Single-Nucleotide Polymorphism of the *MLX* Gene Is Associated With Takayasu Arteritis

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Abstract

BACKGROUND: Takayasu arteritis (TAK) is an autoimmune systemic arteritis of unknown pathogenesis. Genome-wide association studies revealed that single-nucleotide polymorphisms in the *MLX* gene encoding the MLX (Max-like protein X) transcription factor are significantly associated with TAK in Japanese patients. *MLX* single-nucleotide polymorphism rs665268 is a missense mutation causing the Q139R substitution in the DNA-binding site of MLX.

METHODS: To elucidate the hypothesis that the single-nucleotide polymorphism of the *MLX* gene plays a critical role in the development of TAK, we conducted clinical and laboratory analyses.

RESULTS: We show that rs665268 significantly correlated with the severity of TAK, including the number of arterial lesions and morbidity of aortic regurgitation; the latter may be attributed to the fact that *MLX* mRNA expression was mostly detected in the aortic valve. Furthermore, the Q139R mutation caused structural changes in MLX, which resulted in enhanced formation of a heterodimer with MondoA, upregulation of TXNIP (thioredoxin-interacting protein) expression, and increase in the activity of the NLRP3 (NACHT, LRR, and PYD domains-containing protein 3) inflammasome and cellular oxidative stress. Furthermore, autophagy, which negatively regulates inflammasome activation, was suppressed by the Q139R mutation in MLX. The MLX-Q139R mutant significantly induced macrophage proliferation and macrophage-endothelium interaction, which was abolished by the treatment with SBI-477, an inhibitor of MondoA nuclear translocation. Our findings suggest that the Q139R substitution in MLX plays a crucial role in the pathogenesis of TAK.

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Disclosures

None.

CONCLUSIONS: MLX-Q139R mutation plays a crucial role in the pathogenesis of TAK through promoting inflammasome formation.

Keywords

aortitis; autophagy; genetics; polymorphism, single-nucleotide; Takayasu arteritis

Takayasu arteritis (TAK) is an autoimmune systemic arteritis with unclear pathogenesis[1] known to be associated with sex, race, and geographic location. The majority of patients with TAK are young and middle-aged women of the Asian background; the onset of the disease occurs between 10 and 40 years of age,[2] and its prevalence is much higher in Asia than in Western countries.[3–5] In Japan, the annual incidence of TAK is estimated to be ≈150 patients per entire population in a given year.[6] TAK lesions are mainly localized in the aorta and its major arterial branches.[7] Because arterial inflammation progresses with age, at the final stage of the disease, the entire blood vessel can be affected.

Increasing evidence suggests that single-nucleotide polymorphisms (SNPs) of genes encoding specific human leukocyte antigens (HLAs), including *HLA-Bw52, HLA-B39,* and *HLA-B67,* are associated with genetic susceptibility to TAK.[8–10] In our previous genome-wide association study, we have shown that the *HLA-B* region on chromosome 6 and the *IL12B* gene on chromosome 5 encoding IL (interleukin)-12 subunit p40 and the *MLX* gene on chromosome 17 encoding the MLX (Max-like protein X) are significantly associated with TAK in Japanese patients.[11] As earlier reports indicate that *IL12B* is linked to a variety of auto-immune disorders, including psoriasis,[12] Crohn disease,[13] and ulcerative colitis,[14] it is possible that *IL12B* polymorphisms play an important role in TAK development through common molecular mechanisms underlying autoimmune disorders. However, there is little information about the effects of MLX on the immune system, and it remains unknown whether SNPs of the *MLX* gene are associated with TAK pathogenesis.

MLX belongs to the family of basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors and is activated through formation of heterodimers with other bHLH-Zip family members, including MondoA.[15] There is a reason to suggest that the MondoA-MLX complex negatively regulates glucose metabolism by facilitating the expression of TXNIP, an inhibitor of oxidoreductase TRX (thioredoxin), which acts as an antioxidant.[16] Our earlier genome-wide association studies revealed that the A>G (rs665268) SNP of the *MLX* gene (A, nonrisk allele; G, risk allele) encoding the Q139R substitution (Q, no risk; R, risk) was significantly associated with clinical manifestations of TAK.[11] However, the association of MLX with TAK pathogenesis has not been previously investigated. In the present study, we performed clinical and laboratory analyses to test the hypothesis that the rs665268 polymorphism of *MLX* plays a critical role in the development of TAK.

METHODS

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the analyses reported here. Materials and data may be made available on request to the corresponding author. This research conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved

by the Ethics committee of the Tokyo Medical and Dental University for Medical Experiments (permission number: G2000180-01). All patients and healthy volunteers provided written informed consent before the study. Detailed methods are available in the Data Supplement.

RESULTS

MLX Risk Allele Is Associated With the Severity of Aortic Regurgitation and Lesion Number in TAK

Ninety-six patients with TAK (5 men and 91 women; mean age, 46.8 years; mean age at onset, 27.2 years) who visited our hospital were investigated for the association of their clinical characteristics with the presence of the *MLX* risk allele (Table). The distribution of the *MLX* alleles was as follows: no risk alleles (homo-zygote, AA), 21% (n=20); 1 risk allele (heterozygote, AG), 45% (n=43); and 2 risk alleles (homozygote, *GG*), 34% (n=33). The average age, age at TAK onset, and male:female ratios were comparable among the patients of the 3 groups. Patient distribution according to the Numano classification of TAK was as follows: type I, 38%; type IIa, 14%; type IIb, 16%; type III, 0%; type IV, 1%; and type V, 32%. The *IL12B* SNP distribution was as follows: CC, 27%; *CA,* 46%; and AA, 27%. HLA-B52 was detected in 60% of the 96 patients with TAK.

To determine whether the *MLX* polymorphism affected TAK clinical manifestations, we compared clinical characteristics of patients with TAK in the 2-risk allele, 1-risk allele, and no-risk allele groups. The number of patients who had moderate or severe aortic regurgitation in the 2-risk allele group (9 of 33 patients, 27%) and in the 1-risk allele group (11 of 43 patients, 26%) was significantly higher than that in the no-risk allele group (1 of 20 patients, 5%; $P<0.05$; Figure 1A; Table). The mean number of arterial lesions in the 2-risk allele group (4.5) and the 1-risk allele group (4.1) was significantly higher than that in the no-risk allele group (2.8; *AA* versus GG, $P<0.01$; *AA* versus *AG*; $P<0.05$; Figure 1B; Table; Table I in the Data Supplement). Similarly, compared with the no-risk allele group, the 2-risk allele group had many more patients with TAK of Numano type V, who had the entire aortic region, from the ascending aorta to the abdominal aorta and its branch, affected by the disease (*AA*, 15%; *AG*, 28%; *GG*, 49%; $P<0.05$; Table). The patients with TAK who had the *MLX* risk allele were more likely to have moderate or severe aortic regurgitation, have larger total number of lesions, and be classified as Numano type V (Table).

Next, we analyzed the expression of *Mlx* mRNA in the large vessel system of adult mice by in situ hybridization using 3 *Mlx*-specific digoxigenin-labeled RNA probes. Among them, the M/x-long probe had the highest labeling intensity in the villous epithelium and crypts of the mouse jejunum used as positive control for in situ hybridization with *Mlx* mRNA (Figures I and II in the Data Supplement); therefore, it was chosen for the experiment. The results indicated that aortic valves were strongly labeled with *Mlx*-long compared with any other parts of the large vessel system (Figure 1C).

Taken together, these data suggest that the patients with the *MLX* risk allele are more likely to have extensive inflammation in their large vessels, especially in the aortic valve.

