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Anti-inflammatory activity profile of JANEX-1 in preclinical animal models

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Abstract—We examined the biologic activity of the rationally designed JAK3 inhibitor, JANEX-1, in several cellular and animal models of inflammation. Notably, JANEX-1 exhibited potent anti-inflammatory activity in each of these preclinical models, including mouse models of peritonitis, colitis, cellulitis, and systemic inflammatory response syndrome. Therefore, JANEX-1 may prove useful as a broad-spectrum anti-inflammatory agent. The present study may provide the basis for new and effective treatment as well as prevention programs for inflammatory disorders.

1. Introduction

The JAK family of kinases consists of four known members—JAK1, JAK2, JAK3, and TYK2.[1–5] These cytoplasmic protein tyrosine kinases help regulate cellular functions in the lympho-hematopoietic system that are critical for cell proliferation and cell survival. JAKs are involved in the initiation of numerous cytokine-triggered signaling events[5–8] primarily through tyrosine phosphorylation of the *s*ignal *t*ransducers and *a*ctivators of *t*ranscription [STAT] proteins. JAK-mediated signaling is especially crucial for members of the hematopoietin subfamily of cytokines [Reviewed in[9]]. Specifically, the binding of a cytokine to its receptor activates the receptor-associated JAKs, activated JAKs then phosphorylate the cytoplasmic domains of the cytokine receptors, generating docking site[s] for SH-2 containing proteins, including the seven members of the STAT family of transcription factors [Reviewed in[10–12]]. STATs are phosphorylated and thereby activated by JAKs.

JAK3 plays a crucial role in normal lymphocyte development and function.[13–15] It is also expressed in cells of myeloid lineage, including monocytes, and granulocytes.[16] Activation of the JAK3 signaling pathway is associated with myeloid cell growth and differentiation.[16] JAK3 is coupled to several cytokine receptors that share the common gamma (γc) chain and serves a pivotal function in cytokine regulation of lymphocytes as well as monocyte activation and functions that are associated with γc chain-linked biochemical events triggered by engagement of cytokine receptors.[17,18] JAK3 is expressed at low levels in human monocytes but its expression as well as tyrosine phosphorylation levels are strongly enhanced during monocyte activation by lipopolysaccharide.[18] JAK3 has also been implicated as an important regulatory enzyme in various cellular elements of lymphoid and myeloid lineages that are engaged in inflammatory immune responses.[19–21] Therefore, JAK3 has been identified as a candidate molecular target for treatment of inflammatory disorders.[20–22]

Bacterial lipopolysaccharide (LPS) or endotoxin is a glycolipid which is a major component of the outer membrane of Gram-negative bacteria. LPS is a potent activator of monocytes and macrophages, and an initiator of macrophage effector functions. LPS triggers the abundant secretion of many proinflammatory cytokines from macrophages including IL-1, IL-6, and TNFα.[23] The hydrophobic domain of LPS, known as lipid A, is responsible for the proinflammatory effects of LPS.[23] CD14, a glycosylphosphatidylinositol-anchored glycoprotein of leukocytes, binds LPS with affinity. Monocyte/macrophage recognition of lipid A requires TLR4, a Toll-like receptor (TLR) family member and an integral component of the CD14-associated LPS receptor

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complex.[23] TLR4 is a transmembrane protein that initiates a signaling cascade that triggers an innate immune response to endotoxin. The TLR4 cDNA codes for a protein consisting of 799 amino acids, with an approximate molecular weight of 88 kDa. In mammals, TLR4 initiates a signal upon activation by CD14-bound LPS that is transmitted through a series of adapter molecules and protein kinases.[24,25]

The mechanism by which the LPS signal is transduced from the extracellular environment to the nuclear compartment is not well defined. Engagement of the LPS receptor complex induces rapid tyrosine phosphorylation in monocytes and involves activation of multiple tyrosine kinases, including Src family protein tyrosine kinases, Bruton's tyrosine kinase (BTK), and Janus family (JAK) protein tyrosine kinases.[26–29]

Tyrosine kinase inhibitors targeting the Src and JAK pathways have been proposed as a new class of anti-inflammatory agents against LPS-induced severe inflammatory disorders and acute lung injury.[30–32] In recent reports we described the structure-based design of several JAK3 inhibitors, including JANEX-1.[33,34] A novel homology model of the JAK3 kinase domain was used for the identification of this dimethoxyquinazoline compound as a lead compound with potent and specific inhibitory activity against JAK3.[33] JANEX-1 showed a favorable toxicity and pharmacokinetics profile in animal models[35,36] and exhibited promising activity in preclinical models of inflammation, autoimmune diabetes, allergy, and leukemia.[20,22,33,37–41]

In the present study we sought to evaluate the potential of JANEX-1 as a candidate anti-inflammatory agent by examining its activity in several cellular and mouse models of peritonitis, colitis, cellulitis, and systemic inflammatory response syndrome (SIRS). Notably, JANEX-1 exhibited potent anti-inflammatory activity in each of these preclinical models of inflammation. Therefore, this rationally designed JAK3 inhibitor may prove useful as a broad-spectrum anti-inflammatory agent.

2. Results

2.1. Stimulation of a JAK3-linked signaling pathway in LPS-activated macrophages

LPS is a potent activator of monocytes and macrophages and an initiator of macrophage effector functions. Engagement of the LPS receptor complex induces rapid tyrosine phosphorylation in monocytes and involves activation of multiple tyrosine kinases, including Src family protein tyrosine kinases, Bruton's tyrosine kinase (BTK), and Janus family (JAK) protein tyrosine kinases. We first set out to verify that LPS activation triggers tyrosine phopshorylation of JAK3 and its substrate STAT3 in RAW 264.7 cells, a murine macrophage cell line commonly used as a model to elucidate LPS-triggered biochemical and biological monocyte/macrophage responses. To this end, the JAK3 and STAT3 immune complexes immunoprecipitated from RAW 264.7 cells at various time points after LPS exposure were subjected to anti-phosphotyrosine Western blot analysis. As shown in Figure 1a, LPS (25 μg/mL) activation of RAW 264.7 cells triggered tyrosine phosphorylation of JAK3 in a time-dependent fashion with peak tyrosine phopshorylation occurring at 4 min after initiation of LPS activation. As documented in Figure 1b, LPS activation also resulted in enhanced tyrosine phosphorylation of STAT3, a known tyrosine kinase substrate of JAK3. These findings confirm and extend previous studies that implicated JAK3 as a key regulatory kinase in LPS-mediated signaling events. We next examined the effects of the JAK3 inhibitor on LPS-induced tyrosine phosphorylation of TLR4-associated phosphoproteins in RAW 264.7 cells. As evidenced in Figure 1c, LPS activation resulted in enhanced tyrosine phopshorylation of multiple phosphoproteins in TLR4 immune complexes. Presence of the JAK3 inhibitor JANEX-1 (100 μg/mL) reduced the baseline tyrosine phopshorylation level in TLR4 immune complexes and abrogated LPS-induced tyrosine phopshorylation. These results are consistent with the notion that JAK3 plays an important role in LPS-mediated activation of the TLR4 signaling pathway.

