Growth Factors, Cytokines, Cell Cycle Molecules

p21^{Cip1} Is Required for the Development of Monocytes and Their Response to Serum Transfer-induced Arthritis

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One of the central functions of cyclin-dependent kinase inhibitors, such as p21, p27, or p16, is to prevent entry into the cell cycle. However, the question remains as to whether they have other functions in the cell. We previously demonstrated that overexpression of p21 in fibroblasts isolated from patients with rheumatoid arthritis decreases the production of pro-inflammatory molecules. Overexpression of p21 has been also shown to reduce the development of experimental arthritis in mice and rats. To explore the role of endogenous p21 in the development of arthritis, we induced arthritis in p21^{-/-} mice using the K/BxN serum transfer model of arthritis. Mice deficient in p21 were more resistant to serum transfer-induced arthritis (K/BxN) than wild-type (wt) control mice. Fewer macrophages were detected in p21^{-/-} as compared to wt joints following transfer of K/BxN serum. Chemotaxis assays of bone marrow-derived macrophages from p21^{-/-} and wt mice revealed no difference in migration. However, there was a substantial decrease in inflammatory monocytes circulating in peripheral blood and in monocyte precursors in bone marrow of p21^{-/-} mice as compared to wt mice. Adoptive transfer of wt bone marrow-derived macrophages into p21^{-/-} mice restored the sensitivity to serum transfer-induced arthritis. These data suggest a novel role for p21 in regulating the development and/or differentiation of monocytic populations that are crucial for the induction of

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Proper regulation of the mammalian cell cycle is vital for cellular homeostasis, and alterations in the cell-cycle components may lead to pathogenesis. Progression through the cell cycle is dependent on the activities of cyclin-dependent kinases (cdks) bound to their cognate cyclins.¹ Another level of cell-cycle regulation is imposed by the cdk inhibitors. cdk inhibitors bind to cdk or cdk-cyclin complexes and inhibit their kinase activity. The cdk inhibitors are grouped into two categories based on homology and preferential binding to cdk-cyclins (Inks = p15, p16, p18, and p19, and Cip/Kip = p21, p27, and p57). Overexpression of any of the cdk inhibitors will induce G₁-cell cycle arrest.² p21 may have additional roles in regulating transcription, kinase activity, and apoptosis dependent on and/or independent of its ability to inhibit the cell cycle. p21 has been shown to inhibit c-Myc,³ c-Jun NH₂-terminal kinase,⁴ and signal transducer and activator of transcription-3.⁵ Furthermore, deficiency in p21 has been shown to be associated with enhanced or reduced oncogenesis, atherosclerosis, or lupus-like disease.^{6–10}

Rheumatoid arthritis (RA) is a chronic inflammatory and destructive arthropathy of unknown etiology.¹¹ During the pathogenesis of RA, highly activated monocytes/macrophages are directly involved in synovial

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inflammation and destruction of cartilage and bone, such that their number correlates with articular destruction.^{12,13} Further, macrophages are required for collagen-induced arthritis and interleukin-1 (IL-1)/methylated bovine serum albumin-induced arthritis.^{14,15} Although macrophages are unlikely to be the initiators of RA, the increase in the number of macrophages and the enhanced activation of macrophages in the joint indicate that monocytes/macrophages are one of the principal effector cell types in RA. Macrophages are one of the central producers of IL-1 β and tumor necrosis factor α , two essential pro-inflammatory cytokines required for the progression of RA. IL-1 β and tumor necrosis factor α , in turn, are capable of inducing other pro-inflammatory cytokines and activating matrix metalloproteinases in autocrine and paracrine fashions,¹⁶ leading to increased joint destruction. Inhibition of IL-1 β and tumor necrosis factor α activity suppresses synovial inflammation and bone destruction in RA patients.^{17,18} Although macrophages are vital to the pathogenesis of RA, few studies have examined the factors that regulate their development during normal growth and during the induction and development of inflammatory arthritis.

Recently, the role of p21 in the pathogenesis of RA has been investigated. The expression of p21 is reduced in RA as compared to normal or osteoarthritis synovial tissue, particularly in the synovial fibroblast population.¹⁹ Overexpression of p21 inhibits the progression of the cell cycle and the constitutive and IL-1 β -induced production of cytokines, chemokines, and matrix metalloproteinases in synovial fibroblasts isolated from patients with RA.^{19–21} Moreover, articular injection of replication defective adenoviruses engineered to overexpress p21 prevents the development of experimental arthritis in mice and rats.^{20,21} These data suggest that p21 functions to suppress the development of arthritis.

Here, we demonstrate that, contrary to our prediction, p21 is required to sensitize mice to inflammatory arthritis following the transfer of K/BxN serum. p21^{–/–} mice failed to display ankle swelling, which is a physical characteristic of inflammatory arthritis, and showed decreased histological scores of arthritis as compared to wild-type (wt) mice. In contrast, p27-deficient mice developed arthritis equivalent to wt mice. Fewer macrophages were detected in the synovium of p21^{–/–} joints compared to wt mice following serum transfer. However, no differences were seen in the number of macrophages in the joints of p27^{–/–} and wt mice. Additionally, a deficiency in recruitment of monocytes to the peritoneal cavity was also seen in p21^{–/–} compared to wt mice following thioglycollate stimulation. Moreover, p21^{–/–} but not p27^{–/–} mice had a marked decrease in circulating inflammatory monocytes as compared to controls. The reduction of inflammatory monocytes in circulation was associated with fewer monocyte precursor cells in bone marrow. These data suggest that p21 is required for differentiation of monocytes in bone marrow and that these monocytes are necessary for the response to K/BxN serum.

