IL (Interleukin)-1–Mediated Sex Differences in Kawasaki Disease Vasculitis Development and Response to Treatment

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OBJECTIVE: Kawasaki disease (KD) is the leading cause of acute vasculitis and acquired heart disease in children in developed countries. Notably, KD is more prevalent in males than females. We previously established a key role for IL (interleukin)-1 signaling in KD pathogenesis, but whether this pathway underlies the sex-based difference in susceptibility is unknown.

APPROACH AND RESULTS: The role of IL-1 signaling was investigated in the Lactobacillus casei cell wall extract-induced experimental mouse model of KD vasculitis. Five-week-old male and female mice were injected intraperitoneally with PBS, Lactobacillus casei cell wall extract, or a combination of Lactobacillus casei cell wall extract and the IL-1 receptor antagonist Anakinra. Aortitis, coronary arteritis inflammation score and abdominal aorta dilatation, and aneurysm development were assessed. mRNA-seq analysis was performed on abdominal aorta tissue. Publicly available human transcriptomics data from patients with KD was analyzed to identify sex differences and disease-associated genes. Male mice displayed enhanced aortitis and coronary arteritis as well as increased incidence and severity of abdominal aorta dilatation and aneurysm, recapitulating the increased incidence in males that is observed in human KD. Gene expression data from patients with KD and abdominal aorta tissue of Lactobacillus casei cell wall extract-injected mice showed enhanced Il1b expression and IL-1 signaling genes in males. Although the more severe IL-1β–mediated disease phenotype observed in male mice was ameliorated by Anakinra treatment, the milder disease phenotype in female mice failed to respond.

CONCLUSIONS: IL-1β may play a central role in mediating sex-based differences in KD, with important implications for the use of anti–IL-1β therapies to treat male and female patients with KD.

Key Words: aneurysm ◼ arteritis ◼ cell wall ◼ inflammation ◼ phenotype

Kawasaki disease (KD) is an acute febrile disease of unknown cause characterized by systemic vasculitis and myocarditis. KD is the leading cause of acquired heart disease in children in developed countries,1 and if left untreated, KD leads to coronary artery abnormalities in up to 30% of patients.2 Treatment with high dose intravenous immunoglobulin (IVIG) reduces the risk of an aneurysm from 25% to 30% down from 5% to 7%.3–6 However, nearly 20% of patients with KD are resistant to the initial IVIG dose, which leads to a 3-fold increase in risk for the development of coronary artery aneurysms (CAA).6,7

In the Lactobacillus casei cell wall extract (LCWE)–induced mouse model of KD, a single intraperitoneal injection of LCWE results in systemic inflammation, coronary artery vasculitis, aortitis, myocarditis, and abdominal aorta aneurysms (AAA),6,9 closely resembling human KD pathology.9,10 In the LCWE mouse model, genetic
or antibody-mediated blockade of the IL (interleukin)-1 pathway results in protection from vasculitis and AAA formation, indicating a crucial role for IL-1 signaling in pathogenesis. Treatment with an IL-1Ra (IL-1 receptor antagonist, Anakinra) also blocks LCWE–induced AAA formation. Further, pretreatment with Anakinra significantly reduces LCWE-induced myocardial inflammation and NT-proBNP (N-terminal pro-B-type natriuretic peptide) release and improves ejection fraction and left ventricular function. Recent studies also indicate that IL-1β and the NLRP3 inflammasome are required for the Candida albicans water-soluble fraction mouse model of KD vasculitis. In humans, serum levels of IL-1β and IL-1 related genes are upregulated in KD peripheral blood during the acute phase of illness. Taken together, these studies provide strong rationale for therapeutically targeting IL-1 signaling in KD, although whether this approach will be universally effective remains in question.

The incidence of KD is consistently higher in males, with a reported male-to-female KD incidence ratio of 1.5 in the United States, 1.31 in Japan, and 1.62 in Taiwan, and similar trends reported in Finland, Norway, and Sweden. However, the sex-based differences in KD development have remained largely understudied, limiting optimization of health management, and treatment strategies. Here, we find that the LCWE-induced KD mouse model displays sex-based differences that resemble those observed in human patients, specifically increased incidence and severity in males. Patient and mouse transcriptomics revealed IL-1β signaling as a predominant factor in mediating this disparity. Furthermore, while the more severe disease phenotype observed in male mice was treated successfully by Anakinra, the milder phenotype in female mice failed to respond. Given the on-going clinical trials using Anakinra treatment for KD patients, these findings are highly pertinent and have wide-reaching implications for the management of KD.

