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Staphylococcal superantigen-like protein 8 (SSL8) binds to tenascin C and inhibits tenascin C–fibronectin interaction and cell motility of keratinocytes

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ABSTRACT

Staphylococcal superantigen-like protein (SSL), a family of exotoxins composed of 14 SSLs, exhibits no superantigenic activity despite of its structural similarity with superantigens. Several SSLs have been revealed to bind to host immune molecules such as IgA, IgG, complement and cell surface molecules expressed on immune cells, but the physiological function of SSL family has not been fully identified. In this study we attempted to isolate host target proteins of SSLs from human breast milk using SSLs-conjugated Sepharose. SSL8-conjugated Sepharose specifically recovered tenascin C (TNC), a multimodular and multifunctional extracellular matrix protein. Pull down analysis using SSL8-conjugated Sepharose and recombinant truncated fragments of TNC revealed that SSL8 interacts with fibronectin (FN) type III repeats 1–5 of TNC. The interaction of TNC with immobilized FN was attenuated, the scratch wound closure by HaCaT human keratinocytes was delayed and the inhibition of cell spreading on FN by TNC was recovered in the presence of SSL8. These findings suggest that SSL8 binds to TNC, thereby inhibits the TNC–FN interaction and motility of keratinocytes. The present study added a novel role of SSL family protein as an interrupting molecule against the function of extracellular matrix.

1. Introduction

Staphylococcal superantigen-like protein (SSL) is a family of exoproteins composed of 14 SSLs sharing structural similarity with staphylococcal superantigens but no superantigenic activity [1]. The physiological target of several SSLs have been identified; SSL7 binds to IgA [2] and complement C5 [2]; SSL5 binds to P-selectin glycoprotein ligand-1 (PSGL-1) [3], chemoattractant receptors [4], platelet receptors (αIIbβ3, GPIb and GPVI) [5,6] and matrix metalloproteinase (MMP) 9 [7]; SSL10 binds to chemokine receptor CXCL12 [8], human IgG [9,10] and phosphatidylserine [11]; SSL3 and SSL4 bind to toll like receptor 2 [12,13]. These findings imply that SSLs are involved in immune evasion of *Staphylococcus aureus*, however the binding counterparts of remaining SSLs have not been identified.

In this study we attempted to isolate the host target proteins of SSLs from human breast milk because it contains essential nutrients for the growth of infants as well as immune molecules such as lactoferrin, IgA and lysozyme, it would be a good source for isolating target proteins of SSLs.

2. Materials and methods

2.1. Reagents

Reagents were purchased from SIGMA (St. Louis, MO), WAKO pure chemicals (Osaka, Japan) and Nacalitesque (Kyoto, Japan). Oligonucleotides were supplied by Nihon Gene Research Laboratories (Sendai, Japan). Restriction endonucleases and modifying enzymes were products of Roche (Basel, Switzerland), TaKaRa (Osaka, Japan), and Toyobo (Osaka, Japan).

2.2. Cell culture

A human melanoma cell line A375-SM was kindly provided by Dr. Fidler (MD Anderson Cancer Center, University of Texas) and immortalized human keratinocyte cell line HaCaT [14] was a kind gift from Dr. Akimichi Morita (Nagoya City University) with the permission of Dr. Norbert Fusenig (German Cancer Research Center). The cells were grown at 37 °C in DMEM or RPMI1640 medium (Wako) supplemented with 100 unit/ml of penicillin G, 100 μg/ml

Abbreviations: FN, fibronectin; ECM, extracellular matrix; SSL, staphylococcal superantigen-like protein; TNC, tenascin C; EGFL, epidermal growth factor-like repeats; FNIII, fibronectin type III like repeats; FNG, fibrinogen globe.

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Table 1
Primers used for amplification and introduction of mutation of truncated TNC.

