Chemical Genetics Define the Roles of p38α and p38β in Acute and Chronic Inflammation*

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The p38 MAP kinase signal transduction pathway is an important regulator of proinflammatory cytokine production and inflammation. Defining the roles of the various p38 family members, specifically p38α and p38β, in these processes has been difficult. Here we use a chemical genetics approach using knock-in mice in which either p38α or p38β kinase has been rendered resistant to the effects of specific inhibitors along with p38β knock-out mice to dissect the biological function of these specific kinase isoforms. Mice harboring a T106M mutation in p38α are resistant to pharmacological inhibition of LPS-induced TNF production and collagen antibody-induced arthritis, indicating that p38β activity is not required for acute or chronic inflammatory responses. LPS-induced TNF production, however, is still completely sensitive to p38β inhibitors in mice with a T106M point mutation in p38β. Similarly, p38β knock-out mice respond normally to inflammatory stimuli. These results demonstrate conclusively that specific inhibition of the p38α isoform is necessary and sufficient for anti-inflammatory efficacy in vivo.

The ubiquitous involvement of protein kinases in cellular signal transduction pathways makes them attractive targets for pharmacologic intervention. Low molecular weight kinase inhibitors enable titratable control of signaling pathways. However, because of the high degree of structural conservation within protein kinases, the generation of truly specific inhibitors is very challenging (1, 2). In addition, tools are not readily available for comprehensive assessment of inhibitor specificity across the large family of >500 kinases in mammalian species (3, 4). Genetic techniques, such as gene knock-outs and RNA interference, offer alternative ways to study the biological function of kinases. Nevertheless, interpretations of data generated using genetic techniques are not always straightforward because of the potential for compensation within highly adaptable signal transduction pathways. The phenotypes associated with genetic deletion of a kinase can be quite distinct from that associated with pharmacologic inhibition (5). One way to overcome these difficulties is through chemical genetics, which combines specific genetic mutations and pharmacological techniques to manipulate the selectivity of low molecular weight inhibitors. Chemical genetic approaches in which kinases are mutated to enable the binding of specific inhibitors have been described (5–7). In this report, we describe an alternative approach in which genetic knock-in mice are used to render p38α or p38β kinase resistant to the effects of specific inhibitors. Comparisons of the effects of in vivo administration of p38 inhibitors to wild-type versus drug-resistant knock-in mice provide powerful tools to dissect the biological function of these specific kinase isoforms.

The p38 subfamily of MAP3 kinases consists of 4 members, p38α, p38β, p38γ, and p38δ, which share high sequence homology and a signature TGY phosphorylation motif in the kinase activation loop (8). p38α MAP kinase was originally identified as an enzyme that was phosphorylated and activated in LPS-stimulated monocytes and was subsequently shown to be an important mediator of TNFα and IL-1 signaling (9, 10). The function of the various p38 family members has been investigated using a variety of techniques, including overexpression of wild-type and mutant kinases (11–13), antisense knockdowns (14, 15), and pharmacological inhibition (16, 17). The majority of p38 inhibitors that have been described are active against both p38α and p38β with minimal activity against p38γ and p38δ (18). These dual p38α/β inhibitors have potent anti-inflammatory activity in preclinical models, and several compounds have advanced into the early stages of clinical development (16). The dual specificity of p38 kinase inhibitors for both p38α and p38β does not allow the use of these compounds as definitive tools to study the specific functions of the individual p38 isoforms.

The deletion of the p38α gene in mice was reported by four separate groups and, in all cases, was associated with embryonic lethality (19–22). These studies revealed that p38α is required for placental development (19–22) and may play a role in the expression of erythropoietin during early development (22). While tetraploid rescue of the placental defect in p38α knockouts enabled survival of the embryos (19), there is little information available on the phenotype of adult p38α knock-out
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mice. The deletion of the p38β gene in mice was not associated with any known phenotype (23). Mouse embryonic fibroblast cells from p38β knock-out mice displayed normal signaling in response to stress stimuli. p38β knock-out mice had normal in vivo responses to LPS and still showed pathological responses to the overexpression of TNFα. However, it is difficult to draw firm conclusions from the results obtained with p38β knock-out mice because of the potential for compensation between different p38 isoforms. This type of compensation was previously described in p38γ knock-out mice in which phosphorylation of SAP97 was insensitive to a dual p38 inhibitor, SB203580, in wild-type cells but became sensitive to this compound in p38γ knock-out cells (24).