Materials and Methods

Mice

B6;129S2-*Cdkn1a*^{tm1Tyj/J} (p21^{–/–}), B6129SF2/J (congenic control for p21^{–/–} mice), B6.129S4-*Cdkn1b*^{tm1Mf/J} (p27^{–/–}), and C57BL/6 (congenic control for p27^{–/–} mice) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The non-obese diabetic mice were purchased from Taconic (Germantown, NY), and the homozygous KRN TCR transgenic mice (C57BL/6 background) were a kind gift from Drs. D. Mathis and C. Benoist (Harvard Medical School, Boston, MA, and the Institute de Gene-tique et de Biologie Moleculaire et Cellulaire, Strasbourg, France). All experiments on mice were approved by the Animal Care and Use Committee at St. Louis University.

Serum Transfer-Induced Arthritis

The K/BxN serum transfer model shares many features with human inflammatory arthritis, including leukocyte invasion, synovial lining hyperplasia, pannus formation, and cartilage and bone erosion.^{22,23} Progeny from mice transgenic for a TCR (KRN) that recognizes a specific peptide from bovine ribonuclease crossed with non-obese diabetic mice spontaneously develop arthritis.²² The KRN TCR also recognizes the self-peptide, glucose-6-phosphate isomerase (GPI) in the context of A^{g7} major histocompatibility complex class II molecule.²⁴ Transfer of serum or purified immunoglobulin (Ig)G from K/BxN mice leads to induction of a robust and reproducible acute disease in several mouse strains.²³ In these studies 7-week-old progeny from KRN mice crossed with non-obese diabetic mice (K/BxN) were euthanized, peripheral blood was isolated via cardiac stick, and serum was collected via centrifugation and pooled. K/BxN serum (150 μ l) was intraperitoneally injected on each flank of 6-week-old wt, p21^{–/–}, or p27^{–/–} mice. As a control, mice were treated with saline in place of serum (data not shown). At each time point and before euthanasia, the degree of arthritis, as indicated by joint swelling, was quantitated by measuring two perpendicular diameters of the ankles using a caliper (Lange Caliper, Cambridge Scientific Industries, Cambridge, MA). Joint circumference was calculated using the geometric formula of ellipse circumference ($2\pi \times \sqrt{(a^2 + b^2)/2}$), as previously described.²⁵ Ankle joints were removed and either fixed in 10% neutral buffered formalin for 24 hours and then in ethylenediamine tetraacetic acid-decalcification buffer for 2 weeks, embedded in paraffin, and sectioned or placed in liquid nitrogen, ground into a fine powder by mortar and pestle, digested in protein lysis buffer, and homogenized on ice for 20 seconds.²⁶

Immunophenotyping

Peripheral blood and bone marrow were isolated from 5- to 8-week-old mice following euthanasia. Peritoneal cells were isolated by peritoneal lavage from unstimulated mice and from mice 1 and 5 days after intraperitoneal injection of 4% aged thioglycollate. Total leukocyte numbers were determined using an automated hematology

analyzer ABX Pentra 60 (Diamond Diagnostics, Inc., Holidiston, MA). The red blood cells in peripheral blood were lysed, and the remaining cells were fixed with BD FACS lysing solution (BD Pharmingen, San Diego, CA) following incubation with antibodies. Cells were first incubated with Fc Block (BD Pharmingen) and then stained with fluorochrome-conjugated antibodies specific to CD45, CD11b, Gr-1, CD62L, CD16/32, Ly-6C, Ly-6G, or CD31 (BD Pharmingen) or isotype control antibodies for 30 minutes. Bone marrow cells were isolated by flushing Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) through the tibias, and red blood cells were lysed with BD Pharm Lyse (BD Pharmingen) before incubation with antibodies. Cells were acquired on a BD FACSCalibur (BD Pharmingen) using CellQuest Software or BD FACS Aria using Diva Software at the St. Louis University Core Flow Cytometry Facility. All analyses were performed using FlowJo software (Tree Star Inc., Ashland, OR).

Immunohistochemistry and Histopathological Scoring

Following euthanasia, mouse legs were isolated, decalcified with ethylenediamine tetraacetic acid (Sigma-Aldrich, St. Louis, MO) in 10% formalin, embedded in paraffin, and sectioned. To stain for F4/80, antigens were retrieved using the Dako target retrieval solution (Dako, Glostrup, Denmark). Following antigen retrieval, sections were blocked in hydrogen peroxide, incubated with anti-F4/80 antibody (Clone BM8; Caltag Laboratories, Burlingame, CA) or isotype control, and then incubated with secondary biotinylated rabbit anti-rat antibody (Dako). Sections were treated with streptavidin peroxidase conjugate (Dako), color was visualized with diaminobenzidine, and sections were counterstained with hematoxylin. All F4/80 staining was performed on a Dako autostainer. Ankle sections were also stained with hematoxylin and eosin (H&E) or Safranin O and methyl green. Histopathological scoring was performed as previously described in detail.²⁷ A pathologist blinded to the study (GKH) evaluated ankle sections by examining at least three sections/ankle and three fields/section at magnification $\times 400$. H&E ankle sections were scored on a scale of 0 to 5 for inflammation, with 0 = normal, 1 = minimal infiltration, 2 = mild infiltration, 3 = moderate infiltration, 4 = marked infiltration, and 5 = severe infiltration. Bone erosion was scored on a scale of 0 to 5 by viewing H&E ankle sections, with 0 = no or normal bone resorption, 1 = small areas of resorption, 2 = more numerous areas of resorption, 3 = obvious resorption, 4 = full thickness defects in the bone without distortion of the profile, and 5 = full thickness defects in the bone with distortion of the profile. H&E ankle sections were scored on a scale of 0 to 5 for pannus formation, with 0 = no pannus formation, 1 = minimal pannus formation, 2 = mild pannus formation, 3 = moderate pannus formation, 4 = marked pannus formation, and 5 = severe pannus formation. H&E and Safranin O and methyl green sections were scored on a scale of 0 to 3 for cartilage damage, with 0 = no damage, 1 = superficial cartilage destruction, 2 = cartilage destruction to middle zone, and 3 = cartilage destruction to tide mark. Three fields of rep-

