Original Articles

Toll-like receptor signalling induces the expression of serum amyloid A in epidermal keratinocytes and dermal fibroblasts

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Conflict of Interest

The authors have no conflict of interest to declare.

Running head

TLR signals induce SAA expression in the skin

Abbreviations

DAMP, damage-associated molecular pattern; EB, epidermolysis bullosa; NHDF, normal human dermal fibroblast; NHEK, normal human epidermal keratinocyte; PAMP, pathogen-associated molecular pattern; RDEB, recessive dystrophic epidermolysis bullosa; SA, secondary amyloidosis; SAA, serum amyloid A; TLRs, Toll-like receptors

What's already known about this topic?

・Epidermal keratinocytes and dermal fibroblasts express functional TLRs.

・SAA are produced mainly by hepatocytes but also a variety of cells including immune cells, endothelial cells, synoviocytes, and epidermal keratinocytes.

・SAA induces the expression of SAA itself via TLR1/2 in epidermal keratinocytes.

What does this study add?

・TLR ligands induce SAA expression in epidermal keratinocytes and dermal fibroblasts.

・SAA induces the expression of SAA itself via TLR1/2 in dermal fibroblasts.

Abstract

Background: Toll-like receptors (TLRs) play critical roles in innate immune response by sensing pathogen- or damage-associated molecular patterns. Epidermal keratinocytes and dermal fibroblasts also produce proinflammatory cytokines and chemokines under the stimulation with TLR ligands. Serum amyloid A (SAA) is an essential factor in the pathogenesis of secondary amyloidosis, and also has immunomodulatory functions. SAA are produced mainly by hepatocytes but also a variety of cells including immune cells, endothelial cells, synoviocytes, and epidermal keratinocytes. However, SAA expression in human dermal fibroblasts has not been shown so far.

Aim: To investigate the effect of TLR ligands on SAA expression in epidermal keratinocytes and dermal fibroblasts.

Methods: We investigated whether TLR ligands induce the expression of SAA in normal human epidermal keratinocytes (NHEKs) and dermal fibroblasts (NHDFs) by using qPCR and ELISA. The effect of SAA on SAA itself expression in NHDFs was also studied.

Results: SAA expression was induced via NF-κB by TLR1/2, 3, 5, and 2/6 ligands in NHEKs. In NHDFs, TLR1/2 and 2/6 ligands increased the expression. SAA further induced the expression of SAA itself via TLR1/2 and NF-κB in NHDFs, as previously reported for NHEKs.

Conclusions: Our results provide new evidence that the skin's innate immune response contributes to the production of SAA which might lead to the increase in the risk of systemic complication such as secondary amyloidosis of recessive dystrophic epidermolysis bullosa patients.

INTRODUCTION

The skin is the first line of defense against the external environment of the body.[1] Therefore, in the skin, the nonspecific quick inflammatory reaction, or the innate immune system, is necessary, and it is comprised of keratinocytes, neutrophils, macrophages, and more.[1] Skin inflammation by injury starts when Toll-like receptors (TLRs) in keratinocytes at the injured sites recognize molecules derived from microbes that are called pathogen-associated molecular patterns (PAMPs), or molecules derived from injured cells that are called damage-associated molecular patterns (DAMPs).[2-5] PAMPs and DAMPs are also recognized by nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectin receptors, receptor for advanced glycation end products (RAGE), and so on.[6] In addition to keratinocytes, another type of resident structural cells, fibroblasts, also express TLRs, and can also produce proinflammatory cytokines and chemokines, the production of which triggers and enhances skin inflammation.[2]