SNP-Caused Amino Acid Substitution in MLX May Stabilize the MLX-MondoA-DNA Complex

The missense mutation in *MLX* (rs665268: CAG → CGG) leads to the substitution of Gln139 with Arg in the MLX protein (Figure 2A). Because Gln139 is located on the bHLH domain containing the DNA-binding site of MLX, we hypothesized that the Q139R mutation, which increases the positive charge in the protein, may enhance the formation of the MLX-MondoA-DNA complex. Crystallographic analyses suggested that the MAD/MAX heterodimer (protein data bank code: 1NLW) formed by the Myc family proteins strongly interacted with DNA at the bHLH domain containing 3 basic positively charged amino acids (Lys66/Arg69/Arg73 on MAD and Arg33/Arg36/Lys40 on MAX), which contacted negatively charged DNA phosphate groups (Figure 2B). On the contrary, the DNA-binding ability of the MLX-MondoA complex is supposed to be weaker than those of heterodimer complexes formed by other Myc family proteins because both MLX and MondoA have only 2 basic amino acids in their bHLH domain, Arg142/Lys146 and Arg732/Lys736, respectively. However, the Q139R substitution provides another positively charged residue in the MLX mutant, and the resulting 3 basic amino acids, Arg139, Arg142, and Lys146, would enable stronger molecular interaction among MLX-Q139R, MondoA, and DNA (Figure 2C). Thus, in theory, the Q139R mutation would stabilize the MLX-MondoA-DNA complex.

Q139R Enhances the Formation of MLX-MondoA Heterodimers and Increases TXNIP Expression Through Activation of the *TXNIP* Promoter

To test our hypothesis that the Q139R mutation would enhance the formation of the MLX-MondoA-DNA complex in cells, we conducted immunoprecipitation assays. MondoA showed stronger interaction with the MLX-Q139R mutant protein compared with the wild-type MLX (MLX-WT; Figure 3A). To determine whether the Q139R substitution could enhance MLX transcriptional activity, we performed reporter assays for MLX using the *TXNIP* promoter[17] in human aortic smooth muscle cells (hASMCs) transfected with MondoA. The *TXNIP* promoter activity was significantly increased in hASMCs coexpressing MondoA and MLX-Q139R compared with those coexpressing MondoA and MLX-WT (Figure 3B). Consistently, chromatin immunoprecipitation assays demonstrated that MLX was bound to the *TXNIP* promoter and that the Q139R mutation in MLX enhanced the binding of MLX to DNA in hASMCs (Figure 3C). To further evaluate the role of the Q139R mutation in the regulation of TXNIP expression, we determined the effect of the MLX-Q139R mutant on TXNIP protein levels in hASMCs by immunoblotting. Coexpression of MondoA and MLX-Q139R caused a significant increase in TXNIP levels compared with that of MondoA and MLXWT (Figure 3D). Taken together, these results suggest that the Q139R substitution in MLX promotes its binding to MondoA and enhances the DNA-binding ability of the MLX-MondoA complex, which in turn upregulates TXNIP expression.

In our previous study, we have shown that SNP rs6871626 in the *IL12B* region is associated with TAK onset and its resistance to therapy.[18] Therefore, we evaluated serum levels of IL-12p70 (hereafter referred to as IL-12), a heterodimeric cytokine composed of IL-12p40 and IL-12p35 encoded by *IL12.* The results indicated that serum IL-12 levels in patients with TAK were significantly higher than those in normal subjects (Figure 3E), suggesting

that SNP rs665268 in the *MLX* gene would enhance the accumulation of TXNIP in concert with the upregulation of IL-12 secretion by immune cells and vascular smooth muscle cells. To test this hypothesis, we isolated human peripheral blood mononuclear cells (PBMCs) from TAK patients carrying the *MLX* risk geno-type (*MLX*-GG) and healthy individuals and stimulated them with anti-CD3 antibodies to promote PBMC differentiation into T lymphocytes, a major cell target of IL-12. After treatment with 30 ng/mL of IL-12, T lymphocytes from TAK patients carrying the risk genotype showed significantly higher TXNIP expression than those from normal subjects (Figure 3F). Similar results were observed in PBMC-derived macrophages (data not shown). We also found that treatment with IL-12 stimulated the nuclear translocation of MLX in T lymphocytes (Figure 3G), suggesting that IL-12 promoted nuclear accumulation of MLX. Histopathologic examination revealed higher TXNIP expression in the aortic walls of TAK patients carrying the *MLX* risk alleles compared with those without risk alleles (Figure 3H).

MLX-Q139R Promotes Oxidative Stress

As increased expression of TXNIP stimulates intracellular oxidative stress by inhibiting TRX,[17,19] we investigated whether the MLX-Q139R mutant promoted oxidative stress through TXNIP upregulation. JNK (c-Jun N-terminal kinase) is known to be involved in redox signaling,[20] and we found that both JNK expression and its phosphorylation were significantly increased in T lymphocytes derived from TAK patients with *MLX-GG* compared with normal control (Figure 4A). On the contrary, the *MLX* mutation did not affect TRX expression in T lymphocytes (Figure 4B). Similar results were observed in PBMC-derived macrophages (data not shown). As macrophages are one of the most important players in the inflammation during vasculitis, we also evaluated the effect of the MLX-Q139R mutation on macrophages using RAW264.7 cells, a murine macrophage cell line. Immunostaining for 8-hydroxy-2'-deoxyguanosine (8-OHdG), a major product of DNA oxidation and a bio-marker of oxidative stress, demonstrated that the number of 8-OHdG-positive cells was significantly higher among RAW264.7 cells coexpressing MLX-Q139R and MondoA compared with those coexpressing MLX-WT and MondoA (Figure 4C). These results suggest that the MLX-Q139R mutation promotes intracellular oxidative stress, possibly through activation of TXNIP

MLX-Q139R Upregulates NLRP3 Inflammasomes

The NLRP3 (NACHT, LRR, and PYD domains-containing protein 3) inflammasome is a multiprotein complex consisting of caspase-1, ASC (apoptosis-associated speck-like protein containing a CARD), and NLRP3, which is involved in the pathogenesis of various inflammatory diseases through stimulation of the innate immune system.[21] As it was shown that TXNIP plays an important role in the formation of NLRP3 inflammasomes,[22] we investigated whether the MLX-Q139R mutation facilitated the formation of NLRP3 inflammasomes by upregulating TXNIP. Immunoblotting analysis demonstrated that NLRP3 expression was significantly increased in hASMCs coexpressing MLX-Q139R and MondoA compared with those coexpressing MLX-WT and MondoA (Figure 5A). We also found that protein levels of NLRP3 and caspase-1 were significantly elevated in IL-12-stimulated PBMC-derived macrophages from TAK patients carrying *MLX-GG* compared with those

from normal controls (Figure 5B). These results suggest that NLRP3, which is upregulated by TXNIP, is accumulated in PBMCs of TAK patients with the *MLX* risk genotype.

The major end product of the NLRP3 inflammasome is IL-1β, a cytokine that mediates various inflammatory responses.[23] Therefore, we examined whether IL-1 β was increased by the MLX-Q139R mutation by measuring IL-1 β levels in supernatants of cultured PBMC-derived macrophages using ELISA. The results indicated that IL-1 β secretion was significantly upregulated in PBMCs from TAK patients carrying the risk genotype compared with those from normal subjects (Figure 5C). On the contrary, there was no difference between the 2 groups in the secretion of TNF-α (tumor necrosis factor-α) and IL-6, the other major proinflammatory cytokines (Figure 5C). Collectively, these results suggest that the MLX-Q139R mutation promotes the formation of NLRP3 inflammasomes, thereby increasing the production of IL-1 β.

MLX-Q139R Attenuates Autophagy and Enhances Inflammasome Formation in Macrophages

It was suggested that the activity of inflammasomes is negatively regulated by autophagy,[24] and we hypothesized that the MLX-Q139R mutation would suppress the degradation of inflammasomes by inhibiting autophagy. To induce inflammation-mediated autophagy, we treated RAW264.7 cells with lipopolysaccharide and analyzed them by electron microscopy and immunoblotting. The results indicated that in RAW264.7 cells coexpressing MondoA and MLX-Q139R, the number of autophagosomes was significantly decreased (Figure 6A), and the expression of LC3II significantly downregulated, whereas that of p62 was upregulated (Figure 6B) compared with the cells coexpressing MondoA and MLX-WT.