To generate better animal models to study the function of these p38 isoforms, we generated knock-in mice with point mutations in Thr106 of p38α or p38β. These mutations do not affect the kinetic properties of the kinases but render them resistant to certain classes of p38 inhibitors. We used these knock-in mice to demonstrate conclusively that specific inhibitory compartment of the p38γ isoform is necessary and sufficient for anti-inflammatory efficacy in vivo.

EXPERIMENTAL PROCEDURES

Antibodies—The murine monoclonal antibody against p38β and the goat anti-mouse HRP-conjugated antibody were from Invitrogen (Zymed Laboratories Inc.). The rabbit polyclonal antibody against p38α was from Cell Signaling Technology. Donkey anti-rabbit HRP-conjugated antibody was from Santa Cruz Biotechnology. The Arthropen monoclonal antibody mixture was from Chemicon/Millipore.

Compounds—The synthesis of MRK 4g and MRK 48 has been described (29, 30). Compounds were dissolved in Me2SO for the in vitro experiments.

Mice—The Merck Institutional Animal Care and Use Committee approved all animal procedures and were performed in accordance with institutional policy and National Institutes of Health guidelines governing the humane treatment of vertebrate animals. 129/SvEv mice were obtained from Taconic Farms, Inc. (Germantown, NY). Mice were housed at up to ten mice per cage in a temperature- and humidity-controlled room (21 °C, 50%) with a 12 h light/dark cycle (lights on at 0700) either in microisolator cages in a specific pathogen-free barrier facility or in a conventional facility. The mice had access to a standard rodent laboratory diet (Harland Teklad Laboratory Rodent Diet 7012) and reverse-osmosis water ad libitum. Sentinel animals were evaluated quarterly as part of a health surveillance program and were determined to be specific pathogen-free by Charles River Laboratory Assessment Plus profile (Wilmington, MA), endo- and ectoparasite examinations, and gross necropsy. In the LPS-induced TNF production experiments, male mice 12–15 weeks of age were used. In the collagen antibody-induced arthritis (CAIA) experiments, female mice 11–15 weeks of age were used. All mice used for the in vivo experiments were on a 129S6 background.

Cloning and Expression of p38 Proteins—Proteins were expressed, purified, and activated as described previously (47).

Kinase Assays and Km Determinations—Kinase assays were performed as previously described using 2 μM GST-ATF2 as the protein substrate (47). The ATP $K_m$ was determined by measuring the rate at various ATP concentrations. The resulting data were fit using Grafit software version 4.3 (Erithacus Software).

Construction Targeting Vectors and Transfection of ES Cells—Portions of a murine p38β cDNA were used as hybridization probes to screen a mouse genomic library, and several phage clones were isolated that contained fragments of the p38β genomic sequence. One phage clone contained the entire p38β genomic sequence on a single 12.2-kb EcoRI fragment. The p38β allele was confirmed by DNA sequencing.

To generate a MAPK11 (p38β)-null allele, a targeting vector consisting of a 5.4-kb 5′ arm of homology preceded by a thymidine kinase cassette, a neomycin resistance cassette and a 3-kb 3′ arm of homology was constructed as follows: The 5′ arm of the targeting vector was created by inserting a 5.4 BamHI fragment of the MAPK11 cosmid clone into the BglIII site of pKO Scrambler NKT-V-1901 to generate pKOp38βRtkp. The 3′ arm was constructed from a KpnI-EcoRI cosmid fragment containing exons 10–12 inserted into the KpnI–EcoRI sites of pKO Scrambler NTK-V-1902. A KpnI fragment of the MAPK11 cosmid containing part of exon 7 and all of exons 8 and 9 was ligated into the KpnI site of pKOp38βRtkp, and clones in the correct orientation were selected. NotI-Xhol fragments were ligated together to generate the completed targeting vector.

