The Cyclin Dependent Kinase Inhibitor p21 Is Essential for Resolution of Murine Inflammatory Arthritis via its C-Terminal Domain


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Abstract

Objective—The mechanism responsible for persistent inflammation of the synovium that occurs in patients with rheumatoid arthritis (RA) is unknown. Previously, we were the first to demonstrate that expression of the cyclin dependent kinase (CDK) inhibitor p21(WAF1/CIP1) is reduced in synovial tissue from RA patients compared to osteoarthritis patients and that p21 is a novel suppressor of the inflammatory response in macrophages. Here, we sought to determine the role and mechanism of p21-mediated suppression of experimental inflammatory arthritis.

Methods—Experimental arthritis was induced in WT or p21⁻/⁻ (C57BL/6) mice using the K/BxN serum transfer induced model. p21-peptide mimetics were administered to mice as a prophylactic for arthritis development. LPS-induced cytokine and signal transduction pathways were examined in macrophages that were treated with p21-peptide mimetics using Luminex-based assays, flow cytometry, or ELISAs.

Results—p21⁻/⁻ mice exhibit enhanced and sustained development of experimental inflammatory arthritis, which is associated with markedly increased numbers of macrophages and severe articular destruction. Administration of a p21-peptide mimic suppresses activation of macrophages and reduces the severity of experimental arthritis only in p21-intact mice. Mechanistically, treatment with the p21-peptide mimic leads to activation of the serine/threonine kinase Akt and subsequent reduction in the activated isoform of mitogen-activated protein kinase p38 in macrophages.

Conclusion—These data are the first to reveal that p21 plays an important role in limiting the activation response of macrophages in an inflammatory disease such as RA. Thus, targeting p21 in macrophages may be crucial for suppressing the development and persistence of RA.
Macrophages play a central role in the pathogenesis of rheumatoid arthritis (RA). Conventional therapies, including methotrexate and cytokine inhibitors, block pro-inflammatory cytokines primarily produced by macrophages (1). Importantly, synovial macrophage infiltration correlates with subsequent radiographic joint destruction (2). In addition, reduction of RA synovial sublining macrophage number by various therapeutic strategies correlates with clinical improvement, making it a sensitive biomarker for disease activity (3, 4). Intense research is ongoing to elucidate the mechanisms responsible for the increased synovial macrophage number in RA. Thus far, increased chemotaxis (5), reduced emigration (6), and decreased apoptosis of macrophages (7) have all been implicated in RA pathogenesis. In addition to increased numbers, the response of RA synovial macrophages to TLR stimulation is amplified compared to macrophages from other inflammatory joint diseases or normal circulating monocytes differentiated in vitro (8). As is the case for patients, various experimental models of RA, such as collagen-induced arthritis and K/BxN serum transfer-induced arthritis, require monocytes and macrophages for pathology to occur (9–11). Despite the abundant data supporting the crucial role for macrophages in RA, little is known about the factors that control their state of activation.

Cyclin dependent kinase inhibitors (CDKi's) are of central importance in suppressing cell cycle activity. To this end, the vast majority of studies on cell cycle machinery and RA have focused on the suppression of synovial fibroblast proliferation or production of inflammatory cytokines by cell cycle inhibitors such as retinoblastoma (Rb) protein, or cyclin dependent kinase inhibitors (CDKi's) p16, p18, and p21 (12–17). Additionally, multiple intra-articular injections of a replication defective adenovirus expressing p16 or p21 lead to suppression of inflammatory arthritis in rodents (12–14, 16). While these studies focused on the role that p16 or p21 plays in synovial fibroblasts, recently, two studies have shown that the presence of p21 reduces serum cytokine levels and confers a protective effect on survival during lipopolysaccharide (LPS)-mediated endotoxic shock in mice (18, 19), a model dependent on macrophages. Expression of the activation markers CD40 and MHC Class II and secretion of the proinflammatory cytokines IL-6, TNFα, and IL-1β is enhanced in p21-deficient macrophages following toll-like receptor (TLR) ligation, even though these cells have terminally withdrawn from the cell cycle (18, 19). These data suggest that p21 may function as a novel suppressor of inflammation in macrophages.

