The Role of IL-32 in Cutaneous T-Cell Lymphoma

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IL-32 is a pro-inflammatory cytokine expressed by activated natural killer cells, T cells, keratinocytes, and fibroblasts. In this study, we examined the role of IL-32 in cutaneous T-cell lymphoma (CTCL), including mycosis fungoides (MF) and Sézary syndrome (SS). IL-32 mRNA expression levels in lesional skin of MF patch, plaque, and tumor were increased compared with those of normal skin, which positively correlated with CCL17 and CCL18 mRNA expression levels. Serum IL-32 levels positively correlated with disease activity within each patient. Immunostaining showed that keratinocytes expressed IL-32 in the lesional skin of MF patch and plaque, whereas in MF tumor, atypical T cells in the dermis strongly expressed IL-32. We also showed that IL-32 dose-dependently accelerated the proliferation of MF and SS cell lines *in vitro*, which was inhibited by blocking mitogen-activated protein kinase and NF-κB-mediated signaling. The addition of anti-IL-32 antibodies in culture decreased the proliferation of SS cells and the viability of MF cells, suggesting that IL-32 serves as an autocrine growth factor. In conclusion, our results suggest that IL-32 has a role in the formation and maintenance of CTCL lesions, providing a possible therapeutic target for patients with this disease.

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INTRODUCTION

IL-32 was originally described as natural killer (NK) cell transcript 4 found in activated NK cells and T cells and later named IL-32 (Dahl *et al.*, 1992). IL-32 is a pro-inflammatory cytokine, which has a physiological role in T-cell communication. IL-32 is not only induced by various infectious agents, such as mycobacterium, human immunodeficiency virus, and influenza virus (Netea *et al.*, 2006; Rasool *et al.*, 2008; Li *et al.*, 2010), but is also involved in the inflammatory processes of rheumatoid arthritis, chronic obstructive pulmonary disease, and Crohn's disease (Joosten *et al.*, 2006; Shioya *et al.*, 2007; Calabrese *et al.*, 2008). IL-32 is expressed by keratinocytes and fibroblasts and by activated NK cells and T cells (Dahl *et al.*, 1992; Meyer *et al.*, 2010). Recently, IL-32 was reported to be expressed in lesional skin of atopic dermatitis, positively correlating with disease activity (Meyer *et al.*, 2010).

Mycosis fungoides (MF) and Sézary syndrome (SS) are the most common types of cutaneous T-cell lymphoma (CTCL). Patients with MF typically have a prolonged clinical course and only limited cases progress over the years through patch, plaque, and tumor, followed by lymph node and visceral involvement (Girardi *et al.*, 2004). Most cases of MF/SS, especially at an advanced stage, show a T-helper (Th) 2–dominant cytokine environment, characterized by increased IL-4, IL-5, IL-10, and IL-13 production (Vowels *et al.*, 1994; Asadullah *et al.*, 1996a). Quite recently, it was revealed that IL-32 was expressed in MF tumor and in the MF cell line MyLa, but not in early MF, benign controls, and normal skin (van Kester *et al.*, 2012). The aim of this study was to further examine the role of IL-32 in patients with MF and SS. Our findings suggest that IL-32 has an important role as an autocrine and paracrine growth factor in CTCL, providing a possible target for the treatment of CTCL.

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Abbreviations: CTCL, cutaneous T-cell lymphoma; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase extracellular signal-reduced kinase; MF, mycosis fungoides; NK, natural killer; RT, reverse transcription; SS, Sézary syndrome; Th, T-helper

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RESULTS

IL-32 mRNA is expressed in lesional skin of CTCL, especially in MF tumor

We examined IL-32 mRNA expression in lesional skin of MF/SS and in normal skin by quantitative reverse transcription (RT)-PCR. IL-32 mRNA expression in lesional skin of MF patch, plaque, and tumor was increased compared with normal skin (Figure 1a; $P<0.05$, respectively), whereas in lesional skin of erythrodermic MF/SS, IL-32 levels were not elevated. We also measured IL-32 mRNA expression in lesional skin and non-lesional skin from the same patient in 11 MF cases (Figure 1b). In 9 of the 11 cases, IL-32 mRNA expression in lesional skin was increased compared with non-lesional skin (Figure 1b; $P<0.05$). We also found that IL-32 mRNA expression levels positively correlated with CCL17 and CCL18 mRNA expression levels in lesional skin (Figure 1c and d). Thus, IL-32 mRNA was expressed in MF lesional skin, which reflected disease activity.

