

Constitutive Aberrant Endogenous Interleukin-1 Facilitates Inflammation and Growth in Human Melanoma

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Abstract

Interleukin (IL)-1-mediated inflammation is proposed to contribute to the development and progression of some cancers. IL-1 family member proteins are known to be expressed constitutively in many melanoma tumor cells, and we hypothesize that these support molecular pathways of inflammation and facilitate tumor growth. To investigate the expression of IL-1 α and IL-1 β in melanoma patients, and their association with disease progression, immunohistochemical staining was carried out on tissues from 170 patients including benign nevi, primary melanomas, and metastatic melanomas. IL-1 β levels were low (or zero) in benign nevi and higher in primary and metastatic melanomas ($P < 0.0001$). IL-1 α was expressed in about 73% of nevi and 55% of metastatic melanomas, with levels significantly higher in primary tumors ($P < 0.0001$); most (98%) primary melanoma samples were positive for IL-1 α . *In vitro* studies with seven human melanoma cell lines showed that five cell lines expressed IL-1 α and IL-1 β proteins and mRNA. We identified for the first time several important downstream signaling pathways affected by endogenous IL-1, including reactive oxygen and nitrogen species, COX-2, and phosphorylated NF- κ B inhibitor (I κ B) and stress-activated protein kinase/*c-jun*-NH₂-kinase; all of which were decreased by siRNA to IL-1s. Downregulation of IL-1 α , IL-1 β , or MyD88 substantially increased p21 and p53 levels. Treatment with IL-1 receptor type I neutralizing antibody or IL-1 pathway-specific siRNAs led to growth arrest in IL-1-positive melanoma cells. Furthermore, blocking the IL-1 pathway increased autophagy in IL-1-positive melanoma cells. These results indicate that the endogenous IL-1 system is functional in most human melanoma and interrupting its signaling inhibits the growth of IL-1-positive melanoma cells. *Mol Cancer Res*; 9(11); 1537–50. ©2011 AACR.

Introduction

Chronic inflammation not only facilitates the progression of normal cells to malignancy but also supports survival of many malignancies through production of proinflammatory cytokines and reactive oxygen species (ROS) and reactive nitrogen species (RNS). Melanoma is one of these tumor types that is strongly associated with the inflammatory process, as melanoma are known to secrete high levels of cytokines and produce ROS and RNS (1–4). The cytokines expressed by melanoma cells function as paracrine regulators of the microenvironment, and possibly as tumor growth-supporting factors. It has been shown that proinflammatory

molecules, such as interferon- γ and interleukin (IL)-1, can activate and recruit myeloid-derived suppressor cells (MDSC) to the tumor sites, resulting in strong suppression of various T-cell functions (5–9). It is speculated that melanoma-expressed cytokines can stimulate MDSCs and promote highly immune-suppressed microenvironment. It is also well accepted that melanoma-expressed cytokines can also function as autocrine factors controlling tumor growth and metastasis. However, the scope and function of endogenous melanoma cytokines are poorly understood.

The constitutive expression of IL-1 has been shown to stimulate angiogenesis and promote tumor growth and metastasis in some human melanoma cells and mouse melanoma models (10–12). Thus, of all the melanoma-produced inflammatory factors, the IL-1 family of cytokines is of particular interest. The IL-1 family consists of 2 proinflammatory cytokines, IL-1 α and IL-1 β ; an IL-1 receptor antagonist (IL-1Ra); and 2 different receptors, the biologically active IL-1 receptor type I (IL-1RI) and the inert IL-1RII (13). One recent study reports that in the absence of exogenous stimuli, some human melanoma cells spontaneously produce functional IL-1 β , which leads to constitutive activation of the inflammasome (14). On the basis of previous studies, we now hypothesize that the tumor-derived IL-1 promotes endogenous intracellular features of inflammation and thereby

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facilitates growth progression of human melanoma; also the downregulation of IL-1 signaling will suppress the growth of the IL-1-expressing melanoma.

Our group's initial study of 6 human melanoma cell lines and 29 melanoma tissues showed that tumor-secreted IL-1 can function in a paracrine fashion (15). However, a more complete expression profile of IL-1 α and IL-1 β in melanoma has yet to be documented *in vivo*. Furthermore, the mechanisms of melanoma-expressed IL-1 driving inflammation and tumor progression are not yet fully understood. In the immune system, it has been reported that binding of IL-1 to IL-1RI can lead to recruitment of the adaptor molecule MyD88, which subsequently activates several important kinase transduction cascades resulting in activation of NF- κ B and other inflammatory genes such as COX-2 (16–18). Whether autocrine IL-1 affects these inflammatory pathways in melanoma cells is still unclear.

In this study, we addressed the hypothesis presented above by testing the functional role of tumor-derived IL-1 in melanoma growth in a series of *in vitro* experiments. Furthermore, we tested the association of the protein levels of IL-1 with melanoma progression by examining a melanoma tissue microarray (TMA) with cores from 170 patients using immunohistochemical (IHC) techniques. We identify for the first time several important downstream signaling pathways and markers that are affected by aberrant autocrine IL-1 in melanoma cells. We report here that blocking IL-1 signaling by specific siRNAs or IL-1RI-neutralizing antibody leads to the growth suppression in IL-1-positive human melanoma cells. Furthermore, blocking the IL-1 pathway by specific siRNAs can lead to the induction of autophagy in IL-1-positive melanoma cells.

Materials and Methods

Antibodies and reagents

DNA oligomers and MTT were obtained from Sigma. Acrylamide solution, ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Bio-Rad Laboratories. LC3 antibody was purchased from Novus Biologicals; human IL-1RI-neutralizing antibody, goat immunoglobulin G (IgG), and MyD88 antibody from R&D Systems; antibodies κ B- α (H-4) and p- κ B- α (B-9) from Santa Cruz Biotechnology; antibodies for COX-2, phospho-SAPK/JNK (81E11; SAPK, stress-activated protein kinase; JNK, *c-jun*-NH₂-kinase), and SAPK/JNK from Cell Signaling Technology. For IHC, IL-1 α antibody was from LifeSpan Biosciences, and antibody for IL-1 β (H-153) was from Santa Cruz Biotechnology. For Western blotting, IL-1 α antibody (clone 4414) and IL-1 β antibody (clone 8516) were from R&D Systems. Both nontarget and anti-human MyD88 SMARTpool siRNA were purchased from Dharmacon. Chimera RNAi for IL-1 α and IL-1 β were purchased from Novus Biologicals.

Melanoma TMA

The melanocytic tumor progression TMA was developed as a collaborative effort of the Harvard, MD Anderson, and

University of Pennsylvania Skin SPOREs and served as a template for the investigation of IL-1 in human melanocytic tumors. This TMA was designed to provide samples of tumors from each step in melanocytic tumor progression (19). The TMA contains 480 tissue cores from 170 patients, consisting of benign nevi (132 cores from 36 patients), primary cutaneous melanomas (196 cores from 59 patients), melanoma metastases to lymph nodes (60 cores from 29 patients), and melanoma metastases to visceral organs (92 cores from 46 patients). Acral, mucosal, and uveal melanomas were excluded from this TMA (19).

IHC studies

We used an IHC protocol, described by us previously (20), employing primary antibodies for IL-1 α (1:100) and IL-1 β (1:50). Immunolabeling was scored separately for 2 variables, first according to the percentage of melanoma cells with positive staining (<5% = 0, 5%–25% = 1, 26%–75% = 2, and >75% = 3) and then according to the overall intensity of immunoreactivity of the positive cells (no staining = 0, light staining = 1, moderate staining = 2, and marked staining = 3). Cases were considered to be positive if either the percentage or intensity scores were 1 to 3. The slides were manually scored independently by 2 researchers (SE and YQ) without prior knowledge of the tumor stage or data.

