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JNK1 deficiency limits macrophage mediated antigen-induced arthritis

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Abstract

Objective—To elucidate the non-redundant roles of JNK1 and JNK2 in antigen-induced arthritis (AIA).

Methods—Mice that were genetically disrupted in *Jnk1* or *Jnk2* were primed with methylated BSA (mBSA) in complete Freund's adjuvant and then given an intraarticular challenge with mBSA in the knee on day 21. Bone marrow chimeras were generated and similarly treated. Joints were harvested and prepared for histological assessment. T cell responses were verified by cytokine and proliferation responses, and relative immunoglobulin responses were measured by ELISA. Cytokine mRNA expression levels were measured by qPCR. Thioglycollate and zymosan A elicited macrophage recruitment was tested *in vivo* and migration was tested *in vitro*. The peptide inhibitor D-JNKi was injected daily starting four days after intraarticular mBSA injection in wild type (WT) mice and inflammation was histologically scored.

Results—JNK1-deficient, but not JNK2-deficient mice, had a reduction in inflammatory infiltration and joint damage. This effect was primarily restricted to hematopoetic cells, but B and T cell responses were preserved in mBSA-injected mice. JNK1-deficient macrophages produced cytokines and chemokines comparably to WT counterparts. However, macrophage migration was impaired *in vivo* and *in vitro*. Targeting JNK with the peptide inhibitor D-JNKi dramatically reduced inflammation and joint destruction in WT mice.

Conclusions—AIA is dependent on JNK1, but not JNK2. JNK1 is a promising molecular target for reducing autoimmune inflammation as its inhibition impairs macrophage migration.

INTRODUCTION

RA is an autoimmune disease characterized by excessive autoantibody production, aggressive synoviocyte behavior, and abundant cytokine production (1). In addition to synovial inflammation, the aggressive tissue front called pannus invades and destroys local articular structures. The introduction of biological therapy for RA and other inflammatory diseases represents a major therapeutic advance. Biological agents such as tumor necrosis factor-α (TNF) blockers disrupt interactions of effector molecules with their receptors, but

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many patients have an inadequate response (2). However, orally bioavailable small molecules that target key intracellular signaling molecules might regulate both cytokine production and cytokine action, affecting more than one function and thus, be more effective (3).

Mitogen-activated protein kinases (MAPK) are expressed and activated in the RA synovium (4) and have been the focus of drug development efforts due to their prominent role in the regulation of cytokines, chemokines, degradative enzymes, migration, programmed cell death, and cell proliferation (5–7). Jun N-terminal kinases (JNK), which belong to the MAPK family, are highly activated in isolated RA fibroblast-like synoviocytes (FLS) and in the synovium itself (8,9). They play a major role in cytokine production and extracellular matrix regulation through production of matrix metalloproteinases (MMP). However, the functions of JNK extend beyond MMP and cytokine expression and also involve cell proliferation, apoptosis (10), angiogenesis (11), and migration (12).

The JNKs are encoded by three separate loci, *Jnk1-3,* of which *Jnk1* and *Jnk2* are ubiquitously expressed, whereas *Jnk3* is mainly expressed in heart, testis, and brain (13). Separate roles of JNK1 and JNK2 in the pathogenesis of arthritis are yet to be fully defined as in some models they appear to have compensatory and redundant functions. JNK1 is not essential for inflammatory arthritis in TNF-transgenic mice (14), suggesting that signaling through JNK2 might compensate for the deficiency in JNK1 in that model. In addition JNK2-deficient mice exhibited only a modest decrease in cartilage damage in a model of passive collagen-induced arthritis (15). On the other hand, a JNK inhibitor, SP600125, was mildly anti-inflammatory in rat adjuvant-induced arthritis, and also provided striking protection against bone and cartilage destruction (9). However, off-target effects of SP600125 could have potentially contributed to some of these effects (16,17).

In this report, we show that JNK1, but not JNK2, is essential for pathogenesis of antigen-induced arthritis (AIA). JNK1 deficiency attenuates arthritis induction and joint destruction through multiple mechanisms including reduced synovial inflammatory infiltration, inflammatory cytokine production and MMP expression. Of particular importance, the ability of macrophages to migrate was impaired, even in the presence of potent chemokine stimulation. These results indicate that JNK1 plays a key role in the pathogenesis of arthritis and could serve as a new therapeutic target for RA.

METHODS

Mice

Jnk1$^{-/-}$ and *Jnk2*$^{-/-}$ mice were previously described (18,19). Mice used in these experiments were 8–12 weeks age females. C57Bl/6 and CD45.1 congenic mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The mice were bred and maintained under standard conditions in the University of California, San Diego animal facility that is accredited by the American Association for Accreditation of Laboratory Animal Care. All animal protocols received prior approval by the institutional review board.

Reagents

Lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) and zymosan A were purchased from Sigma (St. Louis, MO). Zymosan A was suspended in PBS, boiled for 1 h, rinsed with PBS three times, and resuspended in PBS at 1 mg/ml. Thioglycollate (TG) was from Difco Laboratories. MCP-1 (monocyte chemotactic protein-1) and TNF were purchased from R&D systems (Minneapolis, MN), and IL-1β was purchased from PeproTech, Inc. (Rocky Hill, NJ).

