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The JNK Are Important for Development and Survival of Macrophages

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We report in this study that activation of the JNK by the growth factor, CSF-1 is critical for macrophage development, proliferation, and survival. Inhibition of JNK with two distinct classes of inhibitors, the pharmacological agent SP600125, or the peptide D-JNKI1 resulted in cell cycle inhibition with an arrest at the G2/M transition and subsequent apoptosis. JNK inhibition resulted in decreased expression of CSF-1R (c-fms) and Bcl-xL mRNA in mature macrophages and repressed CSF-1-dependent differentiation of bone marrow cells to macrophages. Macrophage sensitivity to JNK inhibitors may be linked to phosphorylation of the PU.1 transcription factor. Inhibition of JNK disrupted PU.1 binding to an element in the c-fms gene promoter and decreased promoter activity. Promoter activity could be restored by overexpression of PU.1. A comparison of expression profiles of macrophages with 22 other tissue types showed that genes that signal JNK activation downstream of tyrosine kinase receptors, such as focal adhesion kinase, Nek-interacting kinase, and Rac1 and scaffold proteins are highly expressed in macrophages relative to other tissues. This pattern of expression may underlie the novel role of JNK in macrophages. The Journal of Immunology, 2006, 176: 2219–2228.

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itogen-activated protein kinases form part of a signaling cascade linked to many aspects of cell function, including proliferation, differentiation, and apoptosis. In mammals, three major groups have been identified; the ERK and p38 groups of MAPks and the JNK also termed stress-activated protein kinases. JNK can be activated by a number of cytokines (e.g., CSF-1, GM-CSF, TNF-α, and IL-1) and many forms of environmental stress (1–3). The JNK group of protein kinases is encoded by three genes; the jnk1 and jnk2 genes, which are ubiquitously expressed, and the jnk3 gene, restricted in its expression to brain, heart, and testis. All three genes show alternative splicing and encode proteins with and without a C-terminal extension, resulting in 46- and 54-kDa isoforms (4). JNK can phosphorylate Src/Thr-Pro motifs in the activation domains of the transcription factors c-Jun, JunB, JunD, and activating transcription factor-2, resulting in enhanced transcriptional activation (3, 5).

Targeted disruption of either the jnk1 or jnk2 genes resulted in viable mice that exhibit defects in apoptosis and immune responses. These mice were immune deficient due to defects in effector T cell function. There was no apparent defect in T cell proliferation or IL-2 secretion. Instead, T cells failed to differentiate into Th1 effector cells, mediated in part by decreased IFN-γ production (6, 7). Disruption of both jnk1 and jnk2 genes caused early embryonic death associated with decreased apoptosis in the hindbrain and increased apoptosis in the forebrain (8).

JNK can also phosphorylate the PU.1/Spi-1 and Ets-2 transcription factors, important regulatory factors for the development and function of the monocyte/macrophage lineage (9, 10). PU.1 is critical for differentiation of macrophages and is required for expression of numerous macrophage-specific genes, including expression through the macrophage-specific promoter of the c-fms gene (11, 12). Targeted disruption of the PU.1 gene resulted in a block in myeloid and B lymphocyte development (13). Most studies of JNK activation in macrophages have examined their role in activation of inflammation-associated genes by agonists such as LPS. We and others have considered the importance of JNK in lineage-specific growth in response to the cytokine, CSF-1 (10). CSF-1 is required for growth, survival, and differentiation of macrophages and can be found at biologically active concentrations in circulation, where it is critical for survival of mature nondividing macrophages (14). CSF-1 acts through specific binding to the high-affinity CSF-1R, encoded by the c-fms proto-oncogene. Binding leads to receptor dimerization and autophosphorylation of tyrosine residues resulting in activation of multiple signal transduction pathways, including the Ras and PI3K pathways (15–17). Previous work has shown that the p54 isoform of JNK can interact with the Akt/PKB2 (protein kinase B) kinase and catalyze the phosphorylation of Ets-2 in macrophages in response to CSF-1 (10). Ets-2 appears to be involved in CSF-1-dependent survival of macrophages. Overexpression of Ets-2 enhanced survival, and expression of a dominant-negative form of Ets-2 decreased survival upon withdrawal of CSF-1 in macrophages from transgenic mice (18, 19).

The level of functional redundancy in jnk genes and lethality in jnk1−/− jnk2−/− mice complicates the study of JNK in cells, such as macrophages, that express multiple isoforms. A cell-permeable pharmacological inhibitor, SP600125, is available that showed >300-fold selectivity for JNK isoforms over the related MAPks, ERK1, and p38-2 (20). A specific peptide inhibitor has also been developed, based on the JNK-interacting protein, islet-brain-1. This peptide contains the 20-aa sequence that represents the minimal conserved domain of islet-brain-1 capable of inhibiting JNK activity and blocking IL-1-dependent apoptosis in pancreatic

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2 Abbreviations used in this paper: PKB, protein kinase B; BMM, bone marrow-derived murine macrophage; EGFP, enhanced GFP; HPRT, hypoxanthine phosphoribosyltransferase; f, forward; r, reverse; FAK, focal adhesion kinase.
β-cells. Active transport into cells is achieved by fusion of the 11-aa HIV-TAT sequence that directs cellular import. This peptide retains activity when all amino acids are present as D-enantiomers (D-JNK1), and this configuration results in molecules that are stable within cells for at least a week (21).