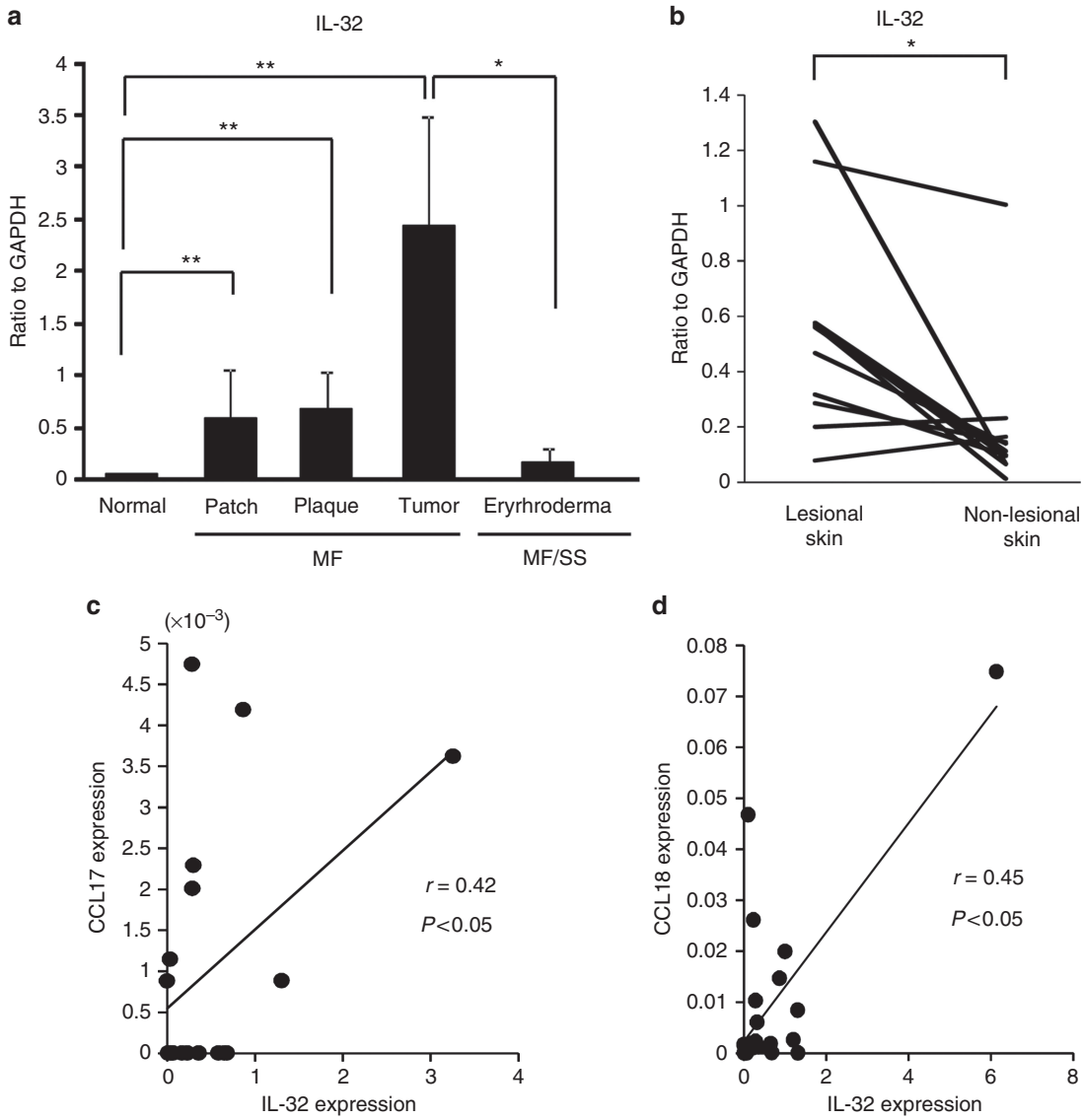

Figure 1. IL-32 mRNA expression in mycosis fungoides (MF) and Sézary syndrome (SS). (**a**) IL-32 mRNA expression in lesional skin of patch, plaque, tumor MF, and erythrodermic MF/SS. (**b**) IL-32 mRNA expression in lesional and non-lesional skin from MF patients. (**c**) Correlation between IL-32 and CCL17 mRNA expression levels in lesional skin. (**d**) Correlation between IL-32 and CCL18 mRNA expression levels in lesional skin. *$P<0.05$ and **$P<0.01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Serum IL-32 levels positively correlate with disease activity within individual CTCL patients

Serum IL-32 levels of patients with patch, plaque, tumor, erythrodermic MF/SS, and healthy controls were 11.4 ± 4.2, 10.3 ± 3.2, 11.2 ± 2.4, 12.2 ± 3.9, and $15.6 \pm 3.1\,pg\,ml^{-1}$, respectively (Figure 2a). There were no significant differences between the groups. Serum IL-32 levels in patients with SS were $13.3 \pm 3.8\,pg\,ml^{-1}$, which tended to be higher than the levels in erythrodermic MF patients ($9.1 \pm 1.9\,pg\,ml^{-1}$), but this did not reach statistical significance. We also measured serum IL-32 levels in five MF/SS cases (three cases of tumor MF and two cases of SS) before and after treatment, which included topical and oral corticosteroids, UV phototherapy, oral etretinate, and/or systemic chemotherapy (Figure 2b). In four of the five cases, serum IL-32 levels after treatment were lower than those before treatment (Figure 2b; $P<0.05$). Thus, serum IL-32 levels may not be a disease-specific marker for differentiating MF/SS from healthy controls, but may be positively correlated with disease activity within each patient with CTCL.