Antigen-induced arthritis (AIA) induction

Experimental AIA was induced by a subcutaneous injection of 100 μg of mBSA emulsified in 100μl of complete Freund's adjuvant (CFA) in the flank, and one week later by an intradermal injection of 100 μg of mBSA/CFA in the tailbase. Two weeks after these injections, arthritis was induced by intraarticular (i.a.) injection of 60 μg of mBSA in 10 μl of saline into the right knee joint. The left knee was injected with PBS to serve as a control. Disease was assessed 10 days post-intraarticular injection by histological analysis as described below.

Histology

Joints were fixed in 10% formalin, decalcified in 10% EDTA for 2–3 weeks, trimmed, and embedded. Sections were prepared from the tissue blocks and stained with hematoxylin and eosin (H&E) or Safranin O-fast green to determine proteoglycan content. A semiquantitative scoring system was used to assess synovial inflammation, extra-articular inflammation, erosion and proteoglycan loss (0–5 scale) as previously described (20). Histological analyses were performed in a blinded manner.

Immunohistochemistry

Sections from decalcified fixed tissues were incubated overnight at 4°C with rat anti-F4/80 antibody (MCA497R) from AbD Serotec (Raleigh, NC) at a 1:200 dilution. Antigen retrieval was with citrate buffer (DAKO S1700) at 96°C for 20 minutes.

Bone marrow chimeras

Adult mice were lethally irradiated with 11 Gy. Bone marrow cells harvested from femurs and tibias of donors were washed in serum-free medium and counted. Recipients were injected with 10^7 cells in 200 μl of serum-free RPMI intravenously. Chimerism was verified by flow cytometry for the appropriate CD45 allele after eight weeks.

Real-time quantitative (q) PCR

Joints were dissected to remove extra-articular tissue, and snap frozen in liquid nitrogen. The specimens were pulverized and total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) and reverse-transcribed with random hexamers and Superscript II Kit (Invitrogen). qPCR was performed with SYBR Green PCR Master Mix Kit (Applied Biosystems). The relative amounts of transcripts were compared to those of 18S rRNA and normalized to untreated samples by the ΔΔCt method. Primer sequences are available upon request.

T cell proliferation assay

Mouse spleen cells were isolated and washed in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 10 mM Hepes, 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 1% L-glutamine, and 100 units/ml of penicillin/streptomycin. Erythrocytes were lysed and after washing, cells were counted and 2×10^5 cells were placed in each well of a sterile, U-bottomed microculture plate in medium with 12.5 or 25 μg/ml of mBSA. Supernatants were harvested for cytokines after 48h or cultures were maintained at 37°C for 4 days for proliferation assays. Sixteen hrs before harvesting, 1 μCi of ^{3}H-thymidine was added in 25 μl of RPMI. Cultures were harvested with a cell harvester and ^{3}H-thymidine incorporation was determined.

Determination of serum antibodies

Methylated BSA-specific antibodies of various isotypes (IgG, IgG1, IgG2a, IgG2b, IgG3) were measured in sera by ELISA. Antigen was coated on microtiter plates at a concentration of 1 μg/ml. Antibody titers were assessed by 2-fold serial dilutions of sera, followed by detection of bound mouse Ig with a 1:500 dilution of peroxidase-conjugated rabbit anti-mouse Ig. O-phenylenediamine was used as substrate for the peroxidase reactions.

Flow cytometry

Antibodies used by flow cytometry included CD45.2-FITC, CD45.1-PE, F4/80-FITC and Gr-1-PE (BD Biosciences, San Jose, CA). Antibodies against integrin-α2-PE, integrin-α4-PE, integrin-β1-PE and integrin-β2-PE were purchased from Sigma (St Louis, MO)

Cytokine and chemokine quantification

MCP-1, IFNγ, IL-4, IL-10 and IL-17 amounts were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN), following manufacturer's protocol.

Zymosan A and thioglycollate-induced peritonitis

Peritonitis was induced by intraperitoneal (i.p.) injection with 2 ml of 3% sterile TG medium or 1 ml with 400ug zymosan A. Mice were sacrificed after 6 hrs or after 3 days and peritoneal cells were removed by lavage with 5 ml of PBS. Lavage fluids were separated by centrifugation and supernatants were used for chemokine analysis. Cell yield was obtained by counting and the relative percentages of neutrophils (measured in mice sacrificed after 6 hrs of TG or zymosan A injection) or macrophages (measured in mice killed after 3 days of TG or zymosan A injection) were determined by flow cytometry after staining with anti-F4/80 and anti-Gr-1 antibodies.

In vitro migration

TG-induced macrophages were harvested from *Jnk1*$^{-/-}$ and WT mice, and 5×10^5 cells per well were dispersed into 96 well plates with central stoppers (Oris™, Platypus Technologies, LLC, Madison, WI) and allowed to settle overnight. The stoppers were removed except for the baseline control wells. The cells were treated with MCP-1 (R&D Systems, Minneapolis, MN), and varying serum concentrations for 4 hrs. The cells were then stained with calcein AM (Invitrogen) and the fluorescence (485/535) of the cells that migrated to the center of the well was measured through a mask with small aperatures aligned with the center of the wells using a fluorimeter (Wallac 1420, PerkinElmer, Waltham, MA).

Immunoblot

Peritoneal macrophages were disrupted in lysis buffer (PhosphoSafe™, Novagen, Gibbstown, NJ) containing a protease inhibitor cocktail. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were probed with antibodies against phospho-JNK (Cell Signaling Technology, Danvers, MA), JNK1 and JNK1/2 (BD, Pharmingen), CCR5 (Novus Biologicals, Littelton, CO) and actin (Sigma, St Louis, MO). Horseradish peroxidase-conjugated anti-IgG (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used as secondary antibody. Membranes were developed using a chemiluminescence system (ECL detection reagent: Amersham Life Science, Aylesbury, UK).