In this study, we used the above inhibitors to show that the JNK group of MAPKs are important for differentiation, growth, and survival of macrophages in response to CSF-1. We provide evidence that at least some of these actions are mediated through the transcription factor, PU.1, the stability of which is dependent on JNK activity.

Materials and Methods

Analysis of cell cycle and apoptosis

Bone marrow cells were isolated from the femurs of adult C57/black mice and cultured in RPMI 1640 medium containing penicillin-streptomycin antibiotics (Invitrogen Life Technologies). Cells were differentiated to macrophages by a 5-day treatment with 1000 U of human CSF-1 (Chiron). Peritoneal macrophages were isolated by lavage of the peritoneal cavity of mice with PBS and culturing with CSF-1 overnight before removal of nonadherent cells. Bone marrow-derived macrophages were rinsed in PBS, and 2 × 10⁶ cells were plated in fresh medium for 4 h in the absence of CSF-1 to synchronize the cell cycle. BMs were then treated with the DMSO vehicle or the PD98059, SP600125, or LY294002 inhibitors (Biomol) for 30 min before addition of CSF-1 for an additional 20 h. Concentrated stocks of 100 mM inhibitor were used to minimize addition of DMSO. For the D-JNK1 inhibitor (Alexis Biochemicals) and aquiferin control, 2 × 10⁵ cells were plated in 200 μl PBS with 100 μM inhibitor for 1 h before dilution in medium to a final concentration of 10 μM and cultured for 3 h before addition of CSF-1 for an additional 20 h. The TAT peptide alone was previously tested on BMs and found to have no effect on growth and survival (data not shown). After 20 h of treatment, cells were rinsed with PBS, permeabilized with 70% ethanol, and stained with propidium iodide according to established protocols. Cells were analyzed on a FACStar flow cytometer (BD Biosciences). Caspase 3 and caspase 7 activity was measured by plating 1 × 10⁶ cells/well into 96-well plates in the presence of CSF-1. Cells were treated for 12 h with the above inhibitors before lysis and measurement of caspase activity using the Caspase-Glo assay system (Promega) with a Trilux plate luminometer according to the manufacturer’s protocol.

Analysis of cell differentiation

Bone marrow cells were isolated from the femurs of Mac-Green transgenic mice (22) and treated with CSF-1 and DMSO or 10 nM SP600125. The media, CSF-1, and inhibitor were changed every 2 days. Cells were analyzed for enhanced GFP (EGFP) expression at day 0, day 2, and day 4 posttreatment by flow cytometry. After 4 days, SP600125 was removed from cells by rinsing with PBS twice, cultured in RPMI 1640 medium with CSF-1, and analyzed for EGFP expression immediately after removal of inhibitor or after 2 days and 4 days of culture. Bone marrow cells from wild-type mice were cultured as described above, but stained with CD11b and F4/80 directly conjugated to PE and FITC, respectively, before analysis (Sorotec).

EMSA and Western blot

BMNs were treated with DMSO or 20 μM SP600125 for 12 h before isolation of nuclei and extraction of protein according to established protocols (23). The concentration of protein in nuclear extracts was determined according to the manufacturer’s protocol. Aliquots of 10 μg of nuclear extracts were electrophoresed across all the tissues.

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Western blotting

nuclear extracts, 25 ng of poly(dI-dC), and −0.1 ng of 32P-labeled probe for 20 min at room temperature. Protein binding was resolved on 5% minipolyacrylamide gels in 0.5 X Tris-borate-EDTA (45 mM Tris base, 54 mM boric acid, and 1 mM EDTA) for 1 h at 100 V. For Western blot, BMNs were treated with DMSO or 10, 20, and 40 μM SP600125 for 12 h before lysis of whole cells and, protein was quantitated by BCA protein assay. Five micrograms of protein from each sample was resolved on precast 12% polyacrylamide gels (Invitrogen Life Technologies) before blotting onto PVDF membrane. Blots were stained with specific Ab to either PU.1 (Santa Cruz Biotechnology), c-Jun (Delta Biolabs), or c-Jun phosphorylated on serine 63 (Cell Signaling). Bands were visualized using a HRP conjugate Ab (Cell Signaling) and chemiluminescence substrate (Amer sham). Bands on x-ray film were quantitated using a Bio-Rad GS 800 densitometer.

RNA isolation and quantitative PCR

Total RNA was prepared using RNeasy isolation kits (Qiagen) according to the manufacturer’s instructions. RNA was treated with DNase 1 (Ambion) and reverse transcribed to cDNA using Superscript reverse transcriptase (Invitrogen Life Technologies). Negative control samples (no first-strand synthesis) were prepared by performing reverse transcription reactions in the absence of reverse transcriptase. cDNA levels of murine hypoxanthine phosphoribosyl transferase (HPRT), CSF-1R, PU.1, Ets-2, and Bcl-xL were quantitated by SYBR Green, real-time PCR using an ABI Prism 7700 sequence detector (Applied Biosystems) according to the manufacturer’s instructions. Amplification was achieved using an initial cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 50°C for 1 min. Specificity of PCR was checked by gel electrophoresis and melting curve analysis. cDNA levels during the linear phase of amplification were normalized against HPRT controls. Determinations were made in duplicate, and mean ± SD was determined. Real-time primers (f, forward; r, reverse) used to detect expression of the corresponding murine genes were as follows; c-fms f: CCAACATCCACTTTGATGCAAAGAT, r: CTCAACACTGTCACCTCTGTT; Bcl-xL f: AGTACCACCTAGGCTTGG, r: AGGAACAGCGGTGAAGCAGC; PU.1 f: GTTCAGGTTTGGCAAG, r: TTAGCTACCTCGCCGTTGCT; Ets-2 f: GTACAGTGGCTTCCTAGTTGGA, r: AGCTGATGAAAGATGGAAGCAGC; HPRT f: GCAGTACAGCCCCAAAATGG, r: AACAAGCTCGGCTGTATCCAA.