In this study we demonstrate that p21 is important not only for limiting the development of inflammatory arthritis, but also for induction of the resolution or wound healing phase that occurs after the arthritic stimulus is withdrawn. Similar to RA patients, the severity of inflammation and destruction of bone correlates with the number of macrophages in the pannus. Further, a p21-peptide mimetic corresponding to the C-terminal domain of p21 is sufficient to reduce the severity of inflammatory arthritis and lower the number of macrophages in the pannus. Isolated macrophages treated with the p21-peptide mimetic displayed enhanced expression of phosphorylated Akt that is associated with reduced p38 activation. Taken together, these data are among the first to identify novel and essential roles for p21 in suppression of inflammation and translate them into clinically significant information that may shed light on the pathogenesis of RA.

MATERIALS AND METHODS

Mice

Male KRN mice were kindly provided by Dr. Diane Mathis and were crossed with female NOD mice purchased from Taconic (Hudson, NY). p21−/− mice were backcrossed onto the C57Bl/6 background for at least 12 generations and tested for microsatellite markers of background contribution (20). All experiments on mice were approved by the Animal Care and Use Committee at Saint Louis University and/or Northwestern University.
**K/BxN serum transfer arthritis**

Serum was harvested via cardiac puncture from male and female 8 week old progeny of KRN and NOD mice (K/BxN) and intraperitoneally (IP) injected into mice (6–8 weeks old). Ankle circumference was calculated from measurements obtained with a caliper. Clinical scores were measured as follows: 0=no swelling, 1=mild in <3 limbs, 2=mild in >2, 3=moderate in >2, 4=severe in >2, 5=compromised mobility. At 7, 14, or 25 days post-injection, mice were euthanized, serum was collected via cardiac puncture, and ankles were harvested and fixed in 10% formalin. Ankles were then subjected to microCT analysis performed by the core facility at Washington University School of Medicine (St. Louis, MO) and/or prepared for immunohistochemistry. For peptide studies, Wt mice (Jackson Laboratory, Bar Harbor, ME) were injected IP with peptide (10 mg/kg) 30 minutes prior to K/BxN serum and daily throughout the experiment.

**Immunohistochemistry**

Fixed ankles were decalcified in ethylenediaminetetraacetic acid (Sigma-Aldrich, St. Louis, MO) in 10% formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin (H&E), with safranin O methyl green, for tartrate resistant acid phosphatase (TRAP), or for CD45, PCNA, F4/80 antigens. All staining procedures were performed by the Saint Louis University core pathology facility except for TRAP staining, which was performed at Washington University School of Medicine Center for Musculoskeletal Biology and Medicine. Histopathological scoring was performed as described (21) by a pathologist blinded to the study using an Olympus BX40CY microscope (Olympus, Tokyo, Japan). Photographs were taken on an Olympus BX41 microscope equipped with a DP20 Digital Camera (Olympus) at 40× magnification.

**Cell culture**

Peritoneal cells were harvested via lavage from male and/or female 6–8 week old mice 3 days following intraperitoneal injection of 4% aged thioglycollate, adhered for 1 hour in serum-free media, then maintained in complete DMEM and used within two days. For cytokine assays, peritoneal macrophages were incubated with peptide (50μM) for 2 hours followed by stimulation with LPS (10 ng/mL; Sigma-Aldrich) in the presence of peptide. For IL-1β secretion assays, macrophages were further treated with adenosine triphosphate (ATP) (5 mM; Sigma-Aldrich) for up to 30 minutes to induce IL-1β release.

**Luminex-based assays**

Cytokine levels in serum or cell supernatants were determined using Luminex-based assays according to the manufacturer's specifications (Invitrogen). Data were collecting on a Luminex 200 (Luminex, Austin TX) using xPONENT software version 3.0 (Luminex) and fitted to a weighted 5-point parameter log standard curve. For the peptide study (50μM), only the Tat-Ctrl peptide was used as a control since Tat and Tat-Ctrl showed no difference in arthritis development. For phosphorylation assays, phosphorylated Akt (BD, Biosciences), phosphorylated p38 (Thr180/Tyr182), and total IκBα protein levels in whole cell lysates (prepared with Cell Lysis Kit; Bio-Rad, Hercules, CA) were measured using a Luminex-based assay according to the manufacturer's specifications (Bio-Rad). Data were collected on a Luminex 200 (Luminex) with IS 2.3 software (Luminex) and analysis was performed.

