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Toll-Like Receptor 4 Signaling Promotes the Migration of Human Melanoma Cells

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Immune cell Toll-like receptors (TLRs) recognize conserved microbial components, leading to immune and inflammatory responses. However, TLRs are also expressed in cancer cells, including melanoma cells, which express TLR2-4. TLR4 ligands have received attention as immunotherapies; therefore, we assessed the expression of TLR4 in human melanoma specimens (29 primary lesions and 28 metastatic lesions) representing different types of melanoma. A high percentage (≥ 90%) of melanoma lesions expressed TLR4, as judged by immunohistochemistry. Next, the role of TLR4 in cell proliferation and migration was assessed using the TLR4-positive ($TLR4^+$) melanoma cell lines 501mel and 888mel, and TLR4-negative ($TLR4^-$) 928mel melanoma cells. Lipopolysaccharide (LPS), a TLR4 agonist, increased the proliferation of $TLR4^+$ melanoma cells but not of $TLR4^-$ 928mel cells. The proliferation-inducing effect of LPS in 888mel cells was abolished by blockade of TLR4 signaling via treatment with short interfering RNA (siRNA) targeting TLR4 or myeloid differentiation primary response gene 88 (MyD88), a molecule downstream of TLR4. However, knockdown of TLR4 or MyD88 expression did not affect the LPS-induced proliferation of 501mel cells, suggesting that residual TLR4 signaling is sufficient to maintain cell proliferation. By contrast, LPS increased the migration of $TLR4^+$ melanoma cells, and this effect was substantially inhibited by TLR4 or MyD88 knockdown. Furthermore, TLR4 knockdown decreased cell migration even in the absence of LPS, suggesting the presence of an endogenous TLR4 ligand(s) in melanoma cells. TLR4 signaling may contribute to melanoma progression, and caution should be exercised when using TLR4 ligands as adjuvant therapy for cancer.

Keywords: innate immunity; melanoma; migration; proliferation; Toll-like receptor 4
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Introduction

Toll-like receptors (TLRs), which form an evolutionarily conserved family of receptors, play a key role in the detection of invading pathogens and in mounting the antimicrobial immune response (Akira and Takeda 2004). TLRs recognize microbial-associated molecular patterns, including lipid-based structures such as mycobacterial lipopeptides (TLR2) or lipopolysaccharides (TLR4), and microbial genetic materials such as viral double-stranded RNA (TLR3) or unmethylated CpG islands (TLR9). Binding of their respective ligands leads to intracellular downstream events that culminate in cell activation, cytokine secretion, and resistance against pathogens. Furthermore, TLR activation has antitumor activity through the activation of both innate and adaptive immune responses. Okamoto et al. (2006) used the Streptococcal agent OK-432 to activate TLR4 signaling, which resulted in an interferon-γ-mediated antitumor immune response. In addition, intratumoral injection of the heterodimeric TLR2/6 agonist showed antitumor activity in some models, including breast cancer (Shingu et al. 2003); however, this agonist does not affect metastatic melanoma, despite successful stimulation of the immune system.

Many TLR studies have focused on immune cells; however, TLR expression is not restricted to immune cells and is shared by many other cell types, including melanocytes (Yu et al. 2009), and is maintained after transformation. TLRs are expressed on tumor cells from a wide variety of organs, suggesting that TLR signaling plays a role in tumor development; however, its functional significance remains unclear. TLRs expressed on tumor cells may influence tumor growth and host immune responses.

Human melanoma cells express TLR2, 3, 4, 7, 8, and 9 (Goto et al. 2008; Saint-Jean et al. 2011), the function of which is not fully understood in cutaneous melanoma. A TLR4 agonist, OK-432, has been used as an immunotherapeutic agent in many types of malignancies, including mel-

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anoma (Kirkwood et al. 1997). To explore the effect of TLR4 activation on melanoma cells, TLR4-positive ($TLR4^+$) and TLR4-negative ($TLR4^-$) melanoma cells were used. This study describes TLR4 expression in human melanoma lesions and shows that TLR4 signaling promotes the migration and proliferation of human melanoma cells.

Materials and Methods

Cell culture

Three melanoma cell lines, 501mel, 888mel, and 928mel, all established from Caucasian patients, were used in this study (obtained from Prof. Y. Kawakami, Keio University School of Medicine, Tokyo, Japan). They were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were fed twice per week and passaged by exposure to 0.05% trypsin prepared in ethylenediaminetetraacetic acid. Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO, USA).

Immunohistochemical staining

Immunohistochemistry was performed on 5 μm-thick paraffin-embedded tissue sections that were deparaffinized in xylene. Rabbit polyclonal antibodies against TLR4 and myeloid differentiation primary response gene 88 (MyD88) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Envision Plus System (Dako, Carpinteria, CA, USA) was used. Paraffin-embedded archival tissue specimens of melanoma (29 primary tumors and 28 metastatic tumors) were obtained from melanoma patients who underwent surgical resection at Shinshu University Hospital (Nagano, Japan) between 1999 and 2009. The study was approved by the Ethical Committee of Shinshu University School of Medicine and was conducted according to the guidelines set out by the Institutional Review Board.