Luciferase reporter assay

RAW264.7 cells were cultured in RPMI 1640 medium described above. The pGL3.3fms, pPCEC-PU.1, and pTK109 plasmids have all been previously described (24). (Cells 5 × 10⁶) in 400 μl of RPMI 1640 medium containing 20 mM HEPES and 5 μg of reporter plasmid or 5 μg of reporter plasmid with 10 μg of pPUEPC plasmid were electroporated at 280 V and 1000 μFarad capacitance on a Bio-Rad Gene Pulsor (Bio-Rad). A total of 1 × 10⁵ transfected cells/well were plated into multiple wells from a 24-well tissue culture plate and allowed to recover for 24 h before addition of the DMSO control or SP600125 to the media. For wells treated with the D-JNK1 inhibitor and aqueous control, media was removed after recovery, and 100 μl of PBS with or without peptide was incubated for 1 h before addition of 1 ml of medium to bring the inhibitor concentration to 10 μM. After 12 h of inhibitor treatment, cells were lysed and assayed for luciferase activity according to the manufacturer’s protocol (Roche). The concentration of protein was determined by BCA assay, and the level of luciferase activity was given as relative light units and calculated as light units per microgram of protein assayed.

Microarray analysis of tissues

Murine kinases tissue microarray expression profile data was retrieved from the RIKEN Expression Array Database (READ) ([http://read.gsc.riken.go.jp/]) as described previously (25). Hierarchical clustering was performed using the Cluster program from Stanford University (de-fault set-up) and visualized using the TreeView program from Stanford University ([http://genome-www5.stanford.edu/MicroArray/SMD/]). From microarray data retrieved from the READ database. The gene list was extracted from the macrophage-enriched cluster of genes. The experimenal design, using 17.5dpC C57BL/6J embryo as a common reference, has been described previously (25). Briefly, cDNA was indirectly labeled with aminooxy-conjugated Cy5 (tissues) or Cy5 (embryo), and hybridized overnight to RIKEN 15-kb single-strand cDNA microarray experiments (Molecularware (Digital Genome)) was used to process the image, and data was corrected for local background, and confidence status flagged for empty spots, signal/noise ratio, spot/CV ratio, and spot morphology. The data were normalized as follows: the ratio of the experimental signal/the control signal for each spot was calculated; and intensity-dependent normalization was also applied, where the ratio was reduced to the residual of the Lowess fit of the intensity vs ratio curve (26). The dataset was restricted to those spots passing confidence status on the control channel of every hybridization, across all the tissues.
Results

JNK activity is required for macrophage proliferation and survival

In contrast to the report by Jaworowski et al. (27), we and others have found that addition of CSF-1 to factor-dependent BMMs caused a rapid activation of both JNK and ERK MAPKs (1, 10). ERK activity is required for maximal proliferative response of these cells to CSF-1 (1). MAPK inhibitors were used to determine whether loss of either JNK activity or ERK activity can impact on proliferation and survival of these cells. The JNK inhibitor (SP600125) described above was used to repress JNK activity, and a similar pharmacological inhibitor PD98059 was used to repress ERK activity. PD98059 is a specific inhibitor of the ERK-selective MAPK kinases, MEK1 and MEK2 and inhibits ERK-1 and ERK-2 by blocking MEK activity (28). The specific PI3K inhibitor, LY294002 was used as a positive control. LY294002 inhibits all isoforms of PI3K equally and does not inhibit related kinases such as PI4K or MAPKs (29, 30). The D-JNKI1 peptide that blocks the active site of JNK was also used to inhibit JNK activity. BMMs were briefly starved of CSF-1 for 4 h to partially synchronize the cell cycle, then treated with inhibitor and CSF-1 for 20 h before analysis of cell cycle and cell death by propidium iodide staining and flow cytometry. A dose response was performed with SP600125 and was based on the levels used (20 μM) to inhibit c-Jun phosphorylation and AP-1 binding in fibroblast-like synoviocytes and the levels required for high-level inhibition of c-Jun phosphorylation in BMMs (20). The high dose, 100 μM concentration of PD98059 and LY294002 was selected to give complete inhibition and was higher than the amount previously shown to give maximum inhibition in PBMC or BMMs, respectively (1, 10).