A recent study demonstrated that MLX and MondoA homologs play a crucial role in autophagy through transcriptional regulation of leucyl-tRNA synthase (*lars-1*),[25] which led to the inhibition of mTOR (mammalian target of rapamycin) homolog TOR and suppression of autophagy in *Caenorhabditis elegans*.[26] On the basis of these findings, we hypothesized that the MLX-Q139R mutant would attenuate the inhibitory activity of MondoA on LARS1 (leucyl-tRNA synthetase 1) expression in RAW264.7 cells through enhancement of protein interaction between MLX and MondoA (Figure III in the Data Supplement). As expected, the expression of LARS1, mTOR, phospho-mTOR (Ser2448), and p62 was significantly increased in RAW264.7 cells coexpressing MondoA and MLX-Q139R compared with that in cells coexpressing MondoA and MLX-WT (Figure 6C).

Taken together, these results suggest that the MLXQ139R mutation inhibits autophagy in cells stimulated with lipopolysaccharide, which would negatively regulate the degradation of inflammasomes.

MLX-Q139R Mutation Promotes the Proliferation and Adhesion of Macrophages

Next, we addressed the functional role of the MLXQ139R mutation in vascular inflammation in vitro because there are currently no suitable animal models of TAK. The growth of a human macrophage THP-1 cell line coexpressing MLX-Q139R and MondoA was significantly increased compared with that of the cells coexpressing MLX-WT and MondoA (Figure 7A). A similar effect was observed for human coronary artery endothelial

cells (Figure 7B). These results suggest that the MLX-Q139R mutation promotes proliferation not only of macrophages but also of endothelial cells.

We next evaluated the effect of the MLX-Q139R mutation on the adhesion of macrophages to endothelial cells. The adhesion of THP-1 cells coexpressing MLX-Q139R and MondoA to human umbilical vein endothelial cells was markedly increased compared with those coexpressing MLX-WT and MondoA, and scanning electron microscopy revealed several pseudopods projected from the surface of THP-1 cells (Figure 7C). After stimulation with 20 ng/mL TNF-α, the level of ICAM-1 (intercellular adhesion molecule-1) was significantly increased in human umbilical vein endothelial cells coexpressing MLX-Q139R and MondoA compared with those coexpressing MLX-WT and MondoA (Figure 7D). These results suggest that the MLX-Q139R mutation enhances the adhesion between macrophages and the endothelium, possibly through upregulation of adhesion molecules.

Inhibition of MondoA Nuclear Translocation Decreases the MLX-Q139R- Mediated Activation of TXNIP and Proliferation of Macrophages

SBI-477 is a small molecule that attenuates lipid accumulation in skeletal myoblasts by promoting nuclear export of MondoA, which downregulates TXNIP expression.[27] Treatment with SBI-477 effectively suppressed MondoA nuclear translocation in RAW264.7 cells (Figure 8A) and significantly inhibited the expression of both TXNIP and NLRP3 in THP-1 cells coexpressing MLX-Q139R and MondoA compared with control cells treated with dimethyl sulfoxide (DMSO; Figure 8B). In addition, SBI-477 significantly inhibited the proliferation of THP-1 cells coexpressing MLX-Q139R and MondoA compared with DMSO-treated cells (Figure 8C). These results suggest that SBI-477 inhibits TXNIP accumulation and NLRP3 inflammasome formation, which decreases the proliferation of monocytes.

DISCUSSION

A recent increase in genome-wide association study application and advances in high-throughput sequencing technologies have resulted in discovering of several risk loci for various autoimmune diseases whose pathogenesis remains unclear. Although the majority of putative causative variants are mapped to noncoding genomic regions and are enriched in distal parts of regulatory elements, some risk-associated polymorphisms were confirmed as critically affecting biological phenotypic traits, including intracellular signaling, immune cell abundance, and serum cytokine levels.[28] In this study, we demonstrate that SNP rs665268 of *MLX* is strongly associated with the severity of TAK, including aortic regurgitation morbidity and the number of arterial lesions. Consistent with the clinical findings, *MLX* mRNA predominantly accumulated in the aortic valve. The MLX-Q139R variant encoded by rs665268 enhanced the formation of a heterodimer between MLX and MondoA, thereby promoting TXNIP transcription, which, in turn, enhanced NLRP3 inflammasome activity, and increased proliferation and adhesion of macrophages. Thus, the MLX-Q139R mutation may play a crucial role in TAK pathogenesis by stimulating the formation of inflammasomes.

Several SNPs linked to disease pathogenesis are located in functional genes involved in biological processes, such as *ATG16L1,* a critical regulator of autophagy machinery. *ATG16L1* polymorphisms have been associated with increased risk of Crohn disease; the most common disease-associated *ATG16L1* SNP, rs2241880, encodes a missense mutation Thr300Ala, which increases ATG16L1 degradation by caspase-3.[28] When SNP-mediated mutation occurs in DNA-binding domains of transcription factors, it may alter their transcriptional activity. Thus, the SNP-associated Val217Met substitution in the DNA-binding domain of tumor suppressor p53 was shown to have an anticancer effect by promoting the expression of several p53 target genes such as *BAX, CDKN1A,* and *PMAIP1.*[29] Crystallographic studies of MAD–MAX complex structure and their critical amino acid motifs suggest that rs665268 increases the number of positively charged residues in the DNA-binding domain of MLX, which was confirmed in the validation assays. These results allowed us to conclude that the MLX-Q139R mutant forms a much stronger molecular complex with MondoA and DNA than the wild-type MLX.

MLX and its dimerization partner MondoA form the MondoA–MLX complex, which stimulates TXNIP expression resulting in the suppression of cellular glucose uptake and aerobic glycolysis and enhancement of oxidative stress.[16] However, it remains to be elucidated whether the MondoA–MLX complex could be associated with inflammatory responses. On the contrary, TXNIP physically interacts with NLRP3 and activates the inflammasome,[22] an important component of the innate immune system. It has been shown that the NLRP3 inflammasome is associated with autoimmunity.[30] On the basis of these findings and our current results, we can conclude that the induction of MondoA-MLX transcriptional activity promotes NLRP3 inflammasome formation. The interaction between MondoA and MLX was markedly increased by the MLX-Q139R mutation, indicating that rs665268 would result in the gain-of-function for MLX regarding its regulation of proinflammatory responses. Furthermore, we showed that MLX was expressed in the aortic valve tissue, suggesting that inflammation in the valve was likely to occur through formation of the NLRP3 inflammasome and that TAK patients with the *MLX-GG* risk genotype have increased chances of aortic regurgitation.

There is evidence to suggest that the activity of inflammasomes is negatively regulated by autophagy, an evolutionarily conserved mechanism of degrading unnecessary cellular components.[31] A previous study demonstrated that systemic inflammation depends on the activity of the NLRP3 inflammasome, which is negatively regulated by cAMP-dependent molecule-specific selective autophagy through activation of MARCH7, an E3 ubiquitin ligase.[32] It was also reported that tripartite motif proteins act as specialized receptors for highly specific autophagy, termed precision autophagy, of key inflammasome components.[33] Recently, Nakamura et al[26] revealed that MXL-2 and MML-1, *C. elegans* homologs of MLX and MondoA, respectively, play a critical role in autophagy through transcriptional regulation of *lars1.* MML-1 attenuates *lars1* expression through binding to its promoter, which decreases the expression of mTOR homolog TOR, a negative regulator of autophagy. [26,34] On the basis of these data, we hypothesized that the Q139R mutation in MLX may facilitate the molecular interaction between MLX and MondoA and positively regulate the expression of LARS1 and mTOR by promoting the sequestration of the transcriptional repressor MondoA and its removal from the *LARS1* promoter. Consistent with this

hypothesis, MLX-Q139R resulted in autophagy suppression as evidenced by the decrease in LC3-II and reduction of the number of autophagosomes in RAW264.7 cells coexpressing MondoA and MLX-Q139R compared with those coexpressing MondoA and MLX-WT. Thus, the MLX-Q139R mutation would promote inflammation through a dual effect of MLX on inflammasome formation and autophagy activity.