Cell proliferation assay

After preparing melanoma cell suspensions, cells were seeded in 96-well plates (Nalge Nunc International, Rochester, NY, USA) in RPMI-1640 medium containing 10% FBS. Melanoma cells were incubated in RPMI-1640 medium containing 2% FBS with or without 10 μg/ml LPS for 4 hours, after which LPS was removed and the cells were incubated in RPMI-1640 medium containing 2% FBS for 48 hours. Thereafter, WST-8 solution (Cell Count Reagent SF, Nacalai Tesque, Kyoto, Japan) was added and the optical absorption was measured at 450 nm with a reference wavelength of 650 nm.

Cell migration assay

For migration assays using transwell chambers, melanoma cells were suspended in RPMI-1640 medium containing 2.5% FBS and seeded onto the porous polycarbonate membrane (8.0 μm pores) of a transwell chamber (Corning Incorporated, Corning, NY, USA). The lower chamber was filled with RPMI-1640 medium supplemented with 2.5% FBS in the presence or absence of LPS (10 μg/ml). After incubation for 24 hours, the inserts were removed and the cells that had migrated to the lower chamber were stained with crystal violet after fixation with 80% ethanol. The number of migrated cells was counted in more than ten randomly selected fields with a light microscope.

For migration assays using the scratch method, cells were grown to confluence in 12-well plates and then serum-starved in RPMI-1640 medium for 24 hours. After serum starvation, a scratch was made through the monolayer with a 200-μl pipette tip. Cells were further incubated, and the scratch areas were filled by migrating cells. Images of more than six fields were captured, and migration into the scratch area was analyzed using ImageJ software.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Tokyo, Japan), as recommended by the manufacturer. cDNAs were synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) and 3 μg of total RNA as the template. qRT-PCR analysis was performed with a LightCycler System and the Universal ProbeLibrary Set (Roche Applied Science, Indianapolis, IN, USA). Specific primers were designed to cross exon-exon boundaries by the Universal ProbeLibrary Assay Design Center (Roche Applied Science) as follows: TLR4, 5′-CCATGGCCTTCCTCTCCT-3′ and 5′-TCAGCT CCATGCATTGATAAGT-3′; MyD88, 5′-CTGCTCGAGCTGCTT ACCA-3′ and 5′-CTTTTGGCAATCCTCCTCAA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GAGTCCACTGGCGTC TTCAC-3′ and 5′-TTCACACCCATGACGAACAT-3′. The Human Universal ProbeLibrary sets #33, #33, and #45 (Roche Applied Science) were used for TLR4, MyD88, and GAPDH, respectively. The expression of TLR4 and MyD88 was normalized to that of GAPDH.

RNA interference

Short interfering RNAs (siRNAs) were designed for TLR4 and MyD88 as follows: TLR4 siRNA#1 (T1), 5′-GGGCAUGCCUGU GCUGAGUUUGAAU-3′; TLR4 siRNA#2 (T2), 5′-GGGUAAGGA AUGAGCUAGUAAAGAA-3′; TLR4 siRNA#3 (T3), 5′-GAGCCU AAGCCACCUCUCUACCUUA-3′; MyD88 siRNA#1 (M1), 5′-AUC GCGGUCAGACACACACAACUUC-3′; and MyD88 siRNA#2 (M2), 5′-UUUCGAUGAGCUCACUAGCAAUAGA-3′. A scrambled siRNA control was purchased from Invitrogen. The siRNAs were transfected using Lipofectamine 2000 (Invitrogen). Block-iT Fluorescent Oligo (Invitrogen) showed that siRNAs were introduced into more than 80% of the melanoma cells.

Statistical analysis

The data were analyzed using the Student's *t*-test for unpaired observations. Differences were considered significant when $p < 0.05$.

Results

Most human melanoma lesions express TLR4 and MyD88

Human melanoma cells express various TLRs at the RNA level (Goto et al. 2008; Saint-Jean et al. 2011). The TLR4 expression of melanomas was assessed in primary tumors ($n = 29$) and metastatic lesions ($n = 28$) by immunohistochemistry. Melanoma is classified into five subtypes based on anatomic location and degree of sun exposure: acral lentiginous melanoma, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, and mucosal melanoma. The clinical characteristics of the patients are shown in Table 1. TLR4 was expressed in 90% of primary melanoma lesions (26/29 samples) and 93% of metastatic lesions (26/28 samples) (Fig. 1A, Table 1). The expression of MyD88, a molecule downstream of TLR4,

Table 1. Expression of TLR4 and MyD88 in primary and metastatic melanoma.