Inhibition of either JNK or ERK kinases resulted in a significant arrest of cells in G1 (Fig. 1, B and D). Growth arrest as a result of JNK inhibition could be distinguished from ERK inhibition by a specific arrest in the G2/M transition. Treatment with 10 μM SP600125 produced an increase in the proportion of cells within G1, with a corresponding decrease in cells at S phase and G2. Higher levels (20 μM) produced a distinct spike in the number of cells at the G2/M transition mainly due to a further decrease in cells in S phase (Fig. 1C). The G2 arrest was also seen in BMMs treated with the peptide inhibitor, D-JNKI1; however, the arrest at G1 was less than in SP600125-treated cells (Fig. 1F). This result may partially involve accumulation of cells at G2 because the number of cells in S phase was decreased by D-JNKI1 treatment. A reduced accumulation of cells in G2 with D-JNKI1 compared with treatment with the highly cell-permeable SP600125 inhibitor may reflect differences in the rate of SP600125 uptake. Treatment with 100 μM ERK inhibitor (PD98059) or PI3K inhibitor (LY294002) caused a significant reduction of cells within both S and G2 phases of the cell cycle.

Activation of the ERK pathway has previously been shown to be important for cell survival in response to a variety of cell signals (31–33). Our experiments with BMMs showed that signaling by ERK kinases was not required for cell survival in response to CSF-1. Inhibition of ERK kinases with maximal levels of inhibitor (PD98059) failed to alter the rate of cell death in CSF-1-stimulated BMMs. By contrast, JNK inhibitor (SP600125) produced a dose-dependent increase in the rate of cell death with the maximum dose (40 μM), causing a 22-fold increase in the proportion of cells undergoing DNA degradation associated with late stages of cell death (<2N DNA; Fig. 2A). Similarly, there was a 7-fold increase in the rate of cell death when BMMs were treated with 10 μM D-JNKI1, peptide inhibitor (Fig. 1H).

To verify that the increased proportion of cells with low DNA content reflected cell death by apoptosis, BMMs or macrophages isolated from the peritoneum were treated with the above inhibitors and assayed for increases in caspase 3 and caspase 7 activities. Macrophages that are deprived of CSF-1 undergo programmed cell death, and we have found that macrophage apoptosis was associated with increased activity of caspase 3 and 7 (Fig. 2, B and C). In addition to BMMs, peritoneal macrophages were used in this assay because sufficient numbers can be flushed from the peritoneum, and these cells represent terminally differentiated tissue macrophages. Additionally, these cells are postmitotic, so the effect of inhibitors is separated from any secondary consequences of antiproliferative activity. There was a dose-dependent increase in
apoptosis with JNK inhibition in both populations. SP600125 produced a 15-fold increase in caspase activity in BMMs and an 8.2-fold increase in peritoneal macrophages. D-JNKI treatment resulted in a 9-fold increase in BMM and a 3.8-fold increase in peritoneal macrophages (Fig. 2, B and C). The consistent loss of BMM survival with two distinct inhibitors of JNK provides strong evidence that JNK activity is required for macrophage survival in response to CSF-1.

The PI3K pathway has previously been shown to be essential for BMM survival and may feed into JNK activation by activation of Akt (10). Inhibition of PI3K with LY294002 resulted in a 45-fold increase in apoptosis. Combined inhibition of both JNK (SP600125) and PI3K (LY294002) resulted in an additive increase (75-fold; Fig. 2A) in the rate of apoptosis. This result indicates that Akt was not the sole pathway for JNK activation and correspondingly that Akt can target other pathways required for macrophage survival.

The data obtained above focused on specific signal transduction pathways activated by the CSF-1R, hence CSF-1 was continuously present during the assay. Macrophages deprived of CSF-1 during the assay display a high rate of cell death that appeared to increase with SP600125 inhibition of JNK. This 1.5-fold increase in cell death was significantly lower than that seen with CSF-1 treatment (Table I). Whether this effect is due to loss of residual JNK activation by CSF-1 or by an outside stimulus such as serum factors or cellular adherence could not be distinguished. The macrophage-like RAW264.7 cell line, although growth factor independent, is transformed at a late stage of macrophage differentiation and displays a significant portion of the biological activity of mature macrophages. This cell line was tested for sensitivity to the JNK inhibitor, SP600125. The results showed a dose-dependent increase in cell death with JNK inhibition, with a 8-fold increase in the rate of cell death at 40 μM (Table I).

**JNK is required for terminal differentiation of macrophages**

We next considered whether JNK inhibitors can also inhibit the proliferation and differentiation of macrophage progenitor cells, as opposed to mature macrophages. Progenitor cells in bone marrow can be induced to differentiate to macrophages in vitro by treatment with CSF-1. To examine the effect of JNK inhibition on macrophage development, we treated the mixed population of bone marrow cells isolated from the femurs of mice with either DMSO vehicle or JNK inhibitor (10 μM SP600125) in the presence of CSF-1. For this analysis, the progress of macrophage differentiation was observed using cells from the Mac-Green transgenic mouse. In these mice, expression of an EGFP transgene is driven by macrophage-specific regulatory elements from the c-fms gene. Previous studies showed that the level of EGFP expression progressively increases as progenitor cells differentiate to macrophages, and macrophages and their precursors can be selectively identified within the mixed population of bone marrow cells by fluorescence (22).