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**Highlights**

- The incidence of Kawasaki disease (KD) is consistently higher in males, however sex-based differences in KD development are largely under investigated.
- Compared with females, males with KD have enhanced Il1b expression and IL (interleukin)-1 signaling genes.
- In experimental Lactobacillus casei cell wall extract–induced murine model of KD vasculitis, anti–IL-1β therapy with Anakinra treatment is more efficient in males than females.
- Differential expression of IL-1β may play a role in mediating sex-based differences during KD.

**Materials and Methods**

The data that support the findings of this study are available from the corresponding author on reasonable request.

**Mice**

Wild-type C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed under specific pathogen-free conditions at the animal center of Cedars-Sinai Medical Center. Experiments were conducted under approved Institutional Animal Care and Use Committee protocols.

**Preparation of LCWE**

LCWE (ATCC 11578) was prepared as previously described. In brief, L. casei were grown in Lactobacillus de Man, Rogosa, and Sharpe broth (DMRS) for 48 hours, harvested, and washed with PBS. The harvested bacteria were disrupted by 2 packed volumes of 4% sodium dodecyl sulfate (SDS)/PBS overnight. Cell wall fragments were washed 8x with PBS to remove any residual SDS. Cell wall fragments were sonicated for 2 hours with a 3 out of 4 horn and a garnet tip at maximum power. During sonication, the cell wall fragments were maintained by cooling in a dry ice/ethanol bath. After sonication, the cell wall fragments were spun for 20 minutes at 11,000 g and 4°C. The supernatant was centrifuged for 1 hour at 180,000g and 4°C, and the pellet was discarded. The total rhamnose content of the cell wall extract was determined by a colorimetric phenol-sulfuric assay as described previously.

**LCWE-Induced KD Model**

Five-week-old male or female mice (as indicated) were injected intraperitoneally with 400 μg LCWE or PBS (day 0). For Anakinra treatment, mice were injected intraperitoneally with Anakinra (500 μg) every 2 days starting the day before LCWE injection (days −1, 1, 3, 5, 7, 9, 11, and 13) for a total of 8 injections. At 7 or 14 days post-LCWE injection (as indicated), mice were euthanized and perfused with PBS. After dissection of the heart, aorta, and the NLRP3 inflammasome are required for the Candida albicans water-soluble fraction mouse model of KD vasculitis. In humans, serum levels of IL-1β and IL-1 related genes are upregulated in KD peripheral blood during the acute phase of illness. Taken together, these studies provide strong rationale for therapeutically targeting IL-1 signaling in KD, although whether this approach will be universally effective remains in question.

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later for RNA extraction as described below or embedded in optimal cutting temperature compound for histological analysis. Hearts were removed and embedded in optimal cutting temperature compound for histological analysis. Serial cryosections (7 μm) were prepared from the tissues and stained with hematoxylin and eosin. Histopathologic examination and inflammation severity scoring of the coronary arteries and aortic root vasculitis were performed by a senior investigator blinded to the experimental groups. KD lesions were assessed with the scoring system as described previously.22

RNA Isolation
Aortas were stored in RNA later (Qiagen) before RNA extraction. RNA extraction was performed using the miRNEasy micro kit (Qiagen) according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction
Quantitative real-time polymerase chain reaction (PCR) was performed with the Power SYBR Green RNA-to-Ct 1 step kit according to the manufacturer’s instructions (Thermo Fisher Scientific). Primer sequences: Il1b: F′: CAGGCCAGCGACTATCCTCA, R′: TGCTCCTCATTCTGAAGGTGTC; Nlrp3: F′: TCCACAAATCTGACCCACAA, R′: ACCTCAGAGGGTCACCCAC; Il1r1: F′: GTGCTACTGGGCTCATTGTG, R′: GGAGTAAAGGACACTTGCGGAAT; Stat1: F′: TCACAGTGTTCAAGCTCTCAG, R′: GCAACGAACATCATAGGCA; Hprt: F′: AATGGATTTTCAAGGGTCAC; Gdtc, R′: AGTTCC, R′: TCCACAATTC

