Understanding the role of inflammasome pathway in AMP secretion from human skin keratinocytes

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20121086

A thesis to be submitted to the Department of Biology in partial fulfilment of requirements of integrated BS-MS dual degree

Indian Institute of Science Education and Research (IISER), Pune

IISER PUNE

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Hindustan Unilever Limited, Bangalore

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Certificate

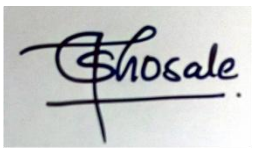
This is to certify that this dissertation entitled "***Understanding the role of inflammasome pathway in AMP secretion from human skin keratinocytes***" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by ***Aishwarya Dipak Bhosale*** *at* ***Hindustan Unilever Limited, Bangalore*** under the supervision of ***Dr. Amitabha Majumdar, Senior Scientist at Personal Care Category Unilever R&D Bangalore*** during the academic year 2016-17.

Signature:

Name of supervisor: Dr. Amitabha Majumdar

Date: 28/04/2017

Signature:



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Declaration

I hereby declare that the matter embodied in the report entitled "***Understanding the role of inflammasome pathway in AMP secretion from human skin keratinocytes***" are the results of the investigations carried out by me at the Department of ***Personal Care Category Unilever R&D Bangalore*** under the supervision of ***Dr. Amitabha Majumdar*** and the same has not been submitted elsewhere for any other degree.

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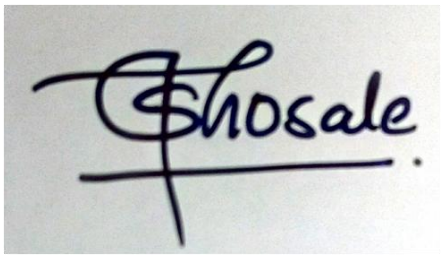

Name of student: Aishwarya Dipak Bhosale

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Abstract

Skin acts as the body's first line of defense protecting us from a host of invading pathogens. It is able to combat invasions by several commensal and pathogenic bacteria by the secretion of specific oligopeptides called antimicrobial peptides. S100a7 particularly, is secreted by healthy human skin keratinocytes in response to invasion by *Escherichia. Coli.* While its secretion is well reported, the mechanistic details regulating the secretion of S100a7 remain largely unknown. In our study, we find certain common players between PAMPs (Pathogen Associated Molecular Pattern) sensing and a wound healing response. One of the principal components in the cultured supernatant of *E.coli* called Flagellin is known to activate TLR5 signalling which causes secretion of IL-1α as an early acute response. IL1α, a potent pro-inflammatory chemokine, is also seen to be activated by the NLRP3 inflammasome via downregulation of Caspase-8 (CASP8). NLRP3 inflammasome further activates P38-MAPk and NFκβ by activating Caspase-1 (CASP1) which causes a prolonged secretion of S100a7, giving way to a chronic response. We also find, CASP8, which was previously reported to be down regulated in a wound scenario to show similar kinetics during *E.coli* invasion further indicating a possible convergence of both pathways. Our findings establish a link between the host response pathways during pathogen invasion and wound healing at CASP8, ultimately leading to S100a7 secretion.

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Acknowledgement

I would like to express my gratitude to my supervisors Dr. Amitabha Majumdar, Dr. Colin Jamora and Dr. Gayathri Pananghat for their venerated help, guidance, useful remarks and providing me with a wonderful research atmosphere to carry out my master's thesis.

This thesis was a collaborative work between Hindustan Unilever Limited, Bangalore and Jamora lab, Institute for Stem Cell Biology and Regenerative Medicine (inStem). I would like to thank Tanay Bhatt, Bhavya Bajantri of Jamora lab and M.S. Mruthyunjaya at HUL for their relentless support, valuable inputs and extensive help to carry out experiments.