		Sequence
EGFL	Sense	5′-GGG GAT CCA AGC GAA AAC AGA AGT TAA AC-3′ (underline; *Bam*H I site)
	Antisense	5′-GGG AAG CTT ATT TCA TTT CTA CTA GAA TTT TTT G-3′ (*Hind* III)
FNIII1–5	Sense	5′-GGG ATC CAA ACA ACG GAA AAA CCA GTT-3′ (*Bam*H I)
	Antisense	5′-GCT GCA GTT AAG CTT TTC TAA CTT TGA T-3′ (*Pst* I)
FNIII6–8	Sense	5′-GGG ATC CAA GAA AA ATA CAA TCA ACT-3′ (*Bam*H I)
	Antisense	5′-GAA GCT TAT TTT ATA TTC ACT TCA AT-3′ (*Hind* III)
FNG	Sense	5′-GGG ATC CAG ACA ACA CCA TCT TCC ACT-3′ (*Bam*H I)
	Antisense	5′-GAA GCT TAT TTT ATA TTC ACT TCA ATG-3′ (*Hind* III)
FNIII1–3	Sense	5′-GGG GAT CCA GAG CGA ACA TGA ATC AAA ATA TG-3′ (*Bam*H I)
	Antisense	5′-GGG GGT CGA CTT ATC TAA TAT TGG CTT CTA TTT TCT C-3′ (*Sal* I)
FNIII4–5	Sense	5′-GGG ATC CAA ACA GAA AGT CAA ACA GT-3′ (*Bam*H I)
	Antisense	5′-GAA GCT TAT TTA TAT TCT AGC TCA AC-3′ (*Hind* III)

of streptomycin, and 5% or 10% fetal calf serum in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of recombinant proteins and SSLs-conjugated Sepharose

Recombinant N-terminal 6xHis-tagged SSLs and SSLs-conjugated Sepharose were prepared as described previously [7]. The genes of truncated TNC were amplified by PCR using cDNA of A375 melanoma cells as a template and primers listed in Table 1, inserted into the prokaryotic expression plasmid pQE-32 (Qiagen, Chatsworth, CA, for FNIII and FNG) or eukaryote expression plasmid pcDNA3.1/His A (Invitrogen, Carlsbad, CA, for EGFL). The *Escherichia coli* strain JM109 was transformed with the resultant constructs derived from pQE32 and the 6xHis tagged recombinant proteins were induced and purified as described previously [7]. Chinese hamster ovary (CHO) was transfected with the resultant constructs derived from pcDNA3.1 using Fugene HD (Roche Applied Science, Indianapolis, IN), and stable transfectants were selected under the cultivating in the presence of G418 for 2 weeks. The 6xHis tagged recombinant protein expressed in the transfectant was purified with Ni Sepharose 6 Fast Flow according to the manufacturer's protocol.

2.4. Isolation and identification of SSLs-binding protein

Human breast milk donated by a healthy volunteer was clarified by centrifugation (13,420*g* for 5 min). The clarified milk (10 ml) was mixed with SSLs-conjugated Sepharose (20 μl of 50% slurry) and incubated at 4 °C for 1 h, and the Sepharose was washed three time with wash buffer (20 mM Tris, 0.5 M NaCl, 1% Nonident P-40; pH 7.5). Proteins bound to SSLs-Sepharose were eluted with 2× Laemmli's sample buffer [15], resolved by SDS–PAGE using 10% polyacrylamide gel, stained with Coomassie brilliant blue (CBB) R250 (Merck, Darmstadt, Germany). The protein specifically recovered by indivisual SSL-conjuigated Sepharose was identified using peptide mase fingerprinting analysis as described previously [7,9].

2.5. Pull down analysis

The mixture (100 μl) containing recombinant truncated TNC (20 μg) and BSA or ovalbumin (OVA, 100 μg) was incubated with SSL8-conjugated Sepharose (10 μl) or unconjugated Sepharose at 4 °C for 1 h. After low-speed centrifugation, the supernatant was recovered and the resin was washed 3 times with wash buffer, and bound proteins were eluted with 2× Laemmli's sample buffer and subjected to SDS–PAGE ("bound fraction", B). The supernatant was mixed with 4× Laemmli's sample buffer and aliquot