To generate a Mapk11 allele containing a T106M mutation in exon 4, a targeting vector consisting of a 4.2 kb 5′ arm of homology preceded by a thymidine kinase cassette, a neomycin resistance cassette and a 5 kb 3′ arm of homology was constructed as follows: A 333-bp Sse83871 fragment of the murine Mapk11 gene containing exon 4 and the T106M mutation (ACG → ATG) was generated by site-directed mutagenesis and confirmed by sequence analysis. The 333-bp Sse83871 fragment containing the T-M mutation replaced the corresponding wild-type sequence in a 9.2-kb p38β genomic EcoRI fragment obtained from a cosmid clone. Xhol linkers were added to the mutated 9.2-kb genomic fragment, and it was subcloned into the Xhol site of pKI-TK3988, a modified pKO Scrambler NTK-V-1901 vector in which the neo cassette and the unique NotI site of the parental vector were removed. To introduce the neo cassette into the p38β genomic segment, a NotI site was introduced into a BsmI-BspEI fragment from the intron between exons 1 and 2 using PCR mutagenesis. Finally, a 1.8-kb neomycin cassette flanked by loxP sites obtained from pBS246neoTK was inserted at the NotI site of the BsmI–BspEI fragment, and the resulting product was used to replace the corresponding BsmI–BspEI fragment in the p38β genomic sequence.

To introduce a T106M mutation into exon 4 of the Mapk14 gene, a targeting vector consisting of a 4.2-kb long arm of homology, a neomycin resistance cassette and a 2.3-kb short arm of homology was constructed as follows: A 6.5-kb HindIII genomic fragment containing exon 4 of the murine Mapk14 gene (21) was isolated and subcloned into pBS-KS. To mutate the fragment, a 1.8-kb KpnI–HindIII genomic fragment was created using PCR to insert the T106M mutation (ACC → ATG) just downstream of the 5′-terminal KpnI site. The corresponding fragment in the original subclone was replaced with the protein substrate (47). The ATP $K_m$ was determined by measuring the rate at various ATP concentrations. The resulting data were fit using Grafit software version 4.3 (Erithacus Software).
the mutated fragment and confirmed by sequence analysis. The resulting mutated 6.5-kb genomic fragment was subcloned into the HindIII site of pBS-KS. Last, a 1.8-kb neomycin cassette flanked by loxP sites obtained from pBS246neoTK was inserted at the PsiI site of Mapk14 located 0.74-kb upstream of the mutation to generate a 4.2-kb 5’ long arm and a 2.3-kb 3’ short arm.

The targeting vectors were linearized and electroporated into the CMTI-1 ES line (obtained from CMTI, Inc.) and seeded on irradiated murine fibroblast feeder cells. The clones were selected in medium containing 300 µg/ml G418 for 3 days following electroporation and 150 µg/ml for routine culture. G418-resistant ES colonies were picked and placed into individual wells of a 96-well tissue culture plate.

Genomic DNA was prepared from ES cells, and the various clones were screened by Southern blot analysis using DNA probes as described in the figure legends. Positive clones were identified and cultured in vitro to obtain adequate cell numbers for blastocyst injections.

Positive ES clones that had the correct karyotype were used for injection into C57BL/6 blastocysts. Coat color chimeras were mated to C57BL/6 females to determine if germline transmission was achieved. PCR was used to genotype mice using DNA isolated from tail snips. For genotyping p38β-null mice, the following set of PCR primers were used: 5’-CCACCACCCGGGTATTGGAAGCTTATAGACATT-3’, 5’-GCCTCCGAGCTCTGAGCCAGCAAA-3’, 5’-GATCCCTCCCCCTGAGGACACCCCCCTTGTAGGCAAAC-3’, 5’-AATCCCCCTCTGTAGGGAGGAG-3’. The PCR reaction produces a 2004-bp product for the wild-type allele and a 1749-bp product for the null allele. A second 2.5-kb product may be generated from the wild-type gene with primers but not under the conditions used.

For genotyping p38β(T106M) knock-in mice, the following set of PCR primers were used: 5’-CCACCACCCGGGTATTGGAAGCTTATAGACATT-3’, 5’-GCCTCCGAGCTCTGAGCCAGCAAA-3’, 5’-GATCCCTCCCCCTGAGGACACCCCCCTTGTAGGCAAAC-3’, 5’-AATCCCCCTCTGTAGGGAGGAG-3’. The PCR reaction produces a 2004-bp product for the wild-type allele and a 1749- and a 1200-bp product for the T106M allele.