Cell culture and RNA isolation

Seven human melanoma cell lines were used in this study: A375, MeWo, SK-MEL-2, WM35, WM793, SB2, and TXM1, which were selected to represent the major somatically mutated and clinically relevant subtypes. Detailed information of each cell line and their authentication are provided in the Supplementary Data. Human melanoma cells were cultured in RPMI medium containing 100 U/mL penicillin, 100 μ g/mL streptomycin, 200 mmol/L L-glutamine, and 5% FBS (Sigma) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Human melanocytes were purchased from PromoCell and cultured in melanocyte growth medium 2 (PromoCell). Cells were maintained in logarithmic growth phase. Total cellular RNA was extracted by the help of a NucleoSpin RNA II kit (Macherey-Nagel).

siRNA transfection

Melanoma cells were plated in 6-well plates at 1×10^5 cells per well and cultured overnight at 37°C in 2 mL of RPMI medium. The next day, each well was transfected with 20 nmol/L siRNA specific for IL-1 α , IL-1 β , or MyD88 with 3.2 μ L of Lipofectamine RNAiMAX (Invitrogen). Cotransfection with 20 nmol/L IL-1 α siRNA and 20 nmol/L IL-1 β siRNA was used to downregulate IL-1 α and IL-1 β simultaneously. Transfection with 40 nmol/L nontarget siRNA with 3.2 μ L of Lipofectamine served as the negative control. Treatment with 3.2 μ L of Lipofectamine without any siRNA served as another negative control. Cells were harvested 24 to 72 hours after siRNA transfection and then subjected to analyses.

Western blot analysis

Cells were lysed in buffer containing 50 mmol/L Tris (pH 7.9), 150 mmol/L NaCl, 1% NP-40, 1 mmol/L EDTA, 10% glycerol, 1 mmol/L sodium vanadate, and protease inhibitor cocktail (Roche). Proteins were separated by 8% SDS-PAGE gels, transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare Biosciences), and blocked in 5% dry milk in PBS. The membrane was then incubated with primary and secondary antibodies, and target proteins were detected with ECL detection reagent (GE Healthcare Biosciences).

Reverse transcriptase PCR

To determine the mRNA levels of IL-1 α and IL-1 β in melanoma cells, we carried out first strand cDNA synthesis with 400 ng of total RNA with a GeneAmp RNA PCR kit (Applied Biosystems) according to the manufacturer's protocol. A 2 μ L cDNA product was used for each 25 μ L PCR reaction. The reverse transcriptase PCR (RT-PCR) primers used to detect human IL-1 α , IL-1 β , and β -actin were adapted from those described previously (21, 22). The PCR protocol consisted of initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 40 seconds, 55.5°C for 30 seconds, and 72°C for 60 seconds; primer extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes. We analyzed 20 μ L of PCR product on a 1% agarose gel.

ROS/RNS detection and quantification

Melanoma cells treated with siRNA for 24 hours were analyzed for ROS/RNS content with the ROS/RNS detection kit (Enzo Life Sciences) according to the manufacturer's protocol. The nitric oxide (NO) detection reagent reacts with NO to yield red fluorescence, and the total ROS detection dye reacts with a wide range of ROS/RNS to yield green fluorescence. We used a Nikon Eclipse TE2000U fluorescence microscope to capture images of the red and green fluorescence. Nikon Elements ROI Statistics software was used for the quantification. The average fluorescence intensity of 6 selected noncell areas was used as the background. The fluorescence intensities of 10 different areas of cells were measured for each siRNA treatment. The background was subtracted from the intensity of each cell area. The adjusted intensity for each cell area was calculated.

Cell proliferation and viability assays

Human melanoma cells were plated in 24-well plates at 1×10^4 cells per well and cultured overnight before treatment. For the cell proliferation assay, melanoma cells were treated with siRNAs for 72 hours, and then MTT reagent was added to cells to a final concentration of 1 mg/mL. After 3 hours, the precipitate formed in cells was dissolved in dimethyl sulfoxide, and the color intensity was measured with a MRX revelation microplate absorbance reader (Dynex Technologies) at 570 nm. For the cell viability assay, melanoma cells treated with antibodies for 72 hours were trypsinized and harvested. The cells were washed with

1 \times PBS and then diluted in 0.2% trypan blue. The number of viable cells was determined.

Quantification of acidic vesicular organelles

Melanoma cells treated with siRNA for 72 hours were stained with acridine orange (AO) and analyzed by flow cytometry to determine the population of cells undergoing autophagy. Melanoma cells were incubated with 1 μ g/mL AO at 37°C for 15 minutes. Then, cells were washed twice with 3 mL per well of PBS, trypsinized, and resuspended in 600 μ L of RPMI medium with 10% FBS. The stained samples were analyzed with a FACSCalibur (Becton Dickinson).

Statistical analysis

We used the Wilcoxon rank-sum test to compare fluorescence intensity for different treatments and to compare cell viability for different treatments. The Fisher exact test was conducted to compare the IL-1 α and IL-1 β expression levels versus tumor type. Levels of statistical significance were set at 0.05. All statistical analyses were conducted with SAS software (version 9.2; SAS Institute Inc).

Results

Expression profile of IL-1 in human melanoma tissues and cell lines

To determine whether melanoma cells express IL-1 *in vivo*, we used IHC to assess IL-1 α and IL-1 β protein levels on a TMA consisting of 480 tissue cores from clinically stratified melanoma specimens from 170 patients. We observed various positive staining intensities and percentages in the cytoplasmic compartment of tumor cells for both IL-1 α and IL-1 β (Fig. 1A). IL-1 α and IL-1 β protein expression characteristics varied with melanocytic tumor progression. We observed positive cytoplasmic staining (percentage score 1–3) for IL-1 α protein in 73.2% of benign nevi, 98.4% of primary melanomas, and 55% of metastatic melanomas (Table 1). We observed the same trend for IL-1 α staining intensity (Table 1 and Fig. 1B). IL-1 α expression in primary melanomas was significantly higher than nevus ($P < 0.0001$) and metastatic melanomas ($P < 0.0001$; Fig. 1B and Tables 1 and 3). Moreover, the IL-1 α expression in thick primary melanomas was higher than thin primary melanomas, but there was no difference in IL-1 α expression between lymph nodes and visceral metastasis melanomas. As shown in Table 1, the positive cytoplasmic staining (percentage score 1–3) for IL-1 β increased significantly from nevus (0%) to primary tumor (13.0%; $P < 0.0001$) and metastasis (9.8%; $P = 0.0001$). Again, we observed the same trend for IL-1 β staining intensity (Fig. 1C and Tables 1 and 4). Due to the low number of positive samples, there was no significant difference of IL-1 β levels between primary and metastatic melanomas. The expression IL-1 α was significantly higher than IL-1 β in melanocytic tumor tissues for staining percentage and intensity ($P < 0.0001$; Tables 1, 3, and 4). The IL-1 α and IL-1 β double-positive samples only appeared in about 10% of primary and metastatic melanoma tissues but not in nevi (Table 2), where the IL-1 β expression was rare.