Activation of the TLR4 signaling pathway after LPS stimulation of macrophages results in production of TNFα and nitric oxide (NO), two recognized mediators and regulators of inflammatory responses.[42–54] Therefore, we next examined the effects of JANEX-1 on TNFα and NO production in LPS-stimulated RAW 264.7 cells at the mRNA level by using Northern blot analyses. Notably, LPS stimulation (100 ng/mL × 4 h) triggered transcriptional upregulation of iNOS (Fig. 1d), and TNFα genes (Fig. 1e). These observed LPS-induced changes in iNOS or TNFα mRNA levels were not owing to differences in amount of RNA in each lane as confirmed by near-equal GAPDH mRNA levels in the RNA samples run in each lane (Fig. 1f). JANEX-1 (1–100 μg/mL) prevented LPS-induced increase in iNOS (Fig. 1d) and TNFα mRNA (Fig. 1e) expression levels in a concentration-dependent fashion. These experimental findings provide direct evidence that inhibition of JAK3 kinase by JANEX-1 blocks the earliest events of the TLR4 signaling pathway in LPS challenged macrophages.

2.2. Anti-inflammatory activity of JANEX-1 in cellular models of acute inflammatory response

Macrophages play an important role in inflammatory responses and have been shown to be activated by LPS.[42–54] LPS stimulates macrophages to produce TNFα and nitric oxide (NO), a recognized mediator and regulator of inflammatory responses.[42–54] We examined the effects of JANEX-1 on LPS-induced TNFα production, as measured by TNF cytotoxicity assays using target murine L929 fibrosarcoma cells, as well as LPS-induced NO production by primary murine peritoneal macrophages and the RAW 264.7 murine macrophage cell line, as measured by nitrite formation. JANEX-1 inhibited NO production and TNFα production by peritoneal macrophages (Fig. 2a) as well as RAW 264.7

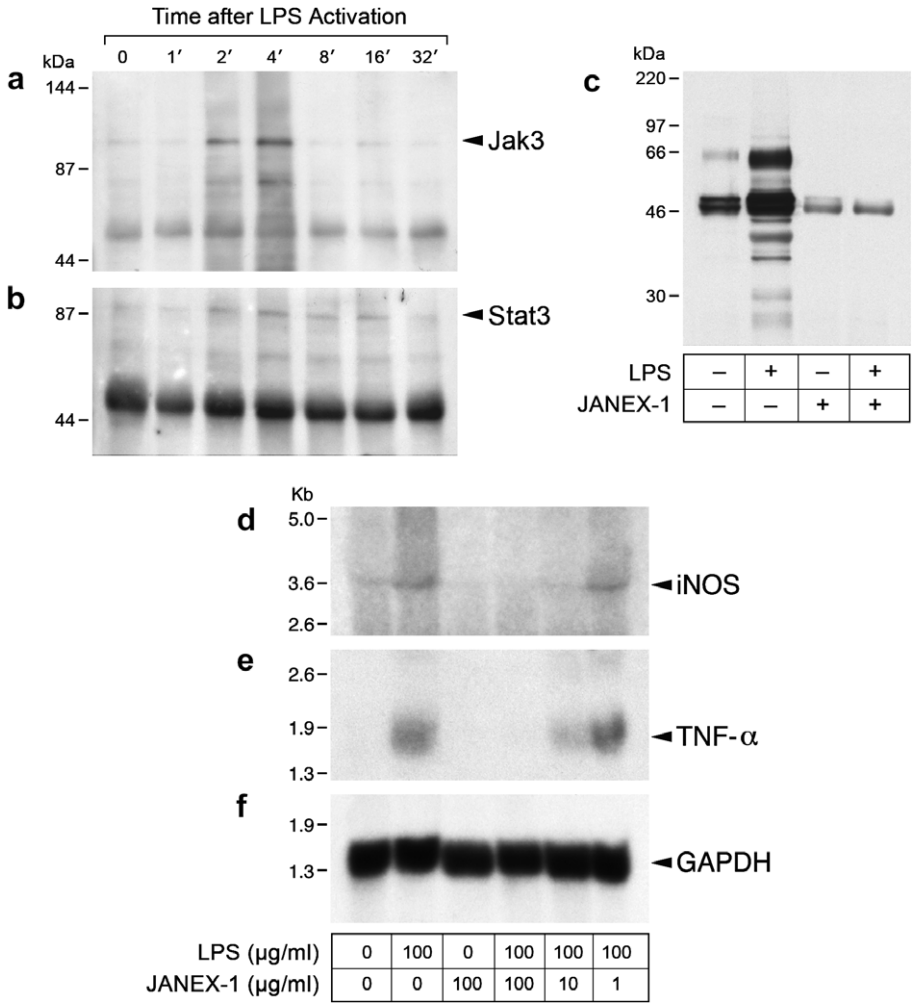


Figure 1. LPS-induced JAK3 activation in macrophages and effects of JAK3 inhibitor JANEX-1 on LPS stimulation of the TLR4 signaling pathway. (a and b) LPS-induced JAK3 activation as measured by increased tyrosine phosphorylation of JAK3 and its substrate STAT3. JAK3 and STAT3 immune complexes immunoprecipitated from RAW 264.7 cells at various time points after LPS exposure were subjected to anti-phosphotyrosine Western blot analysis. LPS activation of RAW 264.7 cells triggered tyrosine phosphorylation of JAK3 in a time-dependent fashion with peak tyrosine phopshorylation occurring at 4 min after initiation of LPS activation (a). LPS activation also resulted in enhanced tyrosine phosphorylation of STAT3, a known tyrosine kinase substrate of JAK3 (b). (c) JANEX-1 inhibits LPS-stimulated activation of the TLR4 signaling pathway. LPS activation resulted in enhanced tyrosine phopshorylation of multiple phosphoproteins in TLR4 immune complexes from RAW 264.7 cells. Presence of the JAK3 inhibitor JANEX-1 reduced the baseline tyrosine phopshorylation level in TLR4 immune complexes and abrogated LPS-induced tyrosine phopshorylation. (d–f) JANEX-1 inhibits LPS-induced transcription of TNFα and iNOS genes. We examined the effects of JANEX-1 on TNFα and NO production in LPS-stimulated RAW 264.7 cells at the mRNA level by using Northern blot analyses. LPS stimulation triggered transcriptional upregulation of iNOS (d) and TNFα (e) genes. These observed LPS-induced changes in iNOS or TNFα mRNA levels were not owing to differences in amount of RNA in each lane, as confirmed by near-equal GAPDH mRNA levels in the RNA samples run in each lane (f). JANEX-1 prevented LPS-induced increase in iNOS (shown in d) and TNFα mRNA (shown in e) expression levels in a concentration-dependent fashion.

cells (Fig. 2b) in a concentration-dependent fashion. The IC_{50} values for inhibition of NO production were 0.5 μg/mL (1.7 μM) for peritoneal macrophages and 0.4 μg/mL (1.4 μM) for RAW 264.7 cells. The IC_{50} values for inhibition of TNFα production were 4.3 μg/mL (14.5 μM) for peritoneal macrophages and 37 μg/mL (124.5 μM) for RAW 264.7 cells. The TNF-mediated cytotoxicity of peritoneal macrophages against L929 cells was 65.1 ± 3.5% at 0 μg/mL, 45.9 ± 2.2% at 1 μg/mL, 25.7 ± 3.0% at 10 μg/mL (P[0 μg/mL vs 10 μg/mL] = 0.006), and 0.0 ± 0.0 % at 100 μg/mL (P[0 μg/mL vs 100 μg/mL] = 0.0002) (Fig. 1a). We next examined the effects of JANEX-1 on LPS-induced TNFα production by RAW 264.7 cells using a quantitative ELISA system.[55] As shown in Figure 2c, JANEX-1 inhibited TNFα production in a concentration-dependent fashion with an IC_{50} value of 16 μg/mL (53.8 μM). The measured TNFα concentrations after JANEX-1 treatment were 5.3 ± 0.6 ng/mL at 0 μg/mL, 4.8 ± 0.5 ng/mL at 1 μg/mL, 3.2 ± 0.0 ng/mL at 10 μg/mL (P[0 μg/mL vs 10 μg/mL]=0.026), and 1.0 ± 0.1 ng/mL at 100 μg/mL (P[0 μg/mL vs 100 μg/mL] = 0.002) (Fig. 1c).