For genotyping p38α(T106M) knock-in mice, the following set of PCR primers were used: 5’-CCACCACCCGGGTATTGGAAGCTTATAGACATT-3’, 5’-GCCTCCGAGCTCTGAGCCAGCAAA-3’, 5’-GATCCCTCCCCCTGAGGACACCCCCCTTGTAGGCAAAC-3’, 5’-AATCCCCCTCTGTAGGGAGGAG-3’. The PCR reaction produces a 2004-bp product for the wild-type allele and a 1749- and a 1200-bp product for the T106M allele.

RPA Assay—Tissue samples were homogenized in 1 ml of TRIzol reagent per 50–100 mg of tissue using a Polytron homogenizer, and RNA was isolated following the manufacturer’s instructions. A 307-bp p38β(T106M) probe containing a single mismatch to the p38β(T106M) sequence and two mismatches to the wild-type sequence was generated by PCR and cloned into the KpnI/PstI sites of pGEM3Z. A 250-bp p38α(T106M) probe that was an exact match for the mutant sequence was generated in a similar fashion. Ribonuclease protection assays were carried out using the RPA III™ Ribonuclease Protection Assay kit (Ambion) following the manufacturer’s instructions.

Western Blot Analysis—Soluble protein extracts were run on 4–20% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk overnight. The blocked membranes were incubated with anti-p38α or p38β antibodies (1:1000 dilution) for 1 h at room temperature with gentle agitation followed by three washes (10 min each) with buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, and 0.1% Tween 20 and then incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies for 1 h at room temperature. Blots were developed using Western chemiluminescent reagent (PerkinElmer Life Sciences) and exposed to x-ray film.

Peritoneal Exudate Cell Preparation and LPS-induced TNF Assay—Peritoneal exudate cells (PECs) were harvested from 2 to 3 mice of each genotype matched for age and gender 3 days after intraperitoneal inoculation of 1 ml of 8% Brewer thioglycollate medium (Sigma). Mice were euthanized and the abdomens flushed with 10 ml of phosphate-buffered saline plus heparin. Cells were collected from the peritoneal lavage fluid by centrifugation for 10 min at 1200 rpm, and resuspended at 5.56 × 10⁶/ml in RPMI supplemented with 10% fetal calf serum (Hyclone), 100 units/ml penicillin, 100 units/ml streptomycin, 0.3 mg/ml l-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1 × non-essential amino acids (Invitrogen), and 10 µM 2-mercaptoethanol (BioRad).

The PEC suspension (180 µl) was dispensed into wells of a 96-well tissue culture plate and 2 µl of MRK 4g dissolved in Me₂SO was added to the cell suspension. The cells were stimulated with 20 µl of a 1 µg/ml solution of LPS (Salmonella minnesota Re 595, Sigma) and incubated overnight at 37 °C in a humidified atmosphere of 5% CO₂. The supernatant was harvested, and the TNFα concentration was determined by ELISA.

LPS Challenge—12-week-old mice were dosed with a p38 inhibitor (p.o., 3 mg/kg in 0.5% methylcellulose) or vehicle 2.5 h prior to injection of 10 µg/mouse LPS (Escherichia coli Sero- type 0111:B4, Sigma) and 800 mg/kg p-galactosamine (Sigma) in saline. Animals were euthanized 90 min later, and plasma TNFα was measured by ELISA. Plasma compound levels were measured by HPLC.

