Introduction

Cytotoxic chemotherapy in cancer patients often results in a myriad of undesirable symptoms that affect physical functioning and quality of life.1-5 These symptoms, including fatigue, decreased appetite, disturbed sleep, cognitive difficulties, changes in body composition, and depression, may also discourage patients from adhering to treatment.6 There is compelling evidence that implicates cytotoxic chemotherapy-induced inflammation in the etiology of these symptoms. Because IL-1β plays a central role as an initiator cytokine in immune responses, we compared doxorubicin, a drug known to induce IL-1β production, with ten other commonly prescribed chemotherapeutic drugs in their ability to lead to processing and secretion of IL-1β by primary mouse macrophages. Seven of them (melphalan, cisplatin, vincristine, etoposide, paclitaxel, methotrexate, and cytarabine) caused the production of IL-1β in cells pretreated with lipopolysaccharide. When delivered in combination with doxorubicin, one of the drugs, vincristine, was also capable of synergistically activating the NLRP3-dependent inflammasome and increasing expression of IL-1β, IL-6, and CXCL1. The absence of TNF-α and IL-1 signaling caused a partial reduction in the production of mature IL-1β. Three small-molecule inhibitors known to suppress activity of kinases situated upstream of mitogen-activated kinases (MAPKs) inhibited the expression of IL-1β, IL-6, and CXCL1 when doxorubicin and vincristine were used singly or together, so specific kinase inhibitors may be useful in reducing inflammation in patients receiving chemotherapy.

Production of IL-1β by bone marrow-derived macrophages in response to chemotherapeutic drugs

Synergistic effects of doxorubicin and vincristine

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Abbreviations: 5-FU, 5-fluorouracil; BMDM, bone marrow-derived macrophages; GAPDH, glyceraldehyde phosphate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; TCA, trichloroacetic acid

Cytotoxic chemotherapeutic drugs, especially when used in combination, are widely employed to treat a variety of cancers in patients but often lead to serious symptoms that negatively affect physical functioning and quality of life. There is compelling evidence that implicates cytotoxic chemotherapy-induced inflammation in the etiology of these symptoms. Because IL-1β plays a central role as an initiator cytokine in immune responses, we compared doxorubicin, a drug known to induce IL-1β production, with ten other commonly prescribed chemotherapeutic drugs in their ability to lead to processing and secretion of IL-1β by primary mouse macrophages. Seven of them (melphalan, cisplatin, vincristine, etoposide, paclitaxel, methotrexate, and cytarabine) caused the production of IL-1β in cells pretreated with lipopolysaccharide. When delivered in combination with doxorubicin, one of the drugs, vincristine, was also capable of synergistically activating the NLRP3-dependent inflammasome and increasing expression of IL-1β, IL-6, and CXCL1. The absence of TNF-α and IL-1 signaling caused a partial reduction in the production of mature IL-1β. Three small-molecule inhibitors known to suppress activity of kinases situated upstream of mitogen-activated kinases (MAPKs) inhibited the expression of IL-1β, IL-6, and CXCL1 when doxorubicin and vincristine were used singly or together, so specific kinase inhibitors may be useful in reducing inflammation in patients receiving chemotherapy.
Many cytotoxic chemotherapeutic drugs such as cisplatin, doxorubicin, etoposide, and 5-fluorouracil (5-FU) activate the mitogen-activated protein kinases cascade, which plays a central role in the production of inflammatory cytokines that may lead to sickness behavior. ZAK, an upstream MAPK, is required for the activation of JNK and p38 MAPK by doxorubicin in epithelial cells and macrophages. IL-1β and IL-18 are first produced as a biologically inactive precursor that requires processing by the inflammasome, a multi-protein complex consisting of caspase 1 or 11 (caspase 4 and 5 in humans), a member of the NLRP family such as NLRP3, and often adaptor molecules such as ASC. Various mechanisms for the activation of the inflammasomes have been proposed. Bacterial pore-forming toxins, bacterial and viral pathogens, asbestos, silica, ATP, double-stranded RNA, uric acid crystals, and translation inhibitors (such as emetine and ribotoxic stressors including doxorubicin) stimulate IL-1β processing via the NLRP3-inflammasome. Of cancer chemotherapeutic agents, gemcitabine and 5-FU have been shown to activate the inflammasome in myeloid-derived suppressor cells; doxorubicin has been shown to do the same in macrophages.