Flow cytometry analysis of bone marrow cells stimulated with CSF-1 and JNK inhibitor showed that development of the high fluorescence population was strongly inhibited by loss of JNK activity (Fig. 3, A and B). The effect of reactivation of JNK activity in the 4-day-arrested bone marrow cells was determined because SP600125 is a reversible inhibitor, and sustained treatment with a dose of 10 μM JNK inhibitor appeared to be nontoxic to bone marrow cells and myeloid progenitors. Cells with inhibitor removed were then treated with CSF-1 for an additional 4 days. The arrested cells showed a progressive increase in fluorescence after the 4- day treatment with CSF-1, suggesting recovery of the ability to differentiate (Fig. 3C). The percentage of high fluorescent cells

| Table I. Effect of SP600125 on BMM and RAW264 survival |
|------------------- | --- | --- | --- | --- |
|                  | CSF-1 Starved BMM | RAW264.7 |
| SP600125 (μM)    | Percentage of apoptosis (mean ± SEM) | Percentage of apoptosis (mean ± SEM) |
| 0                 | 22.8 ± 4.6       | 1.6 ± 0.4     |
| 10                | 23.7 ± 3.2       | 2.2 ± 0.8     |
| 20                | 25.4 ± 6.2       | 8.3 ± 2.7     |
| 40                | 28.1 ± 8.2       | 12.6 ± 4.1    |

**FIGURE 2.** Dose-dependent increases in apoptosis in BMMs treated with either JNK or PI3K inhibitors. A, CSF-1-stimulated BMMs were treated for 20 h with either the DMSO vehicle, V, or 10, 20, or 40 μM SP600125 (JNK inhibitor) and 10, 30, or 100 μM PD98059 (ERK inhibitor) or LY294002 (PI3K inhibitor). Cells were also treated with a combination of 100 μM LY294002 and 10, 20, or 40 μM SP600125. The level of apoptosis in BMMs deprived of CSF-1 for 24 h (-) is shown as a reference. The columns on the graph represent the mean and bars the SE of three independent treatments. Values are given as the percentage of total cells that are apoptotic by propidium iodide assay (<2N DNA). B, CSF-1-stimulated BMMs or CSF-1-stimulated peritoneal macrophages (C) were treated for 12 h with SP600125 as described above or V, 5, 10, or 20 μM D-JNKII peptide inhibitor before lysis and assay for caspase 3 and 7 activity by luminescence reaction. Columns represent the mean and error bars the SEM of duplicate assays from three independent treatments.
was lower relative to differentiated cells that had never been exposed to JNK inhibitor, indicating that arrested cells may not fully regain normal development.

Ab specific for the myeloid cell surface markers, CD11b (Mac-1) and F4/80 were used to examine the developmental state of JNK inhibitor-arrested bone marrow cells. Arrested cells showed an intermediate level of EGFP fluorescence compared with fully differentiated control cells, suggesting that the arrest may occur at specific stages of myeloid development. The morphology of arrested cells was considerably different from normal BMMs, with cells failing to spread on tissue culture plastic. Despite this distinct morphology, the majority of arrested cells were positive for either CD11b or F4/80. The number of JNK inhibitor-treated cells negative for both markers increased by 13%, and the number positive for only the CD11b marker increased by 9% compared with untreated cells.

These data suggest that JNK activation becomes progressively more important for survival as macrophages mature, and that precursor cells become arrested at a preadherent stage of monocyte-macrophage development.

**JNK phosphorylation of PU.1 alters DNA binding**

The apparent selectivity of action by JNK inhibitors during macrophage differentiation suggested that JNK may effect macrophage-specific transcription. The transcription factor PU.1 is selectively expressed in macrophages and is involved in regulation of nearly all of the macrophage-specific promoters described so far (12, 34). Previous analysis of PU.1 phosphorylation by MAPKs showed that PU.1 was specifically phosphorylated by JNK and not by ERK (9). Furthermore, a MAPK phosphorylation site has been defined at serine 142 within the PEST domain of PU.1, which appears to regulate protein stability (35, 36). Western blot of protein from cells treated with JNK inhibitor was used to evaluate the effect of JNK on the level of PU.1, c-Jun, and phosphorylated c-Jun (Jun-P) expression. BMMs were treated with the DMSO vehicle or 10, 20, and 40 μM SP600125 for 12 h, and protein from whole cell extracts was analyzed with specific Ab. The results showed loss of the majority of c-Jun phosphorylation by treatment with 20 μM inhibitor (Fig. 5A). Interestingly, the amount of c-Jun protein expression did not appear to be significantly reduced by inhibition of JNK (Fig. 5B). JNK inhibitor caused a decrease in the amount of full-length PU.1, and an increase in lower m.w. PU.1 degradation products. The ratio of full-length PU.1 (FL) to proteolytically cleaved protein (Fr) was determined by densitometer scan, and this ratio decreased in a dose-dependent manner with JNK inhibition at 10 and 20 μM (FL:Fr; Fig. 5C). At the maximal dose of 40 μM, the level of total PU.1 protein significantly decreased. PU.1 is capable of autoregulating its own expression, and substantial loss of full-length protein may also reduce pu.1 gene expression (37). Similar results were obtained for PU.1 expression.

**FIGURE 3.** JNK inhibition arrests CSF-1-mediated differentiation of bone marrow cells. An EGFP reporter gene was used to monitor the stage of differentiation in CSF-1-treated bone marrow cells from transgenic mice. The lines on the graphs show the level of EGFP fluorescence in untreated bone marrow cells (day 0) or after 2 and 4 days of treatment as indicated on the graph. The 4-day treatments are in bold to emphasize level of fluorescence in differentiated cells. Bone marrow cells were treated with CSF-1 and the DMSO vehicle (A) or CSF-1 and 10 μM SP600125 (B). C, After 4 days of SP600125 treatment, the inhibitor was removed, and the cells were treated with CSF-1 alone for the number of days indicated on the graph. Data is representative of two independent experiments.