Fibroblast like synoviocytes (FLS) and bone marrow-derived macrophages (BMDM)

Briefly, synovial tissue was collected, minced, and incubated with 0.5 mg/ml collagenase VIII (Sigma, St. Louis, MO) in serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco) for 2 hrs at 37°C, washed extensively, and cultured in DMEM supplemented with 10% fetal calf serum, penicillin, streptomycin, and L-glutamine in an humidified 5% CO_2 atmosphere. FLS from passages 3–9 were used. To generate BMDM, bone marrow cells were cultured in DMEM (Invitrogen) with 10% FBS and 20% L929 supernatant containing macrophage-stimulating factor for 6 days and were replated for the assays as indicated.

D-JNKi peptide treatment

D-JNKi, whose sequence is DQSRPVQPFLNLTTPRKPR-PP-RRRQRRKKRG and a TAT control peptide PP-RRRQRRKKRG were synthesized by the Protein Chemistry Department at the Research Institute of Molecular Pathology and were kindly provided by Dr. E. Wagner. Specific inhibition of JNK activation by D-JNKi was shown previously (21). D-JNKi or TAT peptides diluted in PBS were injected i.p. at 20 μg/g body weight daily, starting on day 4 after mBSA i.a. injection and until day 10.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Mann Whitney U test was used for pair-wise comparisons. ANOVA was used for multiple group comparisons with Bonferonni *post hoc* comparisons for multiple pair-wise comparisons. All statistical analyses were performed using PRISM version 4.0b (GraphPad Software, San Diego, California). Results were considered significant for $p<0.05$.

RESULTS

JNK1 is required for antigen-induced arthritis

To examine the role of JNK1 and JNK2 in a model of chronic inflammatory arthritis, C57Bl/6 mice deficient for either JNK isoform were compared to WT mice for incidence and severity of AIA. Histopathological analysis showed a reduction in inflammatory cell infiltration, joint destruction and cartilage damage in *Jnk1*$^{-/-}$ mice (Fig. 1A and B). JNK1-deficient mice had significantly lower scores for all histological indices compared to WT and JNK2-deficient mice ($p<0.05$ by ANOVA with Bonferroni *post hoc* comparison, n=14–16/group).

Effect of JNK1 deficiency on cytokines, MMP and adaptive immunity

To evaluate the influence of JNK deficiency on expression of selected participants in inflammatory arthritis, we determined relative expression of IL-1β, TNF, IL-6 and MMP3 mRNAs in paws from these mice by qPCR on day 10 after i.a, mBSA injection. Amounts of mRNA for all four mediators were statistically decreased in *Jnk1*$^{-/-}$ mice, but not in *Jnk2*$^{-/-}$ mice, compared to WT controls (Fig. 1C).

AIA requires the generation of mBSA-specific $CD4^+$ T cells and antibodies. Although *Jnk1*$^{-/-}$$CD4^+$ T cells showed defects in T cell activation or differentiation *in vitro* (18,22,23), it also was shown that the absence of JNK1 in T cells did not alter their ability to mount a pathogenic autoimmune response to myelin *in vivo* (24). Consistent with the latter results, Fig. 2A shows that quantitatively similar splenocyte proliferative responses against mBSA were found in cells derived from *Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$ and WT mice. In addition there were no differences in splenocyte IFNγ, IL-4, IL-10 or IL-17 release after *in vitro* restimulation between the strains (Fig. 2B). Humoral immunity, tested by the relative levels of mBSA-specific antibodies in sera, was also comparable in all strains (Fig. 2C).

Arthritis is partially dependent on hematopoetic cells and radioresistant cells

To evaluate whether joint inflammation was associated with bone marrow-derived elements other than T or B cells, or with connective tissue, bone marrow chimeras were generated by irradiating WT and *Jnk1*$^{-/-}$ recipients, and reconstituting them with *Jnk1*$^{-/-}$ and WT donor bone marrow. After 8 weeks, bone marrow engraftment was confirmed by flow cytometry and AIA was then induced. Development of arthritis was reduced in mice with *Jnk1*$^{-/-}$ bone marrow-derivedcells (Fig. 3A). As the *Jnk1*$^{-/-}$ recipients of *Jnk1*$^{-/-}$ bone marrow had less inflammation than the WT recipients, a contribution from the radioresistant cells could not be excluded. Concordantly, mice harbouring JNK1-deficient bone marrow trended toward lower levels of IL-1β and IL-6 mRNA expression (Fig. 3B).

JNK1 regulates macrophage migration

In this model, JNK1 deficient mice were able to mount an adaptive immune response to mBSA. (Fig 2). However, JNK deficiency in the bone marrow derived cells reduced inflammation in the chimeras. In the innate immune compartment, JNK1 is markedly down-regulated during neutrophil differentiation and maturation (25). Hence we examined macrophages as a likely candidate amongst bone marrow derived cells. To further analyze the role of JNK1 in macrophages, BMDM were stimulated by TNF and IL-1β. We did not detect any differences in chemokine or cytokine gene expression between JNK1-deficient and WT BMDM stimulation with either TNF or IL-1β (Supplementary Fig. 1).