Collagen Antibody Arthritis Induction in Mice—Female mice were weighed and placed into vehicle or drug treatment groups on day 0. Arthritis-inducing monoclonal antibody mixture
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**TABLE 1**

Comparison of kinetic parameters for wild-type and mutant enzymes

| Wild-type(α) | αT106M | Wild-type(β) | βT106M |
|--------------|--------|--------------|--------|
| V_{max} (arbitrary units) | 19 ± 0.15 | 8 ± 0.2 | 89 ± 14 | 65 ± 9 |
| ATP $K_m$ (μM) | 128 ± 2.4 | 72 ± 4.8 | 69 ± 25 | 35 ± 13 |
| MRK 48 IC$_{50}$ (nM) | 0.08 ± 0.03 (n = 4) | 28 ± 12 (n = 5) | 0.25 ± 0.1 (n = 5) | 260 ± 30 (n = 5) |
| MRK 4g IC$_{50}$ (nM) | 0.1 (n = 1) | 34 (n = 1) | 0.1 (n = 1) | |

(Chemicon International, ECM 1400) was injected intravenously into the caudal portion of each tail following the manufacturer’s protocol for arthritis induction in C57BL/6 mice. Briefly, 0.8 ml of the monoclonal antibody mixture was injected on day 0. On day 3 postinoculation, mice were each given an intraperitoneal injection with LPS. Vehicle or compound was given once daily by oral gavage beginning 24 h post–LPS injections.

Limbs were scored daily using the following system: 0, normal; 1, one or two swollen digits; 2, swelling of more than two digits as well as swelling of the entire carpus/tarsus; 3, deformity; 4, rigidity and immobility of the joints. The lowest possible score was 0 for an asymptomatic mouse, and the highest possible score was 16, which would indicate all four limbs were rigid.

**RESULTS**

**Mutation of Thr$^{106}$ to Methionine Does Not Affect the ATP $K_m$ but Does Decrease Inhibitor Potency**—A variety of studies have demonstrated the important role of Thr$^{106}$ within the ATP binding site of p38α in determining sensitivity to pyridyl imidazole inhibitors (25–28). We expressed mutant p38α and p38β proteins containing T106M mutations in *E. coli* and determined the effect of the mutation on the ATP $K_m$ and the IC$_{50}$ of two structurally distinct p38α/β inhibitors, MRK 4g and MRK 48 (Fig. 1 and Refs. 29, 30). As shown in Table 1, the mutations, even though within the ATP binding site, had no significant effect on the binding of ATP compared with the wild-type proteins. Nevertheless, the mutation of Thr$^{106}$ to Met dramatically decreased the potency of the two tested inhibitors 200–1000-fold. Both of these compounds are highly selective for p38α and β with at least 500-fold decrease in potency against other kinases that have been tested (29, 30). Expression of such mutant proteins in animals would essentially make these dual inhibitors specific for the remaining wild-type kinase and allow us to probe the role of each kinase in vivo pharmacologically. The use of such a pharmacological approach in the presence of a complete complement of normally active kinases would eliminate any possible developmental or other type of compensation.

**Generation of p38α(T106M), p38β(T106M), and p38β-null Mice**—The p38α and β loci were targeted by homologous recombination in murine ES cells. To generate p38β-null mice, we performed a targeted deletion of exons 1–7 in one of the p38β alleles in ES cells (Fig. 2A). Positive clones (Fig. 2B) were used to generate chimeric mice that successfully transmitted the null allele to their offspring. The genotypes of the mice were determined by PCR (Fig. 2C). The p38β$^{-/-}$ mice were fertile with no apparent phenotype in agreement with the observations of Beardmore et al. (23).

For the generation of mice expressing p38β(T106M), exon 4 was replaced with a sequence containing the point mutation with a neomycin-resistance gene flanked by Cre recombination sites inserted downstream within the adjacent intron (Fig. 3A). Successfully targeted clones were identified by Southern blotting (Fig. 3B). Positive ES clones in which the p38β(T106M)
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allele was successfully targeted and transiently transfected with Cre recombinase, and the neomycin resistance gene was deleted by Cre-mediated recombination. Those clones in which the neomycin resistance gene was deleted were identified by Southern blotting (Fig. 3B) and used for the generation of chimeric founders. The founders successfully transmitted the mutant allele to their offspring. The genotypes of the mice were determined by PCR (Fig. 3C).