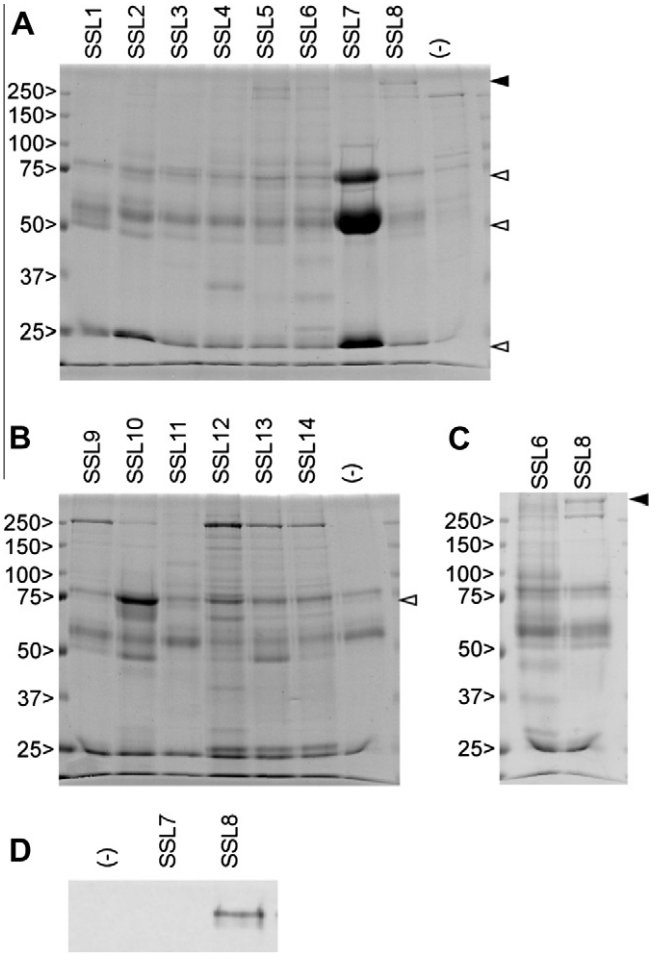

Fig. 1. Isolation of SSL8-binding proteins from human breast milk. (A–C) Isolation of SSLs-binding proteins from human breast milk. The proteins bound to His-tagged SSLs-conjugated Sepharose ((A) SSL1 ~ SSL8-conjugated Sepharose and unconjugated Sepharose, (B) SSL9 ~ SSL8-conjugated Sepharose and unconjugated Sepharose, (C) SSL6 and SSL8-conjugated Sepharose) were separated by SDS–PAGE using polyacrylamide gel (10%) and stained with CBB. Arrowheads indicate the binding proteins of SSL-conjugated Sepharose. The band bound to SSL8-conjugated Sepharose (indicated by a filled arrowhead) was subjected to peptide mass fingerprinting analysis. Open arrowheads show IgA and lactoferrin bound to SSL7- and SSL10-conjugated Sepharose, respectively. (D) SSL7 and SSL8-conjugated Sepharose bound fractions recovered form human breast milk were analysed by immunoblotting using anti-human TNC antibody.

(tenth, 1/10 or eights, 1/8) and was electrophoresed ("unbound fraction", UB). The protein was visualized by staining the gel with

Table 2
The list of observed mass peaks and corresponding amino acid sequences of human TNC detected by the MS analysis.