**ELISA**

For detection of IL-1β from cell supernatants, sandwich ELISAs were performed according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). ELISAs were
quantitated by absorbance at 450 nm on a microplate reader (BioRad) and normalized by number of cells per well.

**Peptides**

A polycationic peptide derived from HIV-1 TAT (22) was fused to the p21-mimetic peptides, which were synthesized by and purchased from the Peptide Synthesis group at Tufts University (Boston, MA). The peptides were as follows: aa 15–40 (Ac-rkkrr-orn-rrr-SKACRRLLFGPVDSQELSRCDCALMAG), aa 46–65 (Ac-rkkrr-orn-rrr-RERWNDFVTETPLEDFW-OH), aa 63–77 (Ac-rkkrr-orn-rrr-AWERTVGLGLPY), and aa 141–160 (Ac-rkkrr-orn-rrr-KRQTSMTDFYHSKRRLLFS). A negative control peptide was designed by incorporating F22K and G23K substitutions: Tat-Ctrl (Ac-rkkrr-orn-rrr-SKACRRLLKKPVDSQELSRCDCALMAG) (23). A Tat (Ac-rkkrr-orn-rrr) peptide was also used as a control. Fluorescein isothiocyanate (FITC)-conjugated peptides were synthesized as above, with FITC replacing the acetyl group on the amino terminus.

**Flow cytometry**

For evaluation of peptide entry, cells were incubated with FITC-conjugated peptides for 2 hours at 4°C or 37°C. Cells were trypsinized to remove surface-bound protein, data were collected on a BD LSRII (BD Biosciences) using FACSDiva V6.1.2 and analyzed using FlowJo V5.5.5 (Tree Star, Inc., Ashland, OR).

**Cell imaging**

Live cell imaging was performed at the Northwestern University Feinberg School of Medicine Cell Imaging Core Facility on a Nikon C1Si laser scanning confocal fitted on a PerfectFocus stand to actively maintain focal plane control. Cells were maintained at 37°C during imaging using a Tokai HIT stage top incubator. Confocal imaging was performed using scan averaging of 12, and laser dwell time of 1.92 μseconds/pixel.

**RESULTS**

**p21 Genetic Ablation Exacerbates the Severity of K/BxN Serum-Transfer Induced Arthritis in Mice**

p21−/− mice backcrossed onto the C57Bl/6 background and verified for over 99% C57Bl/6 (20) have no obvious differences in total leukocyte numbers or in various populations of leukocytes (Supplemental Table 1 and Supplemental Figure 1). Since p21 has been associated with suppression of inflammatory disease, the role that p21 plays in the development of inflammatory arthritis was evaluated using the K/BxN serum-transfer model in Wt and p21−/− mice. p21−/− mice developed significantly worse ankle swelling compared to Wt mice, as measured by the change in ankle circumference following intraperitoneal (IP) injection of K/BxN serum (Figure 1A). p21−/− mice also displayed evidence of more severe disease as assessed by a significant elevation in clinical score, most pronounced at day 25 (3.0-fold increase), as the disease resolved in Wt mice but failed to fully resolve in p21−/− mice (Figure 1A).

**Ankle Joints of p21−/− Mice Display an Increase in Inflammatory Cells and Articular Destruction**

To determine the extent of joint damage in Wt and p21−/− mice, ankles were harvested 7, 14, and 25 days following injection of K/BxN serum and histologically examined. Inflammation and the development of pannus were observed in Wt and p21−/− joints, particularly at days 7 and 14 (Figure 1B and C). There was a detectable pannus at all time...
points (2.9-, 1.5-, and 1.8-fold) in p21−/− as compared to the control mice (Figure 1B and C). The pannus formation was associated with enhanced destruction of cartilage, particularly at day 14 (3-fold), and of bone at all time points (3.7-, 1.5-, and 2.3-fold) in p21−/− mice as compared to Wt mice (Figure 1C). There were increased numbers of TRAP-positive cells, which were localized in pannus, at all examined time points in p21−/− mice as compared to the control mice (Figure 1D, E). Long-term bone destruction was further verified by microCT analysis (Figure 1F), which showed increased bone damage in p21−/− mice.