Murine mRNA-Seq
Library construction was performed using the Lexogen QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen; Vienna, Austria). Briefly, total RNA samples were assessed for concentration using a Qubit fluorometer (Thermo Fisher Scientific; Waltham, MA), and for quality using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Up to 100 ng of total RNA per sample was used for reverse transcription using oligo(dt) priming. After removal of the RNA template, random priming was used to perform second stand synthesis. Next, PCR amplification with indexing adapters for Illumina sequencing was followed by library purification. The concentration of the amplified library was measured with a Qubit fluorometer, and an aliquot of the library was resolved on a Bioanalyzer. Sample libraries were multiplexed and sequenced on the NextSeq 500 platform (Illumina) using 75 bp single-end sequencing. On average, ≈10 million reads were generated from each sample. Raw sequencing data were demultiplexed and converted to FASTQ format by using bcl2fastq v2.20 (Illumina). Then, the raw reads were uploaded to Bluebee Genomics Platform for quality control, alignment, and expression quantification using QuantSeq FWD-UMI Data Analysis Pipeline (Lexogen). Briefly, the umi2indx (Lexogen) process added the 6 nucleotide UMI sequence to the identifier of each read and trimmed the UMI from the start of each read. Reads were then processed by BBduk v35.92 to trim the low-quality tails, poly(A) tails, and adapter contamination. Trimmed reads were aligned to reference genome GRCm38 using STAR (v2.5.2a).23 To remove PCR duplicates, the mapped reads were collapsed if they had the same mapping coordinate and identical UMI sequences. HTSeq-count (v0.6.0) was used for gene expression quantification.24 Normalization and analysis of gene expression data were performed in R using edgeR and Limma-voom. Genes were considered differentially expressed with an adjusted P-value of <0.05 and a fold change of >2. DE genes were analyzed by ingenuity pathway analysis (QIAGEN, Inc; https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) and DAVID Bioinformatics Resources.25,26 Cell composition was analyzed with CIBERSORT27 using the cell signature file as described.28 The murine AA mRNA-seq data have been deposited in NCBI’s Gene Expression Omnibus29 and are accessible through GEO Series accession number GSE141072 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE141072).

Human Transcriptome Data Analysis
Published gene expression data (GSE668004) was analyzed with GEO2R to identify gene expression differences in whole blood between 44 male and 32 female complete KD patients or between healthy controls and patients with KD.30 Differentially expressed genes (FDR < 0.05) were analyzed by ingenuity pathway analysis. For plotting of individual probe counts, significant outliers were removed based on Grubb test. Published gene expression data (GSE7084) was analyzed with GEO2R to identify gene expression differences in AA tissue from controls or patients with abdominal aortic aneurysm.31 Published gene expression data (GSE64486) was analyzed with GEO2R to identify gene expression differences in coronary arteries from controls or patients with KD.32

Western Blotting
AA tissue (pooled from male and female LCWE-injected mice were homogenized in RIPA lysis buffer. Lysates were centrifuged at 13 000g for 10 minutes at 4°C. Twenty-five micrograms of cell lysate was assessed by SDS-PAGE and Western blot using the enhanced chemiluminescent system using the following antibodies: anti-mouse IL-1β (RRID: AB_2233636), anti-rat IgG HRP (RRID: AB_2338128), anti-beta-actin (RRID: AB_476743), and anti-mouse IgG HRP (RRID: AB_10015289).

Statistical Analysis
For data involving single comparisons, a 2-tailed paired t test was used for normally distributed data. For nonparametric data, the Mann-Whitney U test was used. For multiple comparison testing, significance was evaluated by 1- or 2-way ANOVA with Tukey post hoc test where appropriate. Data were tested for normality using the D’Agostino and Pearson normality test, where indicated.

RESULTS
Male Patients With KD Display an Enhanced IL-1β Signature Compared With Females
Since the incidence of KD development is higher in males, we analyzed published and publicly available...
transcriptomic data to assess gene expression difference between male and female patients with KD (GSE686004). We performed pathway analysis of differentially expressed genes (P<0.05) and identified significant enrichment of the IL-1 signaling pathway (P=0.00019), which was enhanced in male patients (Z score =1.387). Male patients displayed significantly enhanced expression of IL1B, NLRP3, MYD88, NFκB2, TRAF6, and TAB2 (Figure 1A through 1G), suggesting higher IL-1β production and signaling compared to females. IL1A expression was not significantly different between male and female patients (data not shown). We, therefore, hypothesized that enhanced IL-1β signaling in males versus females may contribute to the observed sex bias in KD development and pathogenesis. To determine the effect of sex on macrophage polarization, we analyzed the ratio of SOCS1:SOCS3 expression. Males had a significantly lower SOCS1:SOCS3 ratio than females (Figure 1H), indicative of greater M1 polarization. IL1B expression was negatively correlated with the SOCS1:SOCS3 expression ratio in males but not females (Figure 1I and 1J). We, therefore, hypothesized that increased IL1B expression may be due to enhanced M1 macrophage polarization in males.