No.	Lesions	Subtype	Age years	Sex	Stage	Current status	Survival months	TLR4	MyD88
1	Primary	Acral M	68	F	IB	DOD	65	+	+
2	Primary	Acral M	75	F	IB	NED	105	−	+
3	Primary	Acral M	62	M	IIA	DOD	61	+	+
4	Primary	Acral M	79	M	IIA	DOD	14	+	+
5	Primary	Acral M	69	M	IIB	NED	4	+	+
6	Primary	Acral M	83	M	IIC	NED	49	+	+
7	Primary	Acral M	49	F	IIIA	AWD	33	+	+
8	Primary	Acral M	79	M	IIIA	DOD	69	+	+
9	Primary	Acral M	69	F	IIIB	DOD	61	+	+
10	Primary	Acral M	81	M	IIIB	NED	123	+	+
11	Primary	Acral M	44	F	IIIC	NED	143	+	+
12	Primary	Acral M	69	M	IIIC	DOD	28	+	+
13	Primary	Acral M	75	M	IIIC	DOD	75	+	+
14	Primary	Acral M	82	M	IIIC	NED	123	+	+
15	Primary	Acral M	50	M	IV	NED	116	+	+
16	Primary	Acral M	82	M	IV	DOD	23	+	+
17	Primary	SSM	29	M	0	NED	46	−	+
18	Primary	SSM	43	F	IIB	NED	148	+	+
19	Primary	SSM	74	M	IIB	DOD	97	+	+
20	Primary	SSM	57	M	IIIB	DOD	53	+	+
21	Primary	SSM	62	F	IIIB	DOD	33	+	+
22	Primary	NM	68	M	IB	NED	107	+	+
23	Primary	NM	66	M	IIB	NED	98	+	+
24	Primary	NM	56	M	IIIC	NED	20	+	+
25	Primary	NM	67	M	IIIC	DOD	2	+	+
26	Primary	NM	74	F	IIIC	DOD	15	+	+
27	Primary	Mucosal M	79	M	IIC	DOD	28	+	+
28	Primary	Mucosal M	79	F	IIIB	Dead of other causes	3	−	−
29	Primary	LMM	57	M	IB	DOD	29	+	+
1	Metastatic	Acral M	62	M	IIA	DOD	61	+	+
2	Metastatic	Acral M	79	M	IIA	DOD	14	+	+
3	Metastatic	Acral M	61	M	IIB	NED	75	+	+
4	Metastatic	Acral M	49	F	IIIA	AWD	33	+	+
5	Metastatic	Acral M	76	F	IIIB	NED	75	+	+
6	Metastatic	Acral M	81	M	IIIB	NED	123	+	+
7	Metastatic	Acral M	86	F	IIIB	DOD	6	+	+
8	Metastatic	Acral M	47	F	IIIC	DOD	54	+	+
9	Metastatic	Acral M	69	M	IIIC	DOD	28	+	+
10	Metastatic	Acral M	75	M	IIIC	DOD	75	−	+
11	Metastatic	Acral M	81	M	IIIC	DOD	18	+	+
12	Metastatic	Acral M	82	M	IIIC	NED	116	+	+
13	Metastatic	Acral M	82	M	IV	DOD	23	+	+
14	Metastatic	SSM	57	M	IIB	DOD	113	+	+
15	Metastatic	SSM	20	F	IIIA	DOD	90	+	+
16	Metastatic	SSM	64	F	IIIA	DOD	12	+	+
17	Metastatic	SSM	57	M	IIIB	DOD	53	+	+
18	Metastatic	SSM	62	F	IIIB	DOD	33	+	+
19	Metastatic	SSM	63	F	IIIC	DOD	21	+	+
20	Metastatic	NM	53	F	IIB	NED	137	+	+
21	Metastatic	NM	53	F	IIIA	NED	58	+	+
22	Metastatic	NM	76	M	IIIB	NED	89	+	+
23	Metastatic	NM	67	M	IIIC	DOD	10	+	+
24	Metastatic	NM	74	F	IIIC	DOD	15	−	+
25	Metastatic	Mucosal M	79	M	IIC	DOD	28	+	+
26	Metastatic	Mucosal M	42	F	IV	AWD	117	+	+
27	Metastatic	LMM	54	F	IV	DOD	24	+	+
28	Metastatic	UP	68	M	IV	NED	57	+	+

LMM, lentigo maligna melanoma; M, melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma.
AWD, alive with disease; NED, no evidence of disease; DOD, dead of disease; UP, unknown primary.
TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response gene 88.