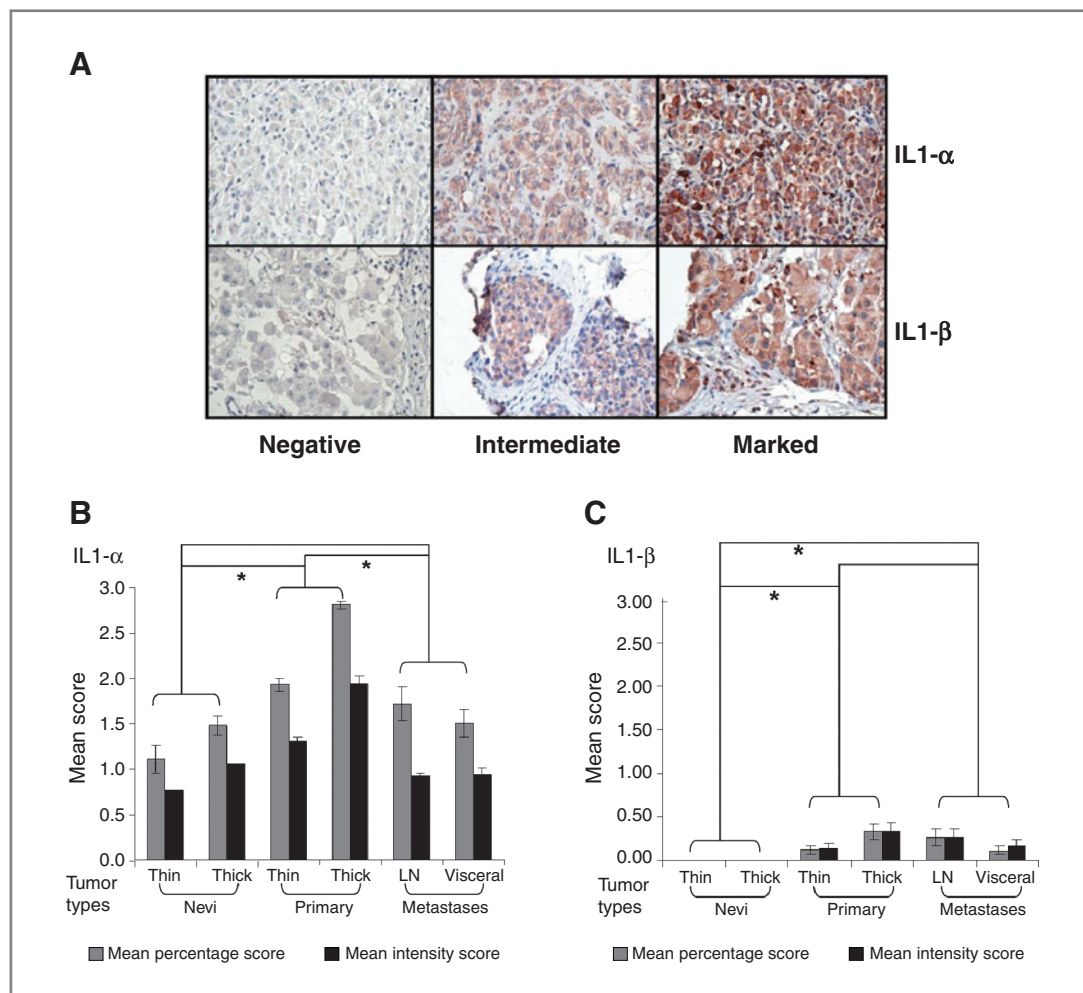


Figure 1. Expression profile of IL-1 α and IL-1 β in human melanocytic tumor tissue cores. Staining intensity increases from left to right. B and C, mean intensity scores and percentage scores of TMA samples of different tumor types: IL-1 α expression (B) and IL-1 β expression (C). Columns, mean; error bars, SD. *, $P < 0.05$. P values from Fisher exact test.

However, the tissue samples, which are double positive to IL-1 α and IL-1 β increased significantly with progression from nevus to primary melanoma ($P = 0.0001$, both percentage

and intensity scores) and from nevus to metastasis ($P < 0.007$, both percentage and intensity scores; Table 2). Interestingly, all but one of the IL-1 β -positive melanoma

Table 1. IHC detection of IL-1 α and IL-1 β in human melanocytic tumors: IL-1 α and IL-1 β versus tumor types, individually

Tumor types	Percentage score			Intensity score		
	Nevus	Primary	Metastasis	Nevus	Primary	Metastases
IL-1α						
Cores number	112	190	151	112	190	152
Negative	30 (26.8%)	3 (1.6%)	68 (44.7%)	29 (34.3%)	2 (1.1%)	67 (44.1%)
Positive	82 (73.2%)	187 (98.4%)	83 (55.0%)	83 (74.1%)	188 (98.9%)	85 (55.9%)
IL-1β						
Cores number	128	146	143	128	146	143
Negative	128 (100%)	127 (87.0%)	129 (90.2%)	128 (100%)	126 (86.3%)	127 (88.8%)
Positive	0 (0%)	19 (13.0%)	14 (9.8%)	0 (0%)	20 (13.7%)	16 (11.2%)

NOTE: Nevus includes thin and thick nevus, primary tissues include thin and thick primary metastases, and melanomas include metastatic lymph nodes and visceral melanomas.

Table 2. IHC detection of IL-1 α and IL-1 β in human melanocytic tumors: IL-1 α /IL-1 β versus tumor types

IL-1 α /IL-1 β	Percentage score			Intensity score			
	Tumor types	Nevus	Primary	Metastases	Nevus	Primary	Metastases
Double negative		30 (26.8%)	3 (1.6%)	58 (40.0%)	29 (25.9%)	2 (1.1%)	59 (40.4%)
1 Negative/1 positive		82 (73.2%)	168 (88.4%)	77 (53.1%)	83 (74.1%)	168 (88.4%)	73 (50.0%)
Double positive		0 (0%)	19 (10.0%)	10 (6.9%)	0 (0%)	20 (10.5%)	14 (9.6%)

NOTE. Nevus includes thin and thick nevus, primary tissues include thin and thick primary melanomas, and metastases include metastatic lymph nodes and visceral melanomas.

samples was also IL-1 α positive; the exception was one metastatic melanoma sample, suggesting that in melanoma cells, IL-1 β expression strongly correlates with IL-1 α expression. We also analyzed IL-1 α and IL-1 β protein levels based on the case number (patient number) of the TMA. The result of statistical analyses based on the case number showed the same trend of IL-1 α and IL-1 β expression profiles in nevi, primary melanoma, and metastatic melanoma as comparing with the result analyzed by core number (Supplementary Data).

To explore the molecular mechanisms of autocrine IL-1 on melanoma cell growth *in vitro*, we examined the expression of IL-1 α , IL-1 β , and IL-1RI in human melanoma cell lines by Western blot, RT-PCR, and IHC. Western blot analysis showed that all 7 melanoma cell lines and the melanocytes expressed IL-1RI (Fig. 2A). A375, SB2, SK-MEL-2, WM35, and WM793 cells expressed high levels of IL-1 α and IL-1 β proteins, whereas the protein levels of IL-1 α and IL-1 β are extremely low in MeWo, TXM1, and melanocytes (Fig. 2A). Semiquantitative RT-PCR confirmed that IL-1 α and IL-1 β mRNA were highly expressed in A375, SB2, SK-MEL-2, WM35, and WM793 cells but undetectable in MeWo and TXM1 cells (Fig. 2B). Therefore, A375 (metastatic melanoma) and WM793 (primary melanoma) cells were included as IL-1–positive lines, and MeWo and TXM-1 as negative lines in our functional studies. The pattern of positive staining for IL-1 α and IL-1 β were predominantly cytoplasmic in melanoma cells, which was shown by IHC staining in A375 and WM793

cells of cytospin preparations (Fig. 2C). The expression of IL-1 α and IL-1 β was confirmed to be negative in TXM1 cells and melanocytes (Fig. 2C) by IHC staining.

Endogenous IL-1 affects the levels of downstream inflammatory markers and oxidative stress in melanoma cells

To investigate the association between autocrine IL-1 and downstream inflammatory molecules in melanoma cells, we used pathway-specific siRNAs to block the IL-1 signaling and examined the effects on NF- κ B inhibitor (I κ B) and COX-2. Under optimized experimental conditions, we could downregulate approximately 75% of IL-1 α , IL-1 β , and MyD88 levels in A375 and WM793 cells by specific siRNA against IL-1 α , IL-1 β , MyD88, respectively, after 24-hour transfection. The effectiveness and specificity of our siRNA experiments were shown by Western blot and RT-PCR in A375 cells (Fig. 3A and B, additional siRNA data for WM793 cells was shown in Supplementary Data). Downregulation of IL-1 α , IL-1 β , MyD88, or both IL-1 α and IL-1 β inhibited I κ B phosphorylation but did not affect the levels of total I κ B in A375 and WM793 cells (Fig. 3C). Lipofectamine, alone, or nontarget siRNA did not affect I κ B phosphorylation. As I κ B phosphorylation can release and activate the inflammatory factor NF- κ B (23), our data suggest that constitutive endogenous IL-1 may constantly activate NF- κ B in human melanoma cells by stimulating I κ B phosphorylation. Furthermore, downregulation of

Table 3. Mean number and intensity of IL-1 α staining in melanoma tissue

Tumor Types	Tissue cores (n)	Mean number	Mean intensity
Nevi (thin)	38	1.11	0.76
Nevi (thick)	74	1.49	1.05
Primary (thin)	71	1.93	1.31
Primary (thick)	119	2.81	1.94
Metastases (lymph nodes, LN)	60	1.72	0.93
Metastases (visceral)	91	1.51	0.95

