Identification of an S100A8 Receptor Neuroplastin-β and its Heterodimer Formation with EMMPRIN

Masakiyo Sakaguchi1,2, Mami Yamamoto2,5, Masashi Miyai1, Tatsuo Maeda2, Junichiro Hiruma2, Hitoshi Murata1, Rie Kinoshita1, I Made Winarsa Ruma1, Endy Widya Putranto1, Yusuke Inoue3, Shin Morizane4, Nam-Ho Huh1, Ryoji Tsuboi2 and Toshihiko Hibino2

We previously reported a positive feedback loop between S100A8/A9 and proinflammatory cytokines mediated by extracellular matrix metalloproteinase inducer, an S100A9 receptor. Here, we identify neuroplastin-β as an unreported S100A8 receptor. Neuroplastin-β and extracellular matrix metalloproteinase inducer form homodimers and a heterodimer, and they are co-localized on the surface of cultured normal human keratinocytes. Knockdown of both receptors suppressed cell proliferation and proinflammatory cytokine induction. Upon stimulation with S100A8, neuroplastin-β recruited GRB2 and activated extracellular signal-regulated kinase, resulting in keratinocyte proliferation. Keratinocyte proliferation in response to inflammatory stimuli was accelerated in involucrin promoter-driven S100A8 transgenic mice. Further, S100A8 and S100A9 were strongly up-regulated and co-localized in lesional skin of atopic dermatitis patients. Our results indicate that neuroplastin-β and extracellular matrix metalloproteinase inducer form a functional heterodimeric receptor for S100A8/A9 heterodimer, followed by recruitment of specific adaptor molecules GRB2 and TRAF2, and this signaling pathway is involved in activation of both keratinocyte proliferation and skin inflammation in atopic skin. Suppression of this pathway might have potential for treatment of skin diseases associated with chronic inflammation such as atopic dermatitis.


INTRODUCTION

Emerging evidence indicates that inflammation in atopic dermatitis (AD) results primarily from a defect of skin barrier function (Chan, 2008; Zheng et al., 2011), which results in excessive loss of moisture from the cornified layer, allowing the skin to become very dry and reducing its protective abilities. Large-scale DNA microarray and proteomics studies of AD skin lesions have shown overexpression of genes located at the epidermal differentiation complex and loss of expression of protective genes in the cornified envelope (Broccardo et al., 2011; Khattri et al., 2014). In particular, S100A8 and S100A9 are highly up-regulated.

The S100 proteins modulate inflammation (Donato, 2001; Donato et al., 2013; Goyette and Geczy, 2011; Schiopu and Cotoi, 2013), and among them, S100A8 and S100A9 form a heterodimer, S100A8/A9 (known as calprotectin), in vivo (Schiopu and Cotoi, 2013). We have shown that multiple proinflammatory cytokines are up-regulated in S100A8/A9-treated keratinocytes (Nukui et al., 2008). Furthermore, S100A8/A9-induced proinflammatory molecules in turn stimulate keratinocytes to synthesize and secrete S100A8/A9, suggesting the existence of a positive feedback loop between S100A8/A9 and these proinflammatory factors.

Receptor for advanced glycation end-products (RAGE) is known to be a general inflammation-related receptor (Bierhaus et al., 2006; Clynes et al., 2007; Leclerc et al., 2009; Sakaguchi et al., 2011). However, its ligands also include advanced glycation end-products, high mobility group box-1 (HMGB1), and amyloid-β. We postulated that more specific receptor(s) might be involved in the positive feedback mechanism for S100A8/A9 (Nukui et al., 2008), and we identified extracellular matrix metalloproteinase inducer (EMMPRIN) as a specific receptor for S100A9 in the inflammatory loop (Hibino et al., 2013). In addition, the action of S100A8 and S100A9 as endogenous damage-associated molecular pattern molecules that link innate immunity and autoimmune responses (Loser et al., 2010) is reported to be mediated by Toll-like receptor 4 (TLR4), which interacts with S100A8 (Vogl et al., 2007). However, S100A8/A9 induce multiple effects, including cell proliferation, migration,
apoptosis, and inflammation (Halayko and Ghavami, 2009), and we hypothesized that other receptor(s) would also be involved.