Enhanced IL-32 protein expression in lesional skin of MF patch, plaque, and tumor

Immunostaining revealed that IL-32 expression in lesional skin of MF patch, plaque, and tumor was enhanced compared with the expression in healthy controls (Figure 3). Keratinocytes strongly expressed IL-32 in lesional skin of MF patch and plaque, although in MF tumor IL-32 expression in keratinocytes was decreased. Interestingly, dermal-infiltrating $CD4^+$

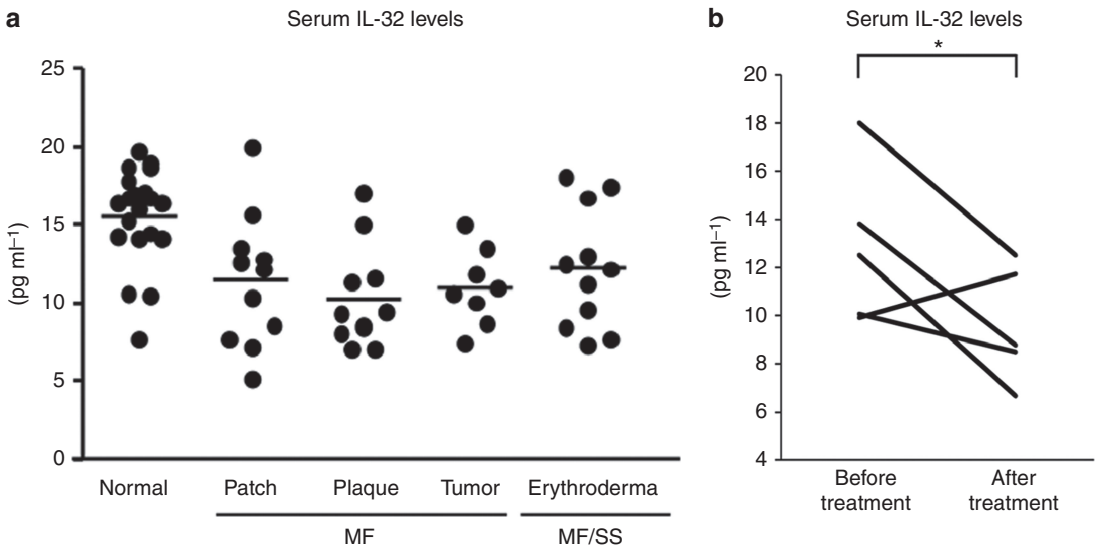

Figure 2. Serum IL-32 levels in mycosis fungoides (MF) and Sézary syndrome (SS). (**a**) Serum IL-32 levels in patients with MF and SS. (**b**) Serum IL-32 levels in MF/SS patients before and after treatment. *$P<0.05$.

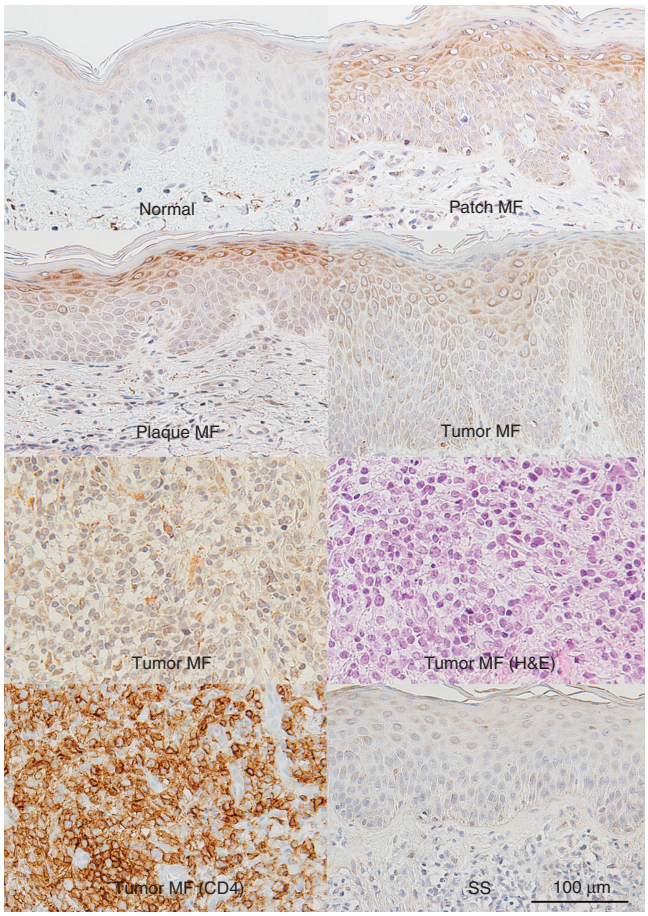

Figure 3. Immunohistochemical staining for IL-32. Representative pictures of five cases in each group (scale bar = 100 μm). Keratinocytes expressed IL-32 in lesional skin of patch and plaque mycosis fungoides (MF), although in tumor MF, dermal-infiltrating $CD4^+$ atypical T cells strongly expressed IL-32. H&E, hematoxylin and eosin; SS, Sézary syndrome.