For generation of mice expressing p38α(T106M), exon 4 was replaced with a sequence containing the point mutation with a neomycin-resistance gene flanked by Cre recombination sites inserted upstream of the targeted mutation within the intron between exons 3 and 4. Successfully targeted clones were identified by Southern blotting (Fig. 4A). As with the p38β<sup>ki/ki</sup> mice, the neomycin gene was removed subsequently by cre-mediated recombination after transient transfection with Cre recombinase. However, none of those clones in which the neomycin resistance gene was deleted gave rise to founders capable of germ line transmission. The original ES clones with the neomycin resistance gene intact, on the other hand, did give rise to founders that were capable of germ line transmission. The presence of the neomycin resistance gene within the intron did not affect expression of p38α (Fig. 5A). The founders successfully transmitted the mutant allele to their offspring, and the genotypes of the mice were determined by PCR (Fig. 4C).

The p38α(T106M) and p38β(T106M) mRNA and Proteins Are Expressed Normally in Mice—To demonstrate that the resulting mice expressed mRNA containing the mutant coding sequence, we used RNase protection assays to discriminate between wild-type and mutant mRNA. Mutant p38α and p38β cDNAs were used to generate antisense RNA probes. If the probe hybridized to a wild-type sequence, the internal mismatch would be digested, and the protected fragment would be half the size of the fragment protected by the mutant sequence.

As shown in Fig. 3D, RNA isolated from mice heterozygous for p38β protected two differently sized bands corresponding to the wild-type and mutant sequences, while mRNA from mice homozygous for either the wild-type or mutant sequence at p38β locus protected only a single band corresponding to the predicted genotype. No bands corresponding to any p38β protected fragment were detected in RNA isolated from p38β<sup>−/−</sup> mice (Fig. 2D). No differences in p38α mRNA expression were observed in any of the p38β mutant mice (data not shown). As with p38β, RNA isolated from mice heterozygous for p38α protected two differently sized bands corresponding to the wild-type and mutant sequences (Fig. 4D).

To demonstrate that the mutant proteins were expressed normally and that the deletion of the p38β gene eliminated protein expression, we performed Western blots on tissue extracts from wild-type, p38α(T106M), and p38β(T106M) mice. Expression of the p38α(T106M) and p38β(T106M) protein was normal in the thymus and spleen (Fig. 5, A and B). Expression of p38β is highest in the brain, and the introduction of the T106M had no effect on p38β expression in the brain (Fig. 5B). No p38β protein was detected in the brain of the p38β<sup>−/−</sup> mice (Fig. 5B). The expression of p38α in the p38β<sup>−/−</sup> mice was unchanged in these tissues.
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FIGURE 4. Construction of p38α(T106M) knock-in mice. A, schematic of p38α knock-in construct for the p38α allele. Restriction sites are indicated (H, HindIII; V, Vspl). The size of genomic fragments and positions of PCR products and Southern blot probes are shown. B, Southern blot confirmation of targeting in ES cells. ES cell genomic DNA was digested with VspI and hybridized to a 700 bp NdeI-EcoRI 5’ probe. Southern blotting yielded a 9.9-kb fragment for the wild-type allele and a 11.7-kb fragment for the correctly targeted p38α(T106M) allele containing neo. C, PCR genotyping of targeted mice. The PCR reaction produces 1200-bp product for the wild-type allele and a 941-bp product for the T106M allele. D, tissue RNA from mice with indicated p38α genotypes were hybridized with an antisense probe for the p38α(T106M) sequences and treated with a mixture of RNase A and RNase T1. The antisense probe protects a 250-bp fragment of p38α(T106M) mRNA and two 124-bp fragments of the wild-type p38α mRNA.

p38 Inhibitors Block LPS-induced Peritoneal Exudate Cell TNFα Production from p38β(T106M)- and p38β-null Mice but Not from p38α(T106M) Mutant Mice—Thioglycollate injected into the peritoneal cavity induces a sterile cellular infiltrate composed of mainly macrophages. Isolated peritoneal exudate cells produce TNFα in response to LPS stimulation in vitro, and this cytokine production is blocked by dual p38α/β inhibitors. As shown in Table 2, MRK 4g is highly effective at blocking LPS-induced TNFα production from wild-type cells with an IC₅₀ = 3.5 nM. MRK 4g is equally effective at inhibiting TNFα production from peritoneal exudate cells derived from p38β+/− and p38β−/−/− mice with IC₅₀ values of 6.8 and 8.3 nM, respectively. However, MRK 4g was significantly less potent at blocking TNFα production from peritoneal exudate cells derived from p38α+/−/− mice with an IC₅₀ > 250 nM. These results indicate that p38α is required for cellular TNFα production in vitro and that p38β does not play any role in the synthesis of this cytokine.