**FIGURE 4.** Ab staining of the CD11b and F4/80 myeloid cell surface markers. Direct immunofluorescence was performed using PE-conjugated Ab to CD11b and FITC-conjugated Ab to F4/80. Graphs show flow cytometry analysis of bone marrow cells differentiated to BMMs with CSF-1 for 4 days (A) or CSF-1-treated bone marrow cells for 4 days and arrested during differentiation with 10 μM SP600125 (B). Arrows show the direction of increasing fluorescence, and quadrants show the line between negative and positive fluorescence. The lower left quadrant was set to include 99% of unstained cells; the upper left quadrant was set to include 96% of cells stained for CD11b only; and the lower right quadrant was set to include 98% of cells stained for F4/80 only. The percentage gated of CD11b/F4/80 stained cells in each quadrant is shown below.
in the RAW264.7 cell line, with a significant loss in expression of full-length protein upon treatment with JNK inhibitor (Fig. 5C). EMSA was used to examine the effect of inhibition of JNK phosphorylation on DNA binding of PU.1. For this analysis, binding of the Sp1 transcription factor was used as a control because previous experiments showed stable constitutive binding that was not significantly altered by CSF-1 stimulation (data not shown). Nuclear extracts were produced from CSF-1-treated BMM cells with or without JNK inhibitor (20 μM SP600125) for 12 h. EMSA was performed using the PU.1 binding site from the c-fms gene promoter (38) or a well-characterized consensus Sp1 binding site (Promega). Protein complexes containing PU.1 were identified by the ability of PU.1-specific Ab to supershift the complex. The results were consistent with the loss of full-length PU.1 in Western blots. Bands that represent binding of full-length PU.1 decreased significantly with JNK inhibitor treatment, with a corresponding increase in binding of high-mobility protein fragments (Fig. 5D). We have shown previously that these products are the result of a defined proteolytic cleavage (39). The binding of protein complexes to the Sp1 consensus element was not significantly altered by treatment with JNK inhibitor. This result indicated that differences in transcription factor binding were not significantly attributed to changes in cell viability. A second low-mobility protein complex (X) was also sensitive to JNK inhibition, and SP600125 treatment resulted in loss of protein binding. The identity of the proteins in this complex is unknown but does not include PU.1 because the complex in untreated BMMs was not supershifted with PU.1 Ab.

**JNK is required for expression of PU.1-regulated genes**

The ability of JNK to enhance PU.1 stability and binding to gene regulatory elements suggested a role in the regulation of several genes that are required for macrophage differentiation, growth, or survival. PU.1 and Ets-2 have been shown to regulate expression of the c-fms and bcl-xL genes, and activation by PU.1 was critical for differentiation and survival of macrophages (40). The effect of JNK inhibition on gene expression was examined by quantitation of mRNA levels with or without treatment of BMMs with 20 μM SP600125. A time course was performed to examine both early and late changes in gene expression. The results showed that JNK inhibition significantly decreased expression of c-fms and bcl-xL mRNA within 2 h in BMMs. A significant level of repression was sustained throughout the 24-h treatment, with a maximum reduction of 4.2-fold for c-fms and 4.4-fold for bcl-xL at 7 h posttreatment with inhibitor (Fig. 6, A and B). JNK inhibition also resulted in decreased expression of both c-fms and bcl-xL in RAW264.7 cells, with a maximum reduction of 2.2-fold for c-fms and 3.3-fold for bcl-xL at 24 h posttreatment (Fig. 6, C and D).

The Ets-2 transcription factor has also been associated with macrophage survival and can be regulated by JNK phosphorylation. Both PU.1 and Ets-2 can activate transcription of Bcl-xL in macrophages, and the results above indicate that JNK activity is required for Bcl-xL expression. The expression analysis of PU.1 suggested that JNK regulation of PU.1 activity was primarily post-transcriptional through phosphorylation and stabilization of PU.1 protein. The above analysis was performed for PU.1 and Ets-2 in BMMs to determine whether JNK can regulate the level of mRNA expression of these genes. The results showed that JNK inhibition has a minor effect on PU.1 mRNA after sustained treatment and no effect on expression of Ets-2 mRNA at any time point post-treatment with inhibitor (Fig. 6, E and F). This expression pattern provides some evidence that JNK acts by direct modification of transcription factors, and the mechanism of JNK-mediated survival involves transcriptional activation of the c-fms and bcl-xL genes.

**JNK activity correlates with activation of the c-fms gene promoter**

The c-fms gene promoter contains a critical PU.1 binding site near the start of transcription, and PU.1 is required for promoter activity (11, 24). The ability of SP600125 to alter PU.1 binding to this
element and repress c-fms mRNA levels suggested that JNK regulated expression of the c-fms gene promoter through PU.1. For this study, the transfectable macrophage cell line, RAW264.7, was used because it is sensitive to JNK inhibitors.