atypical T cells expressed IL-32 in this latter patient group (Figure 3). In lesional skin of erythrodermic MF/SS, neither keratinocytes nor dermal tumor cells expressed IL-32 (Figure 3), which was consistent with the results of IL-32 mRNA expression (Figure 1a). Lesional skin with high IL-32 mRNA expression levels tended to show enhanced IL-32 expression by immunohistochemical analysis. There was no significant correlation between IL-32 mRNA expression levels in lesional skin and serum IL-32 levels. We have summarized the results of ELISA, quantitative RT-PCR, and immunohistochemical analyses of IL-32 in CTCL patients in Table 1.

IL-32 accelerates the proliferation of CTCL cell lines through mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways

In order to determine the effect of IL-32 on CTCL tumor cells, we stimulated MyLa (MF cell line) and SeAx cells (SS cell line) with IL-32 *in vitro*. IL-32 significantly accelerated MyLa and SeAx cell proliferation in a dose-dependent manner (Figure 4a). There were no differences in cell viability between the IL-32-treated cells and control cells (data not shown). We next explored signaling pathways involved in this proliferative effect by adding U0126, a MAPK extracellular signal–reduced kinase 1/2 inhibitor, sc-514, an IκB kinase-2 inhibitor, and LY294002, a phosphatidylinositol 3-kinase inhibitor. On adding U0126, cell proliferation of SeAx was decreased dose-dependently in both untreated and IL-32-treated cells (Figure 4b). When treated with 20 μM of U0126, there were no significant differences in cell numbers between the untreated and IL-32-treated cells (Figure 4b and c). Similar results were obtained when sc-514 was added, although LY294002, which decreased cell numbers both in untreated and in IL-32-treated groups, did not block cellular proliferation induced by IL-32 (Figure 4b and c). These results suggest that MAPK, NF-κB, and phosphatidylinositol 3-kinase/Akt

Table 1. Results of ELISA, quantitative RT-PCR, and immunohistochemical analysis of IL-32 in CTCL patients

						Immunohistochemistry	
Age	Sex	Diagnosis (stage)	Tumor cells in peripheral blood (counts μl^{-1})	ELISA (pg ml^{-1})	Real-time RT-PCR (ratio to GAPDH)	Keratinocytes	Tumor cells
50	M	Patch MF (IA)	0	ND	0.05	+	−
82	F	Patch MF (IA)	0	7.68	1.20	+ +	−
40	M	Patch MF (IB)	0	5.21	0.59	−	−
56	F	Plaque MF (IB)	0	ND	0.29	+	−
64	M	Plaque MF (IB)	0	9.33	0.68	+ +	−
50	F	Plaque MF (IA)	0	11.57	1.31	+	+
40	M	Tumor MF (IIB)	0	7.03	1.55	+	+ +
55	M	Tumor MF (IIB)	0	8.77	3.25	+	+ +
65	F	Tumor MF (IIB)	0	10.95	1.00	−	+ +
70	M	Erythrodermic MF (IIIA)	0	ND	0.30	−	−
64	M	Erythrodermic MF (IIIA)	0	8.49	0.11	ND	ND
67	M	SS (IVA)	30,492	16.73	0.36	−	−
64	F	SS (IVA)	2,812	12.19	0.02	−	+
42	M	SS (IVA)	1,548	12.51	0.04	+	−

Abbreviations: CTCL, cutaneous T-cell lymphoma; F, female; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M, male; MF, mycosis fungoides; ND, not done; RT, reverse transcription; SS, Sézary syndrome.

signaling pathways are all associated with cell proliferation of SeAx, whereas IL-32's ability to accelerate cell proliferation is mainly mediated by MAPK and NF-κB pathways.