Serum amyloid A (SAA) is a 12-kDa acute phage protein encoded by *SAA1-4* located on chromosome 11 in human.[7] *SAA1* and *SAA2* encode acute phage proteins, *SAA3* is a pseudogene, and *SAA4* is constitutively expressed.[7] SAA is induced by

proinflammatory cytokines such as TNF-α and IL-6, synthesized mainly by the liver, and predominantly bound to high density lipoprotein in the blood.[7] Secondary amyloidosis (SA) is a critical disease characterized by the extracellular tissue deposition of the insoluble amyloid fibrils derived from SAA, and it complicates any chronic inflammatory diseases including rheumatoid arthritis.[8] The persistent high concentration of SAA in the sera is reported a prerequisite for the development of SA.[9] SAA are produced by not only hepatocytes but also a variety of cells including immune cells, endothelial cells, and synoviocytes.[7] In addition, SAA is also considered to act as DAMP molecules.[6] In rheumatoid arthritis, SAA expression is increased in synoviocytes of the joints, and it is considered to act as the exacerbation factor of the local inflammation because it has proinflammatory functions.[7,10] Epidermal keratinocytes also express SAA,[11-13] however, the expression in human dermal fibroblasts has not been reported so far.

Epidermolysis bullosa (EB) is an inherited blistering disorder of the skin and mucosal membranes.[14] EB is caused by mutations in keratin or collagen genes, and has various subtypes. [14] Recessive dystrophic epidermolysis bullosa (RDEB) is an uncommon and severe subtype EB characterized by inherently fragile skin and recurrent blister formation occurring over the whole body within weeks after birth, and caused by mutations

in the type VII collagen gene *COL7A1*.[14] Patients with RDEB show persistent, widespread skin ulcers and develop complications such as oesophageal stricture, infection, pseudosyndactyly, renal failure, cutaneous squamous cell carcinoma, and SA.[15,16] The expression of SAA has already been reported to be elevated in the sera of patients with RDEB.[17] In addition, we reported that such elevation is persistent in these patients.[18] However, the mechanism of the increase in SAA expression in patients with RDEB has not been shown so far.

Here we show that TLR ligands induce SAA expression via NF-κB in epidermal keratinocytes and dermal fibroblasts, and that SAA further induces the expression of SAA itself in dermal fibroblasts via TLR1/2 and NF-κB.

METHODS

Cell culture and stimuli

Normal human epidermal keratinocytes (NHEKs) were obtained from Invitrogen/Cascade Biologics (Portland, OR, U.S.A.) and grown in serum-free EpiLife Medium (Invitrogen/Cascade Biologics) containing 0.06 mM Ca^{2+} and 1× EpiLife Defined Growth Supplement (Invitrogen/Cascade Biologics) at 37 °C under standard tissue culture conditions. Normal human dermal fibroblasts (NHDFs) were obtained from Invitrogen/Cascade Biologics and grown in Medium 106S (Cascade Biologics/Invitrogen) containing Low Serum Growth Supplements (Cascade Biologics/Invitrogen). Cultures were maintained for up to eight passages in these media with the addition of 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B.

Cells were stimulated with Pam3CSK4 (10 μg/ml; InvivoGen, San Diego, CA, U.S.A.), polyinosinic:polycytidylic acid (poly(I:C), 10 μg/ml; InvivoGen), lipopolysaccharide (LPS, 10 μg/ml; InvivoGen), flagellin (100 ng/ml; InvivoGen), macrophage-activating lipopeptide (MALP)-2 (100 ng/ml; Alexis Biochemicals, San Diego, CA, U.S.A.), ODN M362 (2 μM; InvivoGen), or recombinant human SAA (10^{-8}-10^{-6} M; PeproTech, Rocky Hill, NJ, U.S.A.) for 24 h. After cell stimulation, the total cell media was

stored at -20°C until analysis. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) after supernatant collection. RNA was stored at -80°C.

Small interfering RNA (siRNA) transfection

NHEKs and NHDFs were transfected with siRNA control or specific siRNA for NF-κB p65 (Cell Signaling Technology, Danvers, MA, U.S.A.), TLR1, or TLR2 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) using Lipofectamine RNAiMax (Invitrogen). After 24 h of transfection, the cells were stimulated with TLR ligands or SAA for another 24 h.

Neutralization of TLR1/2

For the neutralization of TLR1/2, NHDFs were incubated with TLR1- or TLR2-neutralizing antibody (5 μg/ml; InvivoGen) or control IgG for 30 min. Cells were then stimulated with SAA for 24 h.