Therefore, we searched for a putative S100A8 receptor involved in the skin inflammation and abnormal proliferation of keratinocytes. We found that neuropilin-β (NPTNB) forms a heterodimer with EMMPRIN, and this heterodimer is suggested to work as a functional receptor for S100A8/A9, linking to S100A8/A9-induced skin inflammation and abnormal keratinocyte proliferation in AD.

RESULTS

S100A8 specifically binds with NPTNB

We searched for a putative cell-surface receptor for S100A8 based on similarity with EMMPRIN using the protein BLAST program in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and found that NPTNB showed considerable similarity with EMMPRIN. NPTNB has two splicing variants, NPTNB and NPTNB (Langnaese et al., 1997; Owczarek and Berezin, 2012) (see Supplementary Figure S1a and b online). We examined whether these isoforms interact with S100 proteins. S100A8 was co-immunoprecipitated with NPTNB but not NPTNB (Figure 1a). S100A9 was found in the NPTNB fraction. NPTNB possesses two Ig domains in the extracellular region, whereas NPTNB has one extra Ig domain (see Supplementary Figure S1a and b). We found that the additional Ig-3 domain in NPTNB is responsible for the binding with S100A8 (Figure 1b). To assess more precisely the interaction about NPTNB, we used purified recombinant proteins with an aim to rule out any cellular participant on the binding. ELISA with immobilized NPTNB, soluble form (see Supplementary Figure S1c), confirmed dose-dependent bindings of S100A8 and S100A8/A9, which were much higher than that of S100A9 (see Supplementary Figure S1d).

At the mRNA and protein levels, S100A8 and S100A9 are found that NPTNB was the dominant isoform in cultured human keratinocytes, which was more obvious at protein levels, and showed rather constant expression levels under various conditions, whereas EMMPRIN was highly up-regulated by 1.5 mmol/L of calcium ions.

It has been reported that TLR4 transduces S100A8 signals in various cell types (Gan et al., 2014). We found that TLR4 was highly expressed in spleen and HeLa cells but that it was undetectable in cultured keratinocytes (see Supplementary Figures S2a and b and S3 online). Moreover, a TLR4-neutralizing antibody had no effect on S100A8-induced cytokine induction (see Supplementary Figure S2c). In addition, S100A8/A9 did not show any affinity to RAGE (see Supplementary Figure S4 online). Binding of S100A8/A9 with RAGE was weak compared with that of HMGB1, S100A11, and S100A9 (see Supplementary Figure S4b). These results suggest that TLR4 and RAGE would not be key receptors for S100A8, at least in human keratinocytes.