Although *Jnk1*$^{-/-}$ macrophages retained the ability to express cytokines and chemokines, we noticed a marked reduction in F4/80 positive cells in the affected joints of *Jnk1*$^{-/-}$ but not *Jnk2*$^{-/-}$mice (Fig. 4A), suggesting an impairment of inflammatory cell migration. Several prior reports indicated that JNK was critical to cellular migration (12,26). To further study the effect of JNK1 specifically on inflammatory cell migration, we used the TG and zymosan peritonitis models. Neutrophil recruitment was assessed by injecting TG or zymosan into the peritoneum of WT, *Jnk1*$^{-/-}$ *and Jnk2*$^{-/-}$ mice. After 6 hrs the peritoneal cavities were lavaged and neutrophils were quantified. Neutrophil recruitment was reduced in the *Jnk2*$^{-/-}$ mice, but not the *Jnk1*$^{-/-}$ mice (Fig. 4B). The *Jnk2*$^{-/-}$ neutrophils are functionally deficient in both isoforms of JNK as mature neutrophils express minimal detectable JNK1 (25). However, *Jnk1*$^{-/-}$ mice had a significantly reduced influx of macrophages, assessed 3 days after injecting TG or zymosan into the peritoneum suggesting that there is little redundancy in JNK1 and JNK2 in macrophage migration (Fig. 4B). TG elicited JNK1-deficient peritoneal macrophages also did not migrate well when stimulated *in vitro* by non-specific factors in FCS relative to WT macrophages (Fig. 4C). Migration impairment was not due to a decrease in surface integrin expression (Supplementary Fig. 2).

As FCS is not a defined stimulant, we also evaluated migration in response to MCP-1, which is a major chemoattractant for monocytes that acts by binding to a specific cell-surface receptor, CC-chemokine receptor-2 (CCR2). We confirmed a previous report that CCR2 ligation with MCP-1 results in JNK phosphorylation (27) (Fig. 5A). JNK1-deficient macrophages were also compromised in their general migratory response to this stimulus, although their directional response was not directly tested (Fig. 5B). We excluded the possibility that the secretion of MCP-1 from JNK1-deficient fibroblasts or macrophages was impaired; there were no differences in MCP-1 amounts in the peritoneal lavage fluid *in vivo* after TG injection (Fig. 5C). We also could not detect differences in secretion of MCP-1 from peritoneal macrophages and fibroblasts *in vitro* after stimulation with LPS, IL-1β or TNF (Fig. 5D). In addition there were no differences in CCR2 expression between WT and JNK1-deficient macrophages (Supplementary Fig. 3).

Effect of a permeable peptide JNK inhibitor in the AIA model

To test the therapeutic potential of targeting JNK, we used a specific JNK inhibitor that interferes with both JNK1 and JNK2 signaling to treat AIA. The inhibitor is a short D-amino acid peptide (D-JNKi) that has been modified to be cell permeable and has been used to reduce insulin resistance (28), TNF-induced liver failure (28), and hypoxia-induced retinopathy (11). This peptide inhibitor is a more specific JNK inhibitor than the previously reported small molecular weight inhibitor SP600125 (21). D-JNKi and a control peptide (TAT peptide) were injected i.p. (20μg/g/day) daily starting on day 4 after injecting mBSA in the knee joint. D-JNKi treatment successfully interfered with joint inflammation and destruction (Fig. 6A). Although the cellular infiltration was reduced, this inhibitor also reduced the cytokine production from *in vitro* stimulated BMDM (Fig. 6B and C). The D-JNKi treatment did not significantly alter T cell proliferation or antigen specific cytokine secretion (Supplementary Fig. 4). These results suggest that the peptide inhibitor has a greater effect than inhibiting JNK1, and inhibiting both JNK1 and JNK2 is impacting both innate immune cell migration and cytokine production.

DISCUSSION

In chronic arthritis, inflammation is perpetuated by continued recruitment of inflammatory cells to the synovium. There is an initial breech of tolerance to joint-associated self-antigens that might precede the onset of disease by years (29). The acute presentation of joint swelling may involve additional factors, including innate cellular subsets. Histological analysis of synovial samples from RA patients and the profile of inflammatory cytokines in the synovial fluid suggest that macrophages play a key role (30,31). Although biological therapies have targeted cytokines that are produced by macrophages or their receptors, including IL-1β, TNF and IL-6, they are not effective in all patients (2). Another strategy that could successfully intercede with the pathogenesis of inflammatory arthritis would be the use of small molecule inhibitors that disrupt the continued recruitment of inflammatory cells to the synovium and thereby limit the amounts of destructive enzymes and proinflammatory cytokines in the local joint environment.

We sought to examine the efficacy of inhibiting the two major JNK isoforms, JNK1 and JNK2. Using a T cell- and macrophage-dependent arthritis model we examined the impact of JNK1 or JNK2 ablation on pathogenesis. Importantly, JNK1 and JNK2 were not redundant. Where the disruption of *Jnk1* resulted in a reduction in inflammatory infiltration into the synovium and a consequent reduction in bone erosion (Fig. 1A) no effects were seen with *Jnk2* ablation. The role of JNK in T cell priming remains controversial (18,19,22,23). JNK1 deficiency might skew the Th2 phenotype of $CD4^+$ T cells (23) and diminish their proliferative capacity (19). However, it was also shown that alterations in T cell priming were not intrinsic to the T cells and instead were dependent on macrophage behavior (24). In our study we evaluated the lymphocytes for their antigen specific responses to mBSA and found that specific $Jnk1^{-/-}$ splenocytes proliferated to the same extent as WT counterparts. Similarly we did not detect a difference in the antibody profiles of $Jnk1^{-/-}$ or $Jnk2^{-/-}$ mice. As mature neutrophils have little if any expression of JNK1 we investigated macrophages as the cellular subset primary affected by JNK1 deficiency (25).