A number of inflammatory mediators such as PGE2 and IL-8 have been implicated in the pathogenesis of IBD.[56,57] To evaluate whether JANEX-1 can inhibit the production and release of inflammatory mediators by colonic epithelial cells, we exposed the HT29 colonic

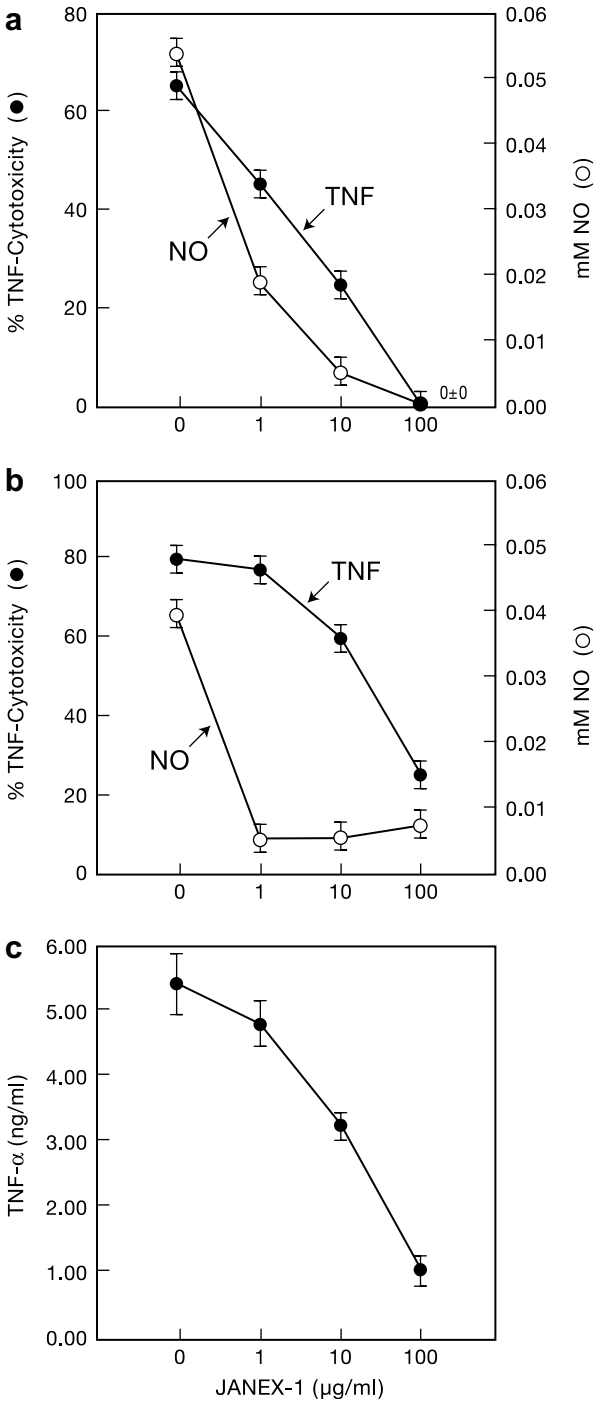


Figure 2. Effects of JANEX-1 on LPS-induced TNFα and NO production by macrophages. Murine peritoneal macrophages (shown in a) or RAW 264.7 cells (shown in b) were cultured in the presence of 100 ng/mL LPS and various concentrations of JANEX-1 for 4 h prior to being examined for TNFα production using L929 cytotoxicity assays (closed circles) and 48 h prior to being examined for NO production (open circles). The effect of JANEX-1 on LPS-induced TNFα production by RAW 264.7 cells was also examined using a quantitative ELISA (shown in c). Each data point depicts the mean ± SE values from three experiments.

epithelial cell line to 5 μg/mL LPS for 6 h in the presence and absence of indicated concentrations of JANEX-1. After LPS stimulation, HT29 cells released significantly higher levels of PGE_2 (2496 ± 224 pg/mL) and IL-8 (4.8 ± 0.9 ng/mL) into the extracellular medium (Fig. 3). Notably, *JANEX-1* prevented LPS-induced release of PGE_2 (Fig. 3a) and IL-8 (Fig. 3b) in a concentration-dependent fashion. Thus, JANEX-1 is a potent inhibitor of LPS-induced proinflammatory mediator released by colonic epithelial cells.

Taken together, these results from three different cellular models of inflammation show that JANEX-1 is a potent inhibitor of cellular inflammatory responses and inhibits the release of inflammatory mediators from macrophages, and colonic epithelial cells.

2.3. Anti-inflammatory activity of JANEX-1 in a mouse peritonitis model of acute inflammation

We next examined the in vivo anti-inflammatory activity of JANEX-1 in a murine model of peritonitis. In the first

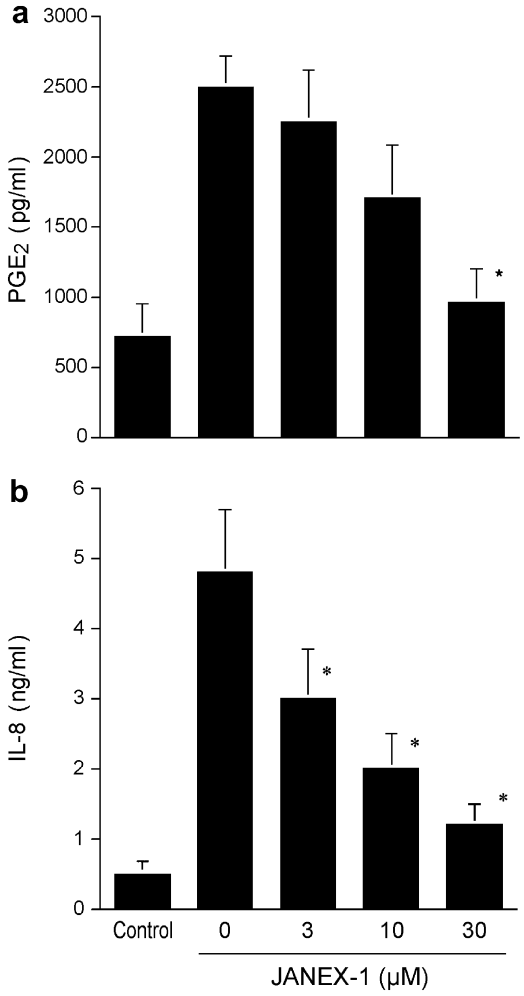


Figure 3. Effect of JANEX-1 on Lipopolysaccharide- (LPS) induced inflammatory mediator release by colonic epithelial cells. Monolayers of colonic epithelial cell line, HT29, were exposed to 5 μg/mL LPS. To examine the effect of JANEX-1 on LPS-induced inflammatory mediator release, monolayers of HT29 cells were incubated with indicated concentrations of JANEX-1 for 1 h prior to LPS challenge. After LPS stimulation of HT-29 cells for 6 h, PGE_2 (a) and IL-8 (b) levels were measured in cell-free supernatants by ELISA. Data are expressed as mean ± SEM. $N = 3$–5 independent experiments. $^*P < 0.05$ compared to control.