S100A8/A9 signaling pathway

Motif analysis showed that the cytoplasmic tails of NPTNB and EMMPRIN contain tumor necrosis factor (TNF) receptor associated factor (TRAF)-binding motifs. In addition, NPTNB possesses an SH3 domain that binds to proline-rich sequences containing a conserved PxxP motif, where P is proline and x is any amino acid (Kay et al., 2000; Mayer et al., 2001) (see Supplementary Figure S5a online). We expressed TRAF2, GRB2, CRK, NCK1, NCK2, and p38x proteins in HEK293 cells, together with EMMPRIN or NPTNB, and examined complex formation (Figure 2a). Upon stimulation with S100A8/A9, EMMPRIN recruited only TRAF2, and NPTNB recruited TRAF2 and GRB2, although NPTNB-TRAF binding seemed to remain at relatively low levels compared with that of EMMPRIN. Mutation of the SH3 binding motif of NPTNB did not affect TRAF2 binding but abolished GRB2 recruitment (Figure 2b, and see Supplementary Figure S5b). When the TRAF binding motif was mutated, only GRB2 was detected as an interactant with NPTNB. S100A8/A9-induced activations of effector kinases p38 and extracellular signal-regulated kinase (ERK) were both suppressed by over-expression of the cytoplasmic domain of wild-type NPTNB. That of mutated SH3 domain specifically inhibited the activation of p38, whereas the mutated TRAF2 domain functioned to mitigate ERK activation (Figure 2c). In these cases, mutated cytoplasmic domains of TRAFs and GRB2 trapped intact TRAF2 and GRB2, resulting in ERK inhibition in the former and p38 inhibition in the latter. Before receptor-mediated downstream analysis using siRNAs (see Supplementary Figure S6 online), we examined kinetics of activation levels of ERK and p38. Under stimulation with S100A8/A9, ERK and p38 were phosphorylated, and peak activity was reached at 30 minutes without noticeable attenuation thereafter (see Supplementary Figure S7a online). Knockdown of NPTNB (see Supplementary Figure S6) caused profound down-regulation of S100A8/A9-mediated ERK phosphorylation, including the steady-state level (Figure 2d). In the case of S100A8 stimulation, knockdown of TRAF2 abolished p38 phosphorylation, and knockdown of GRB2 resulted in loss of ERK phosphorylation. Double knockdown gave essentially the same results as observed with a single knockdown. Similar results were also obtained for S100A9 stimulation and S100A8/A9 stimulation (see Supplementary Figures S6 and S7b). When keratinocytes were treated with small interfering RNA targeted to EMMPRIN (see Supplementary Figure S6), induction of mitogen-activated protein kinase phosphorylation by S100A8/A9 was considerably suppressed (Figure 2d). To rule out any contribution of S100A9-NPTNB axis to the downstream effects on S100A8/A9 in keratinocytes via the S100A8-NPTNB interaction, we used truncated forms of receptors, NPTNB-Δcyt, NPTNB-Δcyt, and NPTNB-Ig-3 Δcyt for the assessment. By this approach, we found that both NPTNB-Δcyt and NPTNB-Δcyt significantly inhibited the S100A8/A9-mediated ERK and p38 activation, whereas NPTNB-Δcyt had almost no effect on ERK activation but impaired p38 phosphorylation, probably because of deprivation of S100A9 binding from EMMPRIN (Figure 2e). Together with the results of slight expression of NPTNB in keratinocytes (Figure 1c), we emphasize that the S100A8-NPTNB axis plays a significant role in keratinocyte behavior upon S100A8/A9 stimulation. We also tested the involvement of endogenous proteins with co-immunoprecipitation studies. We used normal human keratinocytes and HeLa cells for comparison of signals in the
Figure 1. S100A8 binds with NPTNβ, which is endogenously expressed in cultured keratinocytes. (a) Binding of NPTNα, NPTNβ, and EMMPRIN with S100 proteins was examined. After transfection of each S100 family construct into HEK293 cells, conditioned medium was immunoprecipitated with anti-His antibody, and secretion of the expressed S100 protein was verified by Western blot test. The collection of each conditioned medium was then added to another transfected HEK293 cells with NPTNα, NPTNβ, or EMMPRIN. Bindings of S100 proteins to the expressed receptors were analyzed by immunoprecipitation of...
S100A8/A9 receptor interaction is critical for keratinocyte proliferation and cytokine induction

Even in the steady-state condition, double knockdown of these receptors had some effects on cell growth (see Supplementary Figure S8 online). On the other hand, suppression of NPTN or EMMPRIN alone showed little effect on cell proliferation induced by either S100 proteins or by S100A8/A9. When S100A8 was added to the culture medium and incubated for 24 hours, single receptor knockdown had little effect, but suppression of both receptors caused maximal growth inhibition. Similar results were obtained with S100A9. In the case of S100A8/A9 stimulation, double knockdown of these receptors caused more than 50% growth suppression. Analyses of receptor oligomerization showed that NPTNβ bound with itself, as well as with NPTNα (Figure 3a). Similar results were obtained with EMMPRIN. NPTN knockdown significantly suppressed the S100A8-mediated induction of CXCL-1, TNF-α, and IL-8, compared with the control (Figure 3b). Induction by S100A9 or S100A8/ A9 was also suppressed. Knockdown of EMMPRIN had essentially the same effects on cytokine induction. Collectively, these results indicate that NPTNβ-EMMPRIN heterodimer serves as a receptor for S100A8/A9 and induces inflammatory cytokines and keratinocyte proliferation.

Because TRAF2 is also a signal transducer of the NF-κB pathway, we examined whether S100A8/A9 is capable of activating this pathway. After stimulation with S100A8/A9, NF-κB p50 subunit was translocated and accumulated in the nucleus as with TNF-α stimulation. Addition of parthenolide, which inhibits IkB phosphorylation, suppressed nuclear translocation of p50 by S100A8/A9 (see Supplementary Figure S9a and b online). Furthermore, NF-κB luciferase reporter assays showed that S100A9 is responsible for NF-κB signaling (see Supplementary Figure S9c). The effect of S100A8/A9 was less than that of S100A9, and S100A8 alone had little effect. Thus, S100A9-EMMPRIN interaction leads to activation of the NF-κB pathway.