Previous reports described the contribution of macrophages to mBSA-induced arthritis (32,33). Clodronate-mediated ablation of macrophages reduced inflammation and joint destruction in rodents (32). In the AIA model, destruction of bone is due to the release of IL-1, and is not FcγR-dependent (34–36), suggesting that macrophages and not auto-antibodies might play a key role in the chronic phase of disease. Indeed, specific targeting of macrophages severely limited the overall inflammatory effector response (32,33). Although $Jnk1^{-/-}$ macrophages retain the ability to express cytokines and chemokines, we found a

substantial limitation in macrophage migration and recruitment to the synovium in JNK1-deficient mice. Migration of different cell types such as endothelial cells (37), neutrophils (26) or keratinocytes (12) has been associated with JNK1/2 activation. The labile adhesions required for rapid cell migration in response to a variety of stimuli were attributed to the JNK-mediated phosphorylation of paxillin (12). In our study, macrophage migration following different stimuli was significantly impaired in the absence of JNK1. However, neutrophil migration was unaffected as evidenced by their rapid influx to the peritoneum of JNK1-deficient mice following TG and zymosan A injection. This result is not surprising as mature murine neutrophils predominantly express JNK2 and not JNK1 (25).

The inhibition of macrophage migration by targeting MCP-1 with a biological or small molecule CCR2 inhibitor is not effective in RA (38,39). The synovium contains other monocyte or macrophage chemoattractants (40,41). Other receptors or chemokines permit the recruitment of macrophages and a strategy for targeting the converging intrinsic mechanisms of macrophage migration might be more effective. An approach that targets selected protein kinases with small molecules might be effective in multiple pathogenic steps. Here we utilized a cell permeable peptide that reduces the activities of both JNK1 and JNK2. This peptide was effective when initiated after the onset of disease as a treatment and reduced the inflammation and joint destruction in this model. The efficacy of the D-JNKi peptide might not be solely attributable to JNK1 inhibition, as this peptide reduces the activity of both JNK1 and JNK2 and there might have been additional off target effects (21).

The inhibition of both JNK isoforms would likely have pluripotent effects, not only on hematopoietic cells but also on FLS, and endothelial cells. This inhibitor might not only limit the migration of macrophages, but also can reduce inflammatory cytokine and MMP production (9,15), and angiogenesis (11). This notion is supported by a recent report that targeted deletion of both JNK1 and JNK2 in hematopoietic cells markedly reduced TNF production in a hepatitis model (42). Most of JNK functions are redundant between JNK1 and JNK2, as suggested in TNF-transgenic mice, in which JNK1 was not essential for inflammation (14). A dual inhibitor would more potently reduce inflammation as seen with the D-JNKi treated mice compared to the mice genetically disrupted in a single JNK isoform (Figure 1B and 6A). In summary, the JNK1 deficiency or blockade limited the migration of macrophages and reduced joint inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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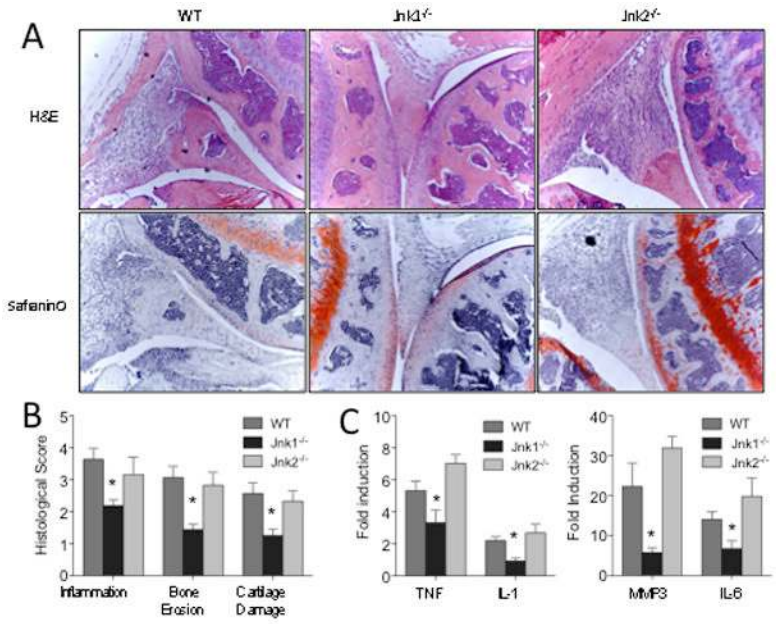

Figure 1. JNK1 is critical for effector function in AIA

A) Representative H&E and Safranin O stained sections of knee joints on day 10 of AIA induction in WT and JNK-deficient mice (n=14–16/group). Original magnification 200x. B) Sections above were scored for inflammatory infiltration, bone erosion and cartilage damage. *Jnk1*$^{-/-}$ mice had significantly lower scores than WT and *Jnk2*$^{-/-}$ mice. Results are expressed as means ± SEM.* p< 0.05 vs. WT and *Jnk2*$^{-/-}$ mice. C) RNA samples from whole joints (6 mice/group) were analyzed by qPCR in triplicate for expression of the indicated genes. mRNA amounts were normalized to 18S rRNA and fold induction is relative to PBS-injected joints of the same genotype. Induction of cytokine and MMP3 expression was less in *Jnk1*$^{-/-}$ mice. Results are expressed as means ± SEM.* p< 0.05 vs. WT and *Jnk2*$^{-/-}$ mice.