Although doxorubicin is able to activate the NLRP3-dependent inflammasome, it is unknown whether other chemotherapeutic drugs also share a common ability to induce the processing of IL-1β. It is also unclear whether cytotoxic chemotherapeutic agents, which are more commonly administered together, have additive or synergistic effects on inflammatory cytokine production by immune cells. In this study we screened 11 cancer chemotherapeutic agents from distinct drug classes for their ability to stimulate the processing and secretion of IL-1β by mouse bone marrow–derived macrophages (BMDM). We found that like doxorubicin, seven other drugs (melphalan, cisplatin, vincristine, etoposide, paclitaxel, methotrexate, and cytarabine) also stimulated the production of IL-1β. Because doxorubicin and vincristine are commonly used together in combination chemotherapy, we determine whether these two drugs have additive or synergistic effects on inflammatory cytokine production. When compared with treatment by each drug singly, the combination of doxorubicin and vincristine synergistically activated NLRP3-mediated processing of IL-1β and synergistically blocked protein synthesis. Moreover, these drugs in combination also induced a synergistic increase in the expression of IL-1β, IL-6, and CXCL1 at both the RNA and protein level. The expression was partially reduced in cells lacking the IL-1 and TNF-α receptors and in the presence of anakinra, an IL-1 receptor antagonist. In contrast, the expression was almost completely inhibited by small-molecule kinase inhibitors nilotinib, ponatinib, and sorafenib. Taken together, these data demonstrate that doxorubicin and vincristine synergistically increase both the expression of IL-1β, IL-6, and CXCL1 and the processing and release of IL-1β and that small molecule kinase inhibitors may prove useful in reducing possible inflammatory side effects of chemotherapeutic drugs.

Results

Mechanistically distinct cytotoxic chemotherapeutic agents activate the NLRP3-dependent processing of IL-1β

Doxorubicin activates the NLRP3-inflammasome to promote the processing of pro-IL-1β to mature IL-1β. To determine whether mechanistically distinct cytotoxic chemotherapeutic agents share a common ability to promote the processing and release of IL-1β, we incubated LPS-primed BMDM with clinically relevant concentrations (corresponding to peak plasma levels) of azacytadine (0.75 μg/mL), cisplatin (17 μM), cytarabine (2 μM), etoposide (33 μM), fludarabine (1.54 μg/mL), melphalan (2.8 μg/mL), methotrexate (2.3 μM), paclitaxel (1 μM), vincristine (0.4 μM), 5-FU (0.5 mM), or doxorubicin (5 μM). Like doxorubicin, (in decreasing ability) melphalan, cisplatin, vincristine, etoposide, paclitaxel, methotrexate, and cytarabine caused the release of mature IL-1β into the culture medium, albeit to a lesser extent (Fig. 1). In contrast, azacytidine, fludarabine, and 5-FU did not induce IL-1β processing and release.

Doxorubicin and vincristine synergistically induce IL-1β production

In this experiment we determined whether cytotoxic chemotherapeutic agents, that are commonly administered together, have additive or synergistic effects on inflammatory signaling. When LPS-primed cells were exposed simultaneously to doxorubicin and vincristine, levels of IL-1β released into the culture medium were significantly increased compared with cells treated singly (Fig. 2A). The production of mature IL-1β by these agents, singly or in combination, was absent in NLRP3-deficient BMDM (Fig. 2B), suggesting a central role for the NLRP3-inflammasome in the conversion of pro-IL-1β to mature IL-1β. The increased release of IL-1β that occurred following cotreatment of BMDM with doxorubicin and vincristine may result from released IL-1β and/or TNF-α acting in an autocrine or paracrine fashion, thereby augmenting the inflammatory effects of the drugs. To examine this possibility, we cultured BMDM from WT and mice lacking both TNFR1 and IL-1R1 (Fig. 2C). Mutant BMDM treated with doxorubicin alone or doxorubicin and vincristine released significantly lower levels of IL-1β into the medium than treated WT BMDM (lanes 2, 4, 6, and 8). Nonetheless, the synergistic effect of doxorubicin and vincristine

![Figure 1. Mechanistically distinct chemotherapeutic drugs caused the release of mature IL-1β by BMDM. After serum deprivation for 0.5 h, BMDM were pre-treated with 50 ng/mL LP5 for 4 h and then washed away. The cells were then treated with the indicated chemotherapeutic drugs for 18 h. LPS-primed BMDM were treated with medium alone (lane 1), azacitidine (lane 2), cisplatin (lane 3), cytarabine (lane 4), etoposide (lane 5), fludarabine (lane 6), melphalan (lane 7), methotrexate (lane 8), paclitaxel (lane 9), vincristine (lane 10), 5-FU (lane 11), or doxorubicin (lane 12). Western blots of cell lysates and medium samples were then processed using antibodies against total p38 MAPK in the cell lysates (as loading control) or IL-1β in the medium.](image-url)
on the production of IL-1β was still observed in cells lacking TNFR1 and IL-1R1 (lanes 6, 7, and 8).