I would also like to thank Ravi, who would never let me go hungry, Rania who organized my schedule for thesis writing, Neha who would motivate me to finish my work by giving dreams of exciting tours after submission, Isha who is a fitness and a shopping expert, Edriss who is a partner in guessing movies, Krithika who would make sure that I keep a check on my time, Husain who is my Swensen's buddy and all time comedian, Achyuth who would keep pestering me, Akhil who would always keep pulling my legs, Dyuti as my partner in crime, Rupali and Sunny for their valuable help and Akshay for his wonderful smile. Lastly but the foremost thing which Abhik has taught me, "Desperation brings creativity".

It has been a great privilege to work with you all.

Introduction

The skin serves as a protective barrier against the external environment. It also serves as the barrier by protecting internal organs from absorption of harmful chemical substances, excessive water loss and invasion of pathogens (Mann, .et. al., 2012). It acts as the first line of defense against a host of invading pathogens and viruses (Salmon, .et .al., 1994). The human skin is mainly structured into three layers: epidermis, dermis and hypodermis, out of which the outermost layer, the epidermis is the one which comes in direct contact with the pathogens. The epidermis is further composed of highly specialized epithelial cells known as keratinocytes. Keratinocytes are continuously replenished at the basal level (stratum basale) and undergo controlled differentiation for its movement to the upper suprabasal layers, stratum spinosum and stratum granulosum (Fig.1) (Blaydon, .et. al., 2014)

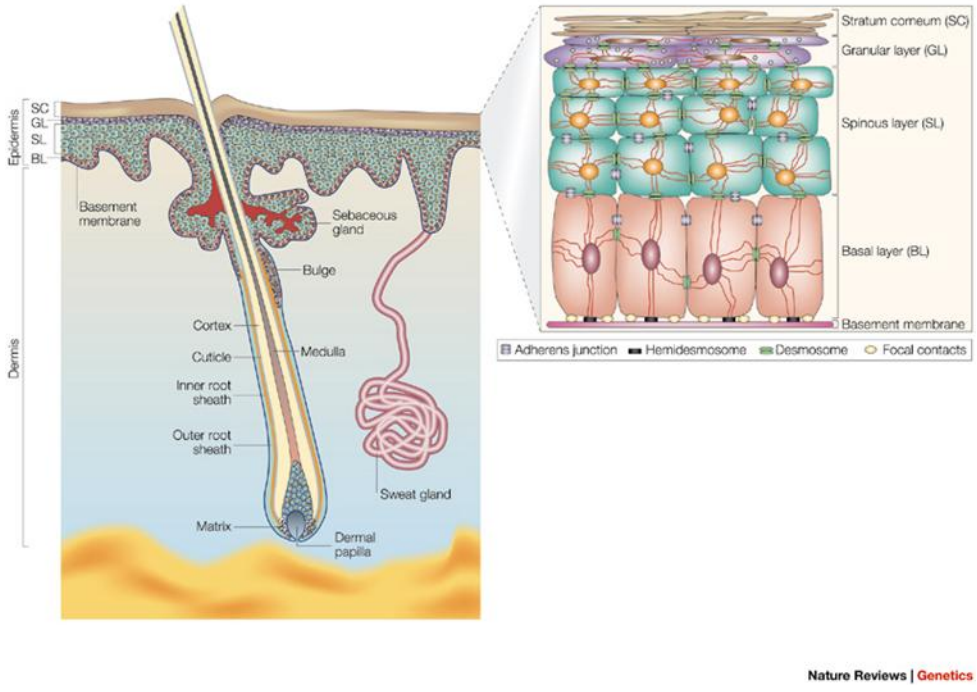


Figure1: Overview of the different layers of skin and epidermis.