Localisation of S100A8, S100A9, NPTN, and EMMPRIN

We next examined the localisation of S100 proteins and their receptors. All of these proteins were barely detectable in normal human skin and nonlesional skin with AD (see Supplementary Figure S10 online). In contrast, heavy staining of S100A8 and S100A9 was always observed in the upper epidermal layers of AD skin (Figure 4a). NPTN and EMMPRIN showed similar localisation, although NPTN was also detected at the basal layer, especially beneath S100A8-positive areas of lesional skin. These results may imply that both molecules were induced at the onset of inflammation (see Supplementary Figure S10b). A monoclonal antibody, 27F10, which recognizes only the S100A8/A9 heterodimer, showed strong staining in the upper epidermal layers (Figure 4b). We also investigated localization of TLR4 and RAGE (see Supplementary Figure S11 online). Although cultured keratinocytes did not express TLR4, we recognized localization of TLR4 in the granular layer of normal epidermis (see Supplementary Figure S11a). However, TLR4 was markedly down-regulated both in the nonlesional and lesional skin with AD (see Supplementary Figure S11b). Expression of RAGE was considerably low in the normal and atopic skin (see Supplementary Figure S11). This was in contrast to the cultured cells, where human keratinocytes expressed a relatively high level of RAGE mRNA (see Supplementary Figure S3b). To investigate ligand-receptor relationships in vivo, we used proximity ligation assay (Figure 4c). Significant reactions between NPTN and S100A8 or S100A8/A9 were detected at epidermis covering the upper granular and the basal layers. Interaction between NPTN and S100A9 was strongly positive in the upper epidermis; EMMPRIN showed bright, almost linear reaction patterns, with S100 proteins at the granular layer (Figure 4d). S100A8/ A9-EMMPRIN interaction showed a broader localization, including the basal layer. We also observed strong positive reactions for NPTN and EMMPRIN from the basal through the upper epidermis of atopic skin (Figure 4e). Significant reaction between NPTN and EMMPRIN in the lower epidermis was evident, especially in areas of acanthotic epidermis, where keratinocyte proliferation and parakeratotic changes took place. Combination of unrelated IgGs did not give any positive signals, showing specificity of these reactions (Figure 4f). The NPTNβ/EMMPRIN interaction was also studied by a co-immunoprecipitation experiment using tissue extract from a fresh atopic skin specimen (see Supplementary Figure S11c).