experiment, C3H-FeJ mice were treated with either vehicle or JANEX-1 daily for 7 days prior to an intraperitoneal injection of paraffin oil (PO) to trigger an inflammatory response. The magnitude of the PO-elicited inflammatory macrophage response was measured 3 days later by macrophage counts of peritoneal lavage specimens as well as in vitro TNFα and NO production by the harvested peritoneal macrophages. Pretreatment of mice with JANEX-1 inhibited the inflammatory response to paraffin oil challenge in a dose-dependent fashion with an ED_{50} value of 2.8 mg/kg. The peritoneal lavage macrophage counts were $6.9 \pm 0.2 \times 10^5$/mL in vehicle-treated control mice, $4.3 \pm 0.4 \times 10^5$/mL in mice treated with JANEX-1 at 2 mg/kg daily dose level (P[JANEX-1, 2 mg/kg vs vehicle] = 0.0032), and $2.5 \pm 0.4 \times 10^5$/mL in mice treated with JANEX-1 at 4 mg/kg daily dose level (P[JANEX-1, 4 mg/kg vs vehicle] = 0.0002) (Fig. 4a).

The peritoneal macrophages isolated from JANEX-1 treated mice had a significantly blunted TNFα response (Fig. 4b) and no detectable NO response to LPS (Fig. 4c). The maximum % TNF-mediated cytotoxicity against L929 cells observed at 100 ng/mL LPS was 61.9 ± 1.0% for vehicle control, 45.3 ± 2.5% for 2 mg/kg JANEX-1 ($P = 0.0005$), and 34.6 ± 2.5% for 4 mg/kg JANEX-1 ($P < 0.0001$) (Fig. 4b). The estimated ED_{50} value was 5.2 mg/kg. The maximum NO output levels triggered by 100 ng/mL LPS were 44 ± 3 μM for vehicle control, 3 ± 0 μM for 2 mg/kg JANEX-1 ($P < 0.0001$), and 4 ± 0 μM for 4 mg/kg JANEX-1 ($P < 0.0001$) (Fig. 4c). The estimated ED_{50} value was 0.01 mg/kg. In the second experiment, JANEX-1 treatments commenced after the intraperitoneal injection of PO and continued for three consecutive days. The magnitude of the PO-elicited inflammatory macrophage response was measured 3 days later by macrophage counts of peritoneal lavage specimens as well as in vitro TNFα and NO production by the harvested peritoneal macrophages. JANEX-1 exhibited a dose-dependent anti-inflammatory effect, as documented by a reduction of macrophage exudation and diminished TNFα and NO responses of the harvested peritoneal macrophages (Fig. 5). The peritoneal lavage macrophage counts were $4.9 \pm 0.7 \times 10^5$/mL in vehicle-treated control mice, $3.1 \pm 1.5 \times 10^5$/mL in mice treated with JANEX-1 at 2 mg/kg daily dose level, and $2.3 \pm 1.2 \times 10^5$/mL in mice treated with JANEX-1 at 4 mg/kg daily dose level (Fig. 5a). The estimated ED_{50} value was 5.7 mg/kg. The peritoneal macrophages isolated from JANEX-1 treated mice had a significantly blunted TNFα response (Fig. 5b) and a significantly blunted NO response to LPS (Fig. 5c). The maximum % TNF-mediated cytotoxicity against L929 cells observed at 100 ng/mL LPS was 60.6 ± 3.3% for vehicle control, 31.8 ± 6.3% for 2 mg/kg JANEX-1, and 18.8 ± 5.1% for 4 mg/kg JANEX-1 (Fig. 5b). The estimated ED_{50} value was 2.1 mg/kg. The maximum NO output levels triggered by 100 ng/mL LPS were 80 ± 2 μM for vehicle control, 40 ± 2 μM for 2 mg/kg JANEX-1 and 13 ± 2 μM for 4 mg/kg JANEX-1 (Fig. 5c). The estimated ED_{50} value was 2 mg/kg.

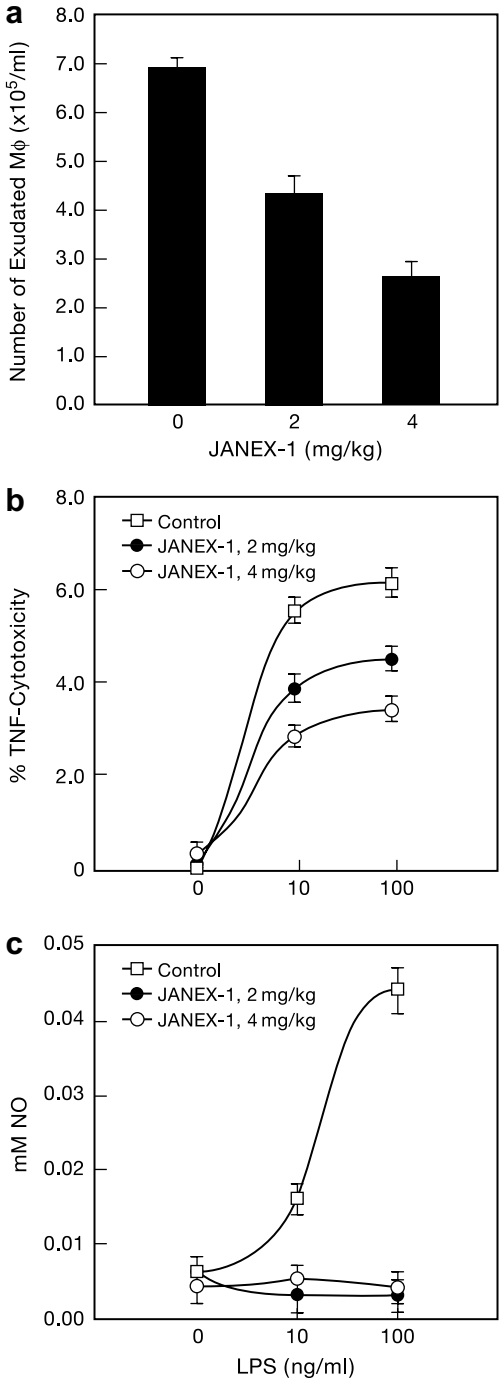


Figure 4. Anti-inflammatory effects of a 7-day treatment with JANEX-1 in a murine peritonitis model. C3H-FeJ mice were treated for seven consecutive days with ip injections of 2 mg/kg or 4 mg/kg JANEX-1. Mice were then challenged with an ip injection of paraffin oil as a peritoneal irritant. Inflammatory macrophages were harvested by peritoneal lavage 3 days after the injection of paraffin oil and cultured in the presence or absence of 1 ng/mL or 100 ng/mL LPS. The numbers of inflammatory macrophages in peritoneal lavage samples are shown in (a). The effect of JANEX-1 treatment on LPS-induced TNF production by peritoneal macrophages from the treated mice is shown in (b). The effect of JANEX-1 treatment on LPS-induced NO production by peritoneal macrophages from the treated mice is shown in (c). Each data point depicts the mean ± SE values from five mice.