m/z	Amino acid sequence[a,b]	*m/z*	Amino acid sequence
3515.36	^{1332}GHSTRPLAVEVVTEDLPQLGDLAVSEVGWDGLR^{1364}	1841.05	^{1638}LSWTADEGVFDNFVLK^{1653}
		1782.86	^{542}CVNGQCVCHEGFMGK^{556}
2823.86	^{1819}WQPAIATVDSYVISYTGEKVPEITR^{1843}	1761.04	^{1042}GLEPGQEYNVLLTAEK^{1057}
2695.47	^{1034}NTTSYVLRGLEPGQEYNVLLTAEK^{1057}	1724.09	^{1659}KQSEPLEITLLAPER^{1673}
2652.71	^{1128}AVDIPGLKAATPYTVSIYGVIQGYR^{1152}	1705.98	^{1698}RSQTVSAIATTAMGSPK^{1714}
2628.51	^{2007}AQALEVFCDMTSDGGGWIVFLRR^{2029}	1662.04	^{2064}ITAQGQYELRVDLR^{2077}
2612.72	^{1492}TAHISGLPPSTDFIVYLSGLAPSIR^{1516}	1654.96	^{927}YAPISGGDHAEVDVPK^{942}
2543.56	^{1844}TVSGNTVEYALTDLEPATEYTLR^{1866}	1644.94	^{1682}EATEYEIELYGISK^{1695}
2515.52	^{1069}ASTEQAPELENLTVTEVGWDGLR^{1091}	1633.95	^{2051}REEFWLGLDNLNK^{2063}
2506.40	^{356}CEEGQCVCDEGFAGLDCSEKR^{376}	1595.96	^{1660}QSEPLEITLLAPER^{1673}
2491.55	^{2}GAMTQLLAGVFLAFLALATEGGVLK^{26}	1548.87	^{902}VSQTDNSITLEWR^{914}
2469.51	^{649}VTEYLVVYTPTHEGGLEMQFR^{669}	1500.87	^{1318}AGTPYTVTLHGEVR^{1331}
2463.50	^{628}DLVVTEVTEETVNLAWDNEMR^{648}	1477.81	^{2052}EEFWLGLDNLNK^{2063}
2421.37	^{387}CVDGRCECDDGFTGADCGELK^{407}	1472.86	^{1401}AVDIPGLEAATPYR^{1414}
2392.62	^{950}TTLTGLRPGTEYGIGVSAVKEDK^{972}	1413.76	^{2174}GHEHSIQFAEMK^{2185}
2371.52	^{2121}SFSTFDKDTDSAITNCALSYK^{2141}	1401.84	^{126}LEELENLVSSLR^{137}
2360.63	^{1401}AVDIPGLEAATPYRVSIYGVIR^{1422}	1352.73	^{2078}DHGETAFAVYDK^{2089}
2350.33	^{356}CEEGQCVCDEGFAGLDCSEK^{375}	1258.75	^{708}VATYLPAPEGLK^{719}
2284.41	^{1892}DLTATEVQSETALLTWRPPR^{1911}	1207.64	^{887}ETFTTGLDAPR^{897}
2144.22	^{970}EDKESNPATINAATELDTPK^{989}	1178.67	^{2064}ITAQGQYELR^{2073}
2128.28	^{1819}WQPAIATVDSYVISYTGEK^{1837}	1146.67	^{1227}AATHYTITIR^{1236}
2110.12	^{1638}LSWTADEGVFDNFVLKIR^{1655}	1128.58	^{2034}ENFYQNWK^{2041}
2095.10	^{878}GDMSSNPAKETFTTGLDAPR^{897}	1105.61	^{1732}APTAQVESFR^{1741}
2057.12	^{2078}DHGETAFAVYDKFSVGDAK^{2096}	1099.65	^{803}LDAPSQIEVK^{812}
2037.26	^{1742}ITYVPITGGTPSMVTVDGTK^{1761}	1083.57	^{2042}AYAAGFGDRR^{2051}
2020.39	^{950}TTLTGLRPGTEYGIGVSAVK^{969}	1061.66	^{692}VFAILENKK^{700}
1954.11	^{1715}EVIFSDITENSATVSWR^{1731}	1051.59	^{1883}FTTDLDSPR^{1891}
1934.14	^{990}DLQVSETAETSLTLLWK^{1006}	927.49	^{2042}AYAAGFGDR^{2050}
1928.28	^{1017}LNYSLPTGQWVGVQLPR^{1033}	906.55	^{1415}VSIYGVIR^{1422}
1901.19	^{1912}ASVTGYLLVYESVDGTVK^{1929}	902.52	^{1310}SMEIPGLR^{1317}
1896.16	^{925}IKYAPISGGDHAEVDVPK^{942}	889.53	^{2186}LRPSNFR.N^{2192}
1863.00	^{58}LPVGSQCSVDLESASGEK^{75}	799.42	^{2142}GAFWYR^{2147}
1859.13	^{1136}AATPYTVSIYGVIQGYR^{1152}	768.51	^{104}INIPRR^{109}

[a] Genebank accession number: match to: A32160 tenascin-C–human.
[b] Mowse score and sequence coverage were 217 and 38%.

CBB or immunoblotting with the combination of anti-His-tag antibody (His-probe H-15, Santa Cruze Biotechnology. Santa Cruz, CA) and Horse radish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA).

2.6. TNC–FN interaction

Ninety-six well microtiter plate (Maxi sorp, Nalgen, Rochester, NY) was coated with human plasma fibronectin (20 μg/ml, Invitrogen) at 4 °C for 16 h. After blocking with PBS-1% BSA, the mixture of A375-SM conditioned medium (containing 727 ng/ml of TNC as determined by human Tenascin-C Large assay kit, Immuno-Biological Laboratories Gunma, Japan) and SSLs (0–200 μg/ml) were added to the wells and incubated at room temperature for 1 h. After washing three times with PBS-0.05% tween 20, the amount of TNC in wells was determined using anti-TNC antibody, HRP-conjugated secondary antibody and TMB substrate.