Neutralization of IL-32 decreases proliferation of SS cells and viability of MF cells

As the tumor cells in lesional skin of tumor MF expressed IL-32 (Figure 3), we next examined whether IL-32 could serve as an autocrine growth factor. We first showed that the MF cell line MyLa and the SS cell line SeAx expressed IL-32 mRNA by real-time RT-PCR (Figure 5a). IL-32 protein was also detected both in MyLa and in SeAx cell culture supernatants (Figure 5b). When anti-IL-32 neutralizing antibody was added to MyLa cells, there was no significant difference in the cell number between the untreated and antibody-treated groups (Figure 5c). On the other hand, cell numbers of antibody-treated SeAx were significantly smaller than the cell numbers of untreated SeAx (Figure 5c). When we analyzed cell viability, IL-32 neutralization significantly decreased the survival rate in MyLa, whereas viability of SeAx was not changed (Figure 5d). Thus, we found that IL-32 produced by CTCL cell lines serves as an autocrine growth factor.

DISCUSSION

Our study revealed that IL-32 expression was increased in lesional skin of MF, which was consistent with a recent report (van Kester *et al.*, 2012). Keratinocytes in lesional skin of early MF (patch and plaque) expressed IL-32, whereas in MF tumor IL-32 expression occurred in atypical T cells located in the dermis and less so in the epidermis (Figure 3). IL-32 was not expressed in the epidermis in erythrodermic MF/SS. We also showed that IL-32 dose-dependently accelerated the proliferation of MF and SS cell lines *in vitro*, which was inhibited by blocking MAPK and NF-κB NF pathways. Finally, we showed that IL-32 serves as an autocrine growth factor in these cell lines.

IL-32 is highly expressed in human keratinocytes after stimulation with Th1 cytokines, including IFN-γ and tumor necrosis factor-α (Meyer *et al.*, 2010). IL-32 is highly expressed in synovial tissue biopsies of rheumatoid arthritis cases and IL-32 mRNA expression is increased in lesional skin of psoriasis (Joosten *et al.*, 2006; Kempuraj *et al.*, 2010). IL-32, however, was reported to be involved not only in these Th1-type diseases but also in CTCL and atopic dermatitis (Meyer *et al.*, 2010; van Kester *et al.*, 2012). Indeed, previous studies have suggested that both Th2 cells and Th1 cells are present within lesional skin of these latter two diseases (Grewe *et al.*, 1994; Asadullah *et al.*, 1996b). In lesional skin of tumor MF and SS, however, Th2 cells are dominant (Miyagaki *et al.*, 2010), which may explain why we saw low or no IL-32 expression in the epidermis in advanced CTCL.

Decreased IL-32 expression could lead to disease progression in CTCL. IL-32 enhanced the cytotoxic effect of NK cells on colon and prostate cancer cells (Park *et al.*, 2012). Intratumoral injections of dendritic cells engineered to express human IL-32β resulted in $CD8^+$ T-cell–dependent suppression of tumor growth in mice (Qu *et al.*, 2011). Thus, low or no IL-32 expression in the epidermis in advanced CTCL may help tumor cells escape from immunosurveillance. Dermal-infiltrating $CD4^+$ atypical T cells strongly expressed IL-32 in tumor MF, suggesting that tumor cells in advanced MF acquired resistance to IL-32-mediated immune responses (Figure 3). On the other hand, in lesional skin of SS, dermal tumor cells did not express IL-32. It has been recently