Table 4. Mean number and intensity of IL-1 β staining in melanoma tissue

Tumor Types	Tissue cores (n)	Mean number	Mean intensity
Nevi (thin)	38	0.00	0.00
Nevi (thick)	90	0.00	0.00
Primary (thin)	58	0.12	0.14
Primary (thick)	88	0.33	0.34
Metastases (lymph nodes, LN)	59	0.27	0.27
Metastases (visceral)	84	0.12	0.17

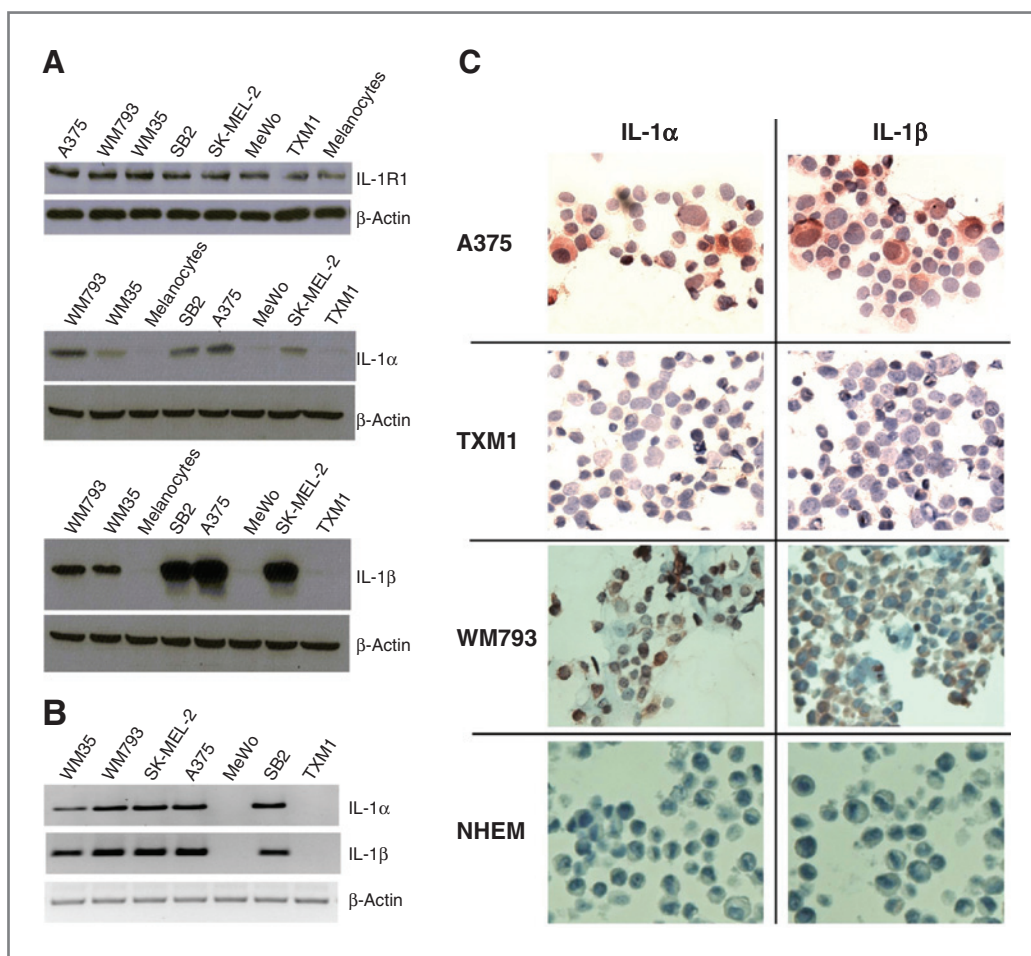


Figure 2. Expression profile of *IL-1* system genes in human melanoma cells. A, Western blot analysis of 40 μ g cell lysates of A375, WM793, WM35, SB2, SK-MEL-2, MeWo, TXM1, and melanocytes with anti-IL-1R1, anti-IL-1 α , and anti-IL-1 β antibodies; β -actin served as control. The concentration for primary antibodies was 1 μ g/mL. B, RT-PCR analysis of the mRNA expression of *IL-1 α* , *IL-1 β* , and *β -actin* in the 7 human melanoma cell lines. C, comparison of IHC staining for IL-1 α and IL-1 β with human melanoma cell lines (A375, TXM, and WM793) and normal human epidermal melanocytes (NHEM). Anti-IL-1 α antibody was used at 1:100 and anti-IL-1 β antibody at 1:50.

MyD88 or IL-1 α and IL-1 β decreased COX-2 protein levels in A375 and WM793 cells compared with controls (Fig. 3C). However, the IL-1 α siRNA alone did not affect COX-2 levels. The IL-1 β siRNA could downregulate COX-2 levels in WM793 but not in A375 (Fig. 3C). These data suggest that elevated IL-1 signaling (both IL-1 α and IL-1 β) can promote COX-2 expression in melanoma cells.

The levels of NO and total ROS/RNS were measured in A375 and WM793 cells before and after downregulating MyD88 or IL-1 α and IL-1 β by siRNAs. Downregulating MyD88 or both IL-1 α and IL-1 β decreased red fluorescence intensity, indicating NO production, by 50% to 60% in A375 cells ($P = 0.0009$; Fig. 4A and B). Similarly, downregulating MyD88 or both IL-1 α and IL-1 β decreased green fluorescence intensity, indicating a reduction of total ROS/RNS levels by 50% to 60% in A375 cells ($P = 0.0007$; Fig. 4A and C). In contrast, nontarget siRNA did not affect the levels of NO or total ROS/RNS as compared with the

Lipofectamine control (Fig. 4A–C). These results indicate that autocrine IL-1 plays a critical role in sustaining oxidative stress in melanoma cells.

Endogenous IL-1 regulates the levels of p21, p53, and phosphorylated SAPK/JNK in human melanoma cells

The tumor suppressors, p21 and p53, are sensors of diverse cellular stresses, including DNA damage and oxidative stress (24). Thus, we examined whether the oxidative stress driven by aberrant endogenous IL-1 affected the levels of p21 and p53 in melanoma cells. Downregulating IL-1 α , IL-1 β , or MyD88 increased p21 and p53 levels in A375 and WM793 cells compared with controls (Fig. 4D), suggesting that aberrant autocrine IL-1 represses p21 and p53 expression in melanoma cells.

As kinase inhibitors have been successfully used in cancer therapy, we attempted to identify the major stress kinase regulated by endogenous IL-1 in melanoma cells. Downregulation of IL-1 β or both IL-1 α and IL-1 β by siRNAs