resentative pannus and synovium stained with anti-F4/80 were viewed under oil emersion at magnification $\times 1000$, and the number of F4/80-positive cells was counted. The number of F4/80-positive cells from the six fields was averaged and compared to counts from untreated mice to determine -fold increase over the time course. Areas of pannus were defined as synovial-looking tissue adjacent to the area of bone erosion and proliferation of synovial-type tissue outside of joint space. Ankle joint sections that did not have areas of pannus were not counted and were not included in the average. Synovium was defined as synovial lining cells and subadjacent tissue, including granulation tissue outside the joint space. Histopathological scoring was conducted by using an Olympus BS40 microscope (Olympus, Tokyo, Japan). Photographs were taken on a Nikon microscope equipped with the Nikon digital camera DMX1200 (Nikon, Tokyo, Japan).

Enzyme-linked Immunosorbent Assay

For detection of GPI autoantibodies in the serum and ankles, sandwich enzyme-linked immunosorbent assays were performed as previously described.²⁸ Plates (96-well) were coated with GPI type IV and XI (Sigma-Aldrich) at 10 $\mu\text{g/ml}$. Serum was diluted in phosphate-buffered saline and incubated overnight. Goat anti-mouse Ig-AP (SouthernBiotech, Birmingham, AL) was used as a detection antibody. Plates were developed with Alkaline Phosphate Yellow (Sigma-Aldrich), and absorption was read at 415 nm on a Microplate reader (Bio-Rad, Hercules, CA). Following euthanasia, serum was isolated from peripheral blood following cardiac stick, and ankles were isolated at various times from the mice following serum transfer. Ankles were snap frozen, ground with a mortar and pedestal, and then homogenized in lysis buffer (150 $\mu\text{mol/L}$ NaCl, 0.5% Nonidet P-40, 50 mmol/L Tris, 2 mmol/L ethylenediamine tetraacetic acid, pH 8.0, with protease inhibitors and phosphatase inhibitors).²⁶ All ankle data were normalized by total protein concentration (micrograms/microliter) of the ankle extracts.

Chemotaxis Assays

Chemotaxis assays were performed with minor modifications to a protocol previously optimized for RA synovial cell chemotaxis.²⁹ Bone marrow cells were isolated from p21-deficient and wt mice by tibia flush. Following tibia flush, bone marrow-derived macrophages (BMDMs) were grown in media containing 20% L-cell supernatant containing macrophage-colony stimulating factor, 10% fetal bovine serum (HyClone, Logan, UT), 5% equine serum (HyClone), 1% L-glutamine (200 mmol/L, Gibco), 1% penicillin-streptomycin (Gibco), and 1% sodium pyruvate (100 mmol/L, Cambrex, Walkersville, MD) for 7 days, then grown in the same media, but with only 10% L-cell supernatant. Cells were passage when they reached confluency and used between passages 3 and 6. Fluorescence-activated cell sorting (FACS) analysis revealed the cells were F4/80⁺, CD45⁺, CD11b⁺, and FC receptor⁺ (CD16/32). wt and p21^{-/-} BMDMs were fed the night before the assay with full

growth media for consistency. Chemoattractants or media without stimulants were added to the bottom wells of a 48-well microchemotaxis chamber (Neuroprobe, Gaithersburg, MD), and wells were covered with a 5- μ m polycarbonate membrane. Chemoattractants included fractalkine (Fkn/CX3CL1, R&D Systems, Minneapolis, MN), leukotactin (Lkn/CCL15, PeproTech, Rocky Hill, NJ), the bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma-Aldrich), and macrophage-colony stimulating factor (M-CSF, derived from dilutions of supernatant from L cells (LCS)). Subconfluent BMDMs were removed with Accutase (eBiosciences, San Diego, CA) and added to the top half of the well (40 μ l at 5.0×10^5 cells/ml) in Dulbecco's modified Eagle's medium containing 0.1% equine and bovine growth serum. The chambers were incubated for 5 hours in a 5% CO₂/95% air atmosphere at 37°C, allowing for cell migration. After migration, non-migrated cells were removed with a cotton swab, and cells that migrated to the opposite side of the membrane were fixed in methanol and stained with Diff-Quik (Dade Behring, Deerfield, IL). Each condition was assayed using eight replicates, and the sum of migrated cells was determined from three randomly selected high power fields per replicate.

Adoptive Transfer of BMDMs

BMDMs were isolated by tibia flush from wt mice and grown in culture conditions described above. 10^6 BMDMs were injected intravenously into tail veins of p21^{-/-} mice 1 day before serum transfer, 1 day after serum transfer, and 3 days after serum transfer. K/BxN serum was administered to mice, and ankle swelling was measured as the increase in ankle circumference over a 7-day period following transfer of K/BxN serum.