Quantitative real time-polymerase chain reaction (qPCR)

Complementary DNA (cDNA) was synthesized from RNA by the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) as described by the manufacturer's

protocol. TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, U.S.A.) were used to analyse expression of *SAA1/2* (assay ID: Hs00761940_s1), *RELA* (Hs00153294_m1), *TLR1* (Hs00413978_m1), and *TLR2* (Hs00610101_m1) as described in the instructions from the manufacturer (User Bulletin #2; Applied Biosystems). *GAPDH* mRNA was assessed as an internal control to validate RNA, and was detected with the probe VIC-CATCCATGACAACTTTGGTA-MGB and the primers 5'-CTTAGCACCCCTGGCCAAG-3' and 5'-TGGTCATG AGTCCTTCCACG-3'. Each mRNA expression was calculated relative to expression of *GAPDH* mRNA, and all data are presented as fold changes against each control (mean from non-stimulated cells).

Enzyme-linked immunosorbent assay (ELISA)

SAA in culture media were measured by commercial sandwich ELISA kits following the manufacturer's instructions (SAA Human ELISA Kit KHA0012, Invitrogen). Culture media were concentrated 20 times by Amicon Ultra-4 (Millipore-Amicon, Bedford, MA, U.S.A.) to detect SAA. Absorbance at 450 nm was determined using SH-1000Lab microplate reader (Corona Electric, Hitachinaka, Japan).

Statistical Analysis

Student's t-test was used to determine significance between the two groups. One-way analysis of variance with Tukey's test was used to determine significance among more than two groups. The analyses were performed by Prism software (version 4; GraphPad Software, La Jolla, CA, U.S.A.). Results are expressed as mean ± standard error of the mean (SEM). Values of $P<0.05$ were considered significant.

RESULTS

TLR ligands induce the expression of SAA in epidermal keratinocytes and dermal fibroblasts

First of all, we investigated the effects of TLR ligands on the expression of SAA in NHEKs and NHDFs. Among a panel of TLR ligands, Pam3CSK4 (TLR1/2), poly(I:C) (TLR3), flagellin (TLR5), and MALP-2 (TLR2/6) significantly induced SAA expression at both the transcript and protein synthesis levels in NHEKs (Fig. 1a, b). In NHDFs, among a panel of TLR ligands, both Pam3CSK4 and MALP-2 significantly induced SAA expression (Fig. 1c, d).

TLR signalling has been reported to induce proinflammatory cytokines through NF-κB. [19] The knockdown of p65 (a subunit of NF-κB) by siRNA significantly suppressed the induction of SAA by Pam3CSK4, poly (I:C), flagellin, and MALP-2 in NHEKs (Fig. 2a, c). As with NHEKs, knock-down of p65 significantly suppressed the induction of SAA by Pam3CSK4 and MALP-2 in NHDFs (Fig. 2b, d).

SAA induces the expression of SAA itself through NF-κB in dermal fibroblasts

SAA, a precursor of amyloid protein, potently activates immunocytes and induces

proinflammatory cytokines and chemokines.[7] Previously SAA is shown to induce IL-6 expression in dermal fibroblasts.[20] We have reported that SAA induces the expression of SAA itself in epidermal keratinocytes.[11] We therefore investigated whether SAA could enhance inflammatory responses in an autocrine/paracrine manner in dermal fibroblasts. As expected, SAA induced the expression of SAA itself in NHDFs in a dose-dependent manner (Fig. 3a). Since SAA is also reported to activate NF-κB signalling,[7,20] we knocked down p65 and confirmed that the induction of SAA by SAA was significantly inhibited in NHDFs (Fig. 3b).

SAA acts through TLR1/2 to induce the expression of SAA itself in dermal fibroblasts

O'Reilly et al showed that SAA induces IL-6 expression via TLR2 but not TLR4 in dermal fibroblasts.[20] In epidermal keratinocytes, SAA is reported to induce the expression of SAA itself via TLR1/2 heterodimer.[11] Our present experiments revealed that TLR1 and TLR2 siRNA significantly inhibited the SAA induction by SAA in NHDFs (Fig. 4a, b, c). TLR1 and TLR2-neutralizing antibody also significantly inhibited the induction of SAA itself by SAA in NHDFs (Fig. 4d). These results suggest that SAA acts through TLR1/2 heterodimer to induce the expression of SAA in dermal fibroblasts, as for epidermal keratinocytes.