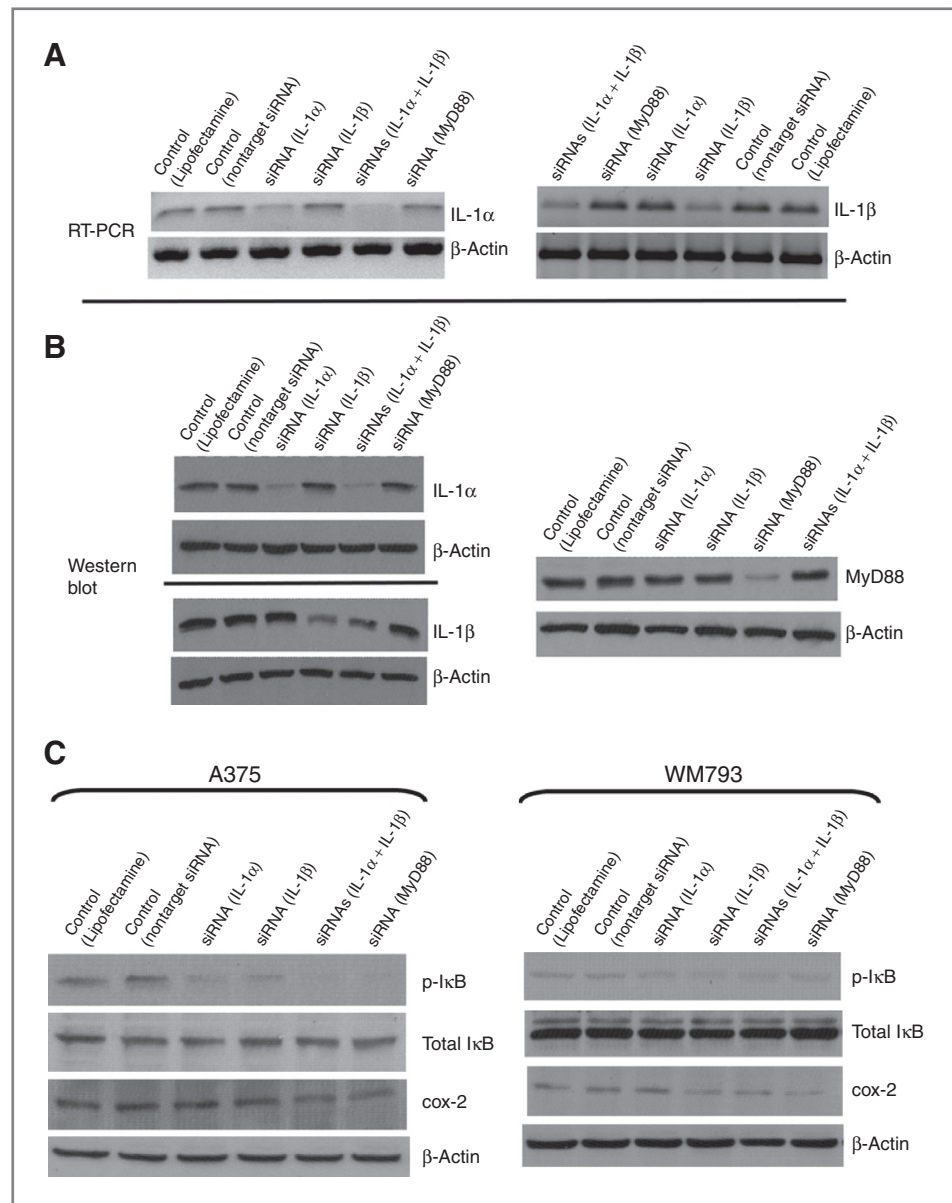


Figure 3. Effects of siRNA downregulation of *IL-1* system genes on several important protein factors in A375 and WM793 cells. A and B, RT-PCR and Western blot analysis showed that anti-IL-1 α , anti-IL-1 β , and anti-MyD88 siRNA could specifically downregulate IL-1 α , IL-1 β , and MyD88, respectively, in A375 cells. C, Western blot analysis of the effect of siRNA downregulation of IL-1 α , IL-1 β , or MyD88 on the levels of inflammatory molecules COX-2, phosphorylated I κ B, and total I κ B. In all experiments, A375 or WM793 cells transfected with nontarget siRNA or treated with Lipofectamine only served as negative controls, and β -actin served as the internal loading control. Shown are representative data from one of 3 experiments.

substantially decreased SAPK/JNK phosphorylation in A375 and WM793 cells but did not affect total SAPK/JNK levels (Fig. 4D). However, the effect of downregulating only IL-1 α or MyD88 on SAPK/JNK phosphorylation varied between A375 and WM793 cells. IL-1 α downregulation inhibited SAPK/JNK phosphorylation in A375 cells but had only a slightly inhibitory effect on SAPK/JNK phosphorylation in WM793 cells. MyD88 downregulation produced similar results, which indicated that MyD88 may not be the major adaptor to mediate the major signaling pathway to activate SAPK/JNK by endogenous IL-1 in WM793 cells (Fig. 4D). These data suggest that SAPK/JNK is one major signal transduction pathway that is regulated by autocrine IL-1 in melanoma cells. Furthermore, autocrine IL-1 may activate SAPK/JNK through signal transduction other than

MyD88 in A375 and WM793 cells. Our data suggest that activation of SAPK/JNK may be conducted through an MyD88-independent signaling pathway in WM793 cells. We also attempted to identify other kinases affected by blocking autocrine IL-1 signaling in human melanoma cells, but we did not observe any significant change on the phosphorylation of AKT or phosphoinositide 3-kinase (data not shown).

Blocking the IL-1 signaling pathway represses human melanoma cell growth

To directly assess whether autocrine IL-1 regulates growth of human melanoma cells, we used IL-1RI–neutralizing antibody to block IL-1 signaling in A375 and WM793 cells and normal melanocytes and measured the cell viability.

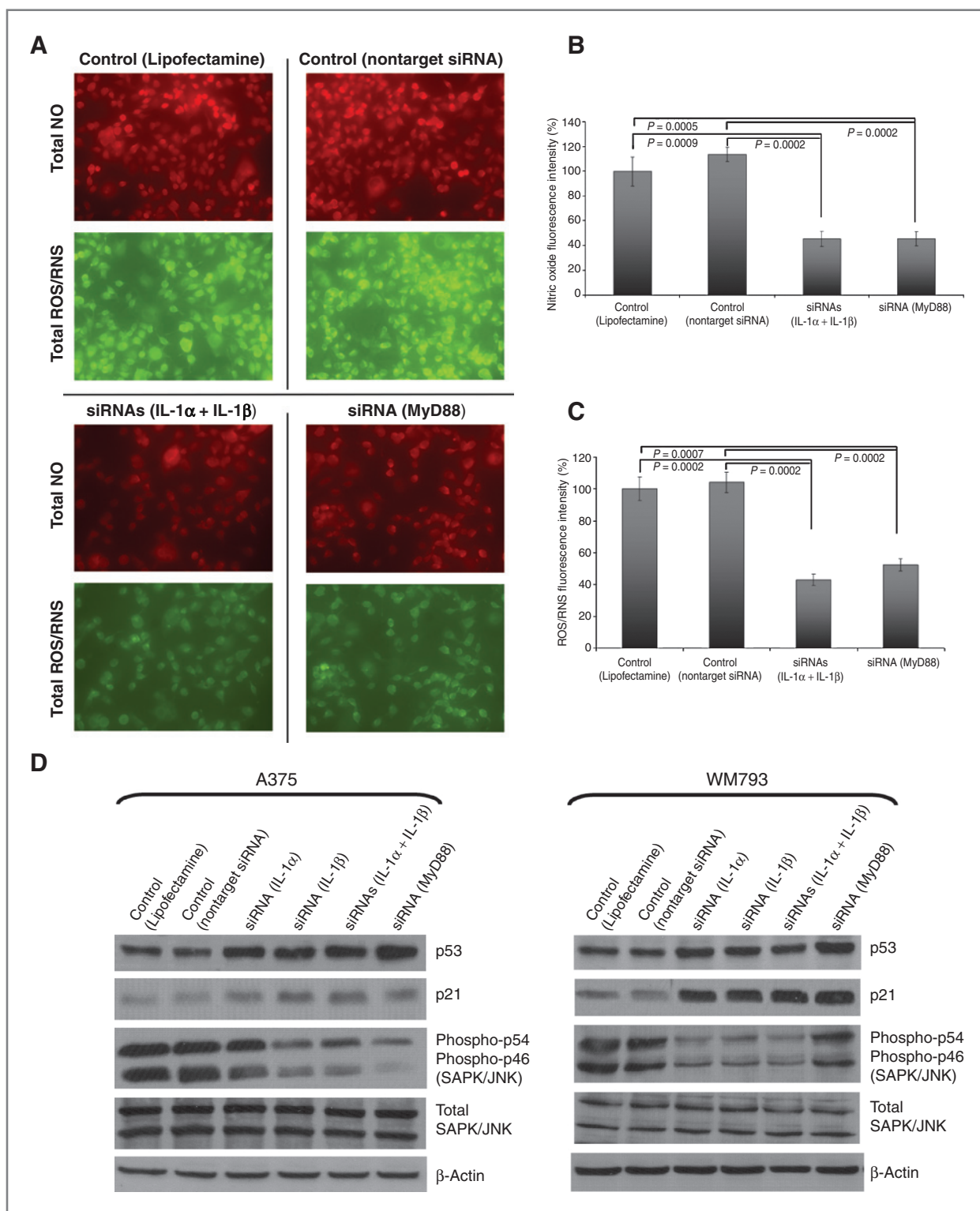
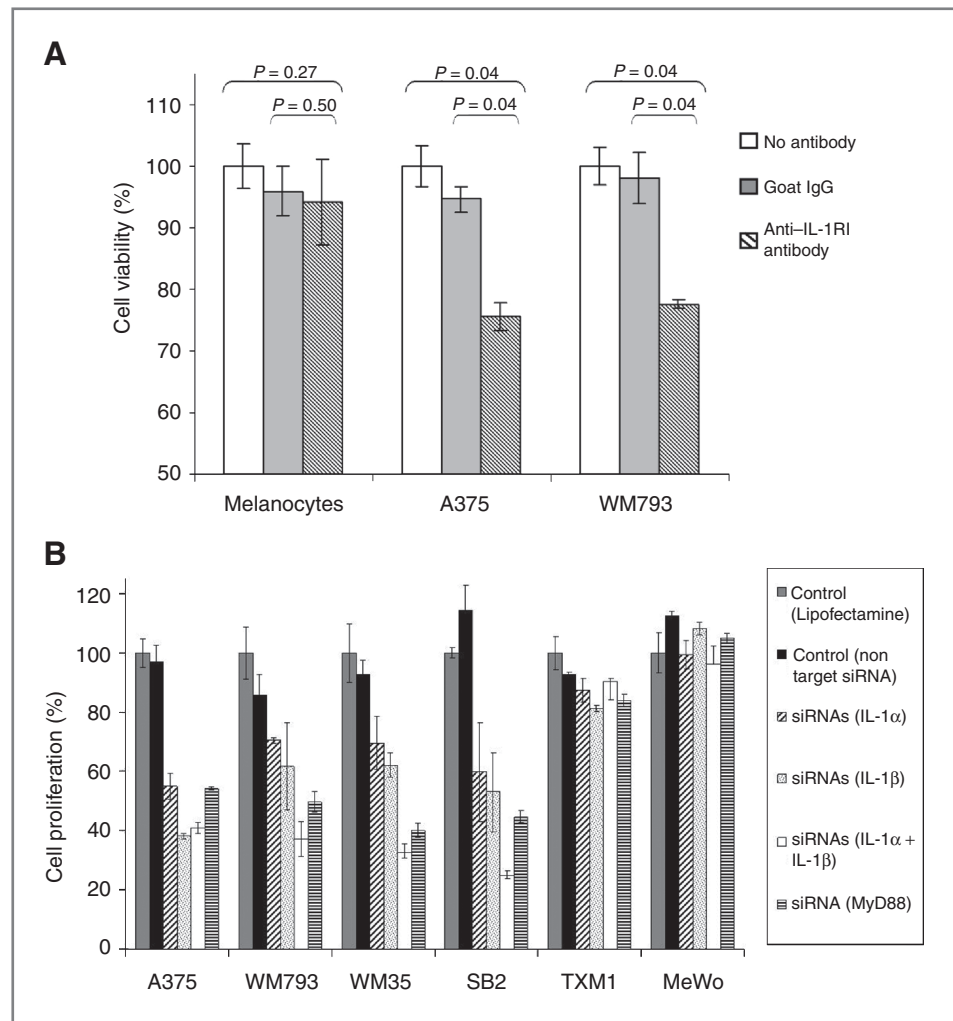


