual staining of a lesional site for IL-1β (dark brown) and the macrophage-specific F4/80 antigen (red). Note the belt of IL-1β$^-$ macrophages (M1) adjacent to the lumen (L). (f) Dual staining of a lesional site for IL-1β (dark brown) and CD4$^+$ T cells (red). (g) Higher magnification of the same section as in e, showing cells (labeled M2) that appear to contain both red stain from the detection of F4/80 antigen and dark brown stain from IL-1β. (h) Higher magnification of the same section as in f, showing CD4$^+$ (red) and IL-1β–expressing cells (dark brown).

notably, a band of cells (labeled M1 in Fig. 4 c) that surrounded much of the lumen were strongly F4/80 positive but contained no IL-1β (labeled M1 in Fig. 4 e). For clarity, no nuclear counterstain was used in panels e and h, but a comparison can be made with panels a–d, in which hematoxylin was used. CD4$^+$ T cells are also abundant, but less so than macrophages, and were found dispersed through the lesional wall. They did not appear to accumulate as densely as the IL-1β$^-$ macrophages (M1) found close to the lumen. In Fig. 4, f and h, it seems likely that IL-1β–expressing cells were associated with CD4$^+$ cells, suggesting that the CD4$^+$ cells might be involved, within the wall of the aneurysm, in activating IL-1β production in the dispersed macrophages and that, in turn, these cells might be activating the CD4$^+$ cells, possibly through the presentation of autoantigens.

We have looked for signs of humoral autoimmunity in the sera of three severely affected animals, a control wild-type

and an MRL/*lpr* mouse, as a positive control, by standard antinuclear antigen assays on Hep-2 cells (Immuno Concepts, Inc.). We detected bound antinuclear antibodies with FITC-conjugated F(ab)$_2$ from goat anti–mouse IgG plus IgM. We found no evidence for antinuclear antibodies in the *Il1m*$^{-/-}$ mouse serum, though they were abundant in the MRL/*lpr* serum.

Discussion

We have eliminated a functional gene for IL-1ra in mice and have found reproducible development of lethal inflammatory lesions at high stress sites in the major arteries. Turbulent flow causes fluctuating shear stress in vessel walls and has been suggested to alter vascular gene activation directly, leading to increased leukocyte adherence to endothelial cells (32), and thus might result in increased leukocyte traffic through the vessel wall. Irregular flow may also allow deposition of bacterial pathogens or proinflammatory debris, including oxidized lipids, at turbulent sites. It has been shown that oxidized lipids can, for instance, activate chemokine expression in endothelium (33). Turbulence probably also causes increased localized damage, particularly desquamation of the endothelium and local inflammation (24). Further studies will be needed to establish whether IL-1 is expressed in mice only at sites of localized arterial damage or whether it is generally induced at sites of stress within healthy vessel walls.

In its transmural destructiveness, the disease that we have observed resembles human giant cell arteritis and polyarteritis nodosa. The former has a similar pattern of distribution within arteries but is normally characterized by frequent giant macrophages, which are rare in the animal disease. In 42 affected *Il1m*$^{-/-}$ animals, we have detected no active inflammation of the arteries of the kidney, which is a common feature of the latter. Human arterial disease and arteritides have been linked to microbial pathogens, and there has been recent interest in *Helicobacter* spp. and *Chlamydia* in coronary disease. Similarly cytomegalovirus causes arteritis in mice, although the pathological features are different from those reported here (34). Our colony was initially established under quarantine (not SPF) conditions, but up to the time of this writing (now seven generations after rederivation into SPF conditions), *Il1m*$^{-/-}$ mice have continued to develop lethal arteritis. Tests for an array of viral and bacterial pathogens (see Materials and Methods) showed that all pathogens investigated were absent, with the exception of *H. hepaticus*, which we have detected in both our colony and that of Hirsch et al. (27), where vascular disease was not found. The bacterium appears to colonize littermates of all genotypes. We are, however, currently investigating the influence of the resident bacterial flora on the occurrence of arteritis.

A pattern of early mortality has emerged among *Il1m*$^{-/-}$ animals (backcrossed onto a C57BL/6 background) from the US group (Hirsch, E., and D. Hirsh, personal communication) and illness in animals from the Japanese group, who bred their mice onto a Balb/c background (Iwakura, Y.,

personal communication). We have now examined specimens from the US group (backcrossed 15 times onto C57BL/6) but have not found evidence for arteritic lesions at the commonly affected sites in six *Il1m*$^{-/-}$ specimens provided (aged 175–427 d), despite overt malaise in four of six cases. The cause of high mortality in the US *Il1m*$^{-/-}$ animals is thus not explained.

It is noteworthy that the *Il1m*$^{-/-}$ Balb/c mice, after the fourth backcross, spontaneously develop a quite different chronic inflammatory pathology, namely arthritis, in 80% of cases before they are 56 d old (Iwakura, Y., personal communication). We have examined bones and joints of 42 *Il1m*$^{-/-}$ animals from our colony, all of which were older than 56 d, and have not detected arthritis, nor have we observed external signs of joint disease in the living animals.

At least a part of the cause of the difference between the three *Il1m*-null colonies is likely to stem from differences in their genetic backgrounds. We have seen that extreme susceptibility is heritable and survives rederivation into SPF conditions, a procedure that would be expected to cause a change of microbial flora. Moreover, the phenotype that we observe is stable and continues to occur at a very high frequency in *Il1m*$^{-/-}$ animals in our colony more than 2 yr since its first detection. It is unlikely that we have selected a susceptibility-causing mutation, unless it is present in the E14 TG2A stem cell line, because our second *Il1m*-null line on an MF1 × 129/Ola background has also developed disease, yet the MF1 came from a separate colony and the chimera was derived from a different G418-resistant clone.

Because heterozygotes develop mild disease, we suggest that the IL-1ra gene is haploinsufficient. This is plausible because of the unusual function of the IL-1ra protein, acting solely as a competitive inhibitor of IL-1. Its concentration at an inflamed site is therefore likely to be critical to its efficacy, and, in the absence of sufficient compensatory regulation, it is likely to achieve a maximum in the heterozygote of only half of the wild-type level. An alternative explanation, however, might be that we have unwittingly created a hypomorphic allele, although there is no obvious reason for the truncated open reading frame of IL-1ra (which is a single domain protein) to be expected to yield a dominant negative species.

We conclude that IL-1ra is required in our mouse colony to prevent the development of lethal arteritis. Because the only known function of IL-1ra is to antagonize the activity of IL-1, our working hypothesis is that the disease is caused by the unopposed action of IL-1. IL-1 induces a wave of release, from many cells within the affected tissue, of chemokines and proinflammatory cytokines that would recruit further inflammatory cells. In the absence of IL-1ra activity, this would amplify the unbalanced response. IL-1 might initially be released spontaneously by stressed smooth muscle or endothelial cells or by macrophages (possibly initially triggered by bacterial infection or oxidized lipid deposition) in the walls of high pressure vessels in areas of high turbulence. We have shown that macrophages are abundant in established lesions, many having cytoplasmic loads of IL-1, and that CD4$^+$ T cells are present. It seems very

likely that later IL-1 production from macrophages in established lesions occurs as a result of their activation by CD4^{+} T cells, and we speculate that the latter might be autoreactive. We have found no evidence of an autoreactive humoral response. We are currently confirming the essential role of IL-1 in the phenotype by crossing *Il1m*-null animals with mice that lack the IL-1 receptor (35). The disease we observe in IL-1ra–deficient mice may offer an opportunity to dissect important pathophysiological mechanisms occurring in large arteries.

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