Previous reports indicate that SNPs are associated with pathogenesis of various human diseases.[28,29] Although recently emerged genome-editing technologies such as CRISPR/ Cas9 would be promising therapeutic strategies for gene polymorphism–related diseases,[35] most of them remain to be validated for clinical significance. SBI-477 is a small compound inhibiting TXNIP accumulation by blocking nuclear translocation of MondoA,[27] and our results demonstrate that SBI-477 effectively decreased the expression of both TXNIP and NLRP3 in THP-1 cells carrying the MLX-Q139R mutation. These findings suggest that SBI-477 could be a promising therapeutic agent for TAK, which does not affect the structure of *MLX*.

Recent clinical investigations revealed the effectiveness of tocilizumab, a humanized monoclonal anti–IL-6 receptor antibody, in treating recalcitrant TAK and giant cell aortitis. [36,37] It has been shown that tocilizumab is also effective in patients with familial Mediterranean fever, an autosomal recessive autoinflammatory disease caused by excessive activation of inflammasomes.[38] Although the detailed mechanism underlying the alleviation of familial Mediterranean fever symptoms by tocilizumab remains unknown, it was hypothesized that tocilizumab could suppress inflammasome activation by inhibiting the priming step of inflammasome formation associated with IL-6 activity. These previous data together with our current results indicate a possibility that TAK patients harboring the MLX-Q139R mutation may be an ideal therapeutic target for tocilizumab treatment.

Our study has limitations. First, we evaluated the effect of the MLX-Q139R mutation on TAK only in vitro, as there are currently no established animal models that can faithfully reproduce the pathological features of TAK. Therefore, it is necessary to create a reliable animal model of TAK to validate our findings. Second, there is currently no evidence of positive association of MLX with TAK in non-Japanese populations,[39,40] and genome-wide association studies of patients with TAK should be conducted in other countries to determine the significance of the MLX-Q139R mutation for TAK pathogenesis.

In conclusion, our study suggests that the SNP-mediated mutation MLX-Q139R plays a crucial role in the pathogenesis of TAK by facilitating inflammasome formation, which in turn promotes the proliferation and adhesion of aorta-resident cells, a pathological manifestation seen in the aortas of patients with TAK. These findings may have potential application in the development of novel therapeutic approaches for the treatment of TAK.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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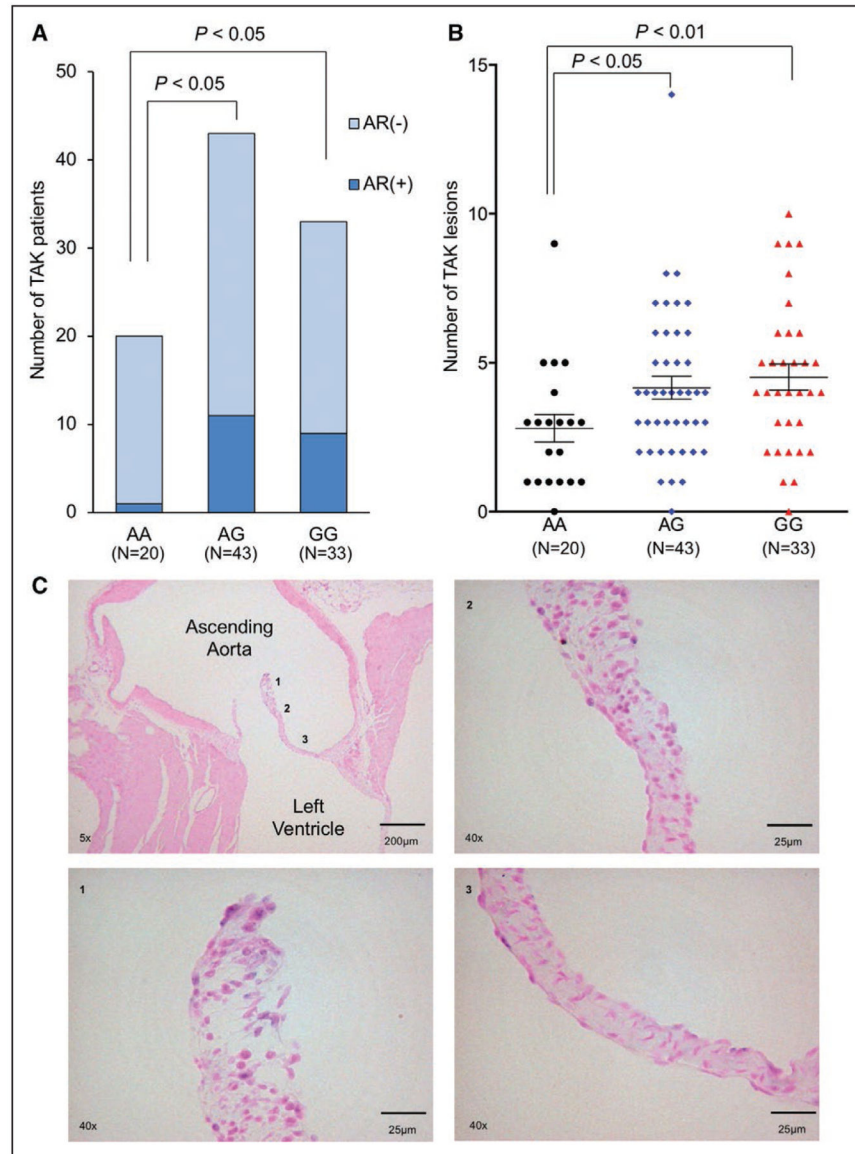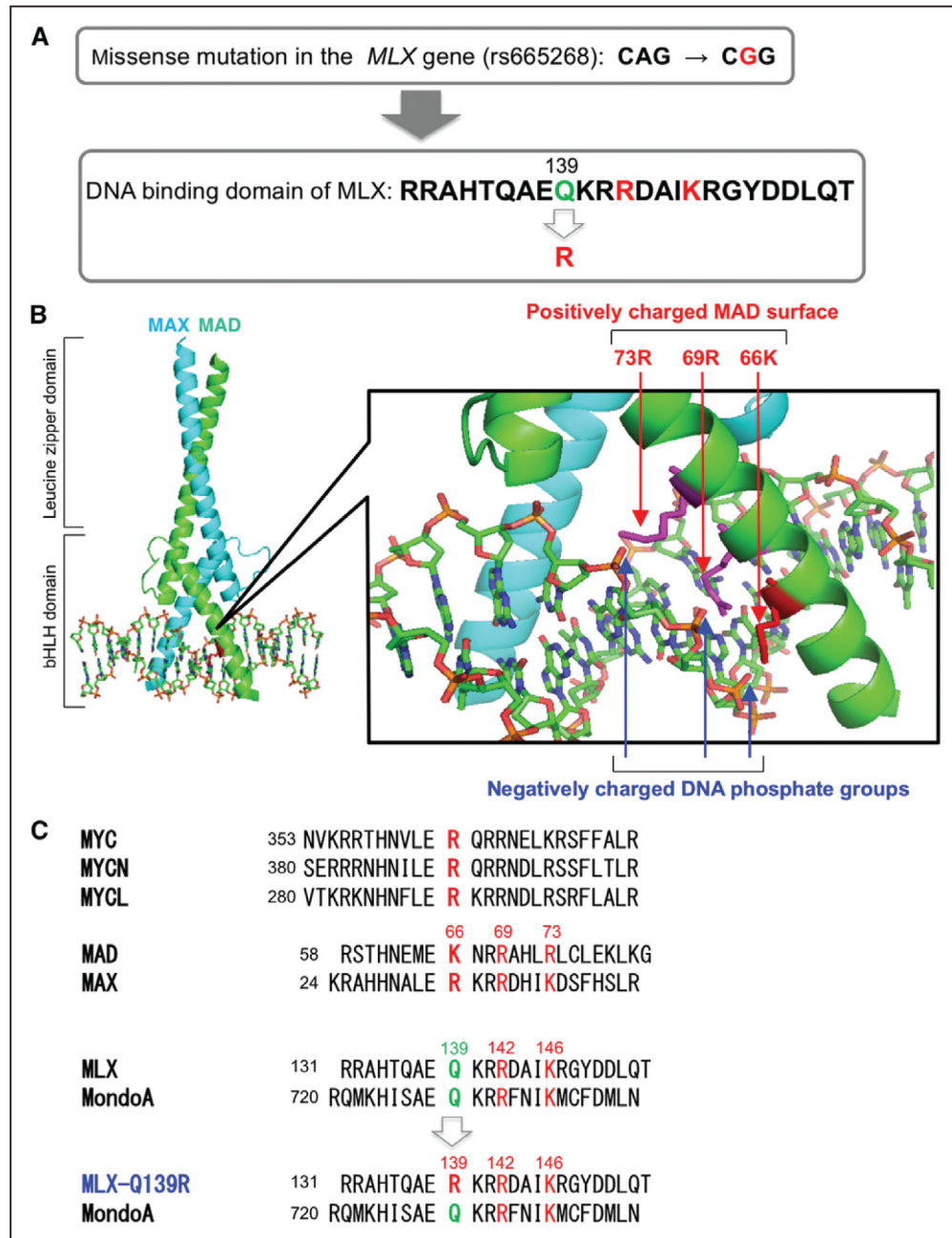
Figure 1.