DISCUSSION

Previous studies have shown that TLR3 and 4 ligands induce SAA expression in airway epithelial cells and hepatocytes, respectively.[21,22] In the present study, we demonstrated that SAA production was induced under stimulation with several TLR ligands in epidermal keratinocytes and dermal fibroblasts. Among a panel of TLR ligands, TLR1/2, 3, 5 and 2/6 significantly induced SAA expression in NHEKs. TLR7 and TLR8 ligands were not evaluated in NHEKs , because these receptors were not constitutively detectable in cultured keratinocytes.[23] In NHDFs, among a panel of TLR ligands, TLR1/2 and 2/6 ligands induced SAA expression at both the transcript and protein synthesis levels. TLR5, TLR7, and TLR8 ligands were not evaluated in NHDFs, because these receptors were not constitutively detectable in cultured fibroblasts. SAA is one of the acute phage proteins and is considered to be synthesized mainly in the liver.[7,12] We also demonstrated that the induction of SAA by TLR ligands in epidermal keratinocytes and dermal fibroblasts was NF-κB-dependent, which is consistent with our previous report of SAA induction in keratinocytes.[11]

The role of SAA in normal skin is not clearly shown so far, but it is thought to contribute to innate immunity in the skin because it has the capacities to activate several types of cells including neutrophils, monocytes, and mast cells, and induce inflammatory

cytokines and chemokines.[7] We further demonstrated that SAA induces the expression of SAA itself in NHDFs as previously reported for NHEKs,[11] suggesting that SAA can enhance inflammatory reactions in an autocrine/paracrine manner. Our results also showed that the induction of both SAA by SAA depends on NF-κB in NHDFs. TLR2, TLR4, CD36, formyl peptide receptor-like 1 (FPRL1), and receptor of advanced glycation end-products (RAGE) have been reported as receptors for SAA.[7] Nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 may also be involved in SAA signalling.[7] The findings of the present study indicate that TLR1/2 is, at least in part, involved in the induction of SAA by SAA in NHDFs.

Yamanaka et al. demonstrated that keratinocyte-specific caspase-1- or mature IL-18-transgenic mice have elevated serum levels of SAA and dense amyloid deposition in the liver, kidney, and spleen,[24] suggesting that persistent skin inflammation could trigger SA. Since IL-18 signalling is able to activate NF-κB[25], epidermal keratinocytes and dermal fibroblasts in the mice might continuously produce SAA via NF-κB.

Patients with RDEB have persistently higher serum levels of SAA.[18] They also have widespread persistent skin ulcers, and keratinocytes and fibroblasts surrounding the ulcer lesions are continuously exposed to PAMPs and DAMPs. Therefore, the elevation of

SAA in the sera of RDEB patients might be due to TLR-mediated SAA production by keratinocytes and fibroblasts of persistent, widespread skin ulcers.

In summary, we have shown here that TLR ligands induce the expression of SAA via NF-κB in epidermal keratinocytes and dermal fibroblasts, and that SAA further induces the expression of SAA itself via TLR1/2 and NF-κB in dermal fibroblasts. Our results provide new evidence that the skin's innate immune response contributes to the upregulation of SAA expression, and the overproduction might lead to the increase in the risk of systemic complication such as SA of RDEB patients.

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FIGURE LEGENDS

Figure 1. TLR ligands induce the expression of SAA in epidermal keratinocytes and dermal fibroblasts.

NHEKs (passage 4) or NHDFs (passage 7) at 80% confluency were stimulated with TLR ligands for 24 h. Relative *SAA1/2* mRNA abundance was analysed by qPCR, and protein expression of SAA in the media was measured by ELISA. (**a-d**) *p<0.05, **p<0.01, ***p<0.001. Data are given as mean ± SEM of triplicate samples and are representative of three independent experiments.

Figure 2. Induction of SAA expression by TLR ligands in epidermal keratinocytes and dermal fibroblasts is NF-κB-dependent.