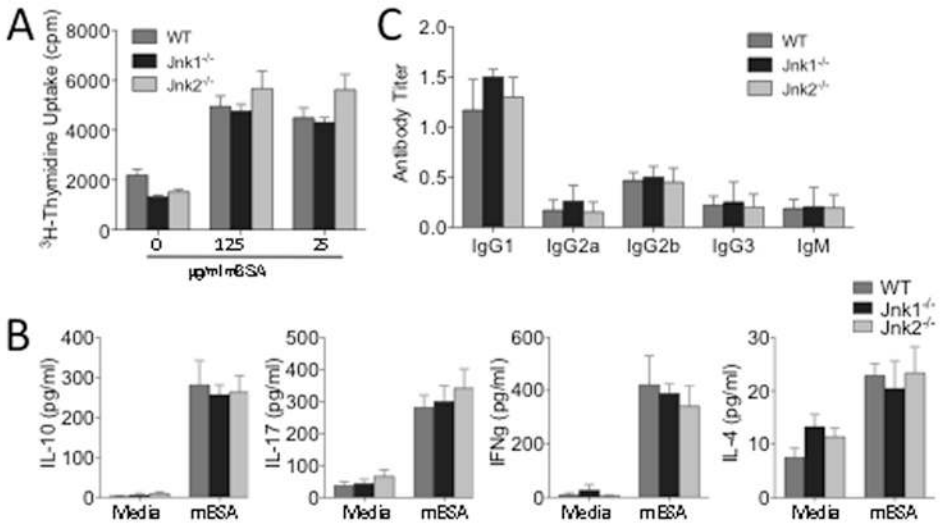

Figure 2. Adaptive immunity is preserved in JNK1 deficiency

A) Cellular immunity was determined by measuring ^{3}H-thymidine incorporation (cpm) after *in vitro* restimulation of splenocytes from arthritic mice with mBSA. (B) Splenocytes were restimulated *in vitro* with mBSA and supernatants harvested after 48 hrs. IL-10, IL-17, IFNγ, and IL-4 were assayed by ELISA. (C) Humoral immunity was determined by measuring amounts of anti-mBSA antibody isotypes by ELISA. Results are expressed as means ± SEM for 6 mice/group.

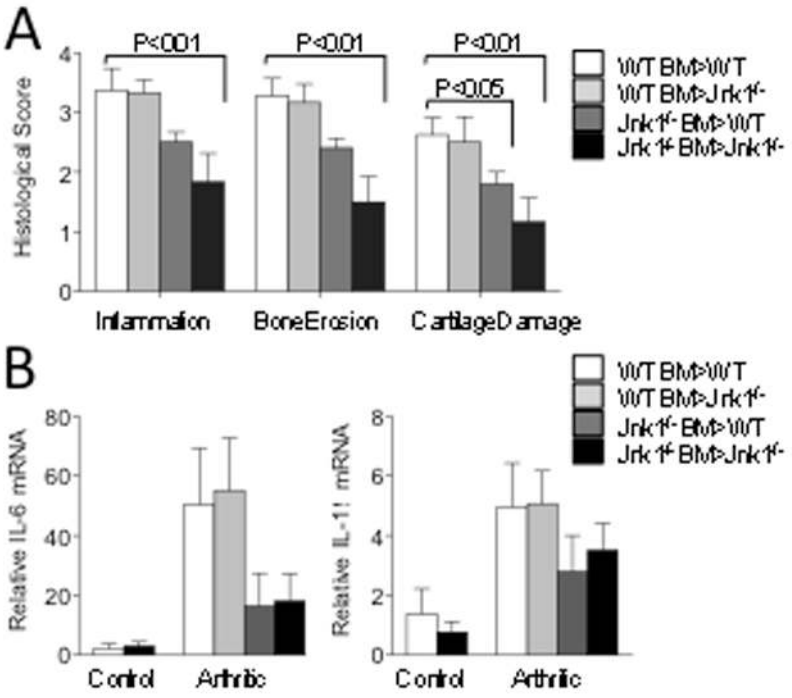

Figure 3. Arthritis depends primarily on JNK1 in bone marrow-derived cells

A) WT and *Jnk1*$^{-/-}$ mice (n=8/group) were irradiated and reconstituted with *Jnk1*$^{-/-}$ or WT bone marrow as indicated. AIA was induced 8 weeks after bone marrow reconstitution and mice were analyzed 10 days later. One knee from each mouse was prepared for histologic scoring with H&E and safranin O staining. Shown are average inflammation, erosion, and cartilage damage scores ± SEM. B) Whole joint RNA was extracted and amounts of mRNAs were analyzed by qPCR. Cytokine mRNA expression decreased in mice reconstituted with *Jnk1*$^{-/-}$ bone marrow. Results are expressed as means ± SEM for 5 mice/group.