Association of MLX polymorphism rs665268 with Takayasu arteritis (TAK) clinical manifestations.**A**, Correlation between *MLX* genotypes and severity of aortic regurgitation. **B**, Correlation between *MLX* genotypes and the number of TAK lesions. The data are presented as the mean±SEM. **C**, *MLX* mRNA expression detected by in situ hybridization in aortic valves of the adult mouse heart. Bars=200 μm (**top left** image) and bar=25 μm (other images). MLX indicates Max-like protein X.

Figure 2.
In silico analysis of the effects of the rs665268 polymorphism on MLX (Max-like protein X) function. **A**, The *MLX* single-nucleotide polymorphism (SNP) causes the Q139R substitution in the DNA-binding domain of MLX, thus changing the neutral residue (Q) to positively charged basic residue (R), which may enhance the formation of the MLX–MondoA–DNA complex. **B**, Crystal structure of the MAD–MAX–DNA complex (protein data bank ID: 1NLW). **C**, Sequence alignment of DNA-binding domains of Myc family proteins. Red letters indicate positively charged basic amino acids; green Q indicates glutamine. bHLH indicates basic helix-loop-helix leucine.
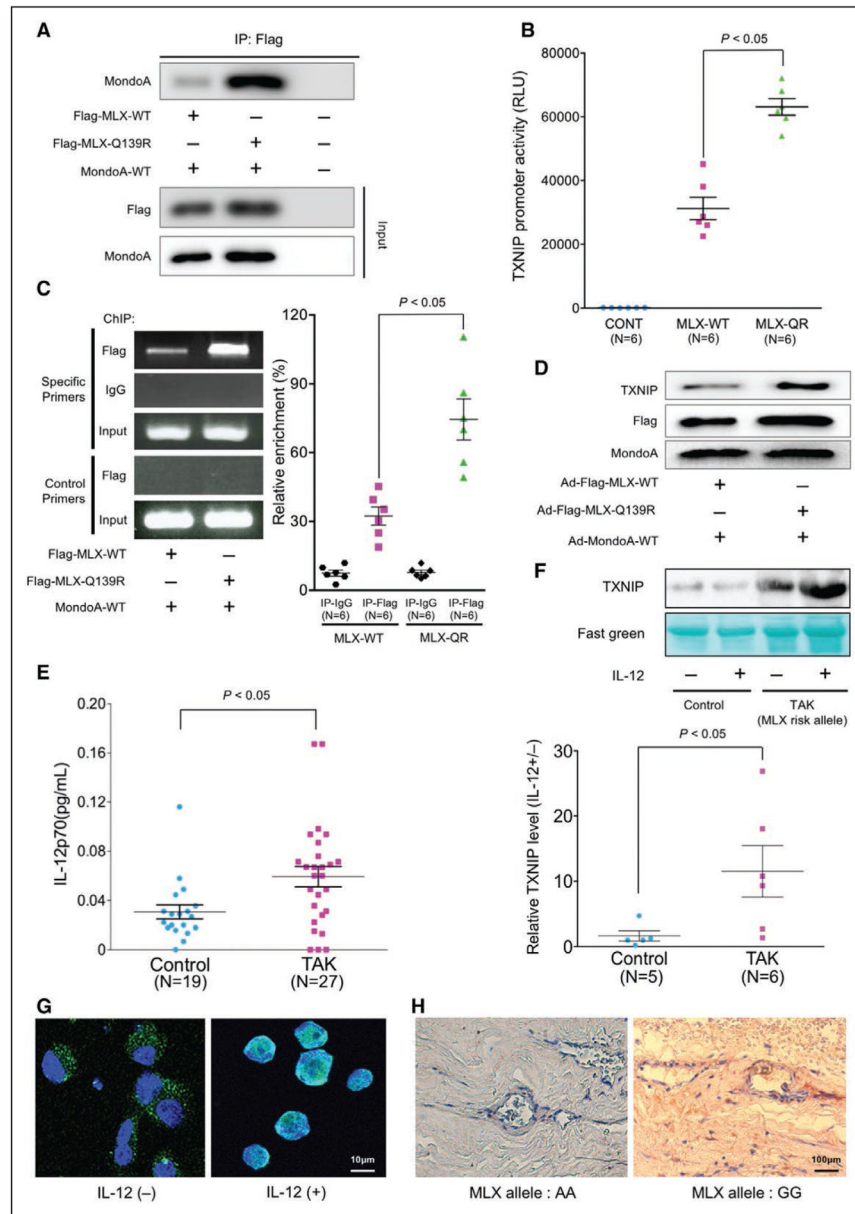
Figure 3.

The MLX (MAX-like protein X) mutant promotes MLX-MondoA heterodimerization and TXNIP (thioredoxin-interacting protein) expression through activation of the TXNIP promoter. **A**, Immunoprecipitation assays using lysates of human aortic smooth muscle cells (hASMCs) coexpressing Flag-MLX-wild-type (WT) and MondoA or Flag-MLX-Q139R and MondoA. (**B**, LUC reporter assays using the *TXNIP* promoter. HASMCs were cotransfected with a reporter gene containing the *TXNIP* promoter together with MondoA and MLX-WT or MLX-Q139R plasmids. **C**, Chromatin immunoprecipitation (ChIP) assay with anti-Flag antibodies; a parallel ChIP assay was performed with rabbit IgG as a control. DNA was amplified and quantified by polymerase chain reaction (PCR) using primers specific for the regions flanking the human *TXNIP* gene promoter, which contained the