[Taken from Fuchs and Raghavan, 2002]

Although the skin constantly remains in contact with various pathogenic and non-

pathogenic microorganisms, it rarely gets infected due to its robust innate immune response. Immune responses emerging from the skin are produced and executed by the cells and molecules of either innate or adaptive system. Rapid innate response indicates quality of response whereas adaptive response shows quantity specific response with developing memory for prolonged time. (Bangert, .et .al ., 2011). Innate response is instantly activated by the host after encountering with pathogens. The skin is equipped with pattern recognition receptors (PRRs), which help in recognizing pathogenic threats via Pathogen Associated Molecular Patterns (PAMPs). PAMPs are the present on the bacteria which are only produced by the bacterial invaders. Keratinocytes expressing certain pattern recognition receptors (PRRs) like Toll-like-receptors (TLRs), detects the pathogen via PAMPs and initiate canonical signaling of NFκB. NFκB activation is mediated via Myd88(myeloid differentiation primary response gene 88) and tumor necrosis factor receptor (TNF-R) associated factor (TRAF) (Mogensen, .et. al., 2009). An encounter with PAMPs often leads to the induction of the expression of particular oligopeptides called antimicrobial peptides (Elinav, .et. al., 2011).

Antimicrobial peptides (AMPs) are a diverse group of cationic and amphipathic polypeptides. They are synthesized mainly in the Stratum granulosum and stratum spinosum. AMPs are stored in the secretory granules and then these peptides are later transported to stratum corneum for their functional activity. They are secreted as a part of the host's immune response, and can preferentially interact with invading microbes thereby directly inhibiting their growth. AMPs exploit their electrostatic interactions to interact with negatively charged bacterial membranes and cross the envelope and membranes of the pathogen by forming pores thereby killing the pathogen (Diamond, .et .al ., 2009).

The Ca^{2+} binding S100a7 was originally discovered in a hyperproliferative inflammatory skin disease, psoriasis thereby getting its common name *Psoriasin*. Since then it has been shown to be upregulated in the epidermis of psoriatic lesions (Anderson, .et .al., 2009). S100a7 has also been associated with inflammatory responses, acting as a potent chemoattractant for the activation of $CD4^+$ T-lymphocytes and neutrophils (Jingquan, .et .al., 1996). Furthermore studies have indicated its role in preferentially killing of *E.coli* by sequestering Zn^{2+} which are expressed by healthy skin keratinocytes (Gläser, .et .al ., 2005).

Although a lot of studies show the effects of these AMPs, including S100a7, in their role as the primary defense response of the body, the mechanism of their regulation has not been studied in detail. In general, it is believed that the expression of AMPs is regulated through signals from various cytokines. Interestingly, cytokines are also secreted in response to PAMP signaling via TLRs, involving NFκB and P38-MAPk pathways. (Kawai, .et .al., 2014). [Fig. 2] This suggests that cytokines can form the common link between pathogen recognition and immune response. Furthermore, evidence suggest that in response to PAMPs human keratinocytes secrete IL1α in a pattern correlating with S100a7 expression (Kupper, .et .al., 2000). A specific study has also shown that IL1α can directly regulate the expression of S100a7 (Bando, .et. al., 2007).

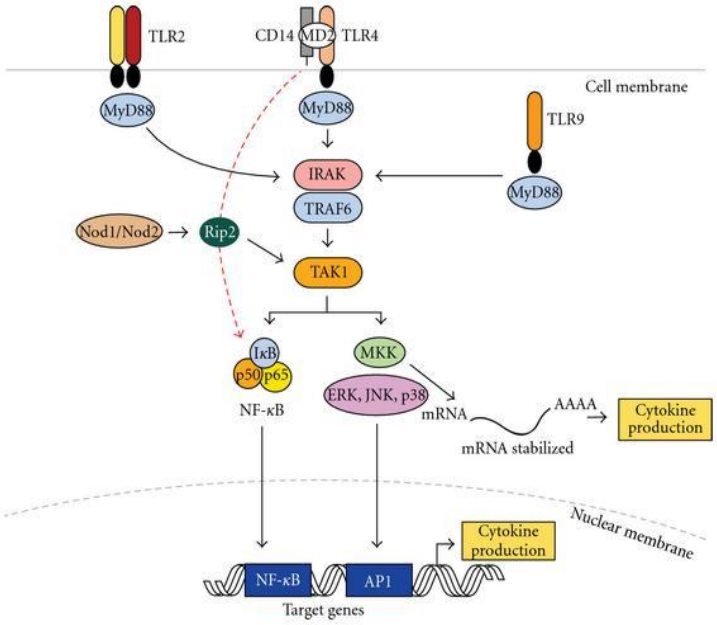


Fig.2. Activation of TLR signalling by Myd88 and TRAF dependent pathways.