Male Have Higher Incidence and a More Severe Disease Phenotype Than Females in a Mouse Model of KD

We next investigated disease development in male and female mice using the LCWE-induced KD mouse model. LCWE-injected male mice showed significantly more inflammation of the aorta and coronary artery than females (Figure 2A and 2B) and had increased incidence of disease (Figure 2C). LCWE-injected male mice also displayed greater dilatation of the AA and increased incidence of AAA than females (Figure 2D through 2F). We performed qPCR analysis of Il1b and Il1r1 expression in male and female LCWE-treated mice. We observed a significant correlation between Il1b and maximum AA diameter in both males and females (Figure 2G) yet to a greater degree in males. While we observed a significant correlation between Il1r1 and maximum AA diameter in males, there was no correlation in females (Figure 2H). Furthermore, Western Blot assessment of IL1-β protein indicates higher expression in males than females (Figure II in the online-only Data Supplement).

We performed mRNA-seq analysis of AA tissue harvested from PBS or LCWE-injected male and female mice and visualized gene expression differences between the groups using principal component analysis (Figure IA in the online-only Data Supplement) and hierarchical clustering (Figure 3A). Gene expression of AA tissue from male and female PBS treated mice appeared almost identical, with only 9 genes differentially expressed (>2-fold) between the sexes (Figure IB in the online-only Data Supplement). AA tissue from male LCWE-treated mice formed a distinct cluster from male PBS controls, with 3273 genes differentially expressed (>2-fold; Figure 3B and 3C). Female LCWE-injected mice showed high variability in gene expression. Two female LCWE-treated mice showed similarity to male LCWE-treated mice, while 2 showed greater similarity to the PBS controls (Figure 3A). This is in consensus with the disease incidence and decreased severity we observed in the female mice (Figure 2D through 2F). Despite broad differences in gene expression among female LCWE samples (as shown by clustering methods), male and female groups still formed distinct clusters based on maximum AA diameter and Il1b expression (Figure IC in the online-only Data Supplement), We, therefore, analyzed the female LCWE mice as a single group to help identify core differences in male and female gene expression. Between female LCWE-injected mice and PBS controls, 204 genes were differentially expressed (>2-fold; Figure 3B and 3D) while 652 genes were differentially expressed (>2-fold) between female and male LCWE-injected mice (Figure 3B and 3E) indicating that on average, female LCWE-treated mice show more similarity to PBS controls than male LCWE-treated mice. Overall, these data indicate that LCWE-induced KD vasculitis results in greater gene expression changes in the AA of male mice than in female mice, reflective of disease incidence and severity.

AA Gene Expression Profiles Indicate Enhanced IL-1β–Mediated Inflammation and Vascular Tissue Disruption in Males

We identified a set of genes associated with disease development based on differential expression between male PBS and LCWE-treated mice (Figure 3C), and a subset that were also differentially expressed between male and female LCWE-treated mice (highlighted red in Figure 3B), which we refer to as the sex difference in disease (SDD) gene set. We performed pathway and upstream regulator analysis of the SDD gene set (Figure 4A and 4B, Tables I and II in the online-only Data Supplement) and plotted heatmaps of selected genes separated into functional groups (Figure 4C through 4F). These functional analyses revealed key sex differences that may impact differential disease development and response to treatment.