2.7. Wound healing assay

HaCaT cells were cultivated in a 12 well plate to form confluent monolayer. A wound was made by scratching the monolayer by a 200 μl pipet tip. After the removal of scratched cells, the adhered cells were cultured for 12 h in the presence or absence of SSL8 or SSL7 (10 μg/ml). The wound closure was observed every 3 h by taking photograph with a phase contrast microscope equipping. The length of wound was measured using graphic software (CANVAS 11, Deneva, Miami, FL).

2.8. Adhesion assay

Ninety-six well microtiter plate (Falcon) was coated with FN (2 μg/ml) overnight, blocked with 1% BSA at room temperature for 1 h. HaCaT cells suspended in serum-free media were adhered to the well in the presence or absence of human TNC (1 μg/ml, Millipore, Temecula, CA) and SSL8 (50 μg/ml) at 37 °C for 1.5 h. The adhered cells were stained with crystal violet (5 mg/ml) in 20% methanol, and then observed under a microscope.

3. Results

3.1. Binding of TNC to SSL8-conjugated Sepharose

We affinity isolated SSL-binding proteins from human breast milk using SSLs-conjugated Sepharose. As shown in Fig. 1A and C, SSL8-conjugated Sepharose recovered the protein having a molecular weight of over 250 kDa on SDS–PAGE under reducing condition (indicated by filled arrowheads). The protein was identified as human tenascin C (TNC) by peptide mass fingerprinting analysis using MALDI-TOF-MS. Major mass peaks detected by the MS analysis and the MASCOT search analysis are summarized in Table 2. The Mowse score and the sequence coverage was 217 and 38%, respectively. Human TNC was detected in the fraction recovered by SSL8-conjugated Sepharose from human milk, but not by SSL7-conjugated Sepharose using anti-TNC antibody (Fig. 1D). SSL7-conjugated Sepharose recovered IgA as previously reported (Fig. 1A, 60 kDa H chain, 25 kDa L chain and 75 kDa Secretory component, indicated by open arrowheads) [2], SSL10-conjugated Sepharose recovered lactoferrin (Fig. 1B, 80 kDa, indicated by

an open arrowhead) however that seemed to be nonspecific, therefore they were excluded from further analysis.

3.2. SSL8 binds to FNIII1–5 repeats of TNC and inhibits TNC–FN interaction

TNC is a multifunctional and modular ECM protein, composed of assembly domain, EGFL, FNIII repeats and FNG (Fig. 2A). Each domain of TNC is reported to have corresponding counterparts [16]. To reveal the effect of SSL8 on the physiological functions of TNC, we attempted to identify the region of TNC that is responsible for the interaction with SSL8. We prepared recombinant truncated TNC fragments, as reported to be successively expressed [17–19], and examined their binding to SSL8-conjugated Sepharose. SSL8-conjugated Sepharose preferentially coprecipitated with the truncated TNC mutant containing FNIII1–5, however, it did only slightly with that of EGFL (Fig. 2B), and did so those of FNIII6–8 and FNG (Fig. 2B–D). Within the region of FNIII1–5 SSL8-conjugated Sepharose specifically coprecipitated with that of FNIII4–5 but not FNIII1–3 (Fig. 2E). As FNIII1–5 of TNC was reported to interact with integrin, heparin and fibronectin [16], we examined whether SSL8 interferes the interaction. The binding of TNC in the conditioned medium of A375-SM (containing 727 ng/ml TNC) to immobilised FN was inhibited by the presence of SSL8 in a dose dependent manner (Fig. 2F). On the other hand, SSL8 did not affect the adhesion of alpha 9 integrin expressing melanoma cells on FNIII1–5 coated substrate and recovery of FNIII1–5 by heparin-conjugated Sepharose (data not shown). These findings suggest that SSL8 is able to bind to TNC via its FNIII1–5 and interfere the interaction between TNC and FN.