The expression of p65 was knocked down by the specific siRNA in NHEKs (passage 7) at 80% confluency and NHDFs (passage 7) at 60% confluency. Cells were then stimulated with TLR ligands for 24 h. The relative p65 (*RELA*) (**a, b**) and *SAA1/2* (**c, d**) mRNA abundance was analysed by qPCR. *p<0.05, **p<0.01, ***p<0.001. Data are given as mean ± SEM of triplicate samples and are representative of three independent experiments.

Figure 3. SAA induces the expression of SAA itself through NF-κB in dermal fibroblasts.

NHDFs (passage 8) at 80% confluency were stimulated with SAA (10^{-8} – 10^{-6} M) for 24 h. Relative *SAA1/2* mRNA abundance was analysed by qPCR. (**a)** The expression of p65 was knocked down by the specific siRNA in NHDFs (passage 7) at 70% confluency. Cells were then stimulated with SAA (10^{-6} M) for 24 h. Relative *SAA1/2* mRNA abundance was analysed by qPCR (**b**). **p<0.01. Data are given as mean ± SEM of triplicate samples and are representative of three independent experiments.

Figure 4. Induction of SAA expression by SAA in dermal fibroblasts is TLR1/2-dependent.

The expression of TLR1 or TLR2 was knocked down by the specific siRNA in NHDFs (passage 5) at 80% confluency (**a, b)**. Cells were then stimulated with SAA (10^{-6} M) for 24 h. Relative *SAA1/2* mRNA abundance was analysed by qPCR (**c**). NHDFs (passage 6) at 80% confluency were incubated with TLR1- or TLR2-neutralizing antibody for 30 min. Cells were then stimulated with SAA (10^{-6} M) for 24 h. Relative *SAA1/2* mRNA abundance was analysed by qPCR (**d**). *p<0.05, **p<0.01, ***p<0.001. Data are given as mean ±

SEM of triplicate samples and are representative of three independent experiments.