Similar to our observations in peripheral blood mononuclear cells from male versus female patients with KD (Figure 1), we found that compared with female mice, LCWE-injected male mice had enhanced upregulation and expression of Il1b as well as components of the IL1-signaling pathway including, Il1r1, Il1r2, Myd88, Nkb2, and the NFκB subunit Rel (Figure 4C and 4D). Validation of selected genes by quantitative polymerase chain
reaction on AA tissues from LCWE-injected male and female mice confirmed this observation (Figure ID in the online-only Data Supplement). While Nlrp3 did not reach significance as a differentially expressed gene between LCWE-injected male and female mice ($P_{adj}=0.105$, fold change=4.04), we observed significant upregulation of
Nlrp3 in LCWE-injected males, but not females, compared with PBS controls (Figure 1E in the online-only Data Supplement). Furthermore, we found enhanced expression of Socs3 in males compared with females (Figure 4D). Functional analysis indicated that IL-1β, IL-1α, MYD88, and NFκB act as upstream regulators of genes within the SDD gene set, and their activation is enhanced in males compared with females (Figure 4B).
Furthermore, we observed enrichment of the inflammasome pathway and NFκB signaling in males (Figure 4A). IL-1α and IL-1β signal through a common receptor, however, we did not observe a significant difference in Il1a gene expression between males and females (Figure IF in the online-only Data Supplement). Overall these results point to an enhanced IL-1β signature within the SDD gene set in males compared with females.

Functional analysis of the SDD gene set indicated a heightened inflammatory state in the AA from LCWE-injected male mice compared with the AA of LCWE-injected female mice (Figure 4, Tables I and II in the online-only Data Supplement). LCWE injection enhanced expression of pathways involved in the innate immune response to a greater degree in males than females, including the acute phase response, TLR signaling and the role of pattern recognition receptors in detection of bacteria and viruses (Figure 4A and Table I in the online-only Data Supplement). Numerous genes involved in promoting these pathways, including Cxcl5, Cxcl14, Stat1, Vcam1, Selp, Sell, s100a9, and Mmp9, were enhanced in the AA of LCWE-injected male compared with female mice (Figure 4C through 4F and Figure ID in the online-only Data Supplement). Furthermore, functional analysis indicated heightened activation of immune cells promoting adaptive immunity, including enrichment of genes involved in dendritic cell maturation as well as Th1, Th2, and Th17 activation (Figure 4A and Table I in the online-only Data Supplement). In addition to IL1β, other cytokines were also predicted regulators of the SDD gene set including TNFα, IL6, IFNγ, and OSM (oncostatin-M; Figure 4B).

Furthermore, we found that IL6, Jak/Stat, OSM, STAT3, and NFκB signaling pathways were enhanced in LCWE-injected males compared with females (Figure 4B and Table II in the online-only Data Supplement). While we did not detect significantly increased expression of Tnf, Il6, Ifngr, or Osm in LCWE-injected males compared with LCWE-injected females, we did find significant upregulation of Tnf, Il6, Osm, and receptors Il6st and Ifngr1 in LCWE-injected males compared with PBS controls, but
this upregulation did not occur in females (Figure ID in the online-only Data Supplement).

Functional analysis also pointed to a gene signature associated with disruption of vascular structure in males. We found enrichment of genes involved in actin cytoskeleton signaling, integrin signaling, ILK (integrin-like kinase) signaling, and inhibition of MMP (matrix-metalloproteinase) signaling in the SDD gene set (Figure 4E and 4F). These pathways were reduced in LCWE-injected males compared with LCWE-injected females, indicative of reduced...
Integrity of the AA vessel structure. Consistent with this, we found enhanced expression of Mmp-3, -8, -9, and -13 in LCWE-injected males (Figure 4E). Furthermore, compared with LCWE-injected female mice, LCWE-injected male mice had a more pronounced downregulation of genes involved in smooth muscle cell function including Acta2, Mhy11, and Mylk (Figure 4F), which play important roles in vascular smooth muscle cell contraction and are associated with thoracic AAA development.36

We next performed CIBERSORT analysis using our mRNA-seq data to infer the immune cell composition of the AA tissue in LCWE-treated male and female mice (Figure 4G). Males exhibited a greater degree of M1 macrophage polarization than females, as predicted, which may account for the enhanced IL-1β expression in males.

Males and Female Mice Show Differential Responses to Anakinra Treatment in the LCWE-Induced KD Mouse Model

Anakinra treatment prevents LCWE-induced coronary lesions, myocarditis, and AAA formation in male mice.9,12,22 Given the differences in Il1b expression and IL-1 signaling observed between males and females in human KD and our murine model, we investigated whether sex influences the response to Anakinra. As expected, Anakinra treatment reduced disease severity (Figure 5A and 5B) and incidence of AAA formation (Figure 5C) in LCWE-injected male mice. In contrast, Anakinra treatment did not significantly affect disease severity (Figure 5A and 5B) or incidence of AAA formation (Figure 5C) in LCWE-injected female mice. These results indicate that Anakinra treatment, while effective in reducing LCWE-induced KD vasculitis in male mice, is not sufficient to inhibit disease in LCWE-injected female mice, which display milder disease and low Il1b expression.