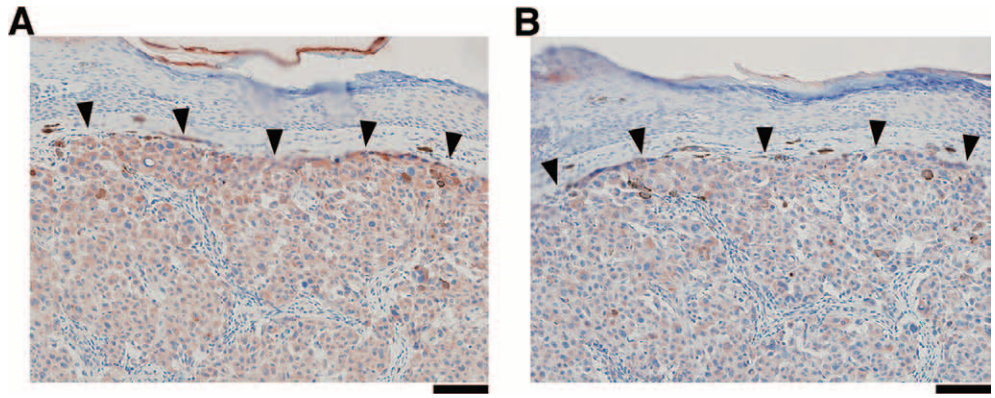

Fig. 1. TLR4 and MyD88 expression in melanoma lesions.
Human melanoma lesions were immunostained with polyclonal antibodies against TLR4 (A) and MyD88 (B) (the primary tumor of patient No. 21 in Table 1 is shown here). Compared to normal epidermis, melanoma lesions expressed TLR4 and MyD88. Arrows show melanoma lesions. Scale bar: 100 μm.

was also assessed. MyD88 was expressed in primary (97%; 28/29 samples) and metastatic (100%; 28/28 samples) melanoma lesions (Fig. 1B, Table 1). Thus, TLR4 and MyD88 are expressed in most, but not all, melanoma lesions.

TLR4 and MyD88 expression in melanoma cell lines

Having shown that TLR4 is constitutively expressed in a large proportion of human melanoma lesions, we investigated whether TLR4 is functional in melanoma cells. We used three human melanoma cell lines, 501mel, 888mel, and 928mel, the doubling time of which was around 11.16 ± 0.66 hours, 13.66 ± 0.40 hours, and 23.73 ± 1.48 hours, respectively. We evaluated the relative expression levels of TLR4 and MyD88 mRNAs. RT-PCR showed that TLR4 was expressed in 501mel and 888mel cells, but not in 928mel cells (Fig. 2A). The three cell lines do not show any amplification or deletion at chromosome 9q33.1, which contains the *TLR4* gene, by comparative genomic hybridization (Tanami et al. 2004). Epigenetic alteration may have led to loss of TLR4 expression in 928mel cells. MyD88 was abundantly expressed in the three cell lines (Fig. 2B). We subsequently used 501mel and 888mel as $TLR4^+$ cell lines and 928mel as a $TLR4^-$ cell line to assess the role of TLR4 in melanoma.

LPS alters the proliferation of melanoma cells

We investigated the effect of TLR4 signaling on melanoma proliferation. LPS, a natural ligand for TLR4, was used to activate TLR4. LPS promoted the proliferation of the $TLR4^+$ cells (501mel and 888mel), but not that of the $TLR4^-$ cells (928mel) (Fig. 3A). The mitogen-activated protein kinase (MAPK) pathway is one of the main intracellular pathways mediating growth control. We investigated whether TLR4 activation induces MAPK activation in melanoma cells using antibodies specific for phosphorylated MAPK. LPS did not affect the activity of extracellular signal-regulated kinase, c-Jun N-terminal kinase, or p38 MAPK in the melanoma cells, irrespective of whether they expressed TLR4 (data not shown).

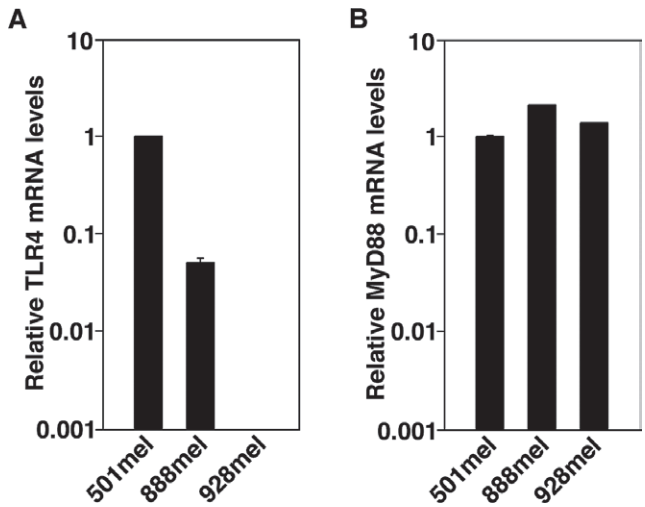

Fig. 2. TLR4 and MyD88 expression in melanoma cell lines.
(A) TLR4 mRNA expression detected by qRT-PCR in human melanoma cell lines (501mel, 888mel, and 928mel). (B) MyD88 mRNA expression detected by qRT-PCR in human melanoma cell lines. mRNA levels were normalized with those of GAPDH. Data are presented as mean ± standard deviation. The results are representative of three independent experiments.