MLX-binding motif. Control primers did not contain the MLX-binding motif; PCR using input DNA as a template served as an internal control. **Left**, representative images; **right**, quantitative analysis of the data shown in **left**. The results are expressed as the ratio of the immunoprecipitated to the input DNA. **D**, TXNIP expression in hASMCs cotransduced with MondoA and MLX-WT or MLXQ139R adenoviruses was analyzed by immunoblotting; representative images are shown. **E**, Serum levels of IL-12p70 in patients with Takayasu arteritis (TAK) and healthy controls evaluated by high-sensitivity ELISA. **F**, Peripheral blood mononuclear cells (PBMCs) isolated from TAK patients carrying the risk genotype (*MLX- GG*) and normal subjects were differentiated into T lymphocytes, which were analyzed for TXNIP expression by immunoblotting. **Left**, representative images; **right**, densitometry of TXNIP protein levels. All data are shown as the mean±SEM. **G**, PBMC-derived T lymphocytes were treated with 30 ng/mL IL-12 and stained with anti-MLX antibodies (green); nuclei were stained with 4′,6-diamidino-2-phenylindole″. (blue). **H**, Surgical aortic wall specimens from TAK patients without (AA) and with (GG) MLX risk alleles were stained with the TXNIP antibody (brown).
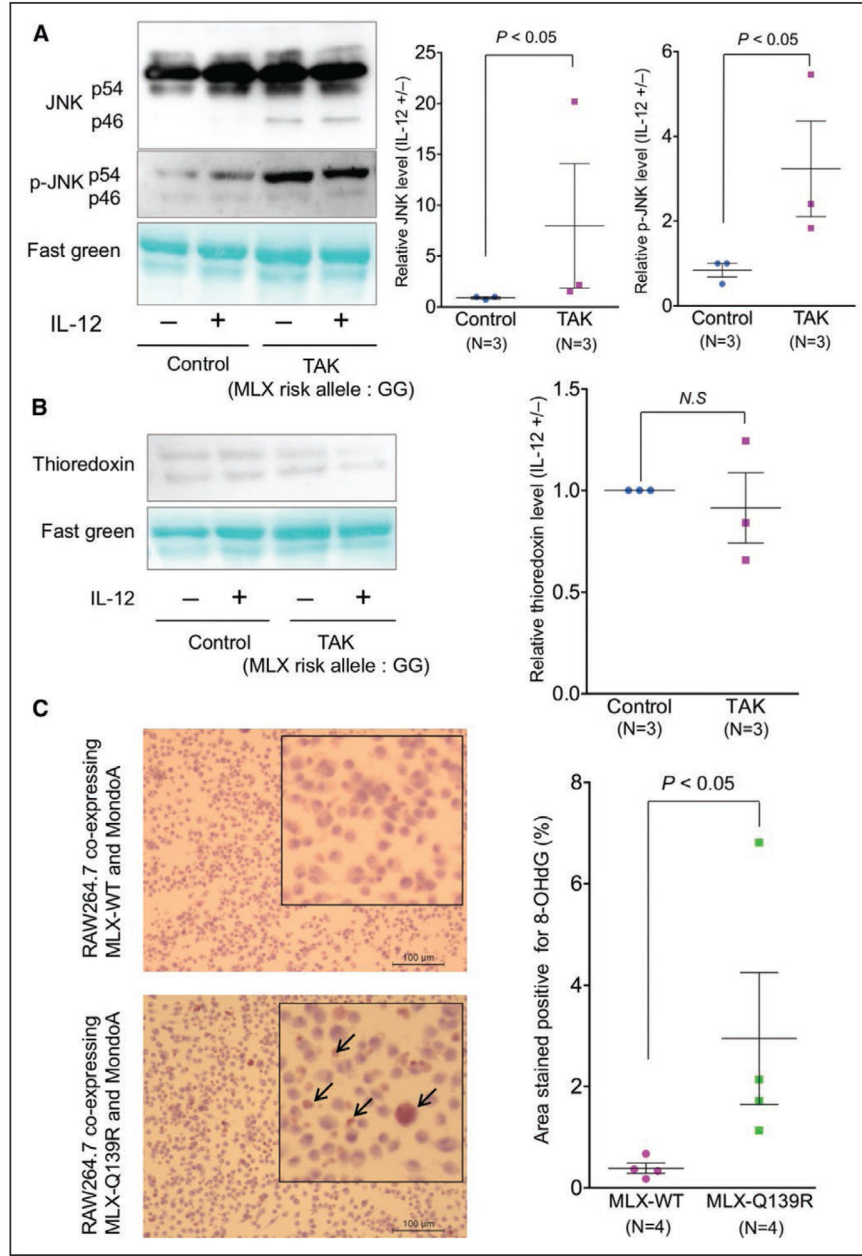
Figure 4.
MLX-Q139R stimulates oxidative stress in immune cells. **A** and **B**, T lymphocytes derived from peripheral blood mononuclear cells of Takayasu arteritis (TAK) patients with the *GG* risk genotype and normal subjects were analyzed for the expression of JNK (c-Jun N-terminal kinase) and p-JNK (**A**) and TRX (thioredoxin; **B**) by immunoblotting. **Left**, representative images; **right**, quantitative analysis by densitometry. **C**, RAW264.7 cells cotransfected with MondoA and MLX-wild type (WT) or MLX-Q139R plasmids were stained with anti-8-OHdG (8-hydroxy-2′-deoxyguanosine) antibodies (dark brown, black arrows). **Left**, representative images; **right**, quantitative analysis of 8-OHdG-positive