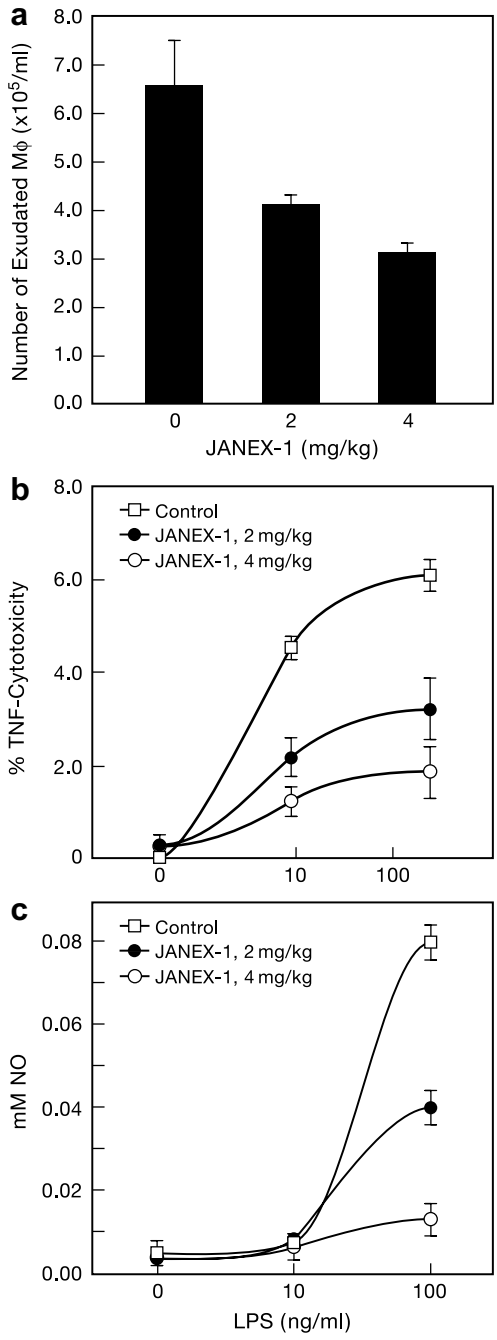


Figure 5. Anti-inflammatory effects of a 3-day treatment with JANEX-1 in a murine peritonitis model. C3H-FeJ mice were challenged with an ip injection of paraffin oil as a peritoneal irritant. Subsequently, mice were treated for three consecutive days with ip injections of 2 mg/kg or 4 mg/kg JANEX-1. Inflammatory macrophages were harvested by peritoneal lavage 3 days after the injection of paraffin oil and cultured in the presence or absence of 10 ng/mL or 100 ng/mL LPS. The numbers of inflammatory macrophages in peritoneal lavage samples are shown in (a). The effect of JANEX-1 treatment on LPS-induced TNF production by peritoneal macrophages from the treated mice is shown in (b). The effect of JANEX-1 treatment on LPS-induced NO production by peritoneal macrophages from the treated mice is shown in (c). Each data point depicts the mean ± SE values from five mice.

2.4. Anti-inflammatory activity of JANEX-1 in the mouse footpad model of soft tissue inflammation

We next set out to evaluate the anti-inflammatory activity of JANEX-1 (30 mg/kg) in the mouse footpad model of inflammation. Hind paw edema was induced by the injection of carrageenan (CAG) into the subplanter region of the left paw of the mice. Right paws were injected with equal volume of PBS. The paw thickness was measured before and 6 h after the challenge. CAG (but not saline) injections triggered paw edema formation and JANEX-1 significantly reduced this inflammatory response (Fig. 6a). JANEX-1 treated mice had significantly less paw swelling than vehicle-treated or ibuprofen-treated control mice. These differences were statistically significant for both CD-1 mice (Fig. 6b) and ICR mice (Fig. 6c).

2.5. Anti-inflammatory activity of JANEX-1 in a mouse model of IBD

It has been shown that administration of intrarectal administration of TNBS in BALB/c mice causes IBD associated with severe diarrhea and extensive wasting.[82,57,58] TNBS caused severe IBD with weight loss in vehicle-treated control mice (Fig. 7a). Treatment of mice daily with 25 mg/kg JANEX-1 prevented TNBS-induced diarrhea and wasting (Fig. 7a). The thickness of the colonic wall (Fig. 7b) and colon weight (Fig. 7c and d), which serve as a surrogate marker of colitis and colitis-associated bowel wall edema, were markedly reduced by JANEX-1 treatment.

2.6. Anti-inflammatory activity of JANEX-1 in a mouse model of systemic inflammatory response syndrome

We next evaluated the anti-inflammatory activity of JANEX-1 in a mouse model of fatal systemic inflammatory response syndrome (SIRS). C57BL/6J mice were challenged with an ip injection of D-galactosamine plus LPS mixed with either vehicle (control mice) or 500 μg JANEX-1 (test mice). At 1 h or 2 h post the LPS challenge, control mice were treated with vehicle and test mice were treated with 500 μg JANEX-1. Control mice rapidly died of a systemic inflammatory response involving multiple organs. The histopathologic evaluation of the control mice revealed severe generalized hepatic necrosis in the livers and a mild-moderate alveolitis with pulmonary edema in the lungs. As shown in Figure 8A, JANEX-1 treated test mice had a significantly improved survival outcome. While 6 of 8 control mice died within 10 h after the LPS challenge, none of the 16 JANEX-1 treated test mice died within the 24 h observation period ($P < 0.001$). Examination of the liver specimens from control mice using in situ TUNNEL assays and confocal laser scanning microscopy revealed extensive apoptosis consistent with previous reports[59] (Fig. 8B). By comparison, very few apoptotic cells were detected in the liver specimens of JANEX-1 treated test mice (Fig. 8C).