To confirm that the increased proliferation induced by LPS is mediated by TLR4 activation, we used siRNA targeting TLR4 or MyD88 to modulate TLR4 signaling (Fig. 3B). Knockdown of TLR4 expression decreased the proliferation of 888mel cells (Fig. 3C), but did not substantially affect that of 501mel cells. Similarly, MyD88 knockdown inhibited the proliferation of 888mel cells, but not that of 501mel cells (Fig. 3D, E). The degree of TLR4 or MyD88 knockdown may be insufficient to inhibit proliferation of 501mel cells i.e., residual TLR4 signaling may be sufficient to maintain the LPS-induced proliferation of these cells. Alternatively, transfection with siRNA can activate the TLR3 pathway in 501mel cells, which may attenuate the effect of the TLR4 knockdown. Although knockdown of TLR4 or MyD88 expression did not substantially attenuate

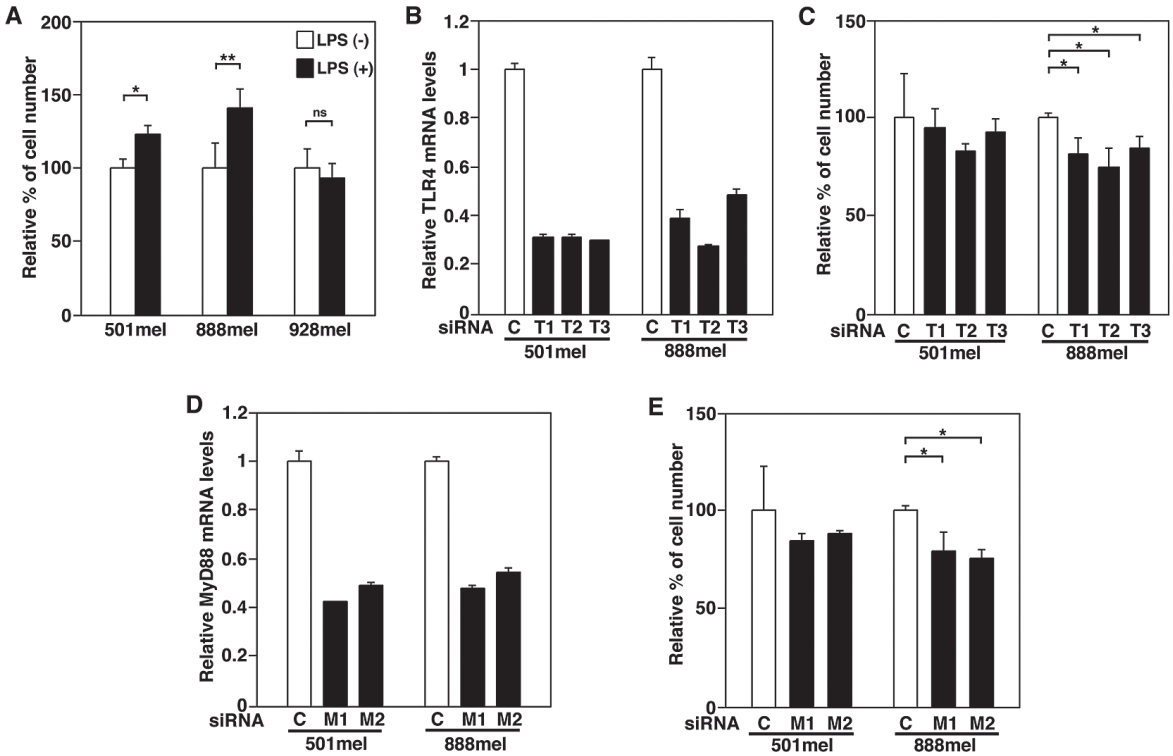

Fig. 3. TLR4 activation promotes melanoma cell proliferation.
(A) Melanoma cell proliferation in the presence (black columns) or absence (white columns) of LPS. (B) Reduction of TLR4 expression 24 hours after transfection with small interfering RNA (siRNA; TLR4 #1-3 [T1-3] and control [C]). Melanoma cells were transfected with TLR4-targeting siRNA (black columns) or control siRNA (white columns), and were incubated without LPS. Cell extracts were collected, and TLR4 mRNA expression levels were determined by qRT-PCR. (C) Effect of TLR4 knockdown on LPS-induced melanoma cell proliferation. Similar to (B), melanoma cells were transfected with TLR4-targeting siRNA (black columns) or control siRNA (white columns). After transfection, cells were incubated with LPS, and the number of cells was counted. (D) Reduction of MyD88 expression by siRNA (MyD88 #1 and 2 [M1 and 2] and control [C]). Melanoma cells were transfected with MyD88-targeting siRNA (black columns) or control siRNA (white columns), and were incubated without LPS. Cell extracts were collected, and MyD88 mRNA expression levels were determined by qRT-PCR. (E) Effect of MyD88 knockdown on LPS-induced melanoma cell proliferation. Similar to (D), melanoma cells were transfected with MyD88-targeting siRNA (black columns) or control siRNA (white columns). After transfection, cells were incubated with LPS, and the number of cells was counted. The cell numbers are relative to the control. mRNA levels were normalized with those of GAPDH. Data are presented as the mean ± standard deviation. $*P < 0.05$; $**P < 0.01$; ns, non-significant. The results are representative of three independent experiments.

the LPS-induced proliferation of 501mel cells, TLR4 activation with LPS promoted proliferation of $TLR4^+$ melanoma cells. It is likely that TLR4 signaling induces the proliferation of melanoma cells.