Doxorubicin and vincristine synergistically increase the RNA levels of IL-1β, IL-6, and CXCL1

In addition to augmenting the processing of pro-IL-1β to mature IL-1β, vincristine and doxorubicin may also increase the level of IL-1β in the medium by elevating the expression of pro-IL-1β. To examine the effects of doxorubicin and vincristine on the expression of RNA encoding pro-IL-1β and other inflammatory cytokines, the RNA of treated BMDM was extracted for real-time PCR analysis (Fig. 3). Combining doxorubicin and vincristine induced a significantly higher level of expression of RNA encoding IL-1β, IL-6, and CXCL-1 (Gro-α or KC), compared with addition of doxorubicin or vincristine alone ($P < 0.001$). Increased expression of TNF-α and CCL2 (MCP-1) was not observed in any treatments (data not shown). Of interest, cells co-treated with doxorubicin and vincristine caused the accumulation of mature IL-1β in the medium even in the absence of LPS priming (lane 4 in Fig. 2A). This finding is consistent with doxorubicin and vincristine being able to enhance the expression of IL-1β resulting in the accumulation of intracellular pro-IL-1β.

**Anakinra partially blocks the expression of IL-6 and CXCL1**

We sought to determine whether the synergistic increase in the expression of IL-1β, IL-6, and CXCL-1 could be due to the paracrine and/or autocrine binding of released IL-1β to the IL-1R receptor. This was achieved using anakinra, a competitive inhibitor of IL-1β signaling.42 Because increased expression of TNF-α was not detected after treatment with doxorubicin, vincristine, or both, we did not use an inhibitor of the TNF-α pathway. In the presence of 10 μg/mL anakinra, the expression of IL-1β caused by the co-incubation with doxorubicin and vincristine was increased by 22%, while the expression of IL-6 and CXCL1 was decreased by 63% and 32%, respectively ($P < 0.001$) (Fig. 3). These results suggest that synthesis and release of IL-1β augment the expression of IL-6 and CXCL1 following exposure to doxorubicin and vincristine.

**Doxorubicin and vincristine inhibit protein translation**

We previously reported that a variety of agents that activate the NLRP3-inflammasome, including doxorubicin, also inhibit protein synthesis.43,44 To determine whether the synergistic effect of doxorubicin and vincristine on IL-1β secretion was due to a synergistic inhibition of protein synthesis, we measured the incorporation of [3H]-leucine into BMDM after treatment with doxorubicin, vincristine, or both. After 1.5 h, the level of leucine incorporation was similar in all treatments (Fig. 4). At 3 h, leucine incorporation was 90% of control levels following exposure to vincristine, whereas leucine incorporation by doxorubicin or doxorubicin plus vincristine was reduced to approximately 60%. At 6 h, vincristine began to enhance the inhibitory effect of doxorubicin, resulting in greater inhibition of protein synthesis in the presence of both drugs than in the presence of either drug added singly. When both doxorubicin and vincristine were present for 12 h, leucine incorporation was reduced to 12%, compared with 40% and 70% in response to single treatments with doxorubicin and vincristine, respectively. These data demonstrate that vincristine augmented the doxorubicin-induced inhibition of translation, but only modestly inhibited translation when added alone.

**Small-molecule kinase inhibitors block the expression of IL-1, IL-6, and CXCL1**

Because anakinra only partially inhibited the inflammatory gene expression following doxorubicin and vincristine, we searched for more effective inhibitors that may have potential therapeutic value in blocking the inflammatory response to cytotoxic chemotherapeutic agents and resulting side effects. By targeting BCR-ABL, nilotinib44 and ponatinib45 are two small-molecule kinase inhibitors that are used in the treatment of chronic myelogenous leukemia. Sorafenib46 inhibits vascular endothelial growth factor receptor and platelet-derived growth factor receptor and is clinically used to treat renal cell carcinoma, hepatocellular carcinoma, and thyroid carcinoma. We have
previously shown that all three of these tyrosine kinase inhibitors block the expression of inflammatory genes by BMDM following exposure to doxorubicin. In the presence of these inhibitors, the expression of RNA encoding IL-1β, IL-6, and CXCL-1 by doxorubicin, vincristine, or both was blocked (Fig. 5A). Media was collected from these cultures for measurement of IL-1β, IL-6, and CXCL-1. Consistent with the expression of RNA, the protein levels of IL-1β, IL-6, and CXCL-1 were highly elevated in the combined presence of doxorubicin and vincristine, compared with either compound alone, and these levels were substantially reduced in the presence of nilotinib, ponatinib, or sorafenib (Fig. 5B).