[Taken from Li, .et .al., 2011]

IL1α is a well-studied protein in context of keratinocyte, and is generally secreted in response to pathogen or injury related signals (Bourke, .et .al., 2014). This signaling generally activates the inflammasome complex. These multiprotein complexes are generally comprising of nucleotide oligomerization domain like receptor proteins (NLRPs), an adaptor protein ASC (apoptosis mediated speck like protein) and pro-caspase1.This serves as the subsequent proteolytic activation of pro-inflammatory

cytokines (eg. IL1α) (Lee, .et .al., 2015). There are different subsets of NLRPs such as NLRP1, NLRP3 and NLRP4 which are shown to be activated with specific parasite triggers (Schroder, .et .al., 2010). In humans, the NLRP3 inflammasome pathway is known to be associated with PAMPs (Haitao, .et .al., 2015).NLRP3 inflammasome assembly induces activation of pro-caspase 1, which further secretes IL-1α. (Lee, .et .al., 2015). Particularly flagellin was seen to activate NLRC4 inflammasome complex (Zhao, .et .al., 2011). This led us to suspect that the inflammasome complex might play a significant role, along with IL1α secretion, in the regulation of S100a7 secretion.

A physiological condition where the inflammasome complex is often encountered is during wound healing. Interestingly, during wound healing, keratinocytes activate NLRP3 inflammasome leading to IL1α secretion. This inflammasome activation requires NFκB and p38 MAPk activation, which is mediated via CASP8 downregulation. In fact, CASP8 downregulation has been shown to be necessary and sufficient for the initiation of wound-healing response. Studies with keratinocytes in a wound healing environment show that CASP8 downregulation activates the NLRP3 inflammasome in a non-canonical manner (Lee, .et .al ., 2009).

Given the common features of both, wound healing and PAMPs sensing, we hypothesized that these pathways may converge at CASP8 downregulation, resulting in inflammasome activation. With these clues we are poised to gain a better mechanistic understanding of the pathogen induced S100a7 expression. Thus, we aim to identify the upstream regulators of the inflammasome activation and AMP secretion, which are as yet unanswered questions in the field. This exploration can also help in understanding the dysregulation of S100a7 in chronic disease conditions like Psoriasis.

Material and Methods

1. Preparation of *E.coli* culture.

For the *E.coli* culture preparation, *E.coli* (strain-10536) were grown in the Tryptic Soy Buffer (TSB) in shaking condition at 37^{o}C for overnight until it reaches optical density 1.0. Then 1ml of culture was diluted with 9mL of TSB media (1:10) in the 75cm^{2} flask (BD, Falcon) and incubated further for next 24hr at 37^{o}C in stable condition (without shaking). Bacteria was further heat killed at 65^{o}C in the water bath for 60 mins. Then culture was centrifuged at 5000g for 15 min by using centrifuge (eppendorf). After the spin, resultant supernatant was collected without touching the pellet. Collected supernatant was filtered by using 0.22µM (Millex-GP syringe filter) and diluted in 1:100 in Epilife medium and then used for the treating human skin keratinocytes (Gläser.et.al.,2005)

2. Cell culture and treatment details

Neonatal human epidermal keratinocytes (NHEK- Invitrogen cell lot no 1760272) were cultured in T75cm^{2} flask (BD,falcon) by using EpiLife medium(sigma). For RNA isolation and western blot cells were splitted into 12 well and 3.5mm plate at the 70-80 % confluency.

Table.1 Different chemical inhibitors treatment.