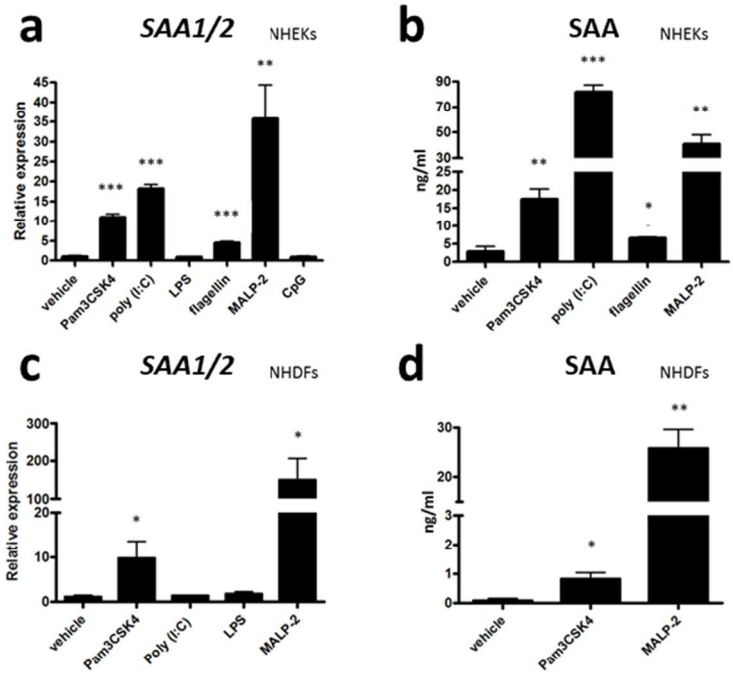

Figure 1

Fig 1

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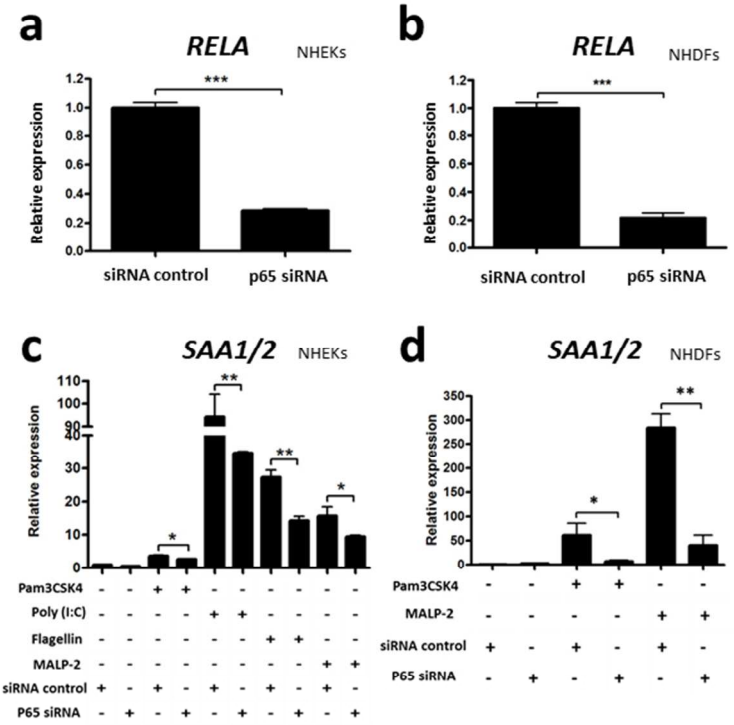

Figure 2

Fig 2

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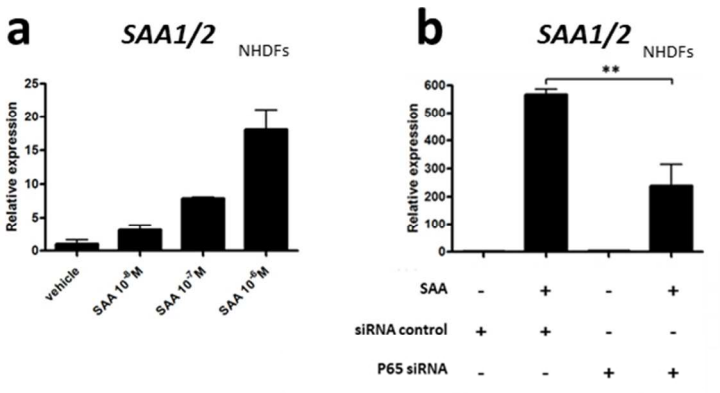

Figure 3

Fig 3

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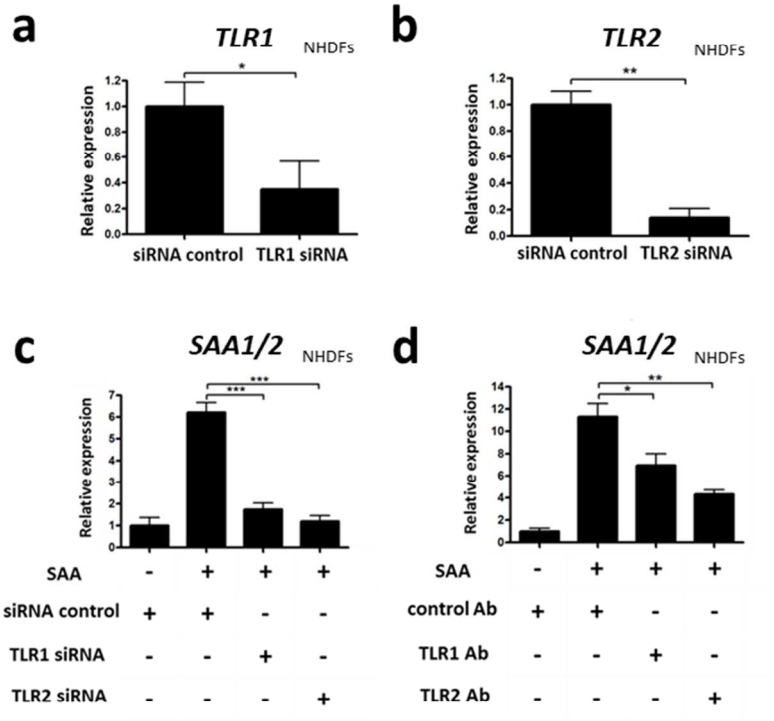

Figure 4

Fig 4

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