Anakinra Treatment Effectively Inhibits LCWE-Induced Gene Expression Changes in Male But Not Female Mice

We further performed mRNA-seq analysis of AA tissue from PBS controls (no LCWE or Anakinra) and LCWE-injected male and female mice treated with or without Anakinra. Principal component analysis, hierarchical clustering, and differential expression analysis demonstrated large changes in gene expression associated with Anakinra treatment in male but not female mice (Figure 6A through 6C and Figure III in the online-only Data Supplement). Clustering and principal component analysis show that the AA expression profile in male LCWE-injected mice treated with Anakinra is highly similar to that of PBS controls and that LCWE-injected male mice treated with Anakinra form a separate cluster from untreated LCWE-injected male mice (Figure 6A and 6C and Figure IIIA in the online-only Data Supplement). One thousand five hundred ninety-two genes were differentially expressed (>2-fold) between male LCWE-injected mice treated with Anakinra or not, while only 7 genes were differentially expressed between PBS only controls and LCWE-injected male mice treated with Anakinra (Figure 6D). Taken together, these results indicate than in male mice, Anakinra treatment inhibits LCWE-induced gene expression changes in AA tissue. In contrast, principal component analysis and clustering analyses show that in female mice, Anakinra treatment did not significantly alter gene expression in the AA after LCWE-injection (Figure 6B and 6C and Figure IIIIB in the online-only Data Supplement). Only 1 gene was found to be differentially expressed between LCWE mice treated with Anakinra and those without, while 54 genes were found differentially expressed between Anakinra-treated LCWE mice and PBS controls (Figure 6E). Thus, Anakinra treatment has limited effect on AA gene expression in females.

Anakinra Is More Efficient in Suppressing Inflammation and Vascular Tissue Disruption in Male Mice Than in Females

We next performed comparative pathway and upstream regulator analysis of genes differentially expressed in the AA across treatment groups and sex (Figure 7A and 7B). LCWE injection resulted in the enrichment of multiple pathways associated with inflammation, immune cell activation, cytokine signaling, and tissue disruption (Figure 7A). As expected, given that male mice show increased severity of LCWE-induced KD vasculitis, these pathways had stronger enrichment (lower P value) and increased activation (higher Z score) in males than in females. A similar pattern was evident with upstream regulator analysis, which identified enrichment of multiple inflammatory mediators such as cytokines, their downstream signaling intermediates and transcription factors (Figure 7B). The majority of these pathways and upstream regulators were also enriched and activated in LCWE-injected mice compared with LCWE-injected male mice treated with Anakinra, indicating that Anakinra treatment is effective at reducing inflammation in male mice (Figure 7A and 7B). In contrast, these pathways were unchanged between LCWE-injected female mice with and without Anakinra (Figure 7A and 7B). Those pathways remained enriched, although to a lesser degree, in the comparison between LCWE-injected female mice treated with Anakinra with female PBS controls (Figure 7A and 7B). The differential change in the enrichment of these pathways further highlights the decreased disease severity and the resulting reduced effectiveness of Anakinra treatment in females. We also generated gene expression plots of selected genes regulated by both LCWE and Anakinra in males, categorized into functional groups (Figure 8A through 8E). Selected genes were further validated by qPCR (Figure IV in the online-only Data Supplement). In males,
LCWE-induced gene expression changes in cytokines, chemokines and their receptors, as well as genes involved in the inflammatory response and the extracellular matrix were regulated by Anakinra. As expected, since LCWE-treated females have mild disease and low Il1b expression, female mice failed to show a significant change in gene expression when treated with Anakinra. To understand the relevance of genes within our data set to human pathology, we compared our gene set to one associated with aneurysms in human AA tissue (healthy versus AAA tissue), a gene set associated with human KD whole blood (healthy versus KD patient whole blood) and a gene set associated with human KD coronary artery (controls versus KD coronary artery; Figure 8). Numerous genes overlapped with our data set (genes shown with a green mark), indicating that Anakinra can suppress the expression of LCWE-induced genes relevant to human KD pathology and aortic aneurysm development. Genes involved in inflammation, as well as many associated with vascular smooth muscle cell function, were rescued by treatment with Anakinra in males but not females. These findings could have wide-reaching implications for the use of anti–IL-1β therapies in human patients with KD.