Because elevated cytokine production may contribute to the increased inflammation and destruction observed in p21−/− ankles, circulating cytokine levels were assessed in Wt and p21−/− mice. Seven days following initiation of arthritis, the serum level of the inflammatory cytokine IL-6 was increased 3.0-fold in p21−/− mice as compared to Wt (Figure 1G). The level of IL-1α was significantly elevated in p21−/− mice as well (Figure 1H).

To further investigate the increased inflammation and destruction observed on hematoxylin and eosin stained sections, infiltration of immune cells into arthritic joints was analyzed. Ankle sections were stained for CD45 (hematopoietic cells), F4/80 (macrophages) and proliferating cell nuclear antigen (PCNA, proliferation). At day 7 and 14, the number of hematopoietic cells (CD45+) was increased 2.8- and 1.4-fold in the pannus and synovium of p21−/− ankles, respectively, as compared to control ankles (Figure 2A, D). There were no differences in the numbers of CD45+ cells at day 25 in Wt and p21−/− mice. In addition, peak PCNA staining occurred at day 7 in the ankles of p21−/− mice and at day 14 in Wt mice (Figure 2B, D), likely representing increased proliferation of synovial fibroblasts as macrophages are terminally differentiated. There were no differences in the number of PCNA-positive cells in the Wt or p21−/− mice at day 25 (Figure 2D). This suggests that synoviocytes may be important at early stages of disease development and infiltration of inflammatory cells at later time points is central for disease progression. As such, macrophages in the pannus were significantly increased in p21−/− mice as compared to Wt mice at days 7 (3.7 fold), 14 (1.6-fold), and 25 (1.8 fold) (Figure 2C, D). Since there was no difference in total CD45-positive cells at day 25 yet there were more macrophages, these data suggest that inability of p21−/− mice to resolve arthritis may be due to the persistence of macrophages in the synovium. Thus, similar to RA patients (4, 24), increased numbers of macrophages in the arthritic p21-deficient mice are associated with more severe articular destruction.

**A Peptidomimetic Corresponding to aa 141–160 of p21 Protects Against K/BxN Serum-Transfer Arthritis In Vivo**

To decipher the critical domain of p21 that plays a vital role in suppression of inflammation, we examined the impact of delivering established domains of p21 to arthritic mice. Previous studies have identified domains on p21 that are critical for interaction with cyclins, CDKs, and PCNA as well as modification of the activities of these peptides leading to alterations in cell cycle progression (21, 25). We have taken advantage of these studies and designed 5 sets of p21-peptide mimetics that were conjugated to a polycationic peptide derived from HIV-1 transactivator of transcription to allow cell entry (Tat, Tat-Ctrl, aa 15–40, aa 46–65, aa 63–77, and aa 141–160; numbers indicate amino acids). We have previously shown that this approach is viable in this model of inflammatory arthritis using BH3 peptide mimetics (21).
The functionality of p21-peptidomimetics in suppression of K/BxN serum-transfer arthritis was evaluated. Mice treated with aa 141–160 had a 36-fold reduction in ankle swelling at day 2, a 6-fold reduction at day 4, and a 4-fold reduction at day 7 as compared to mice treated with control peptide (Figure 3A). Additionally, there was a marked improvement in clinical score in mice treated with aa 141–160 as compared to Tat-control treated mice (Figure 3B). While a mild clinical improvement in ankle swelling or clinical score was observed with aa 15–40 or aa 63–77, these were not significant. Since aa 141–160 treatment led to reduced arthritis in p21-intact mice, we also examined its effect in mice lacking p21. There was no difference in ankle swelling or clinical score in p21−/− mice treated with Tat only or with aa 141–160 (Figure 3C, D).