Statistical Analysis

Results were expressed as the mean $\pm$ SE. Differences between groups were analyzed using Student's *t*-test.

Results

p21-Deficient Mice Are Less Sensitive to Serum Transfer-induced Arthritis

To determine the role of the cell-cycle inhibitory proteins p21 and p27 in the effector phase of arthritis, we used the serum-transfer model of inflammatory arthritis (K/BxN). p21^{-/-}, p27^{-/-}, and congenic control (wt) mice were injected with a single dose of K/BxN serum at a concentration similar to previous studies.³⁰ p21-deficient mice failed to display physical inflammation as indicated by minimal ankle swelling (Figure 1A). In contrast to p21^{-/-} mice, p27^{-/-} developed arthritis similar to wt (C57BL/6) mice (Figure 1B). These data suggest that the inability of p21-deficient mice to develop inflammatory arthritis is unique to p21 and is not due to a general defect in Cip/Kip family members. wt mice demonstrated an increased ankle circumference as early as 3 days after serum transfer, peaking at day 6 and be-

Figure 1. p21-deficient mice do not develop ankle inflammation associated with experimental arthritis. **A:** Quantitative analysis of the differential ankle circumference in p21^{-/-} mice. 300 μ l of pooled serum from K/BxN mice was injected intraperitoneally into wt and p21^{-/-} mice. Ankle joints were examined for arthritis by measuring two perpendicular diameters of both joints (anteroposterior; mediolateral) by calipers. The change in ankle circumference at each time point is defined as the difference between the ankle circumference and the measurement at day 0. wt ($n = 50$) and p21^{-/-} ($n = 60$) mice have been examined in multiple independent experiments. Shown are the composite data from those experiments (p21^{-/-}: day 3, $n = 29$ mice; day 5, $n = 20$ mice; day 6, $n = 21$ mice; day 7, $n = 17$ mice; wt: day 3, $n = 20$ mice; day 5, $n = 22$ mice; day 6, $n = 17$ mice; day 7, $n = 23$ mice). The values represent the mean $\pm$ SE of ankles/time point, which were compared by Student's *t*-test to wt mice under parallel conditions. **, $P < 0.001$ as compared to wt mice under parallel conditions. **B:** Quantitative analysis of the differential ankle circumference in p27^{-/-} mice. wt and p27^{-/-} mice were injected with K/BxN serum, and ankle joints were examined for arthritis as described above. Shown are the composite data from those experiments (p27^{-/-}: day 3, $n = 10$ mice; day 5, $n = 10$ mice; day 7, $n = 14$ mice; wt: day 3, $n = 10$ mice; day 5, $n = 10$ mice; day 7, $n = 16$ mice). The values represent the mean $\pm$ SE of ankles/time point, which were compared by Student's *t*-test.

ginning to resolve by days 7 to 10. There was an eightfold increase ($P < 0.001$) at day 3, a fourfold increase ($P < 0.0001$) at day 5, a sixfold increase ($P < 0.0001$) at day 6, and an 82-fold increase ($P < 0.0001$) at day 7 in the circumference of the ankles from wt as compared to p21^{-/-} mice (Figure 1A). No statistical difference was observed in ankle circumferences of p21^{-/-} mice between days 6 and 10 (data not shown).

p21-Deficient Mice Demonstrate No Difference in Trafficking K/BxN Serum into Ankle Joints

Because increased clearance of GPI autoantibodies within peripheral blood³¹ or a failure to traffic the autoantibody to the joint would also explain resistance to serum transfer-induced arthritis in p21^{-/-} mice,³² serum and ankle joints were isolated from wt and p21^{-/-} mice following transfer of K/BxN serum. Quantitative analysis of GPI autoantibodies

Table 1. Similar Levels of GPI Autoantibodies Are Found in the Serum and Ankles of p21-Deficient Mice following Injection of K/BxN Serum

	Untreated	1 hour after	6 hours after	24 hours after
wt serum*	0.06 ± 0.001	0.40 ± 0.089	0.26 ± 0.107	0.43 ± 0.155
p21 ^{-/-} serum	0.06 ± 0.002	0.33 ± 0.087	0.44 ± 0.028	0.36 ± 0.080
wt ankles [†]	0.02 ± 0.002	0.10 ± 0.014	0.07 ± 0.024	0.09 ± 0.024
p21 ^{-/-} ankles	0.03 ± 0.002	0.10 ± 0.016	0.15 ± 0.010	0.12 ± 0.021
K/BxN serum	0.86 ± 0.042			
K/BxN ankle	0.18 ± 0.014			

*Analysis of anti-GPI autoantibodies in serum. Following euthanasia, peripheral blood was isolated from wt mice (untreated, $n = 8$; 1 hour, $n = 4$; 6 hours, $n = 4$; and 24 hours, $n = 4$) and p21^{-/-} mice (untreated, $n = 7$; 1 hour, $n = 5$; 6 hours, $n = 4$; and 24 hours, $n = 3$) following transfer of K/BxN serum and analyzed for anti-GPI autoantibodies by enzyme-linked immunosorbent assay (described in Materials and Methods). Serum isolated from K/BxN mice ($n = 2$) was used as a positive control. Values represent the mean ± SE.