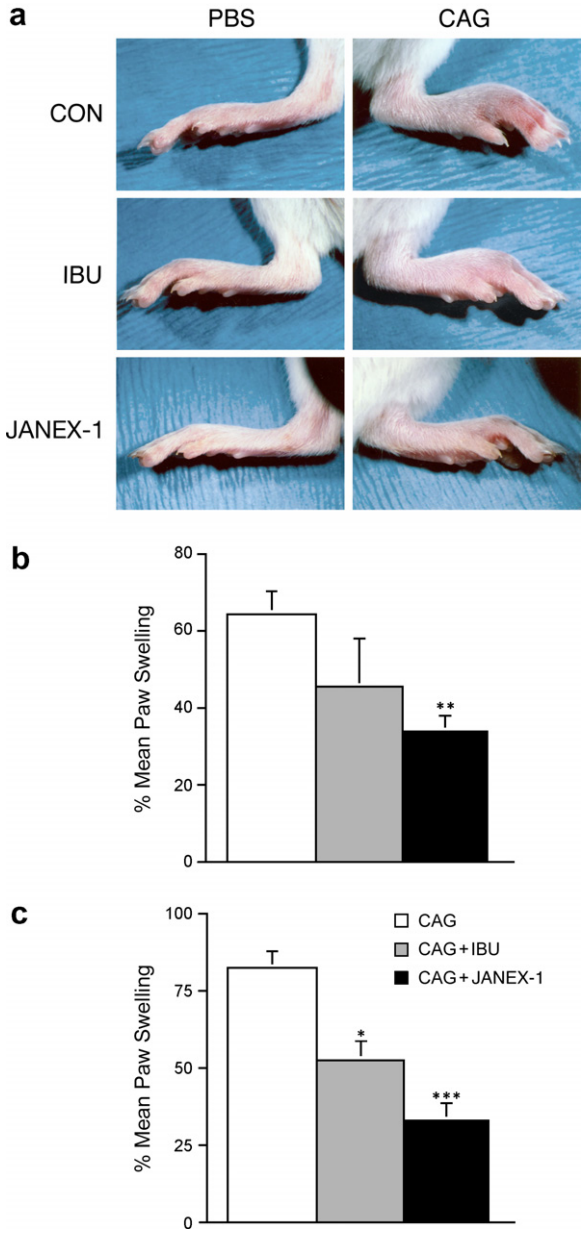


Figure 6. Anti-inflammatory effects of JANEX-1 in mouse model of soft tissue inflammation. Mice were treated with drug or vehicle as has been described in Section 4. Inflammation was induced by the injection of CAG in to the subplanter region of the left paw of the mice. Right paw was injected with equal volume of phosphate buffered saline (PBS). The paw thickness was measured before and 6 h after the challenge. (a) Representative photographs of paws. (b) The effect of JANEX-1 versus ibuprofen on CAG-induced paw edema in CD1 mice. (c) The effect of JANEX-1 versus ibuprofen on CAG-induced paw edema in ICR mice. The results are expressed as percent increase in paw volume. The data are represented as mean ± SEM, $n = 8–33$. $^{**}P < 0.001$ compared to CAG in CD1 mice group. $^{*}P < 0.05$ and $^{***}P < 0.0001$ compared to CAG in ICR mice group.

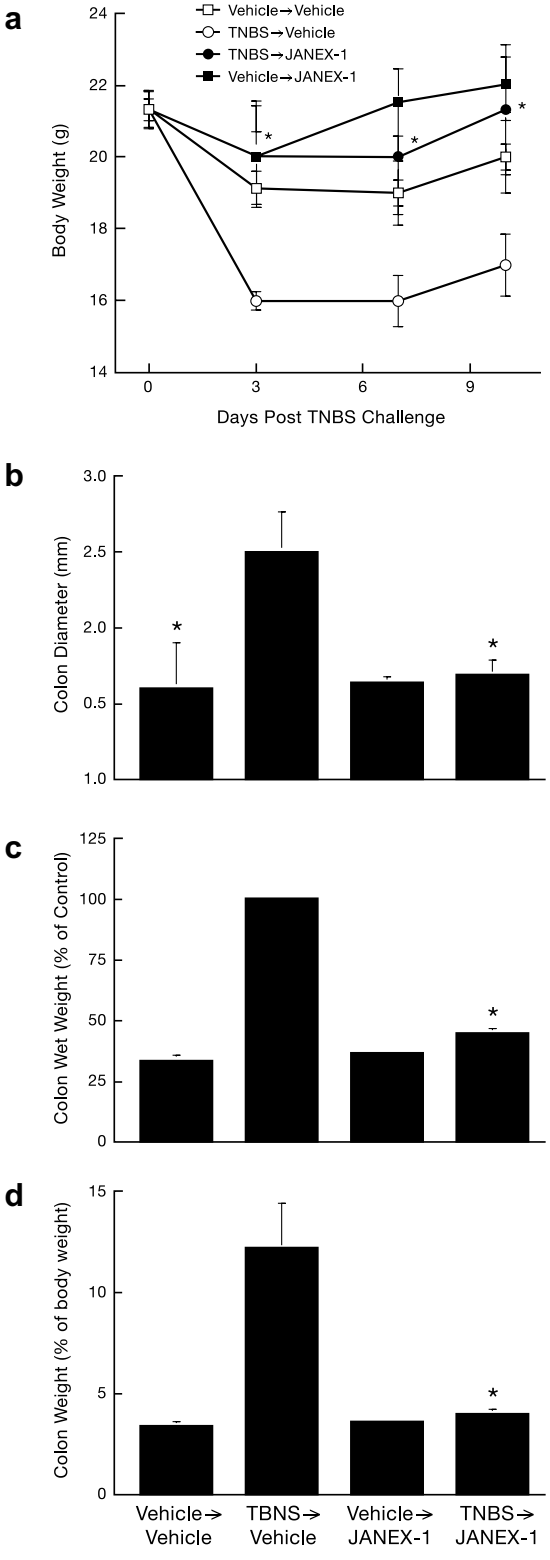
3. Discussion

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed drugs worldwide.[60–67] It is estimated that more than 30 million persons worldwide take NSAIDs daily for treatment of pain and an inflammatory condition. Conventional NSAIDs cause significant gastrointestinal and renal toxicity.[61,63,65] Selective cyclooxygenase (COX)-2 enzyme inhibitors were developed as a new class of NSAIDs to avoid such side effects. Unfortunately, COX-2 inhibitors are associated with significant cardiovascular side effects, including increased risk of myocardial infarction confirmed by meta-analyses of randomized controlled clinical trials.[61,66,67] Consequently, several COX-2 inhibitors have been withdrawn from sale in many countries. Therefore, it is important to identify new molecular targets and new agents for treatment of inflammatory disorders. In the study presented herein, we examined the biologic activity of the rationally designed JAK3 inhibitor, JANEX-1, in several cellular and in vivo animal models of inflammation. Notably, JANEX-1 exhibited potent anti-inflammatory activity in each of these preclinical models of inflammation, including mouse models of peritonitis, colitis, cellulitis, and SIRS. Therefore, this rationally designed JAK3 inhibitor may prove useful as a broad-spectrum anti-inflammatory agent. The present study expands our knowledge of JAK3 functions in inflammation and may provide the basis for new and effective treatment as well as prevention programs for inflammatory disorders using JAK3 inhibitors.

In signal transduction experiments, JANEX-1 prevented LPS-induced tyrosine phosphorylation of JAK3 and its substrate STAT3 in RAW 264.7 macrophage cell line, as measured by anti-phosphotyrosine Western blot analysis of JAK3 and STAT3 immunoprecipitates from whole cell lysates of RAW 264.7 cells. Targeting JAK3 with JANEX-1 was associated with abrogation of LPS-induced iNOS and TNFα responses both at the mRNA and protein levels. These results confirmed and extended previous studies showing the involvement of JAK3 in LPS-triggered TLR4 signaling pathway. The ability of JANEX-1 to prevent LPS-induced macrophage iNOS and TNFα responses provided the biochemical proof of concept that targeting JAK3 may be an effective means of blocking macrophage/monocyte inflammatory responses that follow activation of the TLR4 receptor pathway.

Systemic infection is frequently encountered in immunocompromised cancer patients sometimes leading to severe sepsis and death.[68] Sepsis is characterized by a systemic inflammatory response syndrome (SIRS) caused by infection. This clinical syndrome is triggered by a massive release of proinflammatory mediators and leads to a widespread organ damage.[69,70] SIRS and sepsis occur in approximately 750,000 persons per year in the US and have an associated mortality rate of 30–50%.[69,70] The LPS-galactosamine septic shock model in mice has been broadly used to study SIRS caused by Gram-negative bacteria.[84] JANEX-1 significantly improved the survival outcome in this mouse model of SIRS. The liver tissues from JANEX-1 treated mice revealed much less apoptosis than those from control mice. Thus, the anti-inflammatory activity of JANEX-1 was potent enough to reduce the severity of systemic inflammation in mice challenged with LPS-galactosamine.

In summary, JANEX-1 exhibited broad-spectrum anti-inflammatory activity in multiple cellular and animal models of inflammation. The previously reported favorable safety and pharmacokinetics profile of JANEX-1 and its documented preclinical biological activity at non-toxic dose levels warrants its further development as a potential new anti-inflammatory drug candidate.