Figure 4. Effects of siRNA downregulation of the IL-1 signaling pathway on the production of free NO and ROS/RNS in A375 cells. A, red fluorescence representing free NO and green fluorescence representing total ROS/RNS in A375 cells. Transfection with IL-1 α and IL-1 β siRNAs or MyD88 siRNA for 24 hours decreased the red fluorescence and green fluorescence intensity, indicating decreased NO and total ROS/RNS levels, compared with nontarget siRNA. B, quantitative analysis of red fluorescence intensity of NO in A375 cells for the different treatments. C, quantitative analysis of green fluorescence intensity of in A375 cells for the different treatments. In B and C, columns, mean of 10 visualized fluorescence areas of A375 cells for each treatment; error bars, SE. *P* values from Wilcoxon rank-sum test. D, Western blot analysis of the effect of siRNA downregulation of IL-1 α , IL-1 β , or MyD88 on the expression of p53 and p21 in A375 and WM793 cells. The levels of phosphorylated SAPK/JNK and total SAPK/JNK were also analyzed in A375 and WM793 cells treated with IL-1 α , IL-1 β , or MyD88 siRNA. Cells transfected with nontarget siRNA or treated with Lipofectamine only served as negative controls, and β -actin served as the internal loading control.

Figure 5. Interrupting the IL-1 signaling suppresses growth of human melanoma cells. **A**, normal melanocytes and A375 and WM793 cells were treated with 2.75 $\mu\text{g}/\text{mL}$ IL-1RI–neutralizing antibody or goat IgG for 72 hours. Treatment with no antibody served as another negative control. Percentage of healthy cells was used to determine cell viability for different treatments. Columns, mean of 3 independent experiments; error bars, SD. *P* values from Wilcoxon rank-sum test. **B**, effect of siRNA knockdown of IL-1 α , IL-1 β , and MyD88 on the growth of A375, WM793, WM35, SB2, TXM1, and MeWo cells. Cell proliferation was determined by MTT assay after siRNA treatment for 72 hours. Columns, mean of 3 independent experiments; error bars, SD.



Treatment with 2.75 $\mu\text{g}/\text{mL}$ IL-1RI–neutralizing antibody decreased cell viability in A375 and WM793 cells by approximately 25% compared with treatment with goat IgG or no antibody, which was statistically significant ($P = 0.04$; Fig. 5A). Furthermore, IL-1RI–neutralizing antibody did not significantly inhibit melanocytes growth (Fig. 5A).

We also used a second method to assess the effect of endogenous IL-1 on growth of human melanoma cells. We applied the MTT cell proliferation assay in 6 melanoma cell lines after siRNA treatment for 72 hours to block the IL-1 pathway. For the cell lines with high IL-1 expression—A375, WM793, WM35, and SB2—downregulating IL-1 α , IL-1 β , or MyD88 decreased cell proliferation by 30% to 65% (Fig. 5B). In all 4 cell lines, downregulating both IL-1 α and IL-1 β decreased cell proliferation by 60% to 75% compared with nontarget siRNA treatment (Fig. 5B). For the cell lines with low IL-1 expression, MeWo and TXM1, downregulating IL-1 α , IL-1 β , or MyD88 showed slightly 5% to 15% decreases of cell proliferation compared with the treatment of nontarget siRNA, which

were not statistically significant. Thus, the blockage of IL-1 signaling did not inhibit cell proliferation significantly in MeWo and TXM1 cells. These data suggest that autocrine IL-1 supports melanoma cell growth, and conversely that blocking the IL-1 signaling specifically inhibits the growth of human melanoma cells with elevated IL-1 expression, but not the IL-1–negative melanoma cells or normal melanocytes.

Downregulation of the IL-1 signaling pathway induces autophagy in melanoma cells

We next investigated whether the suppression of melanoma cell growth induced by blocking the IL-1 signaling pathway was due to apoptosis. We did not find any distinct change in the levels of PARP, cleaved PARP, caspase-3, or cleaved caspase-3 in A375 and WM793 cells treated with IL-1RI–neutralizing antibody or siRNAs against IL-1 α , IL-1 β , and MyD88 for 72 hours (Supplementary Data). These results indicate that the suppression of melanoma cell growth induced by blocking the IL-1 pathway was not due to apoptosis.