We found that EMMPRIN co-immunoprecipitated with NPTNβ. Next, we investigated localization of endogenous...
Figure 2. Upon stimulation with S100A8/A9, NPTNβ and EMMPRIN recruit specific adaptor molecules and phosphorylate mitogen-activated protein kinases in cultured human keratinocytes. (a) Identification of adaptor molecules for signal transduction. Cytoplasmic domain of EMMPRIN (Emmprin-cyt) and various adaptor proteins as C-terminal 3xHA-6His-tagged forms were expressed in HEK293 cells and pulled down with anti-His tag antibody after S100A8/A9 stimulation for 30 minutes. (b) Effect of binding motif mutation on binding with adaptor molecules. Cytoplasmic domains of NPTN (NPTN-cyt) were mutated.
either at the SH3 domain or the TRAF binding motif and expressed in cultured keratinocytes. After stimulation with S100A8/A9, expressions of TRAF2 and GRB2 were verified with anti-TRAF antibody and anti-GRB2 antibody, respectively. (e) Inhibition of NPTN-mediated signal transduction in NHK cells by overexpression of NPTN cytoplasmic domains (NPTN-cyt) with or without mutations as decoys. S100A8/A9-induced activations of effector kinases, p38 and ERK, were both suppressed by overexpression of the NPTN-cyt (wt). The NPTN-cyt (mut SH3) specifically inhibited the activation of p38, and the NPTN-cyt (mut TRAF) functioned in the mitigation of ERK activation. (d) Involvement of NPTNβ and EMMPRIN in S100A8/A9 signal transduction. Under conditions of receptor knockdown, the effect of S100A8/A9 on ERK and p38 phosphorylation was examined in cultured keratinocytes. Tubulin was used as a loading control. Each level of phosphorylated ERK and phosphorylated p38 was expressed as p-ERK/ERK and p-p38/p38 ratio, respectively, after normalization with tubulin. (f) Binding profiles of endogenous receptors (RAGE, TLR4, EMMPRIN, and NPTNβ), ligand (S100A8/A9), and downstream adaptor proteins. NHK and HeLa cells (TLR4-positive cells, as shown in Supplementary Figure S3b online) were treated with 10 nmol/L of S100A8/A9 for 30 minutes. The treated cells were lysed and subjected to immunoprecipitation using the indicated biotinylated antibodies. In both NHK and HeLa cells, RAGE showed S100A8/A9 binding, which recruited MyD88 and TRAF6 adaptor proteins. RAGE exhibited no binding with TLR4, EMMPRIN, and NPTNβ or -α. Similar bindings were also shown for TLR4 in HeLa cells. S100A8/A9 was bound with EMMPRIN in both NHK and HeLa cells. Interestingly, EMMPRIN/NPTNβ binding only appeared in NHK cells, at which the complex enabled recruitment of TRAF2 as well as GRB2. In HeLa cells, EMMPRIN did not immunoprecipitate NPTNβ and recruited TRAF2 only. ab, antibody; EMMPRIN, extracellular matrix metalloproteinase inducer; IP, immunoprecipitation; NPTN, neuroplastin; RAGE, receptor for advanced glycation end-products; si, small interfering; TNF, tumor necrosis factor; WB, western blot.
Figure 4. Immunohistochemical localization and PLA analysis of related molecules in lesional AD skin suggest heterodimer formation of both ligands and receptors. (a) Localization of S100A8, S100A9, NPTN, and EMMPRIN in lesional AD skin. Merged figures with nuclear staining are also shown. Scale bar = 100 μm. (b) Localization of S100A8/A9 (calprotectin). To clarify the presence of S100A8/A9, we used 27E10 antibody, which specifically recognizes this dimer.
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NPTNβ and EMMPRIN in cultured keratinocytes with confocal microscopy (Figure 4g). NPTNβ and EMMPRIN showed membrane-associated localization, and these molecules were mostly co-localized, especially on the cell-cell contact area. A merged immunofluorescence image with DAPI staining is also shown in Figure 4h. Co-expression of these receptors resulted in extensive co-localization or association on the cell surface, consistent with heterodimer formation of these receptors.

S100A8 transgenic mice showed abnormal proliferation of keratinocytes

Because S100A8 is able to bind with NPTNβ and recruit GRB2, we examined whether S100A8 is involved in excessive cell proliferation at the onset of inflammation by using involucrin promoter-driven S100A8 transgenic mice (Hibino et al., 2013) (see Supplementary Figure S12a online). We applied SDS as a simple inflammatory stimulant and trinitrochlorobenzene (TNCB) as an inflammatory and sensitizing stimulant. In the transgenic mice, the expression of human S100A8 for SDS and TNCB tended to be induced less than 2-fold and 2.5-fold, respectively (see Supplementary Figure S12b). Only the human transgenic S100A8 showed an increased tendency after stimulation. Intrinsic mouse S100a8 showed rather suppressive tendency in the case of TNCB stimulation. We confirmed that human S100A8 and S100A9 bound with mouse Nptnβ and Emmprin, respectively (see Supplementary Figure S12c). Measurement of ear swelling indicated that SDS treatment tended to have only a very mild effect in wild-type HR-1 mice, whereas a stronger response was observed in S100A8 transgenic mice, peaking at 96 hours after application. There was a strong response to TNCB treatment. Ear swelling gradually increased up to 72 hours and then showed a declined tendency in the wild-type mice. In contrast, ear swelling remained high in S100A8 transgenic mice, even at the 144-hour time point (Figure 5a). Histological sections of the treated dorsal skin exhibited mild spongiosis and dermal edema in TNCB-treated skin of both wild and transgenic mice at the 24-hour time point (Figure 5b), although non-treated S100A8 transgenic mice did not show any noticeable changes compared with wild-type mice (see Supplementary Figure S13a online). Acanthotic epidermis was evident in the skin of S100A8-positive transgenic mice (Figure 5b), and see Supplementary Figure S13b). Numbers of proliferating cells identified with Ki-67 staining were markedly increased at 96 hours, and many basal cells had positive results in the dorsal skin of S100A8 transgenic mice (see Supplementary Figure S14a online). Some spinous cells also had positive results in these mice (see Supplementary Figure S14b). Quantitative analysis clearly showed that Ki67-positive cells were highly up-regulated after stimulation with either SDS or TNCB in the skin of transgenic mice (Figure 5c).