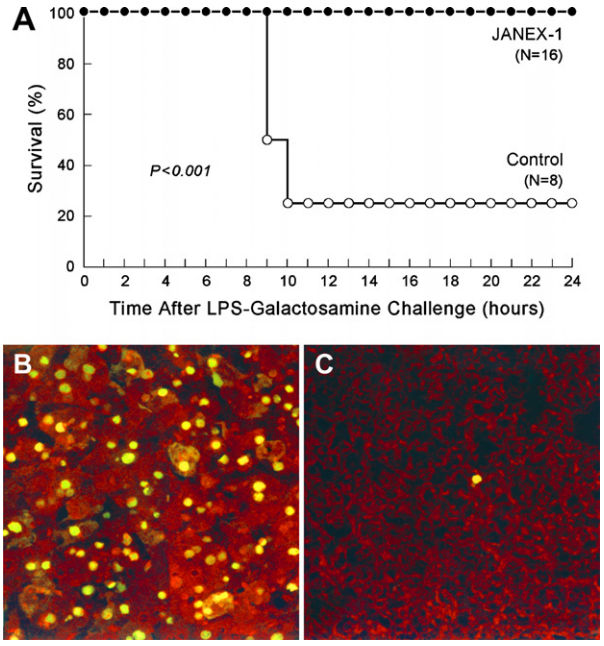


Figure 8. Effects of JANEX-1 in a mouse model of SIRS. C57BL/6J mice were challenged with an ip injection of D-galactosamine plus LPS mixed with either vehicle (control mice) or 500 μg JANEX-1 (test mice). At 1 h or 2 h post the LPS challenge, control mice were treated with vehicle and test mice were treated with 500 μg JANEX-1. The survival outcome of control and test mice is depicted in (A). While 6 of 8 control mice died within 10 h after the LPS challenge, none of the 16 JANEX-1 treated test mice died within the 24 h observation period ($P < 0.001$). Examination of the liver specimens from control mice using in situ TUNNEL assays and confocal laser scanning microscopy revealed extensive apoptosis (B). By comparison, very few apoptotic cells were detected in the liver specimens of JANEX-1 treated test mice (C).

4. Materials and methods

4.1. Signal transduction experiments using RAW 264.7 macrophage cell line

Immunoprecipitations with anti-JAK3, anti-STAT3, and anti-TLR4 antibodies were performed using previously published experimental procedures.[71–73]

Figure 7. (a) Effect of JANEX-1 on TNBS-induced wasting diseases in mice. Chronic intestinal inflammation was induced by delivering 100 μL of 2,4,6,-trinitrobenzene sulfonic acid (TNBS) through a flexible rubber catheter inserted into the colon of unanesthetized BALB/c mice as described in Section 4. Control mice received equal volume of vehicle (50% ethanol). To examine the effect of JANEX-1 on TNBS-induced colitis in mice, a group of BALB/c mice were treated with JANEX-1 (25 mg/kg ip) 1 h before induction of colitis and once daily till the end of the experiment. Data are expressed as mean ± SEM. $N = 10$ mice. $^{*}P < 0.05$ compared to TNBS control. (b–d) Effect of JANEX-1 on TNBS-induced colonic inflammation in mice. Since intracolonic administration of TNBS in mice results in thickening of colonic wall, colonic edema, and an increase in colon weight, we examined the effect of JANEX-1 on TNBS-induced colon edema (b) and increase in colon weight (c and d). Mice were challenged with TNBS as described in (a). Groups of mice were sacrificed 2 weeks after intracolonic administration of TNBS, and colon weight and diameter was recorded. Colon weight refers to the distal 5 cm of the colon. Data are expressed as mean ± SEM. $N = 6$ mice. $^{*}P < 0.05$ compared to TNBS control.

The anti-TLR4 antibody (goat polyclonal IgG, sc-8694, C-18) was purchased from Santa Cruz Biotech. (Santa Cruz, CA). The immune complexes were subjected to Western blot analysis using anti-phosphotyrosine antibodies, as previously reported.[71–73] For Northern blot analysis of tumor necrosis factor alpha (TNFα) and inducible nitric oxide synthase (iNOS) mRNA expression levels, total cellular RNA was extracted with Tri reagent (Molecular Research, Inc. (Cincinnati, OH). Electrophoresis of RNA samples (20 μg/lane) was performed in 1.2% agarose/2.2 M formaldehyde gel which was subsequently transferred onto a Magnagraph nylon membrane (NSI, Westboro, MA) in 10× SSC. Hybridization of the membrane was performed with [^{32}P]-labeled oligonucleotide probes which included a 36-mer probe for the murine TNFα mRNA (5′-CTG GAA GAC TCC TCC CAG CAG GTA TAT GGG CTC ATA CCA-3′) and a 43-mer probe for the murine iNOS mRNA. A [^{32}P]-labeled mouse GAPDH probe (Ambion, Austin, TX) was used as a standard RNA loading control.

4.2. Reagents and supplies

JANEX-1 was synthesized as previously reported.[33,34] For in vivo experiments, JANEX-1 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL and diluted to 1 mg/mL with phosphate buffered saline (PBS) before injection. Dulbecco's modified Eagle's medium (DMEM), RPMI, and HBSS media (Gibco-BRL, Grand Island, NY), Trypsin and recombinant murine TNFα were purchased from Life technologies, Grand Island, NY. LPS-free fetal calf serum (FCS) and fetal bovine serum (FBS) were obtained from Hyclone Laboratories (Logan, UT). Gentamicin, L-glutamine, sodium pyruvate, and non-essential amino acids were purchased from Mediatech, Inc. (Washington, DC). LPS (*Escherichia coli*, 055:B5) was purchased from Sigma (St. Louis, MO) and used for in vitro experiments as well as in mouse models of peritonitis. 6-well and 96-well flat bottom tissue culture plates (Falcon 3046 and 3072) were obtained from Becton Dickinson (Oxnard, CA). Light paraffin oil was purchased from Fisher Scientific (Fair Law, NJ). Pyrogen-free distilled water was from Baxter Healthcare Co., Deerfield, IL. Prostaglandin (PG) E_2 ELISA kits were from Cayman Company (Ann Arbor, MI). Human tumor necrosis factor (TNF)α, and interleukin (IL)-8, ELISA kits were purchased from R&D Systems (Minneapolis, MN). LPS (*E. coli*, 0111:B4) that was used in the mouse model of SIRS, and 2,4,6,-trinitrobenzene sulfonic acid (TNBS) were from Sigma Chemical Company (St. Louis, MO). Ibuprofen, l carrageenan, hexatrimethylammonium bromide, D-galactosamine, and DMSO were purchased from Sigma Chemical Company (St. Louis, MO).

4.3. Mice

Specific pathogen-free 12- to 14-week-old C3HeB/FeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male and female BALB/c (6–8 weeks old) mice were purchased from Charles River Laboratories (Wilmington, MA). CD1 and ICR mice (6–8 weeks old) were purchased from Taconic (Germantown, NY). Female, 6–7 weeks old, hairless albino mice (skh-1) were purchased from Charles River Laboratories (Wilmington, MA). C57BL/6J mice were purchased from Jackson Laboratories, Bar Harbor, ME. Animals were caged in groups of five in a pathogen-free environment in accordance with the rules and regulations of US Animal Welfare Act and National Institutes of Health (NIH). Animal care and the experimental procedures were carried out in agreement with institutional guidelines.