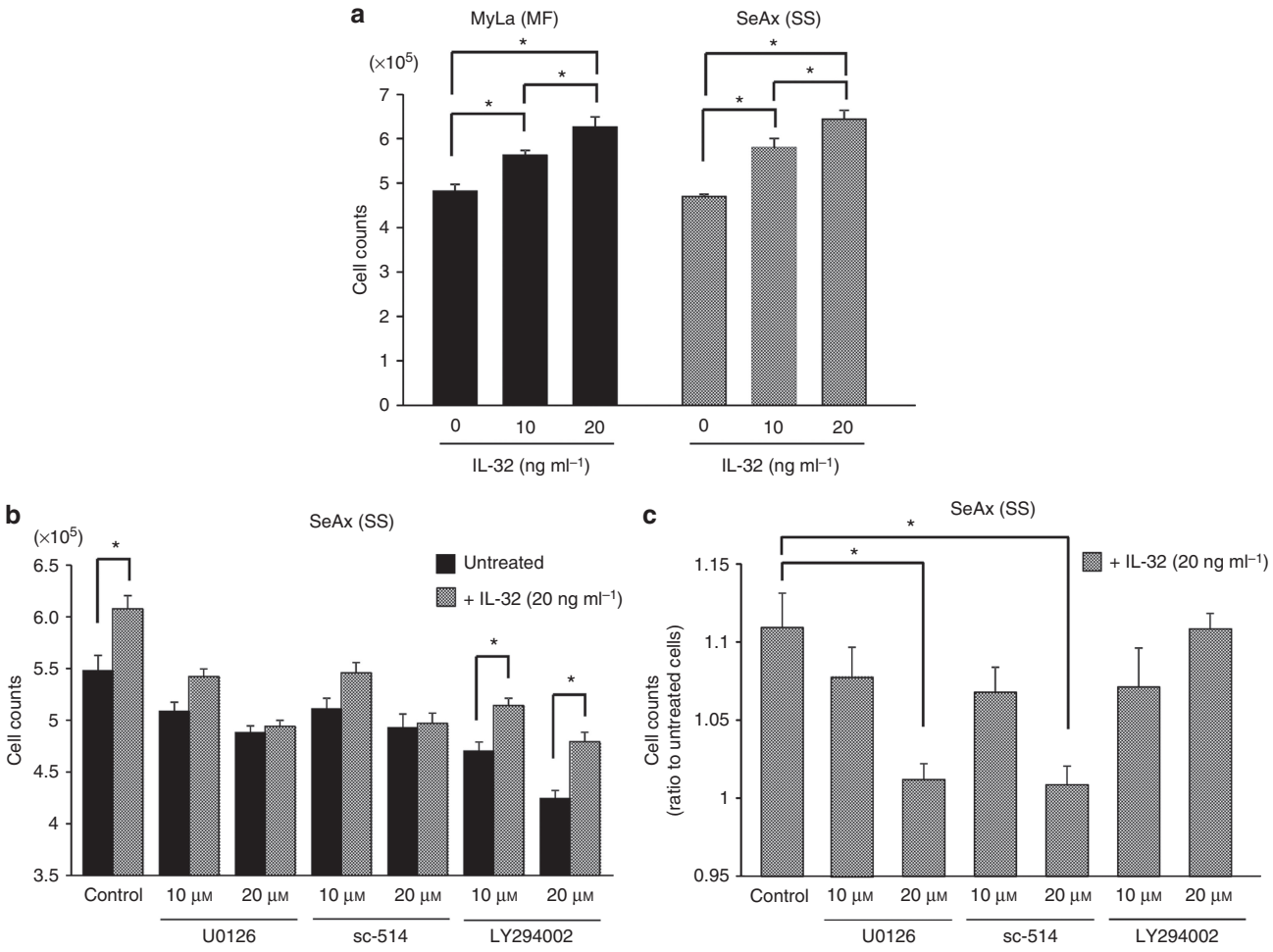

Figure 4. Cell proliferation of MyLa and SeAx accelerated by IL-32. (**a**) IL-32 dose-dependently accelerated cell proliferation of mycosis fungoides (MF) cell line MyLa and Sézary syndrome (SS) cell line SeAx. *$P<0.05$. (**b**) Cell proliferation of SeAx suppressed by U0126, sc-514, and LY294002. (**c**) Cell count ratio between the IL-32-treated groups and untreated groups. U0126 and sc-514 significantly diminished the effect of IL-32.

proposed that MF and SS are distinct clinical entities, arising from different T-cell subsets. MF is a malignancy of skin-resident effector memory T cells and SS originates from central memory T cells (Campbell *et al.*, 2010). The apparent lack of IL-32 expression in tumor cells in SS might support this theory.

IL-32 mRNA expression levels in lesional skin positively correlated with CCL17 and CCL18 mRNA expression levels (Figure 1c and d), which were both disease markers of CTCL (Kakinuma *et al.*, 2003; Günther *et al.*, 2011; Miyagaki *et al.*, 2013). In patients with high IL-32 mRNA expression levels in lesional skin, enhanced IL-32 expression was also detected by immunohistochemical analysis (Table 1). There was, however, no significant correlation between serum IL-32 levels and IL-32 mRNA expression levels in lesional skin. Indeed, there were no significant differences in serum IL-32 levels between CTCL patients and healthy controls (Figure 2a). In eight SS cases, there was no significant correlation between tumor cells in peripheral blood and serum IL-32 levels (data not shown). These results suggested that IL-32 expression in lesional skin may be more important than serum IL-32 in the development of CTCL, especially in the setting of MF.

In vitro experiments using MF and SS cell lines showed that IL-32 accelerated CTCL cell proliferation. When we added cell signaling inhibitors such as U0126, sc-514, and LY294002 to the culture media, U0126 and sc-514 blocked the IL-32-mediated proliferation of these cells. These results suggested that IL-32 induced CTCL cell proliferation through MAPK and NF-κB pathways. There have been several reports regarding the downstream signaling pathways of IL-32 (Kim *et al.*, 2005; Netea *et al.*, 2008; Mabilleau and Sabokbar, 2009; Nakayama *et al.*, 2012). IL-32 induced the production of tumor necrosis factor-α in mouse macrophage cells through MAPK and NF-κB pathways (Nakayama *et al.*, 2012). The IL-32-induced production of tumor necrosis factor-α, IL-1β, and IL-6 by human peripheral blood mononuclear cells was similarly regulated through phosphorylation of p38 MAPK (Netea *et al.*, 2008). On the other hand, others reported that IL-32 induced massive activation of MAPK and PI3-kinase/Akt pathways of human peripheral blood mononuclear cell (Mabilleau and Sabokbar, 2009). Our results were consistent with the former two papers. Interestingly, aberrant activation of MAPK and NF-κB pathways has been reported in CTCL (Izban *et al.*, 2000; Sors *et al.*, 2006; Mao *et al.*, 2008). IL-32