DISCUSSION

We identified NPTNβ, but not NPTNα, as an unreported receptor for S100A8. Physiological roles of NPTNs have been explored in neuronal cells (Owczarek and Berezin, 2012) and include cell adhesion in the plexiform layers during hortogenesis (Kreutz et al., 2001), promoting long-term changes in synaptic activity (Smalla et al., 2000), and promoting synaptic plasticity (Owczarek et al., 2010). To our knowledge, this is the first report that NPTNβ is dominantly expressed in human keratinocytes and transduces signals for S100A8.

Because S100A8 forms a stable heterodimer with S100A9 (Manitz et al., 2003), we considered the possible involvement of the S100A9 receptor EMMPRIN in the present signaling pathway. Immunoprecipitation studies indicated that NPTNβ and EMMPRIN could form heterodimers in cultured keratinocytes and AD skin. Proximity ligation assay also supported the possibility of fashioning the receptors into heterodimers in the granular and basal layers of epidermis in AD skin. Thus, a characteristic feature of this ligand-receptor relationship may appear to be heterodimer formation of both the ligands and the receptors.

We also examined the signaling pathways upon S100A8/A9 stimulation and found that NPTNβ recruits GRB2, whereas EMMPRIN recruits TRAF2. Knockdown of either receptor or adaptor molecules markedly suppressed both ERK and p38 phosphorylation. Double knockdown of the two receptors had a profound effect on mitogen-activated protein kinase activation and keratinocyte proliferation. These results further support the idea that NPTNβ/EMMPRIN heterodimer functions as a receptor for S100A8/A9.

TLR4 and RAGE have been established as general receptors for many S100 proteins. Although we found that TLR4 is expressed in the granular layer of normal skin, it is markedly down-regulated in the atopic skin. These results show sharp contrast to the expression profiles of NPTN, in which it was hardly detectable in the normal skin and highly up-regulated in the lesional skin with AD. As for RAGE signaling (Foell et al., 2007; Heizmann et al., 2007; Ibrahim et al., 2013), RAGE did not bind with S100A8 and did not form a heterodimer with NPTNα and β or EMMPRIN. These findings are consistent with a recent report showing that loss of TLR4 signaling or RAGE deficiency did not appreciably affect S100A9-mediated lung pathology or inflammatory cell infiltration in the alveolar space (Chen et al., 2015). Taken together, we consider that the NPTNβ/EMMPRIN heterodimer, rather than TLR4 and RAGE, has a critical role in S100A8/A9-dependent physiological reactions in human keratinocytes toward the atopic state.

TRAF2 is required for S100A9-induced signaling via EMMPRIN and is also a well-known adaptor for the TNF-α signaling pathway to induce an activation of NF-kB (Bradley...
Figure 5. S100A8 transgenic mice driven by the involucrin promoter are hyperreactive to inflammatory stimuli. (a) Ear thickness of wild-type and transgenic mice after SDS or TNCB treatment. Ear thickness was measured at appropriate times (n = 5, mean ± standard deviation of three measurements). *P > 0.05, **P > 0.01. (b) Hematoxylin and eosin staining of dorsal skin of wild-type and transgenic mice after irritant stimulation. Skin samples were taken from the treated area at 24, 96, and 144 hours. Scale bar = 50 μm. (c) Quantitative analysis of Ki67-positive cells in dorsal skins in Supplementary Figure S14a online. Numbers of Ki67-positive cells were counted in 500 μm-long fields x 8. Mean values of Ki67-positive cells in the 500-μm length were plotted. Error bars indicate standard deviation. **P < 0.01, ***P < 0.001. h, hours; TG, transgenic; TNCB, trinitrochlorobenzene; wild, wild type.
and Pober, 2001; Wajant et al., 1999). Our results show that S100A8/A9 activates NF-κB. This effect is solely S100A9 dependent, because NF-κB luciferase assay clearly showed that S100A9 but not S100A8 has the ability to induce NF-κB pathway activation. Because of the strong proinflammatory activities of the TNF-α–NF-κB system, the S100A9-EMMPRIN system appears to have similar functions to the system. However, we found that S100A8-NPTN binding recruits the adaptor protein GRB2. GRB2 is an essential factor for induction of cell proliferation by various growth factors including epidermal growth factor (Fridell et al., 1996), linking to the Ras–mitogen-activated protein kinase/ERK–ERK pathway. Thus, S100A8 and S100A9 possess distinct and cross-reactive functions. Formation of the receptor heterodimer would be highly effective to integrate complex signals and to promote strong cell reactions leading to proliferation and inflammation.