Melanoma cells migrate in response to LPS

TLR activation promotes leukocyte migration (Fan and Malik 2003); therefore, the effect of TLR4 signaling on melanoma cell migration was assessed using transwell chambers. A greater number of $TLR4^+$ cells than $TLR4^-$ cells migrated across the membrane to the lower chamber containing LPS (Fig. 4A). Furthermore, TLR4 knockdown decreased the migration of 501mel and 888mel cells (Fig. 4B), as did MyD88 knockdown (Fig. 4C). In addition, this result indicates that treatment with the siRNA targeting TLR4 or MyD88 can attenuate TLR4 signaling in 501mel cells. On the other hand, MyD88 knockdown did not affect 928mel cell migration. Thus, TLR4 signaling likely promotes melanoma cell migration.

TLR4 regulates melanoma cell migration in the absence of LPS

We investigated whether TLR4 induces melanoma cell migration in response to an endogenous ligand(s) in the absence of LPS. Migration was not detected in the absence of LPS in the transwell assay. By contrast, TLR4 knockdown decreased melanoma cell migration both in the presence (Fig. 5A, B) and absence (Fig. 5C, D) of LPS in the scratch assay, suggesting the existence of an endogenous TLR4 ligand(s). Thus, human melanoma cells show accelerated motility in response to both exogenous and endogenous TLR4 ligands. In addition, TLR4 knockdown did not lead to any dramatic alteration in cellular morphology regardless of the presence of LPS (Fig. 5E and data not