The p21-Peptidomimetic aa 141–160 Suppresses Articular Destruction and Inflammatory Cell Infiltration

Ankles were harvested and histologically examined at seven days following arthritis induction and treatment with peptide. Only treatment with the peptide corresponding to aa 141–160 consistently caused a significant decrease in pannus development, inflammation, synovial lining thickness, bone erosion, extra-articular inflammation, and infiltration of lymphocytes and polymorphonuclear cells as compared to treatment with a control peptide (Figure 4A and B). No difference was detected in cartilage destruction for any of the groups of mice. Furthermore, decreased infiltration of all hematopoietic cells (CD45) and, in particular, macrophages (F4/80) were observed within the pannus of aa 141–160 treated mice (Figure 5A and B). No differences were noted in areas of normal synovium. PCNA staining also revealed reduced proliferation in the pannus, though this effect was elicited by several of the peptides (Figure 5A and B).

aa 141–160 Suppresses Peritoneal Macrophage Production of Cytokines Following Stimulation with TLR-agonists

We have shown that while TAT-conjugated peptides enter all cells, macrophages appear to preferentially uptake the peptides (21). To explore the mechanism by which aa 141–160 reduces the severity of arthritis, we focused on the effect of this peptide on innate immune responses induced by TLR-agonists. Entry and cytoplasmic localization of Tat-conjugated, FITC-labeled peptide into peritoneal macrophages was confirmed using confocal microscopy (Figure 6A). The percent and amount incorporation was determined using flow cytometry. One hundred percent of the cells equally incorporated the TAT-control and TAT-p21-peptide mimetics (Figure 6B, C) and only a minor reduction in cell survival was observed even at 24 and 48 hours post-administration (unpublished data). The capacity of aa 141–160 to inhibit cytokine production by peritoneal macrophages activated with TLR ligation (LPS) was also assessed. The aa 141–160 peptide, which protected against K/BxN serum-transfer induced arthritis in vivo (Figures 3–5), suppressed production of inflammatory cytokines IL-1β, IL-6, and TNFα in vitro (Figure 6D–F). There was no inhibitory effect by the p21-peptide mimetic on MIP1α and β or RANTES production (unpublished data).

aa 141–160 Increases Active Akt but Reduces p38 Activity in TLR-Stimulated Macrophages

Because the p21-peptide mimetic to aa 141–160 induced a dramatic reduction in production of pro-inflammatory cytokines and development of arthritis, we examined its effect on the upstream signaling events using a Luminex-based assay. Treatment with aa 141–160 led to a marked increase in activation of the serine-threonine protein kinase Akt as compared to Tat-control-treated macrophages beginning at 15 minutes following stimulation with LPS, and Akt levels remained higher throughout two hours of stimulation (Figure 6G). In contrast, a reduction in the phosphorylation of p38 was observed at 30 and 60 minutes following TLR
ligation (Figure 6H). The peptide had little effect on the degradation of IκB as compared to control peptide-treated cells (Figure 6I).

The effect of full-length p21 on intracellular signaling pathways was also explored in Wt and p21−/− peritoneal macrophages activated with LPS using flow cytometry. p21−/− cells displayed decreased phosphorylation and activation of Akt 15 minutes following LPS stimulation as compared to control cells (Supplemental Figure 2A). Subsequently, at 30 and 60 minutes following TLR ligation, an increase in phosphorylated p38 in p21−/− cells as compared to Wt was observed (Supplemental Figure 2B, C). Taken together, these data suggest that p21, via its C-terminal domain, suppresses macrophage function by enhancing the phosphorylation of Akt, thereby reducing p38 activation and subsequently limiting inflammatory cytokine production in TLR-stimulated macrophages.