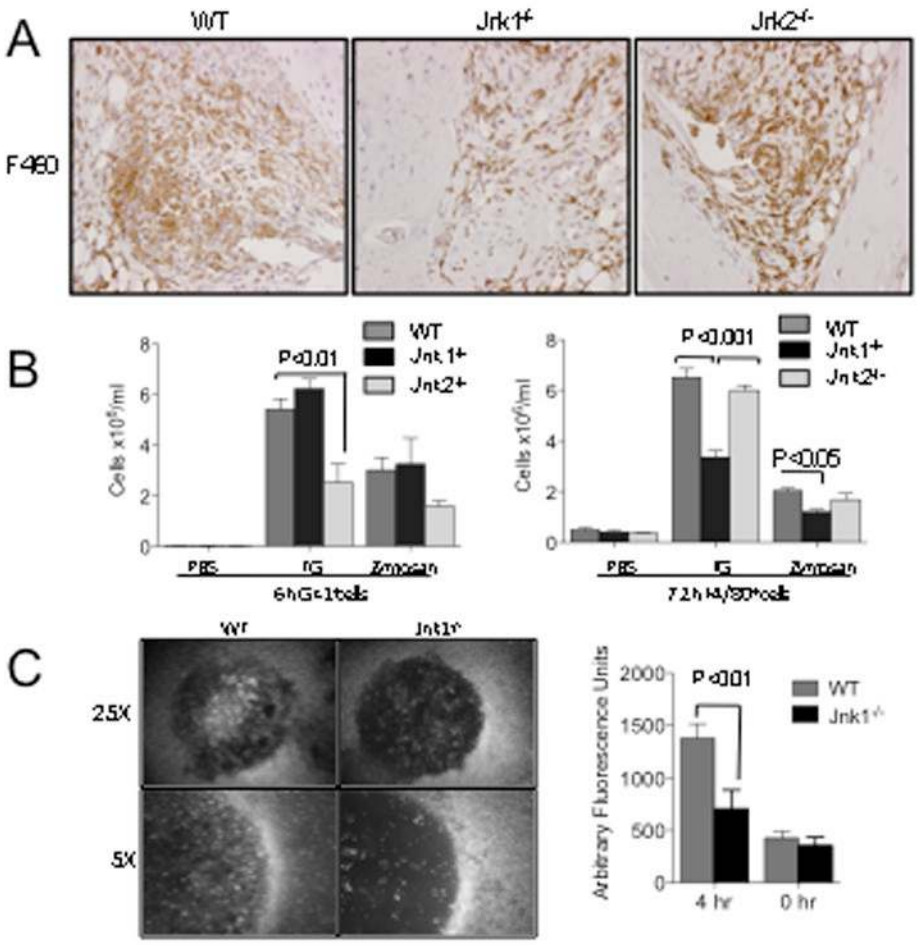

Fig. 4. JNK regulates macrophage migration

A) Knees were harvested on day 10 after mBSA injection and immunostained with F4/80. Original magnification 200x. Shown are representative stains for 5 samples/group. Fewer F4/80^{+} cells were present in joints of arthritic *Jnk1*$^{-/-}$ mice. WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice (n=4–6/group) were i.p. injected with TG or zymosan A. B) The peritoneum was lavaged after 6 hrs and neutrophil influx was determined by cell count and flow cytometry (Gr-1^{+}F4/80^{-}). Alternatively the peritoneum was lavaged after 72 hrs and macrophage influx was determined as above (F4/80^{+}Gr-1^{-}). Shown are means ± SEM. C) TG- elicited peritoneal macrophages were dispersed into 96 well plates with stoppers that obscured the center of the wells overnight. Most of the stoppers were removed and fresh medium with 10% FCS was added. After 4 hrs the cells were fluorescently stained and the central fluorescence measured through a perforated mask provided in the kit. Shown are representative wells and the relative fluorescence measured from the center of the wells. *Jnk1*$^{-/-}$ macrophages migrated significantly less than WT macrophages. Results are expressed as means ± SEM. Representative of four independent experiments.