4.4. Cellular models of an inflammatory response

Raw 264.7 and L929 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Inflammatory peritoneal macrophages (4×10^5 cells/well in 96-well tissue culture plates) from paraffin-oil challenged mice were stimulated with LPS (10 ng in 100 μL) in the presence or absence of increasing concentrations of JANEX-1. In some experiments, Raw 264.7 macrophage cell line (2×10^5 cells/well) was used instead of peritoneal macrophages. After 4 h of incubation at 37 °C, 50 μL of cell culture supernatants was removed for TNF bioassays using TNF-sensitive L929 indicator cells or TNF ELISA tests. Each well was replenished with 50 μL of culture medium and the plates were incubated for another 48 h at 37 °C before 50 μL culture supernatant samples were assayed for nitric oxide (NO) released into the supernatants of mouse macrophages by measuring nitrite formation in a standard Griess reaction.[74,75] In TNF bioassays, supernatants of macrophages were assayed for cytotoxicity against TNF-sensitive L929 indicator cells using a modified photometric crystal violet staining assay.[76] In brief, L929 cells (3×10^4/well) were allowed to adhere to the wells of 96-well tissue culture plates for 3 h at 37 °C. After adherence, L929 monolayers were washed prior to addition of 50 μL of macrophage supernatant or recombinant TNFα (positive reference control) and 50 μL actinomycin D (2 μg/mL). After an 18 h incubation at 37 °C, the supernatants were removed and the adherent L929 cells were examined for viability by staining with 0.5% crystal violet, 30% ethanol, 3% formaldehyde in PBS. After 15 min, cells were solubilized with 1% SDS and the absorbance at 600 nm (A600) was measured on a Multiscan MS microplate scanner (Labsystems, Helsinki, Finland). The magnitude of TNF-mediated cytotoxicity was expressed as: % TNF cytotoxicity = [1 − (A600 of TNF treated cells)/(A600 of untreated control cells)] × 100. TNFα levels were also measured using the commercial Quantikine TNFα ELISA kit (R&D Systems, Minneapolis, MN).

The human colon cancer cell line HT29 was obtained from American Type Tissue Collection (ATCC, Manassas, VA). HT29 cells were maintained as monolayer cultures in 75-cm^2 or 150-cm^2 flask in DMEM (Gibco-BRL, Grand Island, NY) supplemented with 1 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT) as previously described.[77] HT29 cells (0.2×10^6/mL/well) were grown in 24-well tissue culture plates at 37 °C in a 5% CO_2 atmosphere. Conflu-

ent monolayers were washed three times with PBS and 1 mL fresh media was added to each well. Monolayers were exposed to 5 μg/mL LPS or vehicle (PBS) and incubated for 6 h at 37 °C. After incubation the plate was centrifuged at 200*g* for 10 min at 4 °C. Supernatants were removed and saved. To examine the effects of JANEX-1, HT29 monolayers were incubated with indicated concentrations of JANEX-1 or vehicle for 30 min prior to LPS challenge. Prostaglandin (PG) E_2 and IL-8 levels were estimated in cell-free supernatants using standard ELISAs.

4.5. Peritonitis model

C3HeB/FeJ mice were injected ip with 2 mL of sterile light paraffin oil as an inducer of an inflammatory response in a mouse peritonitis model of acute inflammation.[78] Peritoneal lavage was performed 3 days after the paraffin oil injection. Inflammatory macrophages were isolated from the harvested peritoneal lavage samples, washed, and resuspended at 2×10^6 cells/mL in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 25 mM Hepes, and 125 μg/mL gentamicin. Macrophages were plated on 96-well flat bottom microtiter culture plates at 4×10^5 cells/well, allowed to adhere to the plastic for 2 h at 37 °C, and non-adherent cells were removed by two washes with culture medium prior to LPS activation and various bioassays. Raw 264.7 murine macrophage cell line and TNF-sensitive L929 murine fibrosarcoma cell line were purchased from American Type Culture Collection, Rockville, MD. These cell lines were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 125 μg/mL gentamicin, and non-essential amino acids. For prevention of the inflammatory macrophage response, mice were treated with daily ip injections of JANEX-1 at 2 mg/kg or 4 mg/kg dose levels administered either for seven consecutive days between day −7 and day 0 prior to the paraffin oil challenge or for three consecutive days between days 0 and 2 following the paraffin oil challenge.

4.6. Paw edema model

Male ICR and CD1 mice were used in this model of acute inflammation. Hind paw edema was induced by carrageenan as described previously.[79,80] Carrageenan is a sulfated polysaccharide obtained from the alga *Chondrus crispus*. It has been used widely as an inducer of acute inflammatory responses. In brief, left rear paws were injected in the subplanter region with 25 μL of 2% carrageenan using a 30 gauge needle. Contralateral paws were injected with an equal volume of PBS instead. Test mice were treated with JANEX-1 administered at 60 and 30 min prior to carrageenan injection as an intraperitoneal 30 mg/kg bolus dose. Controls included mice treated with ibuprofen (30 mg/kg, po, administered 30 min prior to carrageenan) or vehicle (administered ip at 60 and 30 min prior to carrageenan injection). The thickness of both paws was measured with the help of a plethysmometer (Miyomoto, Japan) immediately before and 6 h after the carrageenan injection. The swelling in the paws was calculated as described by Wang et al.[81]

4.7. Colitis model

Chronic intestinal inflammation induced by 2,4,6,-trinitrobenzene sulfonic acid (TNBS) is characterized by a transmural granulomatous colitis that mimics some characteristics of human IBD. The procedure for the induction of colitis was previously reported by Neurath et al.[82] In brief, a flexible rubber catheter (3.5 French) was carefully inserted into the colon of unanesthetized BALB/c mice such that the tip was 4 cm proximal to the anal verge. To induce colitis, TNBS (0.5 mg in 50% ethanol, 100 μL total volume) was slowly administered into the lumen of the colon through the catheter fitted onto a 1 mL syringe and the mice were held in a vertical position for 45 s for drug delivery. Control mice received an equal volume of vehicle (50% ethanol). Following the treatments, mice were returned to their cages. To monitor the weight changes, the weight of the mice was recorded daily. On days 7 and 14 after administration of TNBS (or vehicle), groups of mice were sacrificed by CO_2 inhalation, and colonic inflammation was assessed. Immediately after sacrifice, the distal colon was excised and examined for macroscopic changes, which was graded on a semi-quantitative scale as described.[82,83] In order to examine the effect of JANEX-1 on the course and outcome of TNBS-induced colitis in mice, BALB/c mice were treated with JANEX-1 (25 mg/kg ip) daily starting on day −1 and continuing until the mice were sacrificed. A group of TNBS-challenged mice were treated with vehicle instead of JANEX-1. The diameter of the colon of each mouse was measured with the help of a caliper. After measurements of the diameter, the distal 8 cm of colon was excised, blotted dry, and weighed.

4.8. Mouse model of systemic inflammatory response syndrome (SIRS)[84]

C57BL/6J mice (4–5 weeks old) (Jackson Laboratories, Bar Harbor, ME) were challenged with an ip injection of D-galactosamine (4 mg) (Sigma, St. Louis, MO) plus LPS (100 ng)[84] mixed with either vehicle (control mice) or 500 μg JANEX-1 (test mice). At 1 h or 2 h post the LPS challenge, control mice were treated with vehicle and test mice were treated with 500 μg JANEX-1. The mortality was monitored for 24 h. At the time of death, lungs and liver were harvested for histopathologic examinations, as previously reported.[35] Liver tissues from control and test mice were examined for apoptosis by the in situ end-labeling method using the ApopTag in situ detection kit, according to the manufacturer's instructions (Oncor, Gaithersburg, MD). Slides were viewed with a confocal microscope (Bio-RAD MRC 1024).

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