**DISCUSSION**

Over the past several years, p21 has gained attention in the fields of inflammation and autoimmunity. Numerous studies have been performed using p21−/− mice to examine the role that p21 plays in murine models of sepsis, lupus, and rheumatoid arthritis. Recently, we and others have shown that p21−/− macrophages, regardless of background, display enhanced activation in response to TLR-agonists as compared to control mice (18, 19). Further, p21−/− mice on either a mixed or inbred background are more susceptible to LPS-induced endotoxic shock (18, 19). These data suggest that in sepsis, the background of the mice is not a contributing factor. However, in spontaneous development of lupus, the background of the mice may be more crucial (26, 27). The potentially conflicting results in the lupus studies may be attributed to the notion that hybrid (C57Bl/6:129) mice are more susceptible to spontaneous autoimmunity due to epistatic interactions between the two genomes (28). To support the argument that p21 may be considered a general autoimmune disease inhibitor, studies by Fornace and colleagues showed that loss of p21 leads to early lethality due to lupus-like disease which is enhanced by the concomitant loss of GADD45a (29). Further, genome-wide scanning studies have now shown that p21 is a susceptibly locus for SLE (30, 31). Taken together, these studies suggest that p21 may be considered a negative regulator of spontaneous autoimmunity. However, the direct role for p21 in inhibiting macrophage function was not examined.

In this study we now show that p21−/− mice backcrossed onto C57BL/6 background for over 12 generations and screened for over 150 loci (20), develop a markedly more severe experimental RA-like disease (Figure 1). The arthritis in p21−/− mice fails to resolve as compared to Wt mice, with continuous articular destruction and a corresponding increase in macrophage number (Figures 1–2). While, we previously obtained opposing data with regard to the role of p21 in the development of K/BxN serum transfer-induced arthritis, these differences are attributable to the mouse background. After extensive phenotyping of the mice, we uncovered that p21−/− mice on a mixed background, but not on an inbred background, develop significantly less inflammatory monocytes when compared to controls or even mice (op/op) lacking macrophage colony stimulating factor (M-CSF) (11, 32). Further, injection of Wt macrophages into p21−/− mice restores their susceptibility to inflammatory arthritis (11). Thus, we were the first to show that p21 cooperates with 129 loci to produce inflammatory monocytes and that inflammatory monocytes are crucial for K/BxN serum transfer-induced arthritis (11). However, p21 is not required for the differentiation of mouse bone marrow-derived macrophages, splenic-derived macrophages or thioglycollate-elicited peritoneal macrophages (17, 33, 34) regardless of the background. Further, p21−/− mice display similar numbers of tissue macrophages as compared to p21-intact mice regardless of the background (C57Bl/6 vs. C57BL/6:129). Collectively, these data are consistent with immunohistochemical studies examining p21 expression in the RA
synovium (17) and therapeutic studies using adenoviral vectors expressing p21 (12–14, 16) which demonstrate that p21 may be an important inhibitor of inflammatory arthritis.

Despite the general success of biologic therapy, many patients continue to suffer from the severely debilitating effects of RA. In addition, the mechanism behind the persistent production of proinflammatory cytokines targeted by these therapies remains to be fully elucidated. We have now demonstrated that a Tat-conjugated peptidomimetic corresponding to the C-terminus of p21 significantly reduces the severity of arthritis development in the K/BxN serum-transfer murine model (Figures 3–5). Further, the aa 141–160 peptide requires the presence of p21 to reduce arthritis development. Previous studies have demonstrated the ability of Tat to carry peptides and other cargo into cells in vitro and in vivo (22, 35), and, in the current study, entry into cells was confirmed by cell imaging, flow cytometry, and functional assays (Figure 6). Wt mice were used for these peptide studies to more closely replicate the condition encountered in humans. Goulvestre et. al. have shown that a peptidomimetic corresponding to aa 141–160 was able to reduce the severity of lupus-like disease in mice (36). This effect was attributed to suppression of lymphocyte proliferation via inhibition of PCNA by the peptide, as this region at the C-terminus of p21 encompasses the PCNA binding domain (25). While we found that the aa 141–160 peptidomimetic reduces proliferation in ankles from Wt mice 7 days following injection with K/BxN serum (Figure 5), the other p21-peptide mimetics also reduced proliferation of synovial fibroblasts in vivo but had no effect on clinical outcome. In vivo, macrophages are terminally differentiated and also appear to preferentially take up Tat-conjugated peptides over other immune cells (21). Therefore, the cells most likely affected by aa 141–160-mediated suppression of proliferation are synovial fibroblasts. Our study also shows a significant decrease in hematopoietic cell infiltration, particularly macrophages, in the ankles of aa 141–160-treated mice (Figure 5). Furthermore, we demonstrate in macrophages that aa 141–160 suppresses inflammatory cytokine production (Figure 6). These data are consistent with previous studies showing p21-mediated reduction in macrophage activation following TLR ligation (18, 19). Goulvestre et. al. further attributed the decreased lupus development in aa 141–160-treated mice to significant proapoptotic effects on lymphocytes (36). However, we observed no difference in apoptosis between Wt and p21−/− macrophages, or following treatment with any of the peptides (unpublished data).