**DISCUSSION**

KD is the leading cause of acquired heart disease in children in the developed world, affecting males at 1.5× the rate of females.\(^1\)\(^,\)\(^18\) Evidence also suggests disease severity is enhanced in males, as sex-based stratification of data published by Hoang et al\(^17\) shows that in patients with acute KD, males have a higher percentage of dilatation (23.5% compared with 17.4% in females) and aneurysms (14.7% compared with 7.2% in females). However, the cause of this sex bias is unknown and largely understudied. The importance of separating clinical trial analysis by sex to identify both appropriate dosing and potential differential responses to therapy was
Figure 6. Sex differences in gene responses following Anakinra (Ank) treatment Kawasaki disease (KD) mice.
Mice were injected intraperitoneally with PBS, *Lactobacillus casei* cell wall extract (LCWE), or LCWE and Ank (*n*=4–5 per group), and abdominal aorta (AA) was harvested 14 days post injection for mRNA-seq analysis. **A and B**, Heatmap and clustering (top 500 variable genes) of gene expression data from mRNA-seq analysis of male (**A**) and female (**B**) mice. **C**, Volcano plots of differentially expressed genes between indicated groups, blue=*P*~adj~ < 0.05 and fold change (FC) < 2, red=*P*~adj~ < 0.05 and FC > 2. **D and E**, Venn diagrams of differentially expressed genes (*P*~adj~ < 0.05, FC > 2) between indicated groups.

highlighted by the finding that females experience more adverse drug responses than males.36 Here, we demonstrate that the LCWE-induced KD mouse model, which closely resembles human KD pathology,10 also recapitulates sex differences observed in the human disease. Male mice displayed enhanced aortitis and coronary
arteritis as well as increased incidence and severity of AAA. Taking a transcriptomics approach, we used this model to study underlying causes of sex bias in KD. Our results point to a central role of IL-1β in mediating sex-based differences in disease, with important implications for the use of anti–IL-1β therapies to treat male and female patients with KD.

The vascular wall consists of a highly structured network of endothelial and smooth muscle cells and involves complex interactions between actin, integrins, and extracellular matrix. Central to the development of KD is an uncontrolled chronic inflammation involving immune cell infiltration into the arterial wall and progressive remodeling and destruction of vascular tissue. IL-1β is a potent inflammatory cytokine involved in autoimmune and chronic inflammatory conditions that has been implicated in regulating many aspects of cardiovascular disease relevant to KD pathogenesis.

Figure 7. Functional analysis of genes differentially expressed in response to Anakinra (Ank) treatment in Kawasaki disease (KD) mice.

Mice were injected intraperitoneally with PBS, Lactobacillus casei cell wall extract (LOWE) or LOWE and Ank (n=4–5 per group). Abdominal aorta (AA) was harvested 14 days post injection for mRNA-seq analysis. A, Comparative canonical pathway analysis of differentially expressed genes between indicated groups. B, Comparative upstream regulator analysis of differentially expressed genes between indicated groups.
**Figure 8.** Gene expression changes in male and female mice in response to Anakinra (Ank) treatment in Kawasaki disease (KD) mice.

Mice were injected intraperitoneally with PBS, *Lactobacillus casei* cell wall extract (LCWE) or LCWE and Anakinra (Ank; n=4–5 per group). Abdominal aorta (AA) was harvested 14 days post injection for mRNA-seq analysis. Heatmaps were generated of selected genes in the following functional categories: (A) Cytokines and chemokines; (B) cytokine and chemokine receptors; (C) inflammatory response; (D) extracellular matrix; and (E) vascular smooth muscle cell function. Green represents genes found differentially expressed \((P_{adj}<0.05, \text{fold change } [FC] >2)\) between human control abdominal aorta and abdominal aortic aneurysm tissue (AAA); genes found differentially expressed \((P_{adj}<0.05, \text{FC} >2)\) between healthy control blood and patient with KD blood (PBMC) and genes found differentially expressed \((P_{adj}<0.05, \text{FC} >2)\) between control and KD coronary artery tissue (coronary).
expression of *IL1B*, *NLRP3*, and IL-1 signaling molecules, including *MYD88*, in male patients with KD. Similarly, in the LCWE-induced KD mouse model, males had enhanced expression of genes encoding IL-1β and components of IL-1 signaling pathways in the AA, including IL-1 receptor subunits and *MYD88*. Our analysis identified IL-1β, IL-1α, *MYD88*, and NFκB as significant regulators of LCWE-induced genes that were differentially expressed between the sexes. Interestingly, the incidence and severity of adult abdominal aortic aneurysms are also greater in males than females, and transcriptional analysis of AAA patient peripheral blood mononuclear cells found enhanced expression of inflammasome components, including *AIM2*, *NLRP3*, *ASC (PYCARD)*, *CASP1*, *CASP5*, and *IL1B* in males compared with females. Thus, a role for IL-1β in mediating sex differences in abdominal aortic aneurysms may be universally relevant.