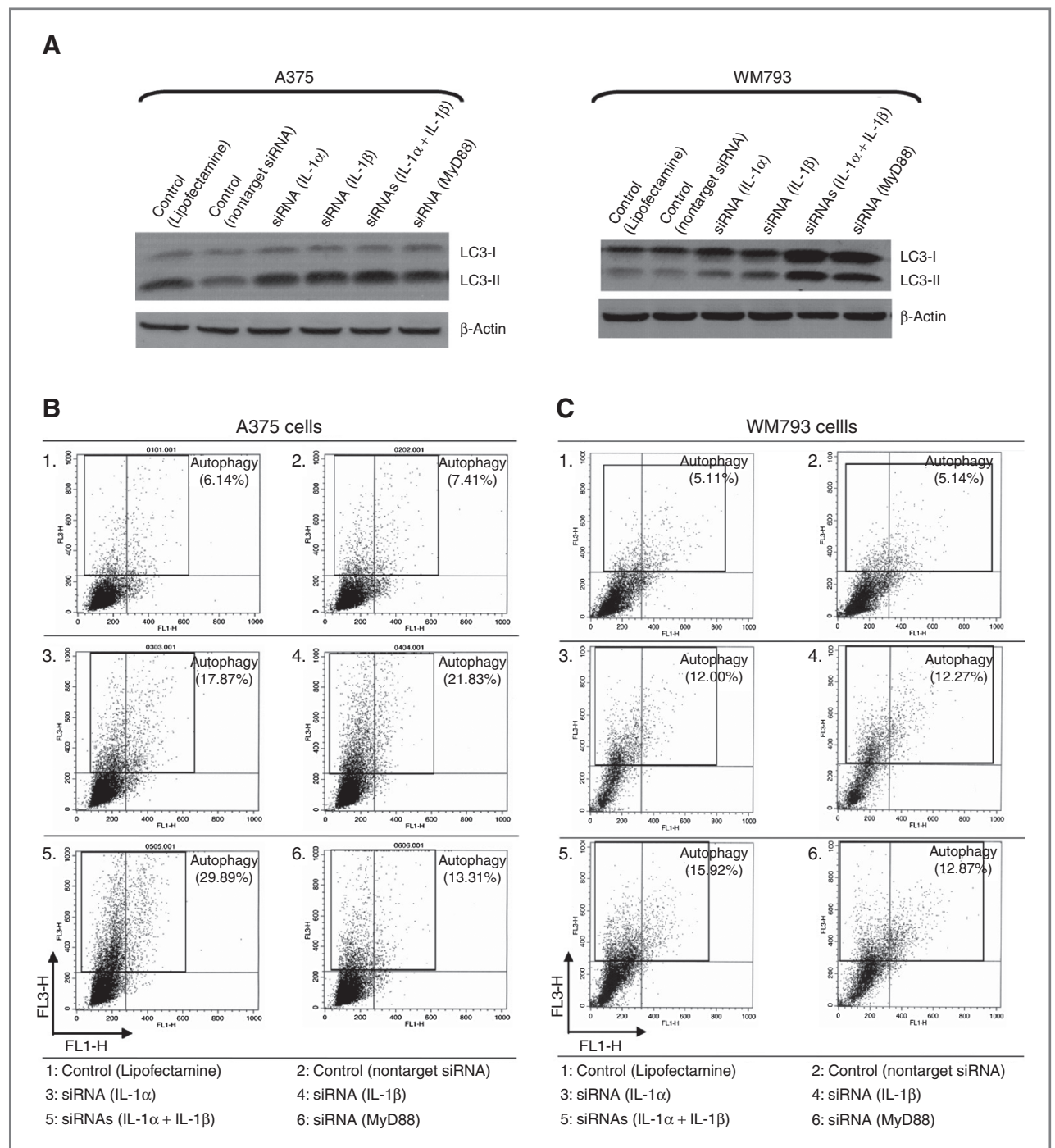


Figure 6. Interrupting the IL-1 signaling pathway can induce autophagy in human melanoma cells with elevated IL-1 expression. A, Western blot analysis of the effect of siRNA downregulation of IL-1 α , IL-1 β , or MyD88 on the levels of LC3-I and LC3-II in A375 and WM793 cells. In all experiments, cells transfected with nontarget siRNA or treated with Lipofectamine only served as negative controls, and β -actin served as the internal loading control. Shown are representative data from one of 3 experiments. B and C, siRNA downregulation of IL-1 α , IL-1 β , and MyD88 increased the percentage of A375 and WM793 cells with AVOs.

Subsequently, we tested whether the downregulation of IL-1 signaling could lead to the induction of autophagy in IL-1-positive melanoma cells. First, we examined the expression of microtubule-associated 1 light chain 3 I

(LC3-I) and its cleavage product LC3-II, which is enriched in the autophagic vacuole fraction during autophagosome formation (25, 26). We found that in A375 cells, both the level of LC3-II and the ratio of LC3-II to LC3-I increased

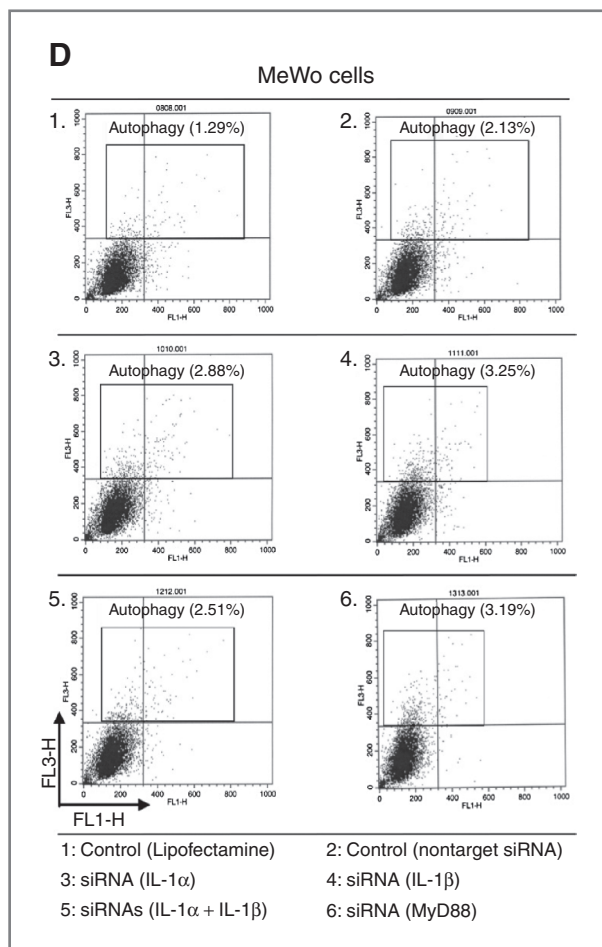


Figure 6. (Continued) D, siRNA downregulation of IL-1 system genes did not affect the percentage of MeWo cells with AVOs. A375, WM793, and MeWo cells were treated with Lipofectamine only (1), nontarget siRNA (2), IL-1 α siRNA (3), IL-1 β siRNA (4), both IL-1 α and IL-1 β siRNAs (5), or MyD88 siRNA (6) for 72 hours. Cells were stained with AO and analyzed by flow cytometry. Approximate locations of autophagic cells are boxed, and the percentage of the autophagic population is indicated. FL1-H, measuring green fluorescence intensity; FL3-H, measuring red fluorescence intensity.

substantially after IL-1 α , IL-1 β , or MyD88 siRNA treatment (Fig. 6A). In WM793 cells, IL-1 α , IL-1 β , and MyD88 siRNA treatment increased levels of both LC3-I and LC3-II (Fig. 6A). Thus, blocking the IL-1 pathway triggered both the conversion of LC3-I to LC3-II and the synthesis of LC3 protein in melanoma cells indicating the increase of autophagy.

To confirm autophagy, we carried out AO staining with analysis by flow cytometry to quantitate the formation of acidic vesicular organelles (AVO), which are associated with autophagy (27). As expected, downregulation of IL-1 α , IL-1 β , or MyD88 in A375 cells increased the percentage of cells with AVOs to 17.87%, 21.83%, and 13.31%, respectively, compared with nontarget siRNA and Lipofectamine controls (7.41% and 6.14%, respectively; Fig. 6B). Downregulation of both IL-1 α and IL-1 β increased the

percentage of cells with AVOs to 29.89% (Fig. 6B), approximately 4 times that of the controls in A375 cells. Similarly, downregulation of IL-1 α , IL-1 β , or MyD88 in WM793 cells increased the percentage of cells with AVOs to 12.00%, 12.27%, and 12.87%, respectively, compared with nontarget siRNA and Lipofectamine controls (5.11% and 5.14%, respectively; Fig. 6C). Downregulation of both IL-1 α and IL-1 β increased the percentage of cells with AVOs to 15.92% (Fig. 6C), approximately 3 times that of the controls in WM793 cells. In contrast, downregulation of IL-1 α , IL-1 β , MyD88, or IL-1 α and IL-1 β together slightly increased about 0.4% to 1.1% of cells with AVOs as compared with nontarget siRNA treatment in MeWo cells (Fig. 6D). In our flow cytometric analysis, 10^4 cells were analyzed for each sample. For MeWo cells, the autophagic population induced by IL-1 pathway-specific siRNAs was around 20 to 110 cells as compared with nontarget siRNA treatment, which was substantially lower than those in A375 and WM793 cells (600–2,200 cells). Therefore, downregulation of IL-1 signaling did not have any significant autophagy-inducing effect in MeWo cells (Fig. 6D). Collectively, our data show that blocking the IL-1 pathway can induce autophagy in melanoma cells with elevated IL-1 expression.