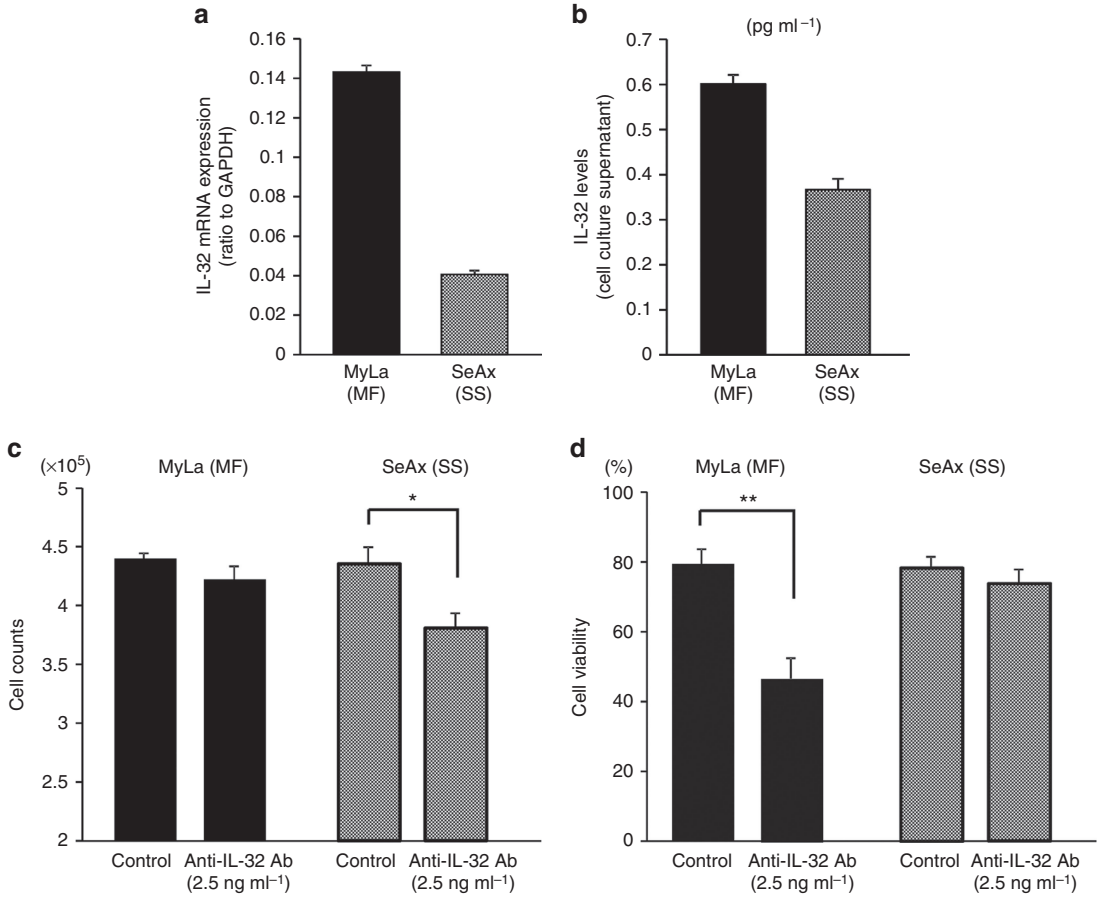

Figure 5. Neutralizing an autocrine effect of IL-32. (**a**) IL-32 mRNA expression by mycosis fungoides (MF) cell line MyLa and Sézary syndrome (SS) cell line SeAx. (**b**) IL-32 in culture supernatants of MyLa and SeAx. (**c**) IL-32 neutralization decreased cell proliferation of SeAx, but not MyLa. (**d**) IL-32 neutralization decreased the viability of MyLa, but not SeAx. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

may be one of the key cytokines activating these signaling pathways, which are known to contribute toward proliferation and resistance to apoptosis. IL-32 neutralization significantly decreased cell numbers of the SS cell line SeAx, while it significantly decreased the survival rate of the MF cell line MyLa. This discrepancy may also support the idea that MF and SS are distinct clinical entities (Campbell *et al.*, 2010).

The role of IL-32 in the setting of other malignancies has been studied. IL-32 expression was associated with a poorer prognosis in head and neck squamous cell carcinoma (Guenin *et al.*, 2013). Expression of IL-32 in human lung cancer correlated with metastasis (Sorrentino and Di Carlo, 2009). Other reports, however, reveal a potential anti-proliferative effect of IL-32. For example, IL-32 silencing in stromal cells *via* siRNA abrogated apoptosis in leukemia cells (Marcondes *et al.*, 2008). IL-32 was also reported to enhance cytotoxic effects of NK cells, inhibiting tumor cell growth (Cheon *et al.*, 2011; Oh *et al.*, 2011). Taken together, aberrant IL-32 expression by tumor cells is associated with tumor progression, whereas the physiological expression of IL-32 enhances tumor immunity. Our study suggests that IL-32 expression is also dysregulated in advanced MF, but not in the erythrodermic variant of MF.