3.3. SSL8 affects cell motility of HaCaT cells

We examined the effect of SSL8 on the cell migration by *in vitro* scratch wound healing assay and adhesion assay using HaCaT keratinocytes. The wound closure of HaCaT cells in melanoma cell-conditioned medium that contains TNC was delayed about 3 h by the addition of SSL8 but not by SSL7 (Fig. 3A). The delay of wound closure was not observed when wound healing assay was performed in the fresh medium supplemented with 5% of FBS (Fig. 3B). HaCaT cells were adhered to and spread on immobilised FN and the spreading was repressed in the presence of purified TNC (Fig. 3C, right upper and left-lower panels, respectively). The repression of cell spreading was recovered by the addition of

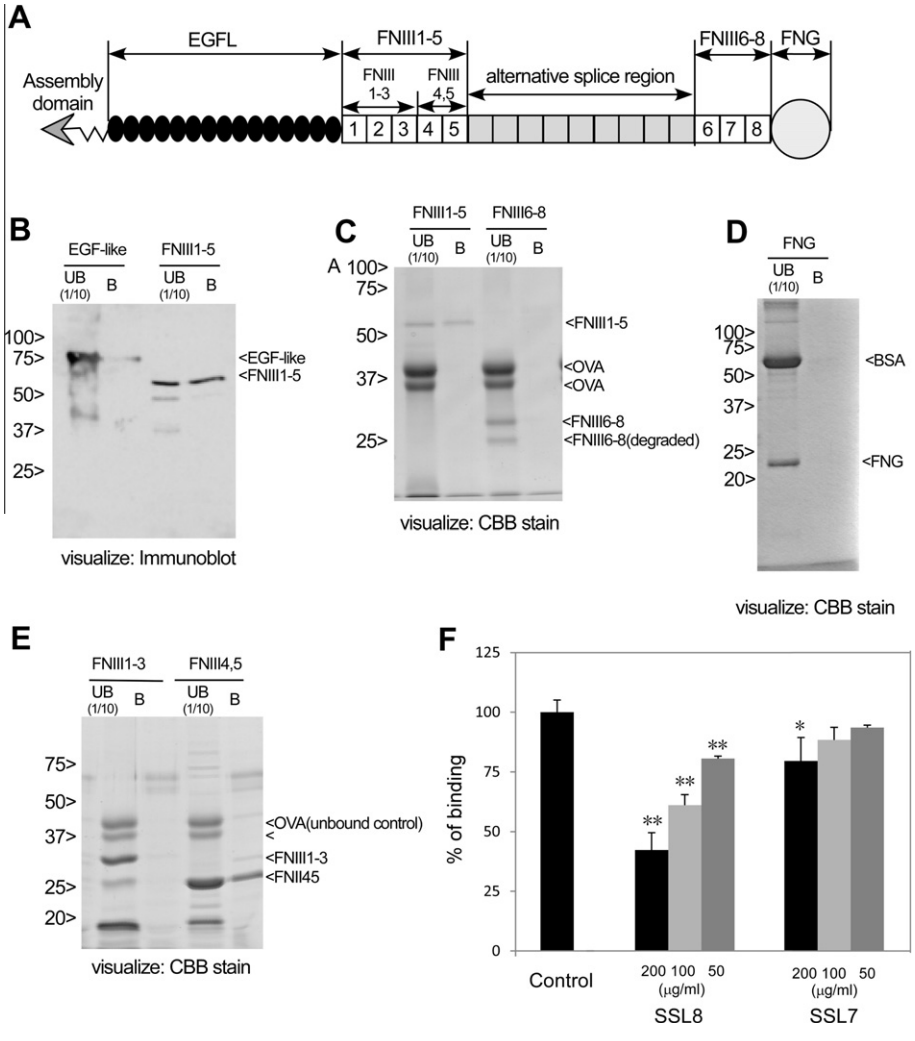

Fig. 2. Identification of SSL8-binding region in TNC and the effect of SSL8 on TNC–FN interaction. (A) Structure of TNC and truncated TNC mutants used in this study. (B–E) The interaction of truncated TNC mutants, (A) EGFL and FNIII1–5, (B) FNIII1–5 and FNIII6–8, (C) FNG and (D) FNIII1–3 and FNIII4–5) with SSL was analyzed by pull down analysis using SSL8-conjugated Sepharose. SSL8-conjugated Sepharose bound or unbound fraction were subjected to SDS–PAGE and truncated TNC mutants were visualized by immunoblotting using anti-His tag antibody (B) or staining with CBB (C–E). (F) The culture supernatant of human melanoma cells that contains TNC was incubated with immobilized FN on 96 well plates, and then TNC bound to immobilized FN was detected by the combination of anti-TNC antibody, HRP-conjugated anti-rat IgG antibody and TMB reagents. UB: aliquot of unbound fraction (1/10 means one-tenth and 1/8 means one-eighth of unbound supernatant, respectively), B: bound fraction.