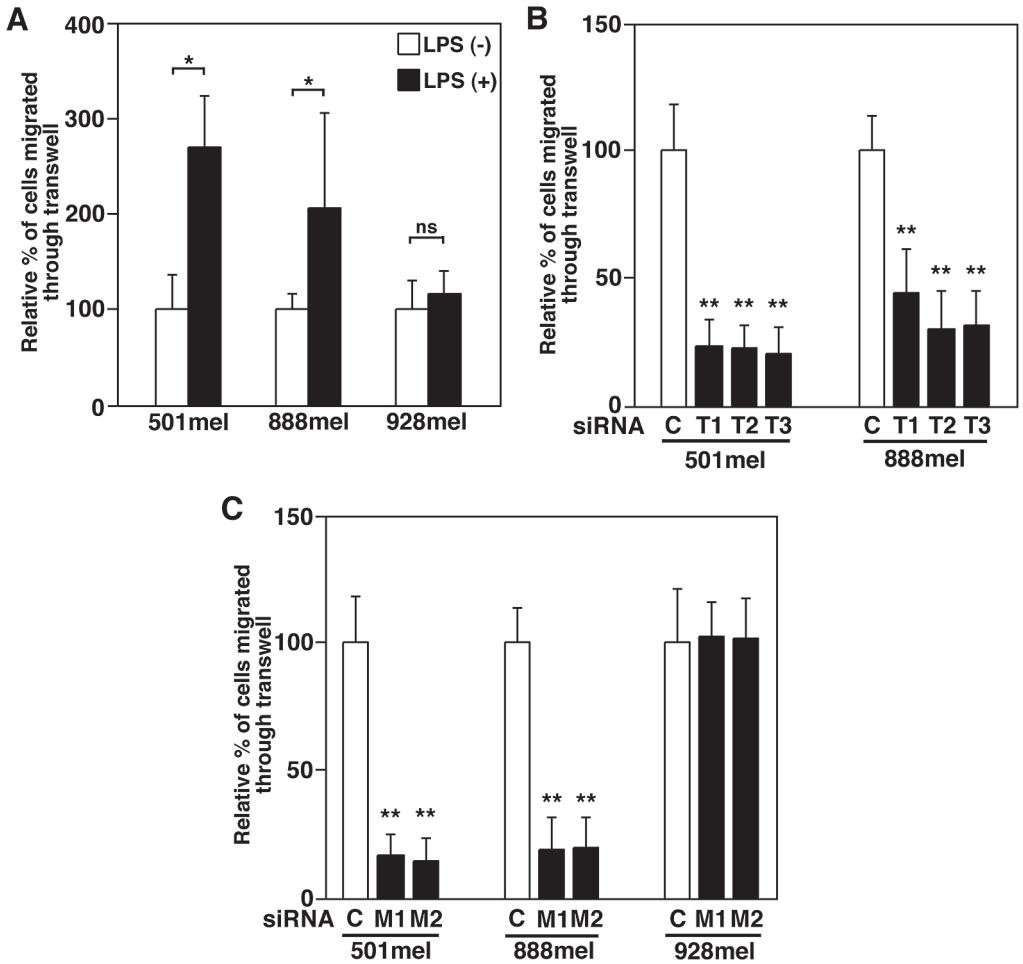

Fig. 4. TLR4 activation induces melanoma cell migration in transwell chambers. (A) Melanoma cell migration in the presence (black columns) or absence (white columns) of LPS. (B) Effect of TLR4 knockdown on LPS-induced melanoma cell migration. Melanoma cells were transfected with small interfering RNA (siRNA; TLR4 #1-3 [T1-3] and control [C]) and migrated across the membrane to the lower chamber containing LPS. The numbers of migrated cells were assessed among melanoma cells transfected with TLR4-targeting siRNA (black columns) or control siRNA (white columns). (C) Effect of MyD88 knockdown on LPS-induced melanoma cell migration. Melanoma cells were transfected with siRNA (MyD88 #1 and 2 [M1 and 2] and control [C]) and migrated across the membrane to the lower chamber containing LPS. The numbers of migrated cells were assessed among melanoma cells transfected with MyD88-targeting siRNA (black columns) or control siRNA (white columns). The numbers of migrated cells are relative to the control. Data are presented as the mean ± standard deviation. $*P < 0.05$; $**P < 0.01$; ns, non-significant. The results are representative of three independent experiments.

shown). How TLR4 signaling activation accelerates melanoma cell migration remains to be elucidated.

Discussion

The present study demonstrates that a large proportion of human melanomas express TLR4. TLR4 activation promotes melanoma cell proliferation, although its contribution may not be robust. Further, TLR4 activation increases melanoma cell migration, which is supported by the fact that knockdown of TLR4 or MyD88 inhibits migration. In addition, TLR4 knockdown delays melanoma cell migration even in the absence of LPS, suggesting the existence of an endogenous TLR4 ligand(s). Thus, melanoma cells use TLR4, a specific defense factor, for their own processes.

The antitumor activity of TLRs is frequently emphasized (Pasare and Medzhitov 2003; Furumoto et al. 2004). In particular, TLR4 supports the development of a strong adaptive immune response (Kasturi et al. 2011). In murine melanoma cells, TLR4 activation before inoculation significantly inhibits in vivo tumor growth through T cell activation (Andreani et al. 2007; Nunez et al. 2012). By contrast, other studies suggest that TLR activation induces tumor cell proliferation and promotes angiogenesis, invasion, and metastasis. TLR4 stimulation induces the production of proinflammatory factors, including nitric oxide, interleukin-6, and interleukin-12, in tumor cells. These factors result in the resistance of tumor cells to immune cell attack and in evasion from immune surveillance (Huang et al. 2005). Furthermore, high TLR4 expression was recently reported to be associated with shortened relapse-free sur-