Sr.no	Inhibitor name	Concentration	Company name
1	anti-hTLR5-IgG inhibitor	5 µg/mL	InvivoGen
2	Caspase 1 inhibitor	2µM	Merck Millipore
3	p38-MAPk inhibitor	10µM	Merck Millipore
4.	IKK inhibitor VII	20µM	Merck Millipore

3. Enzyme Linked Immunosorbent Assay (ELISA) for S100a7

To test the secretion of S100a7 after treating with culture of *E.coli* supernatant in human skin keratinocytes, sensitive ELISA for measuring human S100a7 was developed (CircuLex S100a7 ELISA kit). This was a sandwich enzyme linked immunoassay technique. S100a7 human specific antibody was pre-coated onto a 96- well microplate. Standards were prepared according to CircuLex S100a7 ELISA protocol sheet. Further 100 µL of samples and standards were pipetted into the wells. Human S100a7 was bounded by the immobilized antibody and make sure that unbound substances were removed after washing. After washing, an HRP conjugated antibody was added to the wells followed by the wash to remove any unbound HRP conjugate. Bounded HRP conjugate was allowed to react with the substrate H_2O_2. Reaction was ended with the addition of acidic solution of concentrated H_2SO_4 and measured the absorbance of yellow colored product at 450nm by using Tecan microplate reader (BioRad). To calculate the concentration of S100a7 secretion, a standard curve was obtained by the plotting absorbance values against S100a7 standards calibrators. The detection limit of ELISA was a concentration of 1ng/ µL of S100a7.

4. Quantitative Real Time Polymerase Chain Reaction (qPCR).

CFX384 Touch Real time PCR detection system (BioRad) used for the qPCR experiments following the instructions of manufacturer. Fluorescence labeled amplification was measured by using this technique. RNA isolated from human keratinocytes by using the RNAiso Plus (Takara) as an extraction reagent. Reverse transcription reaction of was carried out by using 1µg of RNA. cDNA equivalent to 100 ng of RNA was used as the template for PCR reaction of volume 10µL. (Total 10µL volume of reaction contains 5 µL of SYBR Green Master mix (Bio-Rad), cDNA template 2 µL followed by addition respective primers (0.06mM) further remaining reaction was diluted with RNAse free H_2O. All qPCR were performed in triplicates.

Table. 2 Primer sequences.

Gene of interest	Forward primer sequence	Reverse primer sequence
CASP 8 (199bp)	CGGACTCTCCAAGAGAACAGG	TCAAAGGTCGTGGTCAAAGCC
RANTES (96bp)	GGCGCTGCTTTGTCAAAAGG	GGCATGCTGACTTCCTTCCT
GAPDH (96bp)	GCCACATCGCTCAGACAC	CCAGAGTTAAAAGCAGCC

5. Western Blot analysis.

For protein expression analysis, keratinocytes were lysed in the RIPA buffer (ThermoScietific). Protein inhibitor cocktail (PIC- labmade) and phosStop (BioRad) were added together with RIPA in (1:100).Samples were lysed and cell debris was removed by the centrifugation at maximum speed 14,000 rpm for 10 min by using centrifuge (eppendorf). Fractionation of cells extracts was done by SDS-PAGE and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer's protocols. (Bio-Rad). After transfer samples were blocked in the 6% BSA(Bovine Serum Albumin) for 60min and primary antibodies diluted with 1X TBST in 1:500 ratio for the of CASP8, pp38-MAPk , pNFκB,GAPDH protein of interest and incubated overnight at 4^{o}C. Membranes were washed three times for 10 min. Secondary antibodies like incubated horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies diluted with 1XTBST in 1:1500 with and incubated for 20 min. Membrane was washed with 1XTBST three times and developed with the commercially available ECL (Luminata Forte substrate-Merck).

6. Enzyme substrate Assay

To check the enzyme activity of Caspase-1, we performed enzyme-substrate kinetic assay. Keratinocytes were lysed in the hypotonic buffer. Hypotonic buffer prepared according to the Lamond lab protocol 2007.Samples were lysed and cell debris was removed by the centrifugation at maximum speed 14,000g for 10 min by using centrifuge (eppendorf) by maintaining temperature at 4^{o}C. 20 µL samples and 80 µL

of hypotonic buffer were added into the 96-well plate and kept it for 37^{o}C for 1 hour. CASP 1 substrate(VYAD-AFC, Caspase-1 Substrate VI,Merck) were added and time kinetics of CASP1 activation was monitored by using Spectramax,M5 (Bio-Rad) for 2hour with each 3 min time interval .Time kinetics was measured at excitation spectra 405 nm and emission spectra 500nm.