To examine further the action of S100A8, we used involucrin promoter-driven S100A8 transgenic mice, because intrinsic S100a8 is expressed mostly in the upper epidermis. The human transgene showed considerable up-regulation after SDS or TNCB treatment, but to our surprise, TNCB treatment rather suppressed the level of endogenous S100A8. These changes may be due to compensatory reactions. The skin of S100A8 transgenic mice consistently exhibited hyperreactive characteristics. S100a8-deficient mice show embryonic lethality, possibly because loss of S100a8 in developmental stages affects essential cell proliferation.

Our results indicate that NPTN/EMMPRIN heterodimer could function as a receptor for S100A8/A9, leading to activation of both keratinocyte proliferation and skin inflammatory pathways via recruitment of specific adaptor molecules, GRB2 and TRAF2. This idea is consistent with increasing evidence that chemokine receptors in general form homo- or hetero-oligomeric complexes, resulting in complex networks and crosstalk with other orthogonal signaling complexes (Kraemer et al., 2013). Thus, our findings offer the latest insight into the roles of S100A8 and S100A9 in chronic inflammation of atopic skin. Targeting this ligand-receptor axis would be a promising strategy to ameliorate skin inflammatory diseases.

MATERIALS AND METHODS

Cell culture
Normal human keratinocytes were purchased from Kurabo (Osaka, Japan). Human embryonic kidney cell line (HEK293) and cervical cancer cell line (HeLa) were purchased from ATCC (Manassas, VA). Details of cell culture are provided in the Supplementary Materials online.

Immunohistochemistry
Immunohistochemical studies using human tissue specimens were approved by the Ethical Committee of Tokyo Medical University. Methods were carried out in accordance with the approved guidelines, and only samples in the university were used. Preparation of tissue specimens and immunostaining were performed as detailed in the Supplementary Materials.

S100A8/A9 proteins
High-purity human S100A8 and S100A9 recombinant proteins were prepared as reported previously (Hibino et al., 2013; Nukui et al., 2008; Sakaguchi et al., 2014a). The amount of contaminating endotoxins (lipopolysaccharides and β-glucans) was confirmed to be less than 0.01 endotoxin units/μg, as determined with a Limulus amebocyte lysate assay (Seikagaku Corporation, Tokyo, Japan).

S100A8-NPTN binding assay
Quantitative binding analysis between S100A8 and NPTNβ was performed as detailed in the Supplementary Materials.

Keratinocyte proliferation
Keratinocyte proliferation was assessed as described in the Supplementary Materials.

Vector constructs
cDNAs were inserted into the pDT-SMART (C-TSC) vector, also named pCMViR-TSC (Sakaguchi et al., 2014b). The inserts are listed in the Supplementary Materials.

Co-immunoprecipitation
Co-immunoprecipitation and Western blot experiments were performed as described in the Supplementary Materials.

Proximity ligation assay
The proximity ligation assay method was used to detect in situ interaction of target proteins. Details are provided in the Supplementary Materials.

Inflammatory response in S100A8 transgenic mice
Responses to simple inflammatory stimulation and inflammation-sensitization stimulation were analyzed using involucrin promoter-driven S100A8 transgenic mice with a hairless phenotype (HR-1, n = 5/group) (Hibino et al., 2013). Details are provided in the Supplementary Materials.

Statistical analysis
Data are expressed as mean ± standard deviation. We used simple pair-wise comparison with Student t test (two-tailed distribution with equal variance in the two samples). P < 0.05 was considered significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.06.617.

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