Both doxorubicin and vincristine activate the phosphorylation of JNK and p38 MAPK

ZAK is a MAP3K whose activation has been tied to expression of inflammatory cytokines. Because nilotinib, ponatinib, and sorafenib each block ZAK-mediated expression of genes by doxorubicin, we employed BMDM isolated from two separate ZAK-deficient mice to determine whether ZAK played a role in MAPK activation following treatment with doxorubicin, vincristine, or both (Fig. 6). In WT cells, doxorubicin and vincristine individually caused phosphorylation of both subunits of JNK, although the increase by doxorubicin was weaker (lanes 2 and 3). When doxorubicin and vincristine were added simultaneously, the level of JNK phosphorylation was similar to that when vincristine was added alone (lanes 3 and 4). Doxorubicin or vincristine, added singly or in combination, induced similar levels of phosphorylation of p38 MAPK (lanes 2, 3, and 4). Compared with WT cells, the phosphorylation of JNK by doxorubicin in Zak−/− cells was undetected (lanes 6 and 10), whereas the phosphorylation of JNK by vincristine (lanes 7 and 11) or by both drugs (lanes 8 and 12) was present but reduced in amount in the ZAK-deficient cells. The phosphorylation of p38 MAPK in Zak−/− cells after treatment with doxorubicin (lanes 6 and 10), vincristine (lanes 7 and 11), or both (lanes 8 and 12) was similar to the level in untreated cells (lanes 5 and 9). These data demonstrate that, like doxorubicin, vincristine activates p38 MAPK in a ZAK-dependent manner, but that activation of JNK is only partly ZAK-dependent.

Cisplatin and vincristine display additive, not synergistic, effects

Among the chemotherapeutic drugs tested in Figure 1A, cisplatin was also able to activate the processing of IL-1β. To test whether cisplatin, a synthetic platinum-containing compound which causes DNA crosslinking, is also capable of synergistically
activating the expression of inflammatory genes and processing of IL-1β, we treated BMDM with cisplatin and vincristine, singly and in combination. Compared with vincristine, cisplatin was a weak activator of the expression of IL-1β, IL-6, and CXCL-1 (Fig. 7A) and of the processing of IL-1β (Fig. 7B). When cells were incubated in both cisplatin and vincristine, the level of IL-1β expression was similar to the level caused by vincristine alone (Fig. 7A). We also observed an additive, but not synergistic, increase in the amount of mature IL-1β secreted into the medium (Fig. 7B).

Discussion

Among a panel of 11 chemotherapeutic drugs that have different mechanisms of action, doxorubicin was the most potent in the ability to induce IL-1β processing and secretion by LPS-primed BMDM (Fig. 1). Seven other drugs were also able to induce release of mature IL-1β. In contrast, azacitidine, fludarabine, and 5-FU, which are all nucleoside analogs, did not stimulate IL-1β processing. Therefore, in addition to pathogens, plant and bacterial toxins, antibiotics, ATP, and crystals, cytotoxic chemotherapeutic drugs (excluding some nucleoside analogs) may now be added as a new class of agents that can activate IL-1β processing. It is known that, compared with lymphocytes, macrophages have much lower intracellular concentrations of the active metabolites of many nucleoside analogs. In addition, macrophages are known to be resistant to physiological concentrations of 5-FU and fludarabine, so the inability of certain nucleoside analogs to induce IL-1β production may be due to poor uptake of these drugs by macrophages. Interestingly, IL-1β was detected after exposure of LPS-pretreated BMDM to cytarabine, which

![Figure 5. Small-molecule kinase inhibitors block the expression of IL-1β, IL-6, and CXCL1. BMDM were treated with doxorubicin, vincristine, or both for 12 h in the presence or absence of nilotinib, ponatinib, or sorafenib as indicated. (A) RNA was extracted from the cells and processed for real-time RT-PCR. (B) Cytokine levels in the medium were quantitated using a multiplex assay.](image-url)
is another nucleoside analog, suggesting that not all nucleoside analogs behave identically.