Results

1. E.coli supernatant induces time dependent S100a7 secretion in differentiated human keratinocytes.

In order to determine the kinetics of S100a7 secretion in human skin, *in vitro*, we treated differentiated human epidermal keratinocytes with cultured supernatant of *E.coli* at different time points (0h, 12h, 24h, 36h, and 48h). The concentrations of secreted S100a7 were determined by the ELISA (Figure. 4a) *E.coli* supernatant treatments show a time dependent S100a7 secretion in differentiated human keratinocytes.

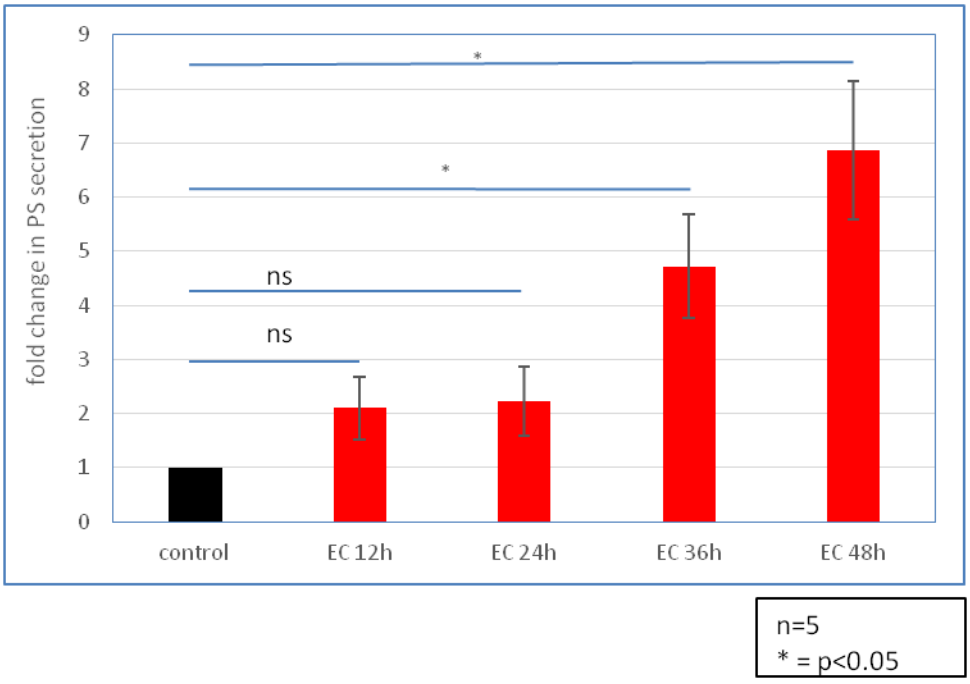


Fig.4.a) Differentiated keratinocytes secrete S100a7 in time dependent manner. Data represents mean fold change, compared to respective controls, of five independent experiments and p-value was calculated by using students paired t-test. * = p<0.05, ns= not significant

Further, to understand whether this surge of S100a7 secretion is accumulated over a period of time or is it a burst-release after a specific time point we measured S100a7 secretion at different time-durations as shown in Table-1.

	24hr	48hr
E.coli supernatant	+	-
E.coli supernatant	+	+

Table.3: *E.coli* treatment for early and late time points.

("**+**" indicated that *E.coli* supernatant treatment was done and "**–**" indicates that keratinocytes replenished with fresh media and *E.coli* supernatant post 24-48hrs)

We observe that S100a7 secretion increases post 24 hours of *E.coli* supernatant treatments and peaks at 48 hour time point. Culture media was collected after 24 hrs of *E.coli* supernatant treatment and replaced with fresh media. We observed that there is surge in S100a7 secretion is observed during 24th to 48th hour post *E.coli* supernatant.