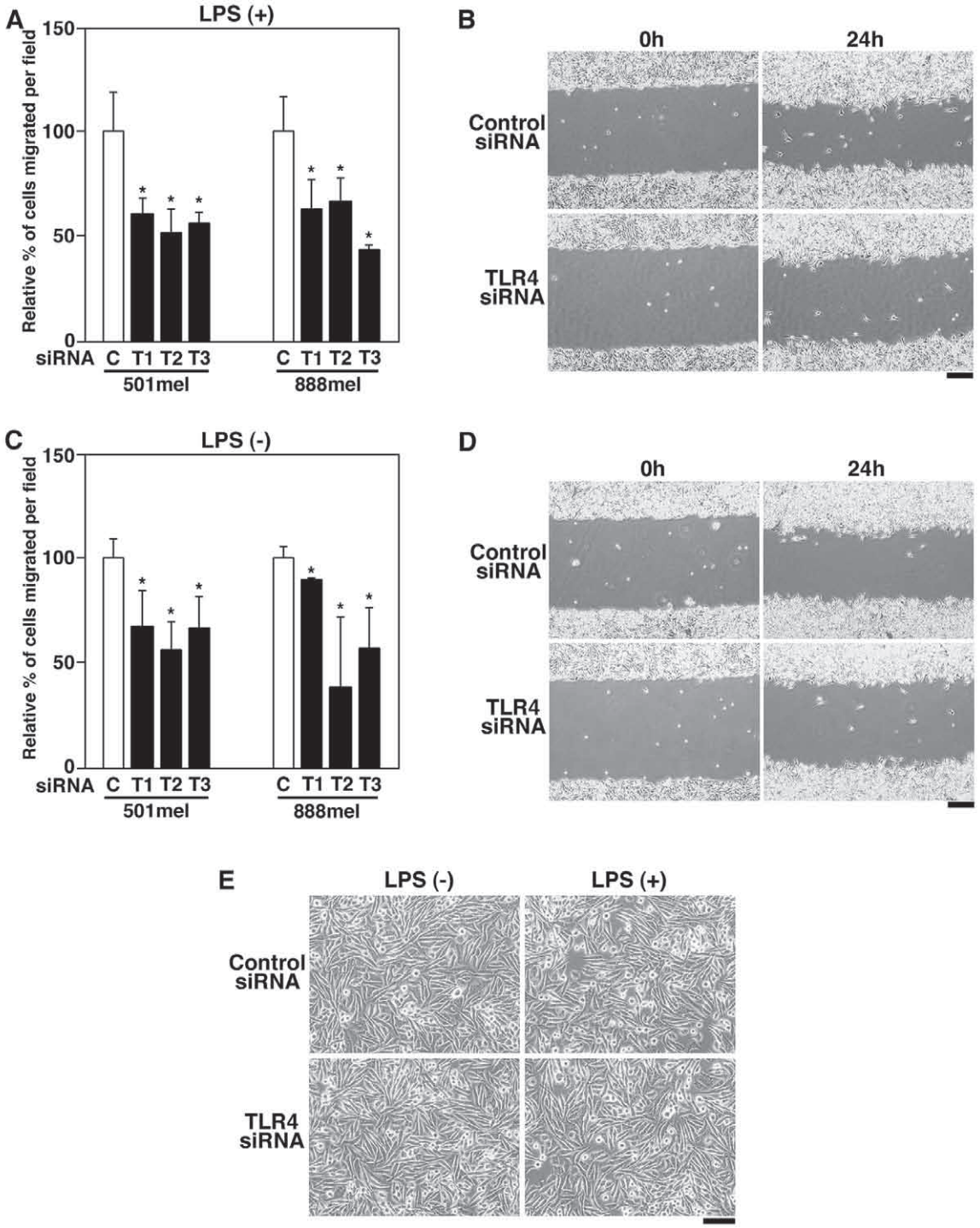

Fig. 5. TLR4 activation induces melanoma cell migration in a scratch assay.
(A) TLR4 knockdown attenuates melanoma cell migration in the presence of LPS. Melanoma cells were transfected with small interfering RNA (siRNA; TLR4 #1-3 [T1-3] and control [C]) and incubated with LPS. Migration was assessed in melanoma cells transfected with TLR4-targeting siRNA (black columns) or control siRNA (white columns). (B) Phase-contrast images of 501mel cell migration into the scratch area in the presence of LPS. Similar to (A), melanoma cells were transfected with TLR4-targeting siRNA or control siRNA. (C) TLR4 knockdown reduces melanoma cell migration in the absence of LPS. Melanoma cells were transfected with siRNA and then incubated in the absence of LPS. Migration was assessed in melanoma cells transfected with TLR4-targeting siRNA (black columns) or control siRNA (white columns). (D) Phase-contrast images of 501mel cell migration into the scratch area in the absence of LPS. Similar to (C), melanoma cells were transfected with TLR4-targeting siRNA or control siRNA. (E) Phase-contrast images showing the morphology of 501mel cells transfected with TLR4-targeting siRNA or control siRNA. 501mel cells were incubated with or without LPS. Scale bar: 200 μm (B, D) and 100 μm (E). Data are presented as mean ± standard deviation. *$P < 0.05$. The results are representative of three independent experiments.

vival in melanoma (Eiro et al. 2013) and with disease progression in colon cancer (Wang et al. 2010). Our results show that TLR4 affects melanoma cell proliferation and motility, although LPS-induced proliferation was not inhibited robustly by TLR4 knockdown. TLR signaling triggers immune cell activation and is indispensable for an efficient immune response against malignant cells. However, when immunosurveillance is unsuccessful against malignant cells, TLR4 expressed on tumor cells may promote tumor cell proliferation, tumor cell migration, or immunosuppression. The outcome of TLR4 activation may be based on the balance between antitumor and tumorigenic factors.

Knockdown of TLR4 or MyD88 suppressed melanoma cell migration even in the absence of LPS, suggesting the existence of an endogenous TLR4 ligand(s). TLRs recognize not only microbial-associated molecular patterns, but also host-derived damage-associated molecular patterns (Medzhitov and Janeway 2002). Damage-associated molecular patterns derived from injured normal cells and necrotic cancer cells appear to be present at significant levels in the tumor microenvironment, and their stimulation of specific TLRs can foster chronic inflammation (Hiratsuka et al. 2008; Sato et al. 2009). A variety of endogenous substances, including fibrinogen, hyaluronic acid, and heat-shock proteins, can trigger TLR4 signaling. Interestingly, low molecular weight fragments of hyaluronic acid, a degradation product of the extracellular matrix, activate TLR4 signaling in melanoma cells and increase their motility (Voelcker et al. 2008).

In summary, this study provides evidence of a TLR4 response in melanoma cells, stressing the importance of TLR4 signaling in melanoma progression. TLR4 signaling may promote melanoma progression; therefore, considerable attention is required when using TLR4 ligands as adjuvant therapy.

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Conflict of Interest

The authors declare no conflict of interest.

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