[†]Analysis of anti-GPI autoantibodies in ankles. wt ($n \geq 8$ /group) and p21^{-/-} ($n \geq 8$ /group) mice were euthanized following K/BxN serum transfer. Ankle joints were isolated, snap frozen, grounded into a fine powder, lysed, and examined for GPI autoantibodies by enzyme-linked immunosorbent assay (described in Materials and Methods). Ankles isolated from K/BxN mice ($n = 2$) was used as a positive control. Data were normalized by total protein concentration. Values represent the mean ± SE.

showed no difference in the levels of GPI autoantibodies in peripheral blood (Table 1) or in ankles (Table 1) between genotypes at any of the time points examined. These data indicate that the inability to develop arthritis in p21^{-/-} mice is not mediated by a defect in trafficking of the autoantibody or a decrease in the half-life of the autoantibody.

p21^{-/-} Mice Display Reduced Histological Scores as Compared to wt Mice

To accurately assess the degree of inflammation and destruction of cartilage and bone, ankle sections were examined using a histopathological scoring system.²⁷ Examination of H&E ankle sections revealed no statistical difference in pannus formation, cellular inflammation, average or median synovial lining, bone erosion, or cartilage destruction in untreated wt or p21^{-/-} mice. In contrast, there was an increase in acute inflammation and soft tissue damage in wt ankles as compared to p21^{-/-} ankles over the 10-day period following injection of K/BxN serum. At 4 days after transfer of serum there was a twofold, a threefold, and a threefold increase in pannus formation ($P < 0.002$), cellular inflammation ($P < 0.001$), and bone erosion ($P < 0.001$) in wt mice as compared to p21^{-/-} mice. Similar results were seen at days 5 and 6; namely, a fivefold increase in pannus formation ($P < 0.001$), twofold increase in cellular inflammation ($P < 0.002$), and fourfold increase in bone erosion ($P < 0.008$) in wt as compared to p21^{-/-} ankle sections (Figures 2 and 3). However, the increase in bone destruction in wt mice was independent of the number of osteoclasts, because there were no differences in the number or location of TRAP-positive cells in wt and p21^{-/-} mice (data not shown). An increase in cartilage destruction was also observed at days 4, 6 (21-fold increase, $P < 0.003$), and 10 after serum transfer. There was a marginal increase in the average number of cell layers in the synovial lining at days 4 (1.2-fold, $P < 0.02$) and 6 (1.3-fold, $P < 0.01$) in wt as compared to p21^{-/-} mice (Figure 2E). By days 7 to 10 there were no differences in histological scores between wt and p21^{-/-} mice, which may be due to the fact that wt mice start to resolve the arthritis after day 6. In contrast to p21^{-/-} mice, wt and p27-deficient mice displayed equivalent histological scores (supplemental data, see <http://ajp>.

[amjpathol.org](http://ajp)). These data suggest that, contrary to the studies that showed overexpression of p21 prevents the development of arthritis,^{20,21,33,34} the endogenous level of p21 is essential for the full development of arthritis.

p21^{-/-} Mice Display Reduced Numbers of Infiltrating Macrophages in the Joint

The infiltrating cells in wt and p21^{-/-} ankles consisted of mainly lymphocytes, granulocytes, and macrophages. However, p21^{-/-} mice had less neutrophils and polymorphonuclear cells compared to wt mice (data not shown). The reduced numbers of infiltrating cells in the p21^{-/-} joint were not mediated by enhanced apoptosis in the joint, because there was no difference in number or location of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells between wt and p21^{-/-} mice (data not shown). Sections stained with F4/80, a marker of macrophages,^{35,36} revealed that p21-deficient mice had significantly fewer macrophages that were recruited to the joint following K/BxN serum transfer (Figure 4). There were five- and threefold increase in macrophages found in wt mice at days 5 and 6 after serum transfer as compared to untreated joints. However in p21^{-/-} ankle joints, the increase in recruited macrophages peaked at 1.5-fold over a 10-day time course. The recruitment of macrophages in wt joints correlated with the level of inflammation and ankle circumference. These data suggest that inflammation and joint destruction seen in p21^{-/-} ankle joints following K/BxN serum transfer may be due to lack of macrophage recruitment.

p21^{-/-} Mice Have a Defect in Recruitment of Macrophages to the Inflamed Peritoneum

To determine whether the decrease in macrophages observed in joints of p21^{-/-} mice following induction of arthritis occurs in other models of acute inflammation, we intraperitoneally injected wt and p21^{-/-} mice with 4% aged thioglycollate. At 1 day following thioglycollate stimulation there was no difference in the fold increase in neutrophils or macrophages between wt and p21^{-/-}

Figure 2. Increased histological scores of ankle sections in wt mice compared to p21^{-/-} mice. Ankles isolated from p21^{-/-} and wt mice as described in Figure 1 were fixed, embedded in paraffin, sectioned, and stained with H&E or Safranin O and methyl green. Ankle sections were evaluated and scored by a pathologist blinded to the study as described in Materials and Methods. Shown are the composite data from two experiments (p21^{-/-}: day 0, *n* = 14 ankles; days 3, 5, and 7, *n* = 8 ankles; days 4, 6, and 10, *n* = 16 ankles; wt: days 0, 3, and 5, *n* = 8 ankles; days 4, 6, and 10, *n* = 12 ankles; day 7, *n* = 6 ankles). The values represent the mean $\pm$ SE of ankles/time point, which were compared by Student's *t*-test to wt mice under parallel conditions. *, *P* < 0.05 and **, *P* < 0.002 as compared to wt mice under parallel conditions.