To examine effects on PU.1-dependent transcription, either the pGL0.3fms luciferase reporter plasmid containing the minimal 300-bp mouse c-fms gene promoter or a control containing 109-bp of the HSV-TK promoter were transfected and cells treated with the DMSO vehicle or 5, 10, or 20 \( \mu \text{M} \) SP600125 for 2, 7, 12, or 24 h before isolation of total RNA. The level of c-fms (A), bcl-x\(_l\) (B), mRNA in RAW264.7 cells and c-fms (C) Bcl-x\(_l\) (D) in BMMs or the level of pu.1 (E) or ets-2 (F) mRNA in BMMs was measured by first-strand synthesis of cDNA with reverse transcriptase and SYBR Green, real-time PCR analysis. Values were calculated relative to an internal mRNA control, HPRT. Columns represent the mean and error bars the SEM of duplicate assays from three independent inhibitor treatments.

**FIGURE 6.** JNK regulates mRNA expression of specific genes. CSF-1-stimulated BMMs were treated with vehicle (0) or 20 \( \mu \text{M} \) SP600125 for 2, 7, 12, or 24 h before isolation of total RNA. The level of c-fms (A), bcl-x\(_l\) (B), mRNA in RAW264.7 cells and c-fms (C) Bcl-x\(_l\) (D) in BMMs or the level of pu.1 (E) or ets-2 (F) mRNA in BMMs was measured by first-strand synthesis of cDNA with reverse transcriptase and SYBR Green, real-time PCR analysis. Values were calculated relative to an internal mRNA control, HPRT. Columns represent the mean and error bars the SEM of duplicate assays from three independent inhibitor treatments.

by treatment with 10 \( \mu \text{M} \) D-JNKII (Fig. 7D). This repression of the c-fms gene by both classes of JNK inhibitors supported our data that PU.1 is an important downstream target of JNK in macrophages and provides a potential mechanism for JNK-induced differentiation of macrophages.

**Expression profiles of elements in JNK signal transduction pathways**

JNK activity is not normally associated with enhanced cell survival. In many cell types, activation of JNK leads to well-characterized apoptotic pathways. JNK may then play a unique role in macrophages, and this role may be related to JNK function in the inflammatory response. This distinction is supported by the above evidence that JNK was not required until later stages of macrophage differentiation. The link between selective use of MAPKs and cell function is also supported by loss of cell survival induced by the endotoxin, LPS upon inhibition of JNK (data not shown). The ability of JNK to signal survival in response to CSF-1 or LPS could involve upstream activators or downstream targets that have specific activities in macrophages. We used expression profiling by microarray to determine whether JNK signal-transducing proteins showed a differential pattern of expression in macrophages compared with 23 other tissue types. Microarray hybridizations were normalized as described in Materials and Methods, and values for each gene were calculated relative to the signal intensity of 17.5-day-old embryonic tissue, assigned a value of 1.0. Many of the components of JNK signal transduction pathways showed a constitutive expression across all cell types, and our analysis focused on the cluster of genes that showed high level expression in macrophages.

The results showed that several important signaling proteins are expressed at significantly higher levels in macrophages. Table II shows the results for genes that showed highly selective expression in macrophages within the cluster analysis of the 24 tissue types examined. Focal adhesion kinase (FAK), also known as protein tyrosine kinase-2, had the most distinct expression profile in macrophages. The level of FAK mRNA was 3.2-fold higher than embryonic tissue and 2-fold higher than the next highest tissue type, uterus. The 22 other tissue types all displayed similar levels of expression, with an average value of 1.1-fold relative to embryonic tissue and a SD of 0.22. FAK has established roles in cell growth, survival, and transformation, and selectively activates the JNK group of MAPKs (41, 42). The Ras/PI3K pathway protein, Rac1 that selectively activates JNK, MAPKs was differentially expressed in macrophages and osteoclasts, 2.4-fold and 2.9-fold, respectively. All other tissue showed an average level of 1.3-fold and a SD of 0.39. Expression of Nck-interacting kinase was high in macrophages (2.5-fold), thymus, heart, and brain (2.0- to 2.3-fold), and highest in spleen (3.1-fold). All other tissue showed an average value of 1.1-fold and a SD of 0.39. Nck-interacting kinase is a selective MAPK kinase kinase for JNK and is activated by tyrosine kinase receptors through Nck, a SOS binding protein that also activates the Ras pathway (43).

**Discussion**

In this study, we have shown that JNK inhibitors can selectively block the proliferation, differentiation, and survival of murine macrophages, implying that JNK activation following CSF-1R ligation is an important event in signaling.

Mutations in specific tyrosine residues have been used to isolate the pathways that are required for proliferation or survival in response to CSF-1. CSF-1 action on macrophages is associated with a rapid increase in nuclear expression of the AP-1 transcription factor, which cooperates with Ets-2 to activate transcription of the...
target gene urokinase (44, 45). AP-1 has also been implicated in cell cycle-associated gene expression in fibroblasts expressing CSF-1R (46). Inhibition of c-Jun by microinjection of antisense RNA or neutralizing Ab blocked entry of cells into S phase, and overexpression of c-Jun promoted S phase transition (47, 48). Fibroblasts derived from the fetuses of c-Jun−/− mice showed a severe proliferation defect associated with low levels of activated cyclin D and cyclin E-dependent kinases (49). There is also evidence that c-Jun directly controls transcription of the cyclin D1 gene (50).