RAW264.7 cells. All data are presented as the mean±SEM. IL indicates interleukin; and MLX, Max-like protein X.
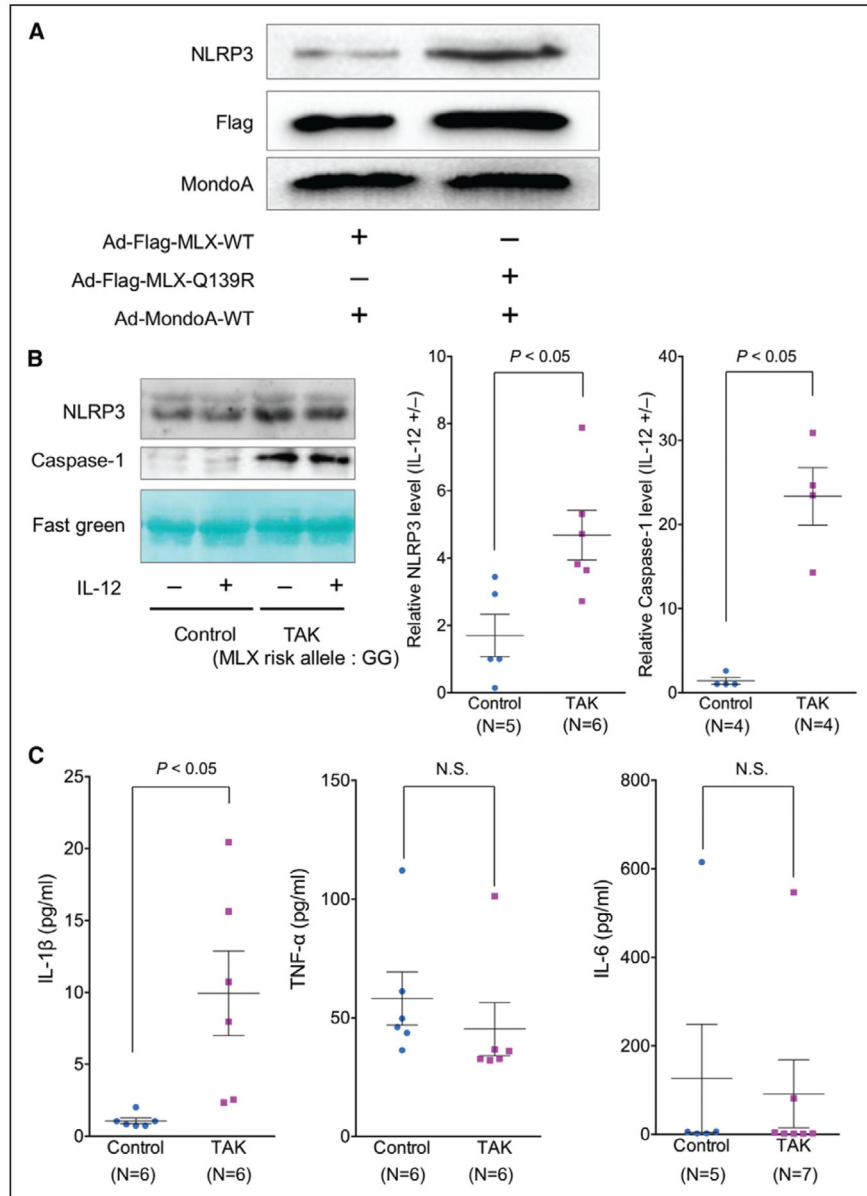
Figure 5.
MLX-Q139R upregulates NLRP3 (NACHT, LRR, and PYD domains-containing protein 3) inflammasomes. **A**, Human aortic smooth muscle cellss cotransduced with MondoA and MLX-wild type (WT) or MLX-Q139R adenoviruses were analyzed for NLRP3 expression by immunoblotting. **B** and **C**, peripheral blood mononuclear cell-derived macrophages of patients with Takayasu arteritis (TAK) with the risk genotype (*MLX-GG*) and normal subjects were analyzed for NLRP3 and caspase-1 expression by immunoblotting (**B**) and for IL (interleukin)-1β, TNF-α (tumor necrosis factor-α), and IL-6 secretion in the supernatants by ELISA (**C**). All data are presented as the mean±SEM. MLX indicates Max-like protein X.
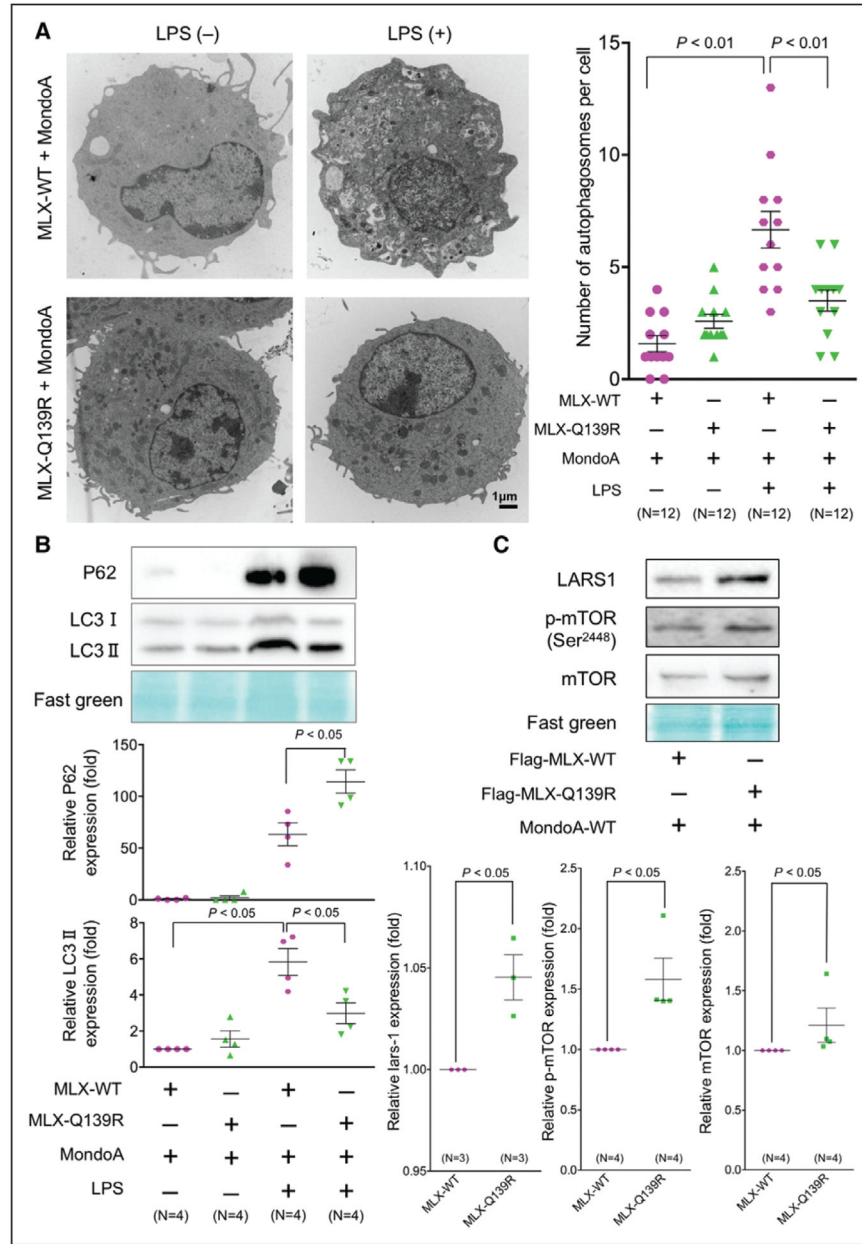
Figure 6.
MLX-Q139R inhibits autophagy and promotes inflammasome formation in macrophages. **A** and **B**, RAW264.7 cells coexpressing MondoA and MLX-WT or MLX-Q139R were stimulated or not with LPS. Cells were analyzed for autophagosome formation by transmission electron microscopy. **Left**, representative images; **right**, quantitative analysis of the number of autophagosomes (**A**). Expression of LC3 and p62 examined by immunoblotting. **Top**, representative images; **bottom**, quantitative analysis by densitometry (**B**). All data are presented as the mean±SEM. **C**, RAW264.7 cells coexpressing MondoA and Flag-MLX-wild type (WT) or Flag-MLX-Q139R were analyzed for LARS1, mTOR (mammalian target of rapamycin), and phospho-mTOR (Ser2448) expression by

immunoblotting. **Top**, representative images; **bottom**, quantitative analysis by densitometry. LARS1 indicates; LPS, lipopolysaccharide; and MLX, Max-like protein X.
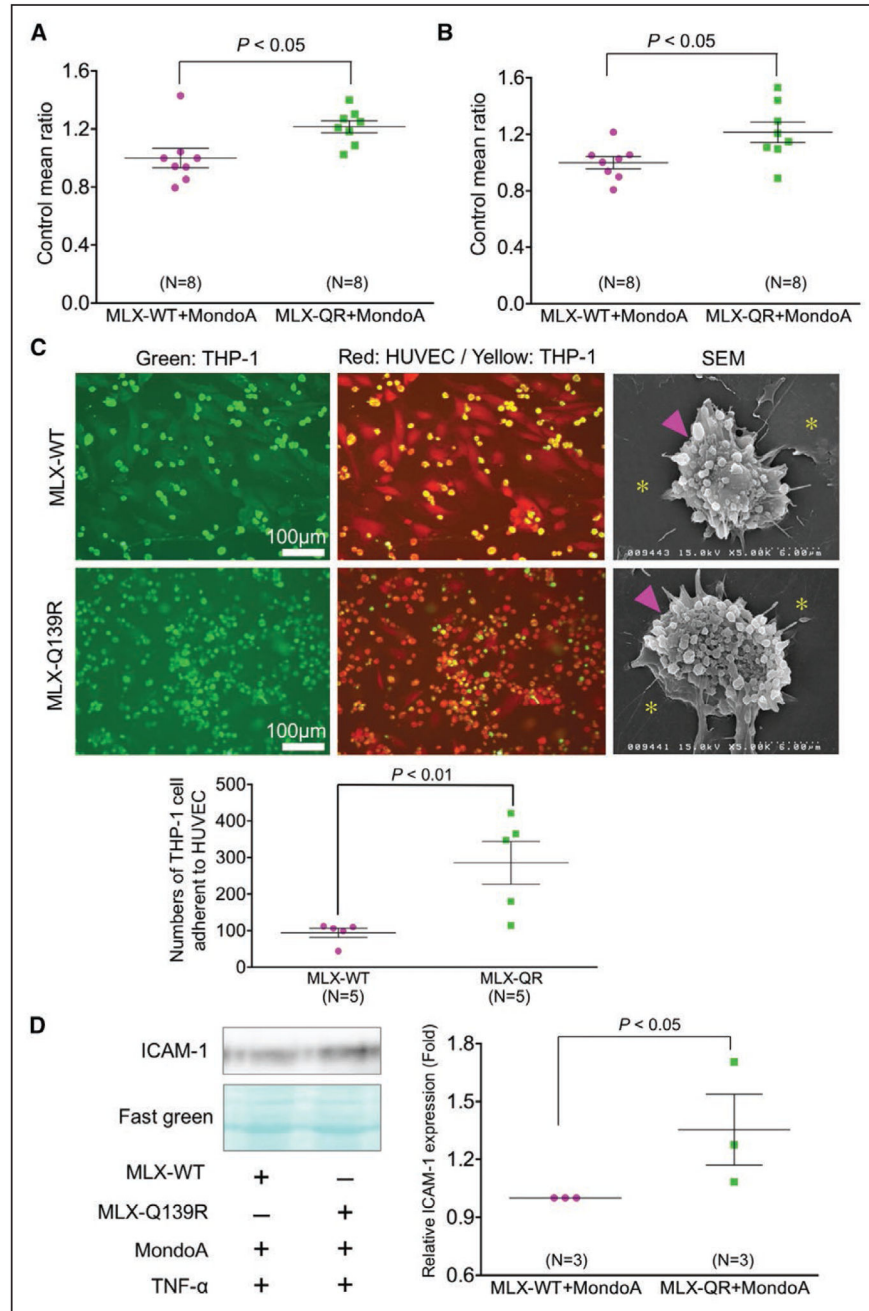
Figure 7.
MLX-Q139R promotes cell proliferation and adhesion to the endothelium. **A** and **B**, Effects of the MLX-Q139R mutation on the proliferation of THP-1 cells (**A**) and human coronary artery endothelial cells (**B**) transfected with MLX-wild type (WT) or MLX-Q139R plasmids were determined by the CCK-8 assay. **C**, Human umbilical vein endothelial cells (HUVECs) transfected with MLX-WT or MLX-Q139R plasmids for 48 h were cocultured with THP-1 cells. **Left**, the 2′−7′-bis(carboxyethyl)-5(6)-carboxyfluorescein″. –labeled adherent monocytes (green). **Middle**, Endothelial cell morphology is indicated by rhodamine-conjugated phalloidin staining (red). **Right**, SEM images show that THP-1 cells cocultured