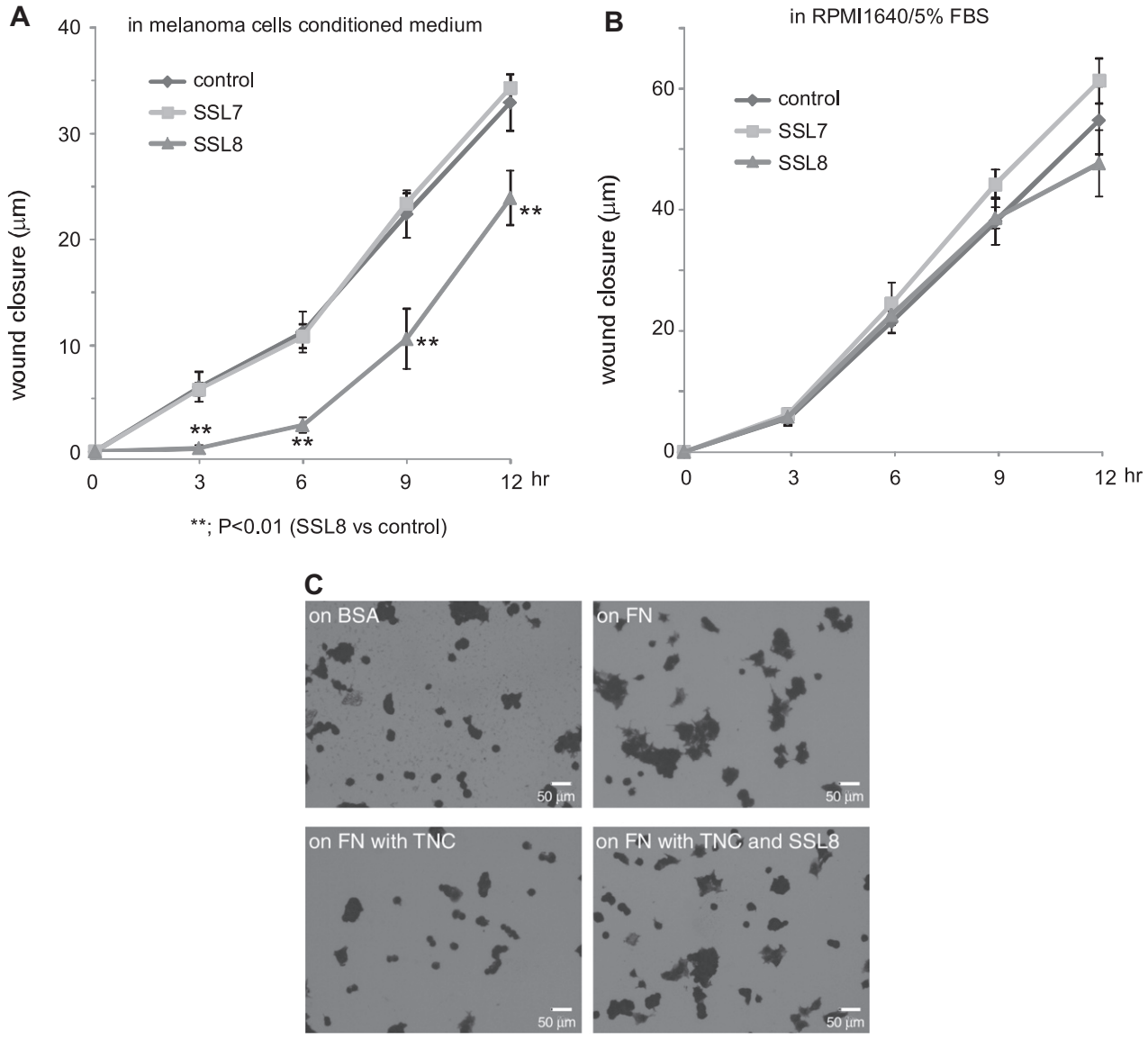

Fig. 3. Effect of SSL8 on cell motility of HaCaT cells. (A and B) wound healing assay. Confluent monolayer of HaCaT cells on 24 well plate was scratch wounded by pipet tip, washed and allowed to regenerate in the presence or absence of SSL8 or SSL7. The scratch wound of HaCaT cells was incubated in melanoma cell conditioned medium that contains TNC (A) or RPIM1640 medium supplemented with 5% FBS (B). The length of wound was measured every 3 h up to 12 h. Data are shown as the mean ± SD of triplicate wells. The statistical difference was assessed by Student's *t*-test: $^{**}P < 0.01$. (C) Adhesion assay. HaCaT cells were allowed to adhere to the immobilised FN in the presence or absence of purified human TNC (1 μg/ml) and SSL8 (50 μg/ml) at 37 °C for 1.5 h. The adhered cells were stained with crystal violet and observed under a microscope. Bar, 50 μm.

SSL8 (Fig. 3C, right-lower panel). These findings suggest that SSL8 is able to interfere the cell motility of keratinocytes in the presence of TNC.

4. Discussion

TNC is a large hexametric extracellular matrix protein which plays a role in embryonic development, tissue repair, inflammation and tumorigenesis [16]. TNC consists of multi-modules and exhibits multiple activities via interacting with a variety of binding counterparts. The responsible regions of TNC for interaction with the partners have been clarified, *e.g.* EGFL of TNC interacts with EGF receptor, FNIII with integrins, heparin, fibronectin and proteoglycans, and FNG with integrin, neurocan and TLR4 [16]. We revealed that FNIII1–5 of TNC and more specifically FNIII4–5 is responsible for the interaction of SSL8 with TNC (Fig. 2B–E). The region was reported to be involved in the interaction of TNC with fibronectin [20], integrins [21–23] and heparin [17]. Among them, SSL8 inhibited the interaction between TNC and FN (Fig. 2F).

TNC inhibits formation of focal contact and matrix contraction in fibroblasts on FN and fibrin matrix [24], enhances migration of fibroblasts from fibrin-fibronectin matrix [25], and FNIII domain of TNC reduces the incorporation of FN produced by NIH3T3 fibroblasts into detergent insoluble matrix [26]. These reports, therefore, suggest that TNC affect the cell motility and character of FN matrix to facilitate the cell detachment and movement. SSL8 delayed the