In addition to IL-1β and inflammasome signaling, we identified many key signaling pathways within the murine expression data that have previously been implicated in human KD and AAA. Furthermore, the top upstream regulators predicted to mediate gene expression changes in the LCWE model—TNF, IFNγ, and TGFβ1—have all been implicated in human KD. These similarities strongly support the translational value of our murine data.

Interestingly, males have shown a higher production of IL-1β in response to lipopolysaccharides in both mouse and human, indicating immune cells from males may be primed to produce a more robust IL-1β response than females. Our data indicate that enhanced IL-1β expression may be a result of a greater degree of inflammatory M1 macrophage polarization in males compared with females. SOCS3 (suppressor of cytokine signaling) promotes M1 polarization, whereas SOCS1 promotes M2 polarization. Recently, Barrett et al. showed that in atherosclerosis, the ratio of SOCS1/SOCS3 expression negatively correlates with IL1β expression. Similarly, we found that the SOCS1/SOCS3 ratio negatively correlated with IL1β expression in peripheral blood mononuclear cells from male KD patients, and the SOCS1/SOCS3 ratio was lower in male patients. Furthermore, analysis of the murine AA revealed more M1 macrophages and monocytes in male mice treated with LCWE than in females, and LCWE-treated male mice had enhanced SOCS3 expression compared with females. In support of a more reactive phenomenon, males in the LCWE-treated male mice had enhanced *Socs3* expression compared with females, and *Socs3* expression was not investigated in those studies. In the LCWE model, we identified the related gene *FCGR2B* as differentially regulated between males and females and differentially modulated by Anakinra treatment.

Recently, much attention has been focused on hormonal differences impacting cardiovascular disease, in relation to both immune and vascular cell function. However, KD occurs mainly in prepubescent children under the age of 51 and displays a consistent sex bias across infancy and childhood. Similarly, our studies were performed on 5-week-old sexually immature mice. Given that sex-hormone levels are similar in male and female prepubescent children, sex-hormone signaling is unlikely to play a direct role in mediating the sex differences observed in KD. However, given the important role of sex hormones during fetal development, we cannot rule out the possibility that those developmental differences have long lasting effects on the immune response, perhaps due to epigenetic modifications. Indeed, sex differences in epigenetic modifications of genes associated with cardiovascular disease pathogenesis have been identified, and the expression of DNA methylation modifiers DNMT1 and TET2 is altered in patients with KD. Future studies investigating epigenetic modifications and their relationship to KD, particularly in regard to IL-1β-mediated pathways, should be stratified by sex to help identify such potential mechanisms.

The microbiome plays a central role in regulation of the immune system and has been implicated in patients with KD and the LCWE-induced KD mouse model. Sex differences in the composition of gut microbiota have been described in both mouse and human, although whether they contribute to IL-1 signaling or KD pathogenesis is unknown.

Given our data pointing to altered IL-1β signaling in male and female KD mice, we hypothesized that there may be differential responses of male and female mice to treatment with the IL-1R antagonist Anakinra. Indeed, we
found that Anakinra was only effective at reducing AAA development and severity and associated gene expression changes in male mice. A number of case reports have highlighted the successful use of Anakinra in IVIG-nonresponder patients with KD, leading to 2 Phase II clinical trials for Anakinra in IVIG-resistant patients. Case reports to date have shown effective Anakinra treatment in both male and female IVIG-resistant patients. Overall, our results highlight the importance of incorporating independent analyses of male and female patients with KD in trials testing the therapeutic efficacy of Anakinra as well as other emerging therapeutics. Additional biomarkers, that is, serum IL-β or presence of polymorphisms, may also be beneficial in determining which patients will respond to Anakinra treatment. Ongoing research in KD, both in human and mouse, should incorporate sex-based stratification of results to further tease out the mechanisms driving differences in KD development and response to treatment between the sexes.

ARTICLE INFORMATION
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