mice (Figure 5, A and B). However, p21^{-/-} mice displayed a marked reduction in the fold increase in macrophages but not neutrophils as compared to wt mice at day three (Figure 5, A and B). To determine whether the differences in the two models of acute inflammation were the result of a defect in macrophage migration, chemotaxis assays were performed using BMDMs from wt and p21^{-/-} mice and several known chemoattractants, including fractalkine (CX3CL1, Fkn), leukotactin (CCL15, Lkn), fMLP, or M-CSF, which is contained in LCS. There was no significant differences in the migration of BMDMs derived from wt and p21^{-/-} mice in response to Fkn (0.001 to 10 nmol/L), Lkn (1 nmol/L), fMLP (10 nmol/L), or M-CSF (LCS) (Figure 5C). Taken together, these data suggest the reduction in recruitment of monocytes to the inflamed joint or to the peritoneum is not attributed to a defect in migration of macrophages.

p21^{-/-} Mice Have a Significant Decrease in Inflammatory Monocytes in Circulation and in Monocyte Precursors in Bone Marrow

Because there is a severe reduction in the number of macrophages in p21^{-/-} joints following induction of arthritis, we examined whether there is a deficiency in monocytes that circulate in blood. wt and p21^{-/-} mice had equivalent num-

ber of leukocytes in peripheral blood (data not shown). As recently reported, monocyte populations may be subdivided into two categories: resident monocytes (CD45⁺CD11b⁺Gr-1⁻CD62L⁻) and inflammatory monocytes (CD45⁺CD11b⁺Gr-1⁺CD62L⁺).^{26,29,37} As compared to wt mice, p21-deficient mice displayed a marked reduction (3.4-fold; *P* < 0.001) in inflammatory monocytes (Figure 6A). The reduction in inflammatory monocytes was not attributed to increased death, because there was no difference in the percent apoptotic (annexin V-positive) monocytes in peripheral blood (data not shown). The reduction in inflammatory monocytes was specific to p21, because no differences were observed in the monocytic or neutrophil populations in mice lacking p27 (supplemental data, see <http://ajp.amjpathol.org>). These data suggest that the lack of infiltrating monocytes into the inflamed joint of p21^{-/-} mice may be mediated by a severe reduction in the circulating pool of inflammatory monocytes.

To determine whether the reduction of monocytes in p21^{-/-} mice is a result of a deficiency in bone marrow, flow cytometric analyses were performed on isolated bone marrow from wt and p21^{-/-} mice. Based on previous studies, the myeloid populations in bone marrow may be separated into different subpopulations based on surface expression of CD45, CD31 (ER-MP12 and PECAM), CD11b, Ly6C (ER-

Figure 3. Increased inflammation and destruction in wt mice as compared to $p21^{-/-}$ mice following transfer of serum. At 4, 6, and 10 days following serum transfer, ankles as described in Figure 1 were harvested, sectioned, and stained with hematoxylin (blue) and eosin (pink). Shown are representative photomicrographs (40 $\times$ or 200 $\times$) of the joint from wt and $p21^{-/-}$ mice. **Arrows** denotes invading pannus. SL, synovial lining; B, bone; P, pannus.

MP20), and Ly-6G.^{38,39} Further, cell-sorting analyses followed by culturing in media containing M-CSF showed that the CD11b⁺CD31⁺Ly-6C⁺Ly-6G⁻ and CD11b⁺CD31⁺Ly-6C⁺Ly-6G⁻ populations were responsible for the development of macrophages in culture (data not shown) as indicated by positive staining for F4/80, CD11b, and FcR (CD16/CD32) of adherent cells. There was a twofold decrease ($P < 0.002$) in the CD11b⁺CD31⁺Ly-6C⁺Ly-6G⁻ and CD11b⁺CD31⁺Ly-6C⁺Ly-6G⁻ populations in $p21^{-/-}$ mice as compared to wt mice (Figure 6B). Although there was also a reduction (twofold, $P < 0.002$) in the CD11b⁺CD31⁺Ly-6C⁺Ly-6G⁺ population in $p21^{-/-}$ as compared to wt mice (data not shown), this population failed to de-

velop into macrophages in culture and Giemsa-Wright staining of the sorted cells, demonstrating that the vast majority of these cells were mature neutrophils (data not shown). Collectively, these data suggest that p21 is required for the differentiation of monocyte progenitors and/or precursors independent of its inhibitory role in apoptosis.

Adoptive Transfer of wt BMDMs Restores the Development of Arthritis in $p21^{-/-}$ Mice

Because p21-deficient mice have a deficiency in the inflammatory monocyte population in peripheral blood,

Figure 4. Fewer macrophages are recruited to p21^{-/-} joints following injection of K/BxN serum. **A:** Quantitative fold increase of macrophage recruitment in ankle sections. Ankles from mice as described in Figure 1 were harvested, sectioned, and stained with antibodies that recognize the F4/80 antigen (brown staining) or isotype control (data not shown) and counterstained with hematoxylin. Macrophage counts were determined by a pathologist blinded to the study as described in Materials and Methods. Shown are the composite data from two experiments (p21^{-/-}: day 0, *n* = 14 ankles; days 3, 5, and 7, *n* = 8 ankles; days 4, 6, and 10, *n* = 16 ankles; wt: days 0, 3, and 5, *n* = 8 ankles; days 4, 6, and 10, *n* = 12 ankles; day 7, *n* = 6 ankles). The values represent the mean $\pm$ SE of ankles/time point. The fold increase was defined as increase over untreated conditions. The data are representative of three independently performed experiments. *, *P* < 0.004; **, *P* < 0.0001 as compared to wt mice under parallel conditions. **B:** Photomicrographs (200 $\times$) of ankle sections from wt and p21^{-/-} mice stained for F4/80 antigen. Ankles from mice as described in Figure 1 were harvested, sectioned, and stained with antibodies that recognize the F4/80 antigen (brown staining) or isotype control (data not shown) and counterstained with hematoxylin. SL, synovial lining; B, bone.