with HUVECs stretched out pseudopodia (arrowheads) and adhered to HUVECs (asterisks). The graph presents quantitative analysis of THP-1 cells (indicated as yellow dots in the images) adhered to HUVECs. **D**, HUVECs coexpressing MondoA and MLX-WT or MLX-Q139R were analyzed for ICAM-1 (intercellular adhesion molecule-1) expression by immunoblotting. **Left**, representative images; **right**, densitometry analysis. All data are presented as the mean±SEM. CCK-8 indicates Cell Counting Kit-8; and SEM, scanning electron microscopy.
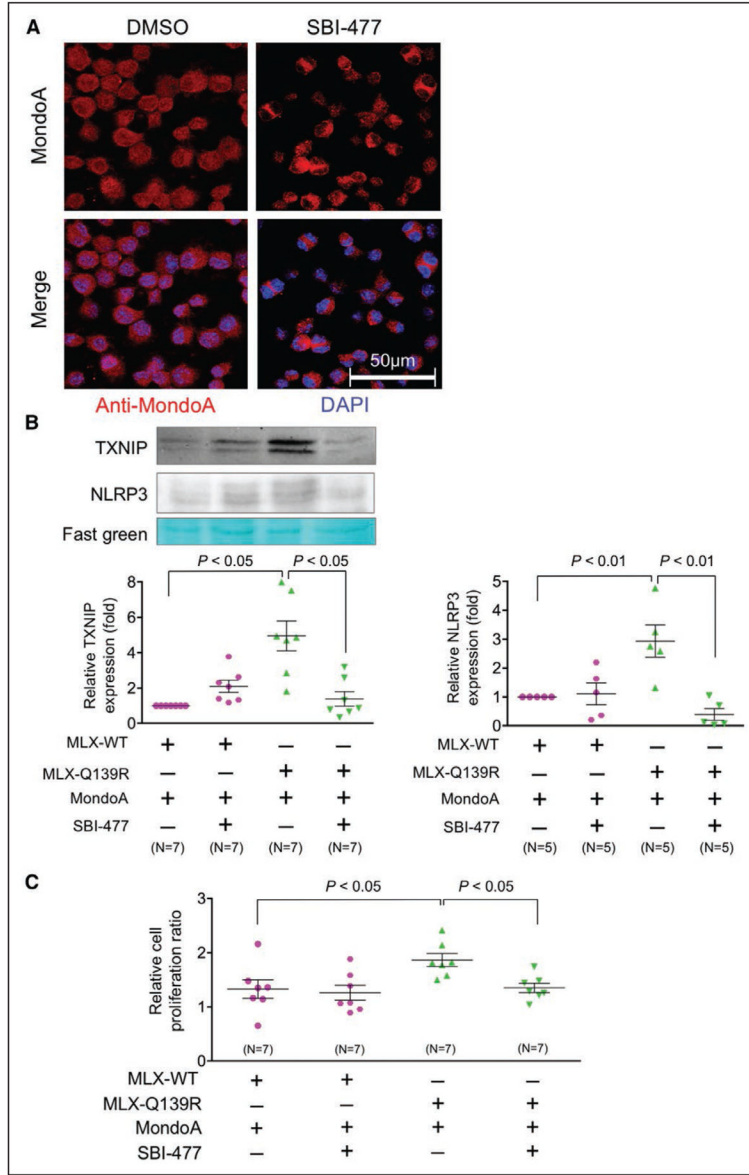
Figure 8.
SBI-477 inhibits MondoA nuclear translocation, MLX-Q139R-inducted TXNIP (thioredoxin-interacting protein) expression, and monocyte proliferation. **A**, RAW264.7 cells were treated with 20 µmol/L SBI-477 or dimethyl sulfoxide (DMSO) for 24 h and analyzed for MondoA expression (red); nuclei were stained with 4′,6-diamidino-2-phenylindole″. (blue). **B**, RAW264.7 cells coexpressing MondoA and MLX-wild type (WT) or MLX-Q139R were treated or not with 20 µmol/L SBI and analyzed for TXNIP and NLRP3 by immunoblotting. **Top**, representative gel images; **bottom**, densitometry analysis of protein expression. **C**, THP-1 cells transfected with MLX-WT or MLX-Q139R plasmids were treated or not with 20 µmol/L SBI-477 and analyzed for proliferation by the CCK-8 assay. All data are presented as the mean±SEM.

Table.

Clinical Characteristics of Patients With TAK

MLX Genotype (SNP ID: rs665268)	AA (%)	AG (%)	GG (%)	Total (%)
No. of patients	20 (21)	43 (45)	33 (34)	96 (100)
HLA-B52 (%)				
+	10 (50)	32 (74)	16 (48)	58 (60)
−	10 (50)	11 (26)	17 (52)	38 (40)
IL12B (%)(SNP ID: rs6871626)				
CC	5 (25)	13 (30)	8 (24)	26 (27)
CA	7 (35)	19 (44)	18 (55)	44 (46)
AA	8 (40)	11 (26)	7 (21)	26 (27)
Numano classification (%)				
I	9 (45)	17 (40)	10 (30)	36 (38)
IIa	3 (15)	6 (14)	4 (12)	13 (14)
IIb	5 (25)	7 (16)	3 (9)	15 (16)
III	0 (0)	0 (0)	0 (0)	0 (0)
IV	0 (0)	1 (2)	0 (0)	1 (1)
V	3 (15)	12 (28)	16 (49)	31 (32)
Surgery	3 (15)	8 (19)	3 (9)	14 (15)
Relapse	9 (43)	15 (35)	11 (33)	35 (36)
IBD complication	3 (15)	4 (9)	1 (3)	8 (8)
Immunosuppressant	8 (40)	14 (33)	9 (27)	31 (32)
Infliximab	3 (15)	3 (7)	4 (12)	10 (10)
Tocilizumab	2 (10)	2 (5)	1 (3)	5 (5)
AR (>moderate)	1 (5)	11 (26)	9 (27)	21 (22)
Mean HbA1c(%)	5.8	5.9	5.6	5.8
Mean onset age, y	24.9	28.1	27.4	26.8
Mean first dose of prednisolone, mg/d	32.2	33.5	29.4	31.7
Mean no. of lesions	2.8	4.1	4.5	3.8

Associations between the risk allele of *MLX* and clinical characteristics of patients with TAK (n=96) are shown. There were significant differences between the MLX risk allele–carrying group and the no-risk group in the morbidity of aortic regurgitation (>moderate) (*AA* vs *AG*: $P<0.05$; *AA* vs *GG*: $P<0.05$) and the number of lesions (*AA* vs *AG*: $P<0.05$; *AA* vs *GG*: $P<0.01$). AR indicates aortic regurgitation; HbA1c, hemoglobin A1c; HLA, human leukocyte antigen; IBD, inflammatory bowel disease; IL, interleukin; MLX, Max-like protein X; SNP, single-nucleotide polymorphism; and TAK, Takayasu arteritis.