It is unknown which characteristics are shared by melphalan, cisplatin, vincristine, etoposide, paclitaxel, methotrexate, cytarabine, and doxorubicin that contribute to their ability to induce the production of mature IL-1β. The ability to inhibit protein synthesis (Fig. 4) may underlie this effect. Our finding that doxorubicin and vincristine synergistically enhance IL-1β production and inhibit protein synthesis is consistent with this idea. These data demonstrated for the first time that two agents can synergistically activate the NLRP3-dependent inflammasome in LPS-primed BMDM (Fig. 2). In contrast, when vincristine and cisplatin were used together, the effect on IL-1β processing was additive and not synergistic (Fig. 7). It is clear that chemotherapeutic drugs differ in their ability to augment the processing of IL-1β and the expression of inflammatory mediators. Additional experiments are needed to determine systematically which drugs, especially when used in combination, can induce an inflammatory response.

Doxorubicin and vincristine are commonly used together in the treatment of Hodgkin lymphoma, B-cell lymphomas, lung cancer, Wilms tumor, multiple myeloma, thymic carcinoma, and Kaposi sarcoma, raising the concern that patients undergoing such therapy may express elevated levels of inflammatory cytokines, which in turn may lead to increased cancer treatment-related symptoms. The advantages of combination chemotherapy need to be balanced with consideration of serious side effects. Because the inhibition of protein synthesis appears to be mechanistically linked to the production of IL-1β, assays that measure protein synthesis in cytokine-producing cells may be effective in predicting the ability of drugs to activate the inflammasome and cause inflammation-induced symptoms. Ultimately, using drug combinations that enhance tumor killing without enhancing side effects would be clearly be optimal.

Doxorubicin-treated BMDM from Tnfrc−/− Iletr−/− mice expressed lower amounts of IL-1β than WT BMDM (Fig. 2C), and anakinra was partially able to block the increase in gene expression following co-treatment with doxorubicin and vincristine (Fig. 3). These results suggest that IL-1β may act in an autocrine or paracrine fashion to increase the production of IL-1β, perhaps by activating NFκB, a transcription factor important in the expression of many pro-inflammatory genes. Therefore, doxorubicin and vincristine each activate JNK and p38 MAPK (Fig. 6). Should secreted IL-1β result in activation of NFκB in these cells, the participation of both MAPK and NFκB pathways may prove to be responsible for the observed synergistic increase in gene expression.

Unlike anakinra, the three small-molecule kinase inhibitors (nilotinib, ponatinib, and sorafenib) were able to suppress the expression of IL-1β, IL-6, and CXCL1 nearly to baseline levels (Fig. 5), suggesting that these drugs may potentially be useful in lowering the level of inflammation in patients. These three kinase inhibitors have been identified as putative ZAK inhibitors that block doxorubicin-mediated increase in the phosphorylation of MAPKs in keratinocytes and BMDM and in the expression of inflammatory proteins in BMDM. Indeed, because BMDM deficient in ZAK showed decreased phosphorylation of p38 MAPK (Fig. 6), ZAK may regulate the pro-inflammatory effects of doxorubicin and vincristine, at least in part. Additional experiments are needed to determine whether ZAK may play a role in the pro-inflammatory effects of other chemotherapeutic drugs and whether these inhibitors may be beneficial in countering side effects. Interestingly, nilotinib has been shown to reduce the cardiotoxicity due to doxorubicin in rats and may even contribute to reversing multidrug resistance by sensitizing cancer cells to killing by doxorubicin or vincristine. Therefore, these studies provide additional incentives to introduce ZAK inhibitors such as nilotinib with chemotherapeutic drugs such as doxorubicin and vincristine to increase tumor killing and reduce systemic toxicity and other side effects.

Reducing IL-1β levels may lead to a reduction of the undesirable side effects of some chemotherapeutic drugs. However, IL-1β may also promote the eradication of the tumor that the drugs are targeting, perhaps due to changes in the tumor microenvironment and in the immune responses of the body. Additional research, especially employing in vivo models, needs to be performed to study any effects on tumor responses and animal survival.