In pursuing the mechanism by which the aa 141–160 peptidomimetic mediates a reduction in arthritis severity and suppression of cytokine production, we found that the peptide enhances Akt phosphorylation and inhibits p38 activation (Figure 6). Similarly, p21−/− cells display reduced Akt phosphorylation and increased p38 activation as compared to Wt (Supplemental Figure 2). These data are consistent with our previous work demonstrating increased levels of IL-6 and IL-1β mRNA in p21−/− macrophages, which suggest that the mechanism by which p21 inhibits inflammatory cytokine production is likely to be suppression of intracellular signaling pathways, leading to a reduction in transcription of these cytokines (18). This effect of p21 on activation of Akt and suppression of p38, and subsequent reduction in secretion of proinflammatory cytokines, is not surprising given previous studies showing that sustained Akt activation leads to decreased production of these cytokines (37, 38). Furthermore, enhanced signaling through the MAPK pathway leads to increased production of inflammatory cytokines and has been shown to play a role in RA pathogenesis (39). In fact, the targeting of intracellular signaling pathways is a prominent focus of studies exploring novel therapeutic mechanisms in the treatment of arthritis (40). Our data which suggest that the peptidomimetic corresponding to the C-terminus of p21 is sufficient to enhance Akt activation and suppress p38 activation (Figure 6) is supported by previous studies demonstrating that p21 interacts with Akt at the C-terminus (41). Interestingly, Akt has been shown to induce retention of p21 in the cytoplasm and enhance its stability by phosphorylating p21 on its C-terminus (42). Thus, one could envision a

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feedback loop in which p21-mediated Akt activation further activates p21 cytoplasmic functions, thus perpetuating p38 suppression in order to turn off inflammatory reactions. Additional work is required to determine whether p21 also regulates other intracellular signaling pathways. Previous studies have shown that NF-κB DNA binding is increased in p21 deficient macrophages, along with increased IkB degradation and IkB kinase (IKK) complex activity (19). Other studies have shown that p21 may in fact promote transcriptional activation by NF-κB via activation of p300 through derepression of a repression motif in a promoter-dependent manner; however, these studies were not conducted in macrophages (43). We found that the aa 141–160 peptidomimetic has little to no effect on IkBα degradation (Figure 6I). In addition, direct inhibition of JNK by p21 has been previously demonstrated in cell-free systems, 293 cells, and synovial fibroblasts (13, 17, 44). Thus, further research is necessary to determine the exact role of p21 in regulating intracellular signaling, particularly in the context of macrophage activation. Elucidating the mechanisms by which macrophages are activated or inhibited remains crucial to promote development of new treatments for inflammatory disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Increased and prolonged inflammatory arthritis and elevated cytokine in p21−/− mice

(A) Shown is the change in ankle circumference and clinical score of arthritic mice (n=15/group at day 7, 10/group at day 14, and n=5/group at day 25). Data shown are representative of at least two independent experiments. (B) Representative day 7 ankle section stained with H & E. (C) H&E stained ankle sections were scored by a pathologist blinded to the study. Inflam.=inflammation, Synov.=synovial, Cartil.=cartilage, Lymph=lymphocytes, PMN=polymorphonuclear cells, Extra-articular=extra-articular inflammation. (D) Representative day 25 ankle sections stained for TRAP. (E) TRAP stained ankle sections were scored for TRAP-positive cells per 40× field. (F) microCT imaging of p21−/− ankles. (G, H) Serum levels of IL-6 (G) and IL-1α (H) were measured using Luminex-based assay on serum collected at day 7 from arthritic ankles. Values represent mean ± SE as compared by Student’s t-test (*p<0.05).
Figure 2. p21<sup>−/−</sup> mice display increased inflammatory cell infiltrate