Discussion

We hypothesized that the tumor-derived IL-1 promotes endogenous inflammatory products that further facilitate growth and progression of human melanoma. To determine the IL-1 family expression in human tumor samples, we carried out biostatistical analysis of IL-1 α and IL-1 β protein expression in a TMA with 480 tissue cores from 170 patients. By using this TMA, we were able to carry out biostatistical analysis of IL-1 α and IL-1 β expression in melanocytic tumor progression for the first time. We found IL-1 α and IL-1 β were significantly elevated in primary melanoma, and that IL-1 α was predominant. Such data also suggest that the elevated autocrine IL-1 signaling may be crucial for primary melanoma development. Studies were then devised to interrupt the IL-1 signaling pathway, which resulted in decreasing reactive oxidative species and other proteins levels of COX-2, and phosphorylation of I κ B and SAPK/JNK. Our studies indicate that the elevated oxidative stress induced by constitutive autocrine IL-1 is a driving force in chronic inflammation in human melanoma cells. The study from Okamoto and colleagues showed that melanoma cells spontaneously secreted IL-1 β leading to constitutive activation of the NALP3 inflammasome, which contributed to the autoinflammation in human melanoma (14). Along with the data from Okamoto and colleagues, our studies further confirm that the tumor-derived IL-1, including IL-1 α and IL-1 β , promotes inflammation and thereby facilitates growth progression of human melanoma. Our results also suggest that autocrine IL-1 suppresses p21 and p53 expression to low levels even under elevated oxidative stress. Furthermore, the interruption of endogenous IL-1 signaling could inhibit the growth of IL-1-expressing melanoma cells. Our results suggest that selectively inhibiting

IL-1 signaling may be a promising therapeutic strategy in the subgroup of melanoma patients with constitutively high IL-1 expression.

In contrast to our finding, several studies showed that exogenous IL-1 could suppress the proliferation of some melanoma cells *in vitro* (28–31). These studies were mainly conducted in melanoma cell lines, A375.S2 and A375-6, which were subclones derived from A375 and have been well established as IL-1-sensitive lines. However, our results showed that melanoma tumor expressed high levels of IL-1, and the endogenous IL-1 could promote inflammation and support the growth of melanoma. The discrepancy between our studies and other studies may reflect the heterogeneity and clonal evolution of melanoma, as clearly A375.S2 and A375-6 cell lines represent a unique subset of melanoma cells that are sensitive to the cytotoxic effect of IL-1 *in vitro*. It is still not clear whether endogenous IL-1 and IL-1R exist in A375.S2 and A375-6 cells, and whether there was any genetic alteration in these cells different from their parental cell line, A375. To date, there is a lack of evidence that exogenous IL-1 inhibits melanoma growth *in vivo*. Further investigation is needed to determine whether the IL-1-sensitive melanomas exist *in vivo* under physiologic conditions.

Although IL-1 β was expressed to a smaller extent (about 10%) in primary and metastatic melanoma than IL-1 α , it is strongly correlated with IL-1 α expression. Our studies indicated that downregulating both IL-1 α and IL-1 β inhibited melanoma cell growth more than blocking only IL-1 α or IL-1 β . We also found that IL-1 α and IL-1 β were expressed in both *BRAF* mutant (A375, WM35, and WM793) and *BRAF* wild-type (SK-MEL-2 and SB2) melanoma cells (Supplementary Data). Furthermore, the expression of IL-1 α has been found in above 98% of primary melanoma from different genetic backgrounds. Thus, IL-1 is likely active in a large subpopulation of melanoma patients, and not related to *BRAF* mutations. We are now interested in conducting studies to determine why melanoma cells produce predominantly IL-1 α rather than IL-1 β as well as have initiated a study to investigate the expression profile of IL-1Ra and its biological role in human melanoma cells.

For the first time, we report an association between the upregulation of IL-1 signaling and activated SAPK/JNK in human melanoma cells. Interestingly, MyD88 knockdown had a slight inhibitory effect on SAPK/JNK activation in WM793 cells, which is much weaker than the siRNA knockdown of IL-1 α and IL-1 β (Fig. 4D and Supplementary Data). These results suggest heterogeneity of endogenous IL-1 downstream signaling in different melanoma cell lines. We speculate that IL-1 may activate SAPK/JNK through the other Toll-like receptor adaptors binding to IL-1RI, which are MyD88 independent. Previous studies have shown that Toll-like receptor domain-containing adapter-inducing interferon (TRIF)-related adaptor molecule (TRAM) was specifically involved in the Toll-like receptor (TLR)-mediated MyD88-independent signaling pathway (32, 33). On the basis of this unexpected data, we

initiated a study to examine the role of TRAM on activating SAPK/JNK by IL-1 in WM793 cells. In our preliminary study, the knockdown of TRAM by specific siRNA did not show any significant inhibitory effect on the phosphorylation of SAPK/JNK in WM793 cells (Supplementary Data). This data further indicates that in addition to MyD88, TRAM is not likely to be involved in activation of SAPK/JNK in WM793 cells. TLR signaling is very complicated. In addition to MyD88 and TRAM, TRIF and MyD88 adaptor-like (Mal) can also directly bind to Toll-like receptors and conduct signaling transduction in cells (33, 34). Currently, it is still not clear whether SAPK/JNK is affected by other signaling pathways other than IL-1 in WM793 cells. We are now conducting experiments to test Mal and TRIF involvement in regulating SAPK/JNK activation in WM793 cells. As siRNA knocking down MyD88 can lead to a significant reduction of the phosphorylated I κ B in WM793 cells (Fig. 3C), the IL-1 signaling conducted through MyD88 is considered as one major pathway to stimulate downstream inflammatory genes in WM793 cells.

The expression of IL-1 α and IL-1 β is significantly elevated in primary melanoma indicating that the elevated endogenous IL-1 and related downstream signaling may be crucial for primary melanoma development. Recent studies from L. Chin and colleagues showed that the mutated *BRAF* alone was not sufficient to transform melanocytes *in vivo* (35). However, the UV-induced robust activation of JNK might be considered as part of the additional factor(s) needed to transform *BRAF*-mutated melanocytes (35). The observation of IL-1 elevation in primary melanoma suggests that the autocrine IL-1-induced activation of JNK may play a critical role in melanocytes transformation and tumor initiation in the absence of risk factors, such as UV radiation. We are currently investigating the role of JNK in mediating oncogenic behavior driven by aberrant endogenous IL-1 in melanoma and the potential of using JNK inhibitors to inhibit melanoma cell growth.

To our knowledge, this is the first study to show that the IL-1 signaling pathway regulates autophagy in human melanoma cells. Recent studies showed that SAPK/JNK activation occurred downstream of autophagy induction and was dependent on the autophagic process in mice (36, 37). Our results suggest that IL-1 may be a key link between SAPK/JNK and autophagy, however, further investigation is needed to determine whether the linkage between IL-1 signaling, SAPK/JNK, and autophagy exists in human melanoma cells.

In clinical studies, the accumulation of MDSCs has been shown to functionally suppress the immune response in melanoma patients (38). It is known that IL-1 can stimulate MDSCs (7). Therefore, we speculate that the constitutive melanoma-expressed IL-1 can activate and recruit MDSCs to tumor sites resulting in immunosuppression. This mechanism may, in part, explain the high resistance to immunotherapy and the aggressive behavior of some melanomas. For melanoma patients with aberrantly high levels of IL-1 in tumors, blocking the IL-1 pathway may inhibit tumor growth by interrupting the

autocrine stimulation and may also be able to reverse immunosuppression by inhibiting the paracrine activation of MDSCs in the tumor microenvironment. Further studies are needed to determine whether this can be achieved safely without immunosuppressive toxicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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