The G1 arrest seen with inhibition of JNK was consistent with a requirement for c-Jun activation in cell cycle progression in macrophages. JNK inhibition with 10 μM SP600125 showed a comparable decrease in the number of cells in cycle to treatment with the maximal 100 μM

| Table 2. CDNA microarray profiles of JNK pathway genes in macrophage and mouse tissues

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* Values represent the normalized signal intensity ratio of the mRNA abundance in each tissue compared with the mRNA abundance in the common reference (17.5 dpc H9253 Pik3c).
concentration of the ERK inhibitor, PD98059. Higher levels of JNK inhibitor did not result in further inhibition at G1, possibly due to a second arrest in the G2 phase of the cell cycle.

The function of JNK in progression through the G2/M checkpoint of the cell cycle could be related to phosphorylation of c-Myc. A recent report (51) showed that blocking JNK phosphorylation of c-Myc resulted in a G2/M transition arrest. JNK and the upstream activator mixed lineage kinase-3 can both be localized to centrosomes throughout the cell cycle, and active, phosphorylated JNK is associated with the centrosome from S phase to anaphase (52). Mixed lineage kinase-3 is a selective MAPK kinase for JNK and has been shown to be a homologue of the fungal protein NIMA (never in mitosis A) that regulates the G2/M transition (53).

The PU.1 transcription factor has been shown to effect proliferation of BMMs in a CSF-1-dependent manner, and decreased proliferation of BMMs in response to JNK inhibition may partially occur through repression of c-fms gene expression (54) (Fig. 6). Analysis of the level of CSF-1R on the surface of BMMs over the course of inhibitor treatment was difficult due to the acute down-regulation of receptor in cells stimulated with CSF-1. Despite the down-modulation of the receptor, its continued re-expression and ligation is absolutely required for CSF-1 responsiveness (55), hence loss of c-fms mRNA expression could still be significant. Our data could not distinguish growth inhibition through loss of receptor signaling from direct regulation of the cell cycle by JNK. A direct link between JNK activity and cell cycle entry and progression is supported by the G2/M transition arrest and the ability of downstream targets of JNK to act as cell cycle regulators, as described above.

Our data that showed JNK activity and not ERK activity promoted CSF-1-mediated survival of macrophages was surprising given the quantity of literature that defines a proapoptotic role for JNK (56). Although many growth factors that promote survival activate JNK, it is believed that proapoptotic signaling by JNK is blocked by activation of the NF-κB, Akt/PI3K, and ERK survival pathways. Inhibition of PI3K and the downstream Akt/PI3K survival pathway with LY294002 did significantly effect survival of BMMs; however, the combination of JNK and PI3K inhibition resulted in an additive increase in apoptotic cells. The ability of Akt/PI3K to form a catalytically active complex with JNK and phosphorylate Ets-2 in macrophages may in part explain cooperation between the two pathways (10).

Although JNK-induced cell survival may partially involve activation of CSF-1R expression, lymphocyte studies in jnk−/− mice and our studies with other activation signals support the ability of JNK to deliver a direct antiapoptotic signal. Cell death in the growth factor-independent RAW264.7 cell line also supports JNK survival signaling. This cell line is transformed by the v-Abl oncogene, and BCR-Abl/v-Abl has been shown to signal in a similar manner to growth factor receptors, including the CSF-1R (57). JNK signaling was required for BCR-Abl-mediated transformation of B cells (58). A second possibility is that the RAW264.7 cell line represents cells transformed at a late stage of macrophage differentiation, and the requirement for JNK signaling may result from a closely related pattern of gene expression to primary macrophages. Both of these proposals are supported by high level PU.1 expression and loss of Bcl-xL expression with JNK inhibition. The ability of JNK to signal cell survival in macrophages is likely to involve enhanced expression of Bcl-xL. In contrast to other hemopoietic lineages, Bcl-xL expression continues to increase as cells differentiate from progenitors to macrophages, and expression can be activated by stimulation with CSF-1 (19).

The high level expression of FAK in macrophages may also contribute to the central role JNK plays in macrophage biology. Cell adhesion acts as a strong survival signal in macrophages, and the high level expression of FAK is likely to promote this response. FAK may also mediate survival signals in response to CSF-1 stimulation by receptor activation of Src and Crk, which can activate FAK through p130CAS (59). FAK has been shown to activate JNK through interaction with the JIP-3 (JNK-interacting protein 3) scaffold, which showed high level expression in macrophages (Table II) (60). These expression profiles may then provide a basis for future study on how JNK signal transduction pathways are distinctly regulated in macrophages.

Regulation of CSF-1R expression by PU.1 is likely to play a key role in the ability of JNK inhibitors to arrest differentiation in long-term culture of bone marrow cells. The level of CD11b and F4/80 staining indicated that inhibition of JNK predominately affected late-stage differentiation of myeloid precursors. A block at late stages of macrophage differentiation without effecting lineage commitment is consistent with the immature monocyctic cells seen with deletion of either the c-fms or pu.1 genes (61, 62). Analysis of macrophage populations in pu.1−/− mice using cell surface markers showed a depletion of CD11b-positive cells in blood leukocytes and in the peritoneal and pleural cavities and depletion of F4/80-positive cells in many tissues.

The JNK pathway has been considered as a therapeutic target in inflammatory disease (20). JNK is phosphorylated at high levels in response to proinflammatory agents such as LPS (63, 64). The results presented in this study suggest that mature macrophages have a selective requirement for JNK for survival, proliferation, and differentiation. Anti-inflammatory actions of such inhibitors may also be based upon production and recruitment of macrophages as well as their subsequent activation.

Disclosures
The authors have no financial conflict of interest.

References