In conclusion, this study highlights IL-32 as a cytokine functioning as an autocrine and paracrine growth factor in CTCL, especially in MF. Keratinocytes are a main source of this cytokine in patch and plaque MF, although IL-32 is expressed by malignant cells in lesional skin of tumor MF and by CTCL cell lines. Our study suggested that IL-32 may have a role in the formation and maintenance of CTCL lesions, providing a possible therapeutic target for patients with this disease.

MATERIALS AND METHODS

Patients and samples

Skin samples were collected from 26 patients with MF/SS (seven cases of patch MF, eight cases of plaque MF, five cases of tumor MF, and six cases of erythrodermic MF/SS; seven cases of stage IA, eight cases of stage IB, four cases of stage IIB, two cases of stage IIIA, three cases of stage IVA, and two cases of stage IVB) and six healthy control subjects. Blood samples were collected from 42 patients with MF/SS (25 male and 17 female patients; SD age: 59.9 ± 13.1 years; 11 cases of patch MF, 11 cases of plaque MF, 9 cases of tumor MF, and 11 cases of erythrodermic MF/SS; 12 cases of stage IA, 10 cases of stage IB, 7 cases of stage IIB, 4 cases of stage IIIA, 7 cases of stage IVA, and 2 cases of stage IVB) and 20 healthy control subjects (11 male and 9 female subjects; 42.6 ± 17.3 years). No tumor cells in peripheral blood (B0)

were detected in any MF patient. The tumor cell load in the peripheral blood for SS patients ranged from 1,548 to 42,543 counts μl^{-1} (mean: 14,273 counts μl^{-1}). All the patients were untreated or treated with only topical corticosteroid at the time of biopsy or blood test. The diagnosis of MF/SS was based on the World Health Organization classification and on the criteria of the International Society for Cutaneous Lymphomas (Olsen *et al.*, 2007). The healthy controls had no history of MF/SS or any skin diseases. All samples were collected after written informed consent was obtained. The medical ethics committee of the University of Tokyo approved the study, and it was conducted according to the Declaration of Helsinki Principles.

Quantitative RT-PCR

RNA was obtained from human skin samples with RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA). Complementary DNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Berkeley, CA). Quantitative RT-PCR was performed, as described previously, based on the SYBR Green assay (Sugaya *et al.*, 2012). Primers for human IL-32 and glyceraldehyde-3-phosphate dehydrogenase were as follows: IL-32 forward 5′-GGAGACAGTGGCGGC TTAT-3′ and reverse 5′-GGCACCGTAATCCATCTCTT-3′; glyceraldehyde-3-phosphate dehydrogenase forward 5′-ACCCACTCCTCCA CCTTTGA-3′ and reverse 5′-CATACCAGGAAATGAGCTTGACAA-3′.

ELISA

Quantification of serum IL-32 levels was performed using the human IL-32 Quantikine ELISA kit (Biolegend, San Diego, CA), according to the manufacturer's instructions.

IL-32 immunohistochemical staining

We also performed immunohistochemical staining for IL-32 using lesional skin of patch MF ($n=5$), plaque MF ($n=5$), tumor MF ($n=5$), erythrodermic MF/SS ($n=5$), and with normal skin ($n=5$). We used rabbit anti-human IL-32 polyclonal antibody (Sigma-Aldrich, St Louis, MO), followed by ABC staining (Vector Lab, Burlingame, CA).

CTCL cell lines

MyLa (an MF cell line) and SeAx (an SS cell line) were kind gifts from Dr Kazuyasu Fujii (Department of Dermatology, Okayama University, Okayama, Japan). MyLa and SeAx (4×10^5) were cultured in 1 ml of serum-free RPMI1640 (Millipore, Billerica, MA).

IL-32 stimulation and blocking of cell signaling pathways

Recombinant human IL-32α was purchased from BioVision (Milpitas, CA). To inhibit the cell signaling pathway, U0126 (MAPK extracellular signal–reduced kinase 1/2 inhibitor), sc-514 (IκB kinase-2 inhibitor), and LY294002 (phosphatidylinositol 3-kinase inhibitor) were purchased from Cell Signaling Technology (Danvers, MA). Cell counting was performed 48 hours after incubation using the Coulter Counter. Trypan blue solution was used for cell viability analysis. To neutralize IL-32 in culture supernatants, we used an anti-IL-32 rabbit polyclonal antibody purchased from Acris Antibody (San Diego, CA). Isotype-matched antibody was used as a control.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test and Student's *t*-test for the comparison of values. *P*-values <0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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