we attempted to reconstitute this population by adoptively transferring cultured wt BMDMs into p21^{-/-} mice and then administering K/BxN serum. There was a ninefold increase (*P* < 0.02) in ankle swelling in p21^{-/-} mice that received wt BMDMs as compared to uninjected p21^{-/-} mice at 7 days after serum transfer (Figure 7). These data suggest that reconstitution of the macrophage population in p21^{-/-} mice partially eliminates their resistance to serum transfer-induced arthritis.

Discussion

To date all studies that have examined the function of p21 in RA have specifically focused on the synovial fibroblast population. We demonstrated a twofold reduction in the number of synovial fibroblasts expressing p21 in synovial tissue isolated from RA patients as compared to control osteoarthritis patients.¹⁹ Articular injection of replication-defective adenoviruses engineered to overexpress p21 prevents development of experimental arthritis.^{20,21,33,34} Based on these data we anticipated that p21^{-/-} mice would have a marked increase in the development of experimental arthritis. However, our data reveal a surprising role for endoge-

nous p21 in the effector phase of arthritis. The p21-deficient mice are resistant to K/BxN serum transfer-induced arthritis, a model system for the effector phase of arthritis.³⁰ Although these data suggest that p21 may be pro-arthrogenic, the paradoxical role for p21 may be explained by our observation that p21 is required for the development of inflammatory monocytes. Our data also indicate that the level of inflammatory monocytes influences the sensitivity of arthritis following transfer of K/BxN serum. In support of this concept, Wipke et al⁴⁰ showed that the addition of anti-Gr-1 antibody (Clone RB6-8C5), which binds to neutrophils and to inflammatory monocytes,³⁷ results in their depletion (data not shown), and prevents serum transfer-induced arthritis.⁴⁰ Thus, these data suggest that both neutrophils and inflammatory monocytes are central contributors to the pathogenesis of inflammatory arthritis following transfer of K/BxN serum. Because there are no differences in the number of neutrophils in p21^{-/-} mice as compared to wt mice, we can infer from these data that in this model the inflammatory monocytes may be necessary for arthritis development. Recently, a study showed that depletion of macrophages by clodronate liposome treatment leaves mice completely resistant to K/BxN serum transfer-induced arthritis.⁴¹ Fur-

Figure 5. Reduced migration of monocytes to the inflamed peritoneum of $p21^{-/-}$ mice. $p21^{-/-}$ and wt mice were given intraperitoneal injections of 4% thioglycollate. Peritoneal cells were isolated 0 ($n > 10$), 1 ($n = 8$), and 3 ($n > 12$) days after injection and examined by flow cytometry as described in Materials and Methods. **A:** The fold increase of neutrophils recruited. Mice were treated as described above, neutrophils ($CD45^{+}Gr-1^{+}$) were identified using FACS analysis. **B:** The fold increase of macrophages recruited. Mice were treated as described above. Macrophages ($CD45^{+}F4/80^{+}$) were identified using FACS analysis. The data were a combination of four independently performed experiments. The values represent the mean $\pm$ SE of cells/time point, which were compared by Student's *t*-test. The fold increase was defined as increase over untreated conditions. **C:** wt and $p21^{-/-}$ BMDMs respond similarly to chemotactic signals. Equal numbers of wt and $p21^{-/-}$ BMDMs were introduced to various chemoattractants to induce migration. Cell counts were expressed as -fold increase over background migration (no stimulus). Fkn, fractalkine/CX3CL1; Lkn, leukotactin/CCL15; fMLP, formyl-methionyl-leucyl-phenylalanine; LCS, L-cell supernatant containing macrophage-colony stimulating factor.

ther, adoptive transfer of BMDMs from wt mice restores sensitivity to K/BxN serum transfer-induced arthritis. Taken together, these data suggest that failure to recruit macrophages following an inflammatory response is one of the primary defects in $p21^{-/-}$ mice that may be responsible for the lack of development of inflammatory arthritis.

The lack of inflammatory monocytes suggests a mechanism for decreased sensitivity to serum transfer-induced arthritis in $p21^{-/-}$ mice. These data also indicate that a potential function of inflammatory monocytes may be to traffic the GPI-anti-GPI antibody immune complex to the articular surface. A reduction in anti-GPI antibody deposition on the articular cartilage surface is observed in mice deficient in Fc receptors or in mice depleted of