Materials and Methods

Chemicals and antibodies

Azacitidine (Sigma), cisplatin (Teva), cytarabine (Sigma), etoposide (Teva), fludarabine (Sagent), melphalan (Sigma), methotrexate (Hospira), paclitaxel (Hospira), vincristine (Hospira), 5-FU (Teva), doxorubicin (APP Pharmaceuticals), and anakinra (Amgen) were stored according to manufacturer’s recommendation. Lipopolysaccharide (LPS) from E. coli serotype 0111:B4
was purchased from Enzo Life Sciences. Nilotinib and sorafenib (LC Laboratories) and ponatinib (Tocris Bioscience) were dissolved in DMSO and stored at -80 °C. Insulin was purchased from Sigma. Trichloroacetic acid (TCA) was purchased from Fisher Scientific. Antibody against IL-1β (ab9722) was purchased from Abcam, phospho-JNK (9251) and phospho-p38 MAPK (9211) from Cell Signaling Technology, and p38 MAPK (sc-535) from Santa Cruz Biotechnology.

Mice
All animal procedures were approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University and Massachusetts General Hospital. C57BL/6J mice, mice lacking NLRP3, and mice lacking both the TNF-α type 1 receptor and IL-1 type 1 receptor (B6;129S-Tnfrsf1atm1Imx Il1r1tm1Imx/J) (10–12 wk of age) were purchased from the Jackson Laboratory. Generation of mice lacking ZAK is described in a separate manuscript under preparation.

Isolation and treatment of bone marrow-derived macrophages (BMDM)
Mice, 8–10 wk of age, were used throughout the experiments. Bone marrow cells from the femurs and tibias of wild-type C57BL/6 (WT), Nlrp3−/−, Tnfr1−/− Il1r1−/−, and Zak−/− mice were flushed with and cultured in α-Minimum Essential Medium (Corning Cellgro), supplied with 10% fetal bovine serum (Hyclone), 50 μg/mL gentamicin (Life Technologies), and 100 ng/mL recombinant mouse colony-stimulating factor 1 (R&D Systems) for 72 h on non-tissue culture-treated 10-cm petri dishes. BMDM were passaged and cultured for an additional 72 h. Cells were plated onto 12-well or 24-well tissue culture plates and cultured for 24 h before initiating experimental treatment. Nilotinib, ponatinib, sorafenib (all at 1 μM), or anakinra (10 μg/mL) was added 30 min before the addition of doxorubicin, vincristine, or both.

Immunoblotting
BMDM were lysed in 2x electrophoresis sample buffer. Proteins were separated on a denaturing polyacrylamide gel in the presence of sodium dodecyl sulfate and transferred onto polyvinylidene difluoride membranes according to standard laboratory procedures. Proteins from BMDM media supernatants were first precipitated using TCA plus 200 μg insulin carrier protein and separated by 13% SDS-PAGE gels. Membranes were incubated with the indicated antibodies and the corresponding horseradish peroxidase-conjugated secondary antibodies. Signals were detected by using enhanced chemiluminescence. Experiments were repeated at least three times and representative experiments are shown.

Measurement of protein synthesis by incorporation of [3H]-leucine
BMDM were cultured in 24-well tissue culture plates. Treatments were performed in leucine-free/serum-free Dulbecco modified Eagle medium (BioWhittaker), for the indicated times. Prior to harvesting, the cells were pulse-labeled with 1 μCi [3H]-leucine (Perkin Elmer) in leucine-free medium for times specified in the figure legends. An equal volume of 10% TCA was added to terminate incorporation. Wells were washed in water, and 88% formic acid was added to solubilize the TCA-insoluble proteins. The samples were counted in a liquid scintillation counter. In each experiment, triplicate wells were used per experimental point.

Real-time RT-PCR
Total RNA from frozen tissues was isolated using TRIzol (Invitrogen) following the manufacturer’s instructions. RNA was treated with DNase I (Invitrogen) and reverse-transcribed with SuperScript II and oligo dT primer (Invitrogen). Real-time PCR was performed using SYBR Green reagents on a ViiA 7 Real-Time PCR System (Applied Biosystems); fold induction was calculated with the absolute quantification method using levels of glyceraldehyde phosphate dehydrogenase (GAPDH) for normalization. The nucleotide sequences of the primers used in this study have been previously published.56 Experiments were repeated at least three times, and representative data are shown. Statistical analyses were performed using t test.
Measurement of inflammatory cytokines from culture supernatant

Levels of IL-1β, IL-6, and CXCL1 were measured using bead-based immunassays (EMD Millipore). Data were collected and analyzed using the Luminex-100 system Version IS (Luminex). A four- or five-parameter regression formula was used to calculate the sample concentrations from the standard curves.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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