Arthritis was induced in Wt and p21<sup>−/−</sup> mice as in Figure 1 and ankles (n=10/group) were prepared for histological analysis. (A–C) Representative antigen-retrieved paraffin-embedded ankle sections (Day 7) were stained with antibody to (A) CD45, (B) F4/80 or (C) PCNA. SL=synovial lining, P=pannus, B=bone, BM=bone marrow, C=cartilage. (D) The number of positive cells in the indicated region per high powered field was counted by a pathologist blinded to the study. Values represent the mean of at least 3 sections/ankle and 3 fields/section ± SE, compared by Student’s t-test (*p<0.05). Macs=macrophages. Prolif.=proliferating cells.
Figure 3. aa 141–160 reduces the severity of arthritis in vivo
Arthritis was induced in Wt mice as in Figure 1. Mice were IP injected with peptide (10mg/kg) 30 minutes prior to injection of K/BxN serum and daily throughout the experiment (n=8/group). Shown is (A, C) the change in ankle circumference and (B, D) clinical score for p21+/+ (Wt) and p21−/− mice, respectively. Values represent mean ± SE as compared by Student's t-test (*p<0.05 for aa 141–160 as compared to Tat-Ctrl, † p<0.05 for aa 141–160 as compared to Tat, data not significant for aa 15–40, aa 46–65, or aa 63–77 as compared to Tat-Ctrl or Tat at any time point). Data shown are representative of at least two independent experiments.
Figure 4. Treatment with aa 141–160 suppresses articular destruction

Wt mice were treated with peptide, arthritis was induced as in Figure 3 and ankles (n=16/group) were prepared for histological analysis. (A) Representative ankle sections were stained with H & E. SL=synovial lining, P=pannus, B=bone, BM=bone marrow, C=cartilage. (B) H&E stained ankle sections were scored on a 0–5 scale by a pathologist blinded to the study as previously described (11, 21, 45, 46). Values represent the mean of at least 3 sections/ankle and 3 fields/section ± SE, compared by Student’s t-test (*p<0.05 as compared to Tat-Ctrl, †p<0.05 as compared to Tat). Data shown are representative of at least two independent experiments.
Figure 5. Reduced proliferation and inflammatory cell infiltrate is observed in ankles from aa 141–160 treated mice

Wt mice were treated with peptide and arthritis was induced as in Figure 5 and ankles (n=16/group) were prepared for histological analysis. (A) Representative antigen-retrieved paraffin-embedded ankle sections were stained with antibody to CD45, F4/80 or PCNA. (B) The number of positive cells in the indicated region per high powered field was counted by a pathologist blinded to the study. Values represent the mean of at least 3 sections/ankle and 3 fields/section ± SE, compared by Student’s t-test (*p<0.05 as compared to Tat-Ctrl, †p<0.05 as compared to Tat). Macs=macrophages. Prolif.=proliferating cells. Data shown are representative of at least two independent experiments.
Figure 6. aa 141–160 enters into macrophages and reduces production of inflammatory cytokines following TLR stimulation in vitro

(A) Peritoneal macrophages were incubated with no peptide, Tat-Ctrl, or aa 141–160 peptide conjugated to FITC for 2 hours and images were taken. (B, C) Peritoneal macrophages were incubated with no peptide or with a Tat-peptide corresponding to various domains of p21 conjugated to FITC for 2 hours at (B) 37°C or (C) 4°C or 37°C. Shown in (B) is the overlay of all the cells treated with the TAT-conjugated, FITC labeled peptides at 37°C. (D–F) Supernatants from LPS-stimulated peritoneal macrophages incubated aa 141–160 or control peptide were analyzed by ELISA for (D) TNFα, (E) IL-6, and (F) IL-1β. (G–I) Cell lysates from (D–F) were analyzed for expression of (G) phosphorylated AKT, (H) phosphorylated p38, (I) and total IκB by Luminex-based assay. Data were normalized to cell number. Values represent mean ± SE of fold change relative to untreated cells as compared by Student’s t-test (*p<0.05). Data shown are representative of two independent experiments.