scratched wound closure by keratinocytes only in the presence of TNC (Fig. 3A and B), and SSL8 recovered the inhibition of cell spreading on FN by TNC (Fig. 3C) suggesting that SSL8 is able to attenuate cell motility via interferring TNC–FN interaction. TNC is thought to be involved in the process of wound healing; TNC is transiently expressed in tissue injury sites [27]; keratinocytes express TNC during the early phase of wound healing [28]; TNC knock-out mice were born alive but showed abnormality in wound

healing, namely the process of wound healing in TNC-knockout mice is apparently normal, however deposition of fibronectin was reduced [29]; TNC knockout mice showed impairment of keratinocytes migration in cornea wound [30]. Present findings that SSL8 is able to attenuate the interaction of TNC with FN and motility of keratinocytes would indicate a novel role of SSL8 in the wound infection by *S. aureus*.

Several reports indicate the relationship between TNC and inflammation and immunity. TNC is induced in chemically induced dermatitis and deficiency of TNC exacerbated and prolonged the dermatitis [31,32]. Deficiency of TNC attenuates bronchial asthma in mouse treated with allergen [33]. Midwood et al. reported that TNC acts as endogenous ligand of TLR4 and expression of TNC mediates persistent joint inflammation [34]. They showed that FNG of TNC is responsible for the induction of cytokines from fibroblasts. In the present study, the truncated mutant that contains FNG did not interact with SSL8 (Fig. 2C). Therefore, it is unlikely that SSL8 inhibits activation of TLR4 by TNC. The role of SSL8 in the inflammation induced by *S. aureus* infection is the focus of future study.

Low amino acid sequence homology among SSL family suggests that each SSL has corresponding target protein. The identification of all the SSLs targets would lead to the comprehensive understanding of their roles in host-*S. aureus* interactions. SSL family widely interferes the host immunity against *S. aureus* infection by binding to immunoglobulins and complement [2,9,10], adhesion molecule and chemoattractant receptors [3,4,8], a protease [7], and a pattern recognition receptor [12,13]. The present study added a novel role of SSL family protein as an interrupting molecule against the function of ECM.

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