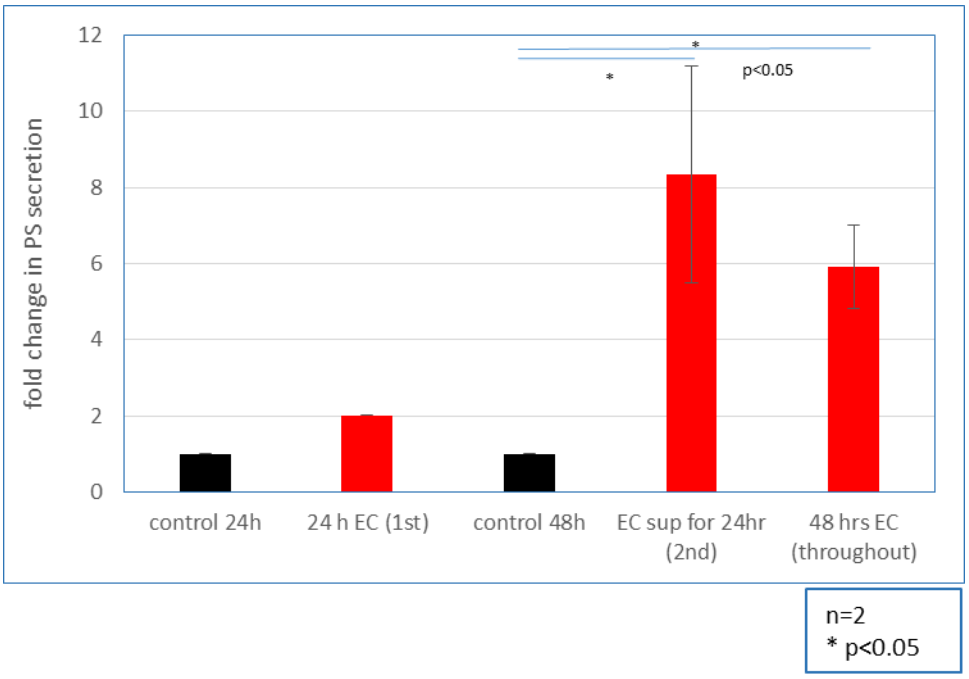


Fig.4.b) Surge in S100a7 secretion is observed during 24th to 48th hour post *E.coli* supernatant treatment. Data represents fold change, compared to respective controls, of two independent experiments and p-value was calculated by using students paired t-test. *=p<0.05

To understand whether flagellin, an essential component in the *E.coli* supernatant,

follows a similar trend as observed with the *E.coli* supernatant treatment, keratinocytes were treated with recombinant flagellin for 48h, and we saw that flagellin can induce S100a7 secretion.