p38 Inhibitors Do Not Block LPS/ω-Galactosamine-induced TNFα Production in p38α(T106M) Mutant Mice, but Are Efficacious in p38β(T106M)- and p38β-null Mice—LPS when given in vivo will induce a proinflammatory cascade that leads to fever, myalgia, and even death. One of the first cytokines induced by LPS is TNFα with peak expression around 90 min after an intravenous dose of LPS in mice and rats. A role for p38α and/or β in this process has been demonstrated using a variety of structurally diverse p38 inhibitors that inhibit LPS-induced TNF production in mice, rats, and man (30–33). However, unequivocally defining the contribution of each isoform to proinflammatory cytokine production has been impossible due to the problems and caveats associated with interpreting genetic deletions in mice.

Beardmore et al. (23) demonstrated that LPS-induced TNF production is normal in p38β−/− mice, but as they point out, the possibility of compensation cannot be ruled out, preventing a definitive conclusion that p38β plays no role in TNF expression. In agreement with the observations of Beardmore et al., we have shown in an LPS/ω-galactosamine challenge model that TNF production in p38β−/− mice is similar to wild-type mice and that TNF syn-
thesis is sensitive to p38 inhibitors (Fig. 6A). To avoid any possibility of compensation and to answer definitively any question regarding the role of p38β in LPS-induced TNFα production, we tested the efficacy of dual p38 inhibitors in p38α^ki/ki and p38β^ki/ki mice. As shown in Fig. 6B, both strains of mutant mice produce TNFα in response to LPS at levels comparable to wild-type mice. However, the p38 inhibitor MRK 48 has no effect on TNFα production in the p38α^ki/ki mice but inhibits TNFα production in the p38β^ki/ki mice as potently as wild-type mice. The plasma levels of MRK 48 were determined at the end of the experiment, and the average plasma concentration was slightly higher in the p38α^ki/ki than the p38β^ki/ki or wild-type mice (data not shown) indicating that the failure to inhibit TNFα production in the p38α^ki/ki was not because of differences in the pharmacokinetics of MRK 48 in these mice.

p38 Inhibitors Do Not Block Collagen Antibody-induced Arthritis in p38α(T106M) Mutant Mice, but Are Efficacious in p38β-null Mice—Although p38β does not play a role in TNFα production in response to LPS stimulation in vivo, it might still have an important function downstream of the initial proinflammatory cytokine cascade. To determine if p38β has a role in more complex inflammatory responses, CAIA was used as a model. Unlike collagen-induced arthritis, CAIA can be used in a much wider variety of mouse strains. CAIA is induced by injection of a mixture of monoclonal antibodies to type II collagen followed by an injection of LPS 24–72 h later (34, 35). The arthritis usually peaks around day 7 and begins to resolve by day 14. CAIA is dependent upon TNFα, IL-1β, and MIP-1α but not IL-6 and is characterized by a neutrophil infiltration of the joints and fibroblast proliferation around the synovium (36). Neutrophils are essential for the induction and maintenance of arthritis in this model, but T and B cells are dispensable (36, 37). The dosing of the p38 inhibitor, MRK 4g, was initiated 24 h after LPS treatment to avoid any possibility that the efficacy of the p38 inhibitor in this model might be caused by inhibition of the LPS-induced cytokine cascade.

The response of both mutant mouse strains to the monoclonal antibody mixture and LPS stimulation was comparable to wild-type mice (Fig. 7, A and B). The time to disease onset, severity, and time to resolution in the p38β^-/- group was similar to the wild-type group, suggesting that p38β did not play a role in any of these processes. Consistent with this observation, Beardmore et al. (23) observed no differences between p38β^-/- and wild-type mice in the Tnf^AARE model of arthritis.

To address the issue of potential compensation, the p38α^ki/ki mice were treated with the p38 inhibitor, MRK 4g, after the initiation of arthritis to determine if loss of p38β activity pharmacologically would affect induction, maintenance or resolu-
mice, p38β must not play a role in the induction or maintenance of CAIA.