Figure 6. **A:** Immunophenotyping of peripheral blood reveals a deficiency in inflammatory monocytes in $p21^{-/-}$ mice. Peripheral blood was isolated by cardiac sticks from 5 to 8 week old wt ($n = 23$) and $p21^{-/-}$ ($n = 16$) mice. Cells were blocked for 10 minutes with Fc block and then stained with anti-CD45, anti-CD11b, anti-Gr-1, and anti-CD62L antibodies for 30 minutes. Following incubation with antibodies red blood cells were lysed and cells were fixed with BD Biosciences FACS lysing solution. Resident monocytes: $CD45^{+}CD11b^{++}Gr-1^{-}CD62L^{-}$; inflammatory monocytes: $CD45^{+}CD11b^{++}Gr-1^{+}CD62L^{++}$; neutrophils: $CD45^{+}CD11b^{++}Gr-1^{++}CD62L^{++}$. Values represent the mean $\pm$ SE, which were compared by Student's *t*-test. *, $P < 0.001$ as compared to control under parallel conditions. **B:** Immunophenotyping of bone marrow reveals a deficiency in monocyte precursor populations in $p21^{-/-}$ mice. Bone marrow cells were isolated by tibia flush from 5 to 8 week old wt ($n = 18$) and $p21^{-/-}$ ($n = 13$) mice. Red blood cells were lysed, and the remaining cells were blocked for 10 minutes with Fc block and then stained with anti-CD45, anti-CD11b, anti-Gr-1, anti-CD31, anti-Ly6C, and anti-Ly-6G antibodies. Values represent the mean $\pm$ SE, which were compared by Student's *t*-test. *, $P < 0.002$ as compared to wt under parallel conditions.

Gr-1-positive cells.³⁰ Because inflammatory monocytes have Fc receptors on their cell surface and because they express Gr-1, these data suggest that one of the functions of inflammatory monocytes may be to traffic the GPI-anti-GPI-immune complex. Thus, it is possible that the lack of inflammatory monocytes contributes to a reduction in the total levels of Fc receptor. However, this is unlikely because all of the monocytes and neutrophils in $p21^{-/-}$ and control mice were positive for Fc receptor, and there were no differences in the mean fluorescent intensity of the receptors on the cell surface (data not shown). These data indicate that a similar concentration of Fc receptors are present on myeloid cells of $p21^{-/-}$ and control mice. Alternatively, there might be an increase in the clearance of the anti-GPI autoantibody in serum of $p21^{-/-}$ mice. Mice lacking FcRn, which is required for stabilization of IgGs, display increased clearance of the anti-GPI autoantibody, and excessive amounts of serum lead to the induction of arthritis.⁴² Thus, the lack of inflammatory monocytes or the lack of p21 in other hemopoietic cells may affect the stabilization

Figure 7. Adoptive transfer of wt BMDMs restores sensitivity to serum transfer in p21^{-/-} mice. wt BMDMs were grown in culture as described in Materials and Methods and intravenously injected via tail vein into p21^{-/-} mice at 1 day before serum transfer, 1 day after serum transfer, and 3 days after serum transfer. Ankle circumference was measured as described in Figure 1A. The values represent the mean $\pm$ SE of ankles/time point, which were compared by Student's *t*-test to mice not receiving BMDMs under parallel conditions. (p21^{-/-}; day 2, *n* = 12 mice; day 7, *n* = 20 mice; wt; day 2, *n* = 16 mice; day 7, *n* = 23 mice).

of the anti-GPI antibody. However, we observed similar levels of GPI autoantibodies over time in the joints isolated from wt and p21^{-/-} mice. These data suggest that the resistance to arthritis observed in p21^{-/-} mice is not due to a defect in the binding of Fc receptors or in the trafficking of the anti-GPI autoantibody to the joint.

Similar to RA, the inflammatory response is a major contributor to the pathogenesis of atherosclerosis.⁴³ Monocytes and macrophages are required for the development of experimental atherosclerosis in mice.⁴⁴ Recently, p21-deficient mice have been shown to be resistant to experimental atherosclerosis.¹⁰ On the other hand, p27^{-/-} mice show increased arterial inflammation, macrophage accumulation, and lesion size when crossed with ApoE^{-/-} mice.⁴⁵ We show that p21^{-/-} but not p27^{-/-} mice are resistant to K/BxN serum transfer-induced arthritis. Thus, these data demonstrate a differential role for the cdk inhibitor family members during the development of experimental atherosclerosis and arthritis.

p21 is known to be essential for differentiation of skeletal muscle cells,⁴⁶ oligodendrocytes,⁴⁷ keratinocytes,⁴⁸ and neuronal cells.⁴⁹ p21 has also been shown to be required for differentiation of monocytic cells and for maintaining their survival in culture.^{50,51} Moreover, in human monocytes isolated from peripheral blood, antisense oligonucleotides to p21 block differentiation into macrophages.⁵² These data suggest that p21 is required for differentiation and the survival of monocyte cells that remain in the cell cycle. The effect of p21 on BMDMs is somewhat different from monocytic cell lines and primary monocytes, because p21 is not required for the differentiation of BMDMs. Additionally, in BMDMs, which also proliferate, p21 is essential for prevention of apoptosis but not for arresting the cell cycle in response to interferon γ or decorin.^{53–55} However, p21 has no effect on survival or growth arrest in BMDMs deprived of M-CSF or treated with lipopolysaccharide.⁵⁵ Here we show that p21 is also necessary for the development of monocytes *in vivo*, independent of its role in apoptosis. Although there are substantially less inflammatory monocytes in cir-

ulation and myeloid progenitors or precursors in bone marrow in p21^{-/-} mice, the apoptotic indices are not increased in bone marrow and blood of p21^{-/-} mice as compared to control mice (data not shown). Because we observe a decrease in myeloid precursors and because there is a similar complement of neutrophils in p21^{-/-} and control mice, these data suggest that p21 may be required for differentiation of monocytes in bone marrow at a developmental stage that is distinct from granulocytes. Further, these monocytes are necessary for the effector phase of arthritis.

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