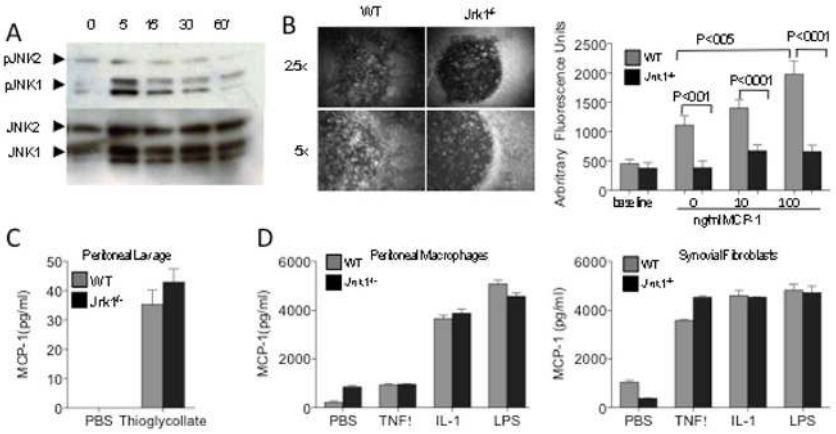

Figure 5. JNK1-deficient macrophages migrate poorly after MCP-1 stimulation

A) Peritoneal macrophages were stimulated *in vitro* with 100 ng/ml MCP-1 for the indicated times. Cells were lysed and analyzed by immunoblotting for presence of phospho-JNK1/2. B) TG-elicited peritoneal macrophages were dispersed into 96 well plates with stoppers overnight. Most of the stoppers were removed and graded amounts of MCP-1 were added to the medium. Migration into the central clearing was assessed by fluorescently staining the cells after 4 hrs. Baseline fluorescence was measured by removing the stoppers from control wells at 4 hrs. Results are expressed as means ± SEM. Shown are representative examples of macrophage migration into the central area, and the relative fluorescence of wells compared to wells where the stopper was removed at the end of the experiment. *Jnk1*$^{-/-}$ macrophages migrated significantly less than WT macrophages. Representative of four independent experiments. C) MCP-1 amounts in lavage fluids were assayed by ELISA. Shown are means ± SEM for 5 mice/group. D) MCP-1 amounts in peritoneal macrophages (5×10^5 cells/ml) and fibroblasts (plated at 1×10^5 cells/ml) supernatants, 24 hrs after stimulation with IL-1β (1 ng/ml), TNF (100 ng/ml) and LPS (50 ng/ml),. MCP-1 was measured in triplicate by ELISA.

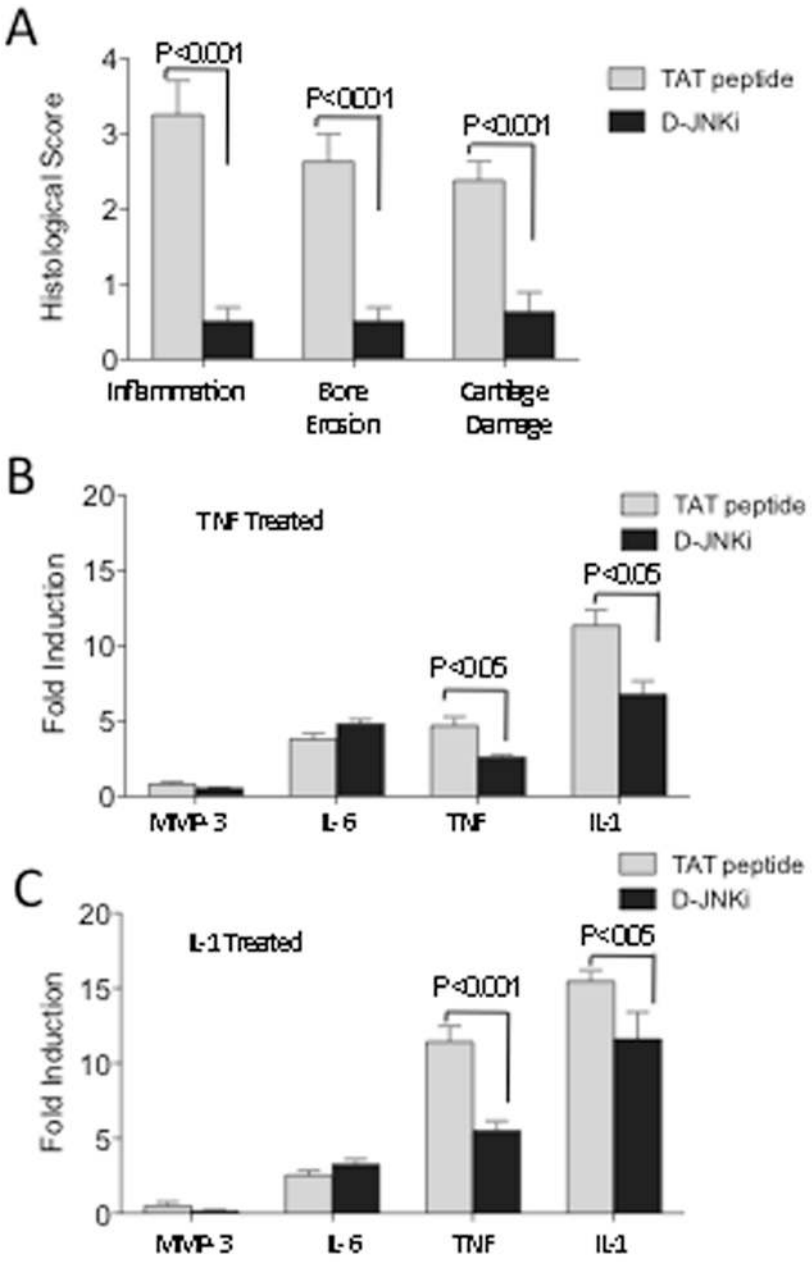

Figure 6. D-JNKi treatment successfully abrogated joint inflammation and destruction

A) AIA was induced in WT mice. D-JNKi or TAT peptides diluted in PBS were injected i.p. at 20 μg/g body weight daily starting on day 4 after mBSA or saline i.a. injection until day 10. Both knees from each mouse were prepared for histological scoring by H&E and safranin O staining. Shown are the average inflammation, erosion and cartilage damage scores ± SEM for 8 mice/group. Sections from D-JNKi treated mice had significantly lower scores (Mann Whitney U test). BMDM were pretreated with 10μM TAT peptide or D-JNKi for 1 hr and then stimulated with B) TNF (10 ng/ml) or C) IL-1β (2 ng/ml) for 4hrs. The cells were lysed, RNA extracted and analyzed by qPCR for expression of the indicated amplicons. The fold induction relative to unstimulated BMDM are shown. Data are pooled from two experiments and significance assessed by Mann Whitney U test.