DISCUSSION

We used a chemical genetics approach to dissect the specific roles of p38α versus p38β in mouse models of inflammation. Previous in vitro studies demonstrated that replacement of Thr106 with Met in p38α had no measurable affect on enzymatic activity but decreased the affinity of certain p38 inhibitors by several orders of magnitude (25–28). We generated in vivo models to study the specific roles of these p38 isoforms by creating genetically engineered mice expressing a Thr106 to Met substitution in either p38α or p38β. These knock-in mice were healthy and viable with no readily apparent phenotypic abnormalities. These mice exclusively expressed p38 mRNA containing the mutated alleles and had normal levels of the variant proteins in the brain and spleen.

Plasma levels of TNFα after in vivo challenge with LPS were indistinguishable in either knock-in mouse lines compared with wild-type mice. We found that the dual p38α/β inhibitor, MRK4g, lost the ability to block LPS-induced TNFα release in p38α/knock-in mice but retained this ability in p38β/knock-in mice. These results provide firm evidence that the TNFα blocking activity of dual p38 inhibitors in this in vivo assay are derived exclusively from their ability to inhibit the p38α isoform. Inhibition of p38α is both necessary and sufficient to achieve this effect.

These data are consistent with the ability of p38β knock-out mice to mount normal responses after challenge with LPS (23). Collagen antibody-induced arthritis was used to study the role of p38α and p38β in a pathological autoimmune disease model. Both the p38α/knock-in and p38β-knockout mouse lines remain completely susceptible to arthritis in this model. Similar to the LPS challenge experiments, the dual p38α/β inhibitor, MRK4g, completely lost the ability to protect against disease in p38α/knock-in mice but retained this ability in p38β-knockout mice. Because a dual p38α/β inhibitor is essentially as a highly selective p38α inhibitor in p38β-knockout mice, these data suggest that p38α-selective inhibitors would be fully efficacious at treating arthritis and that additional inhibition of p38 may not provide any additional benefit.

These knock-in mice provide powerful tools to distinguish between on-target and off-target effects of p38 kinase inhibitors. Any toxicology findings that occur in wild-type but not in knock-in mice could be clearly classified as on-target while any findings that occur in both wild-type and knock-in mice must be due to off-target activity. Cross-breeding to obtain p38α/p38β double knock-in mice would provide a very useful tool for toxicology studies of dual p38α/β inhibitors.

p38 kinases have been implicated in many processes in addition to inflammation, such as cell survival and apoptosis (38), myogenesis (39, 40), neuronal excitability (41), and ischemia/reperfusion injury (42, 43). However, much of the information on p38 function comes from the use of the p38 inhibitors of uncertain specificity. For instance, SB203580, originally thought to be a potent and specific p38 inhibitor (44), also has measurable activity against cyclin G-associated kinase (GAK), casein kinase II, JNK, Raf, and RICK (30, 45). In fact, SB203580 is as potent an inhibitor of RICK as it is of p38α (45). Thus results obtained using these inhibitors to elucidate the biological role of p38α or p38β kinase must be interpreted with caution. The transgenic knock-in mice described in this report provide a means to definitely address the function of p38α or p38β in any biological process that can be monitored in mice.

The substitution of p38 residue Thr106 into the corresponding position of other kinases such as Erk2 can render these kinases sensitive to p38 inhibitors (25, 27, 46). Introduction of genes expressing such altered kinases into the double p38α/β knock-in background would enable the use of well-studied p38 inhibitors with excellent pharmacokinetic properties for probing the function of the newly sensitized kinases in the absence of any p38 inhibition. This could provide a model to study the effects of pharmacological inhibition of a given kinase even if suitable specific inhibitors were not available. Such an approach has been very successful in yeast (6) and there is one report of a similar approach taken to study JNK2 function in mice (5).

In summary, we report the generation of novel p38α and p38β knock-in mouse lines that can be used in conjunction with non-selective p38 kinase inhibitors to definitively study the specific biological functions of these individual p38 isoforms. We used these mice to demonstrate that p38α is the relevant therapeutic target that accounts for the anti-inflammatory activity of dual p38α/p38β inhibitors.
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