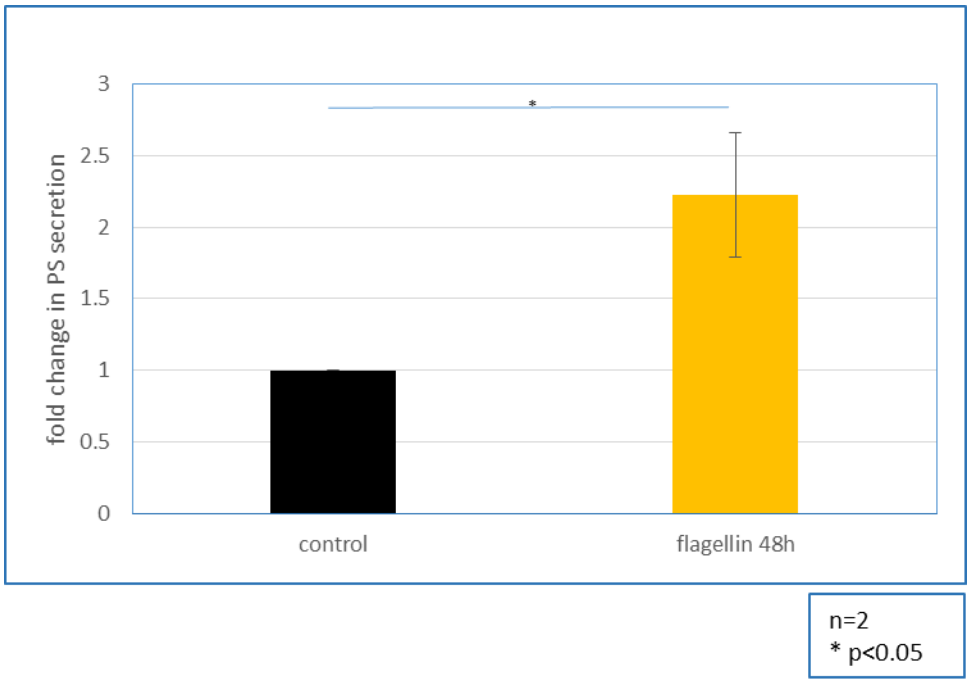
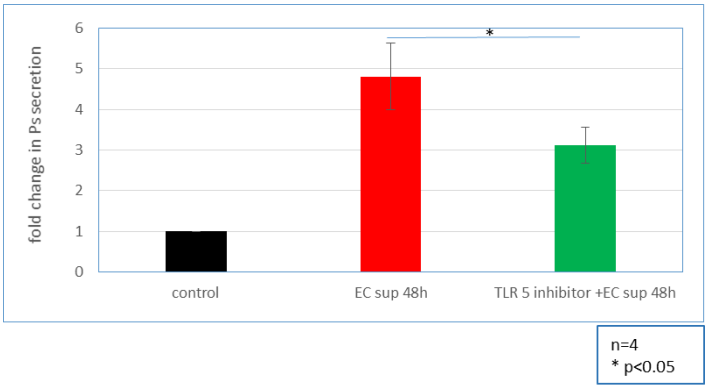


Fig.4 c) Flagellin is sufficient to induce S100a7 secretion in a differentiated human keratinocytes. Data represents fold change, compared to respective controls, of two independent experiments and p-value was calculated by using students paired t-test. *=p<0.05

2. TLR5 signaling is necessary to induce S100a7 secretion.

It is known that TLR5 is a receptor for flagellin (Schroder et al., 2008). We next wanted to determine if TLR5 signaling was essential in the sensing of flagellin. Differentiated keratinocytes were treated with *E. coli* supernatant, while the TLR5 receptor was neutralized with anti-TLR5 antibody. Cultured medium was collected after 48h and processed for ELISA to measure secreted S100a7. We saw that inhibition of TLR5 can significantly scale down S100a7 secretion in both treatments, however not as low as isotype controls.



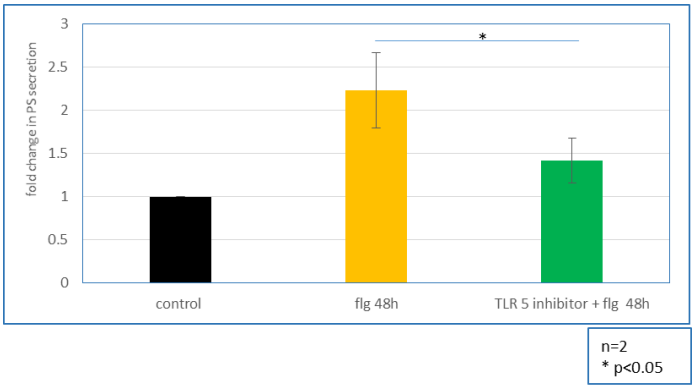


Fig. 5 The increase in S100a7 secretion induced by both *E. coli* supernatant and flagellin is reverted upon neutralization of the TLR5 receptor. Data represents fold change, compared to respective controls, of four independent experiments for *E. coli* supernatant treatment and two independent experiments for flagellin treatment. p-value was calculated by using students paired t-test. *=p<0.05

3. IL-1α is sufficient to induce the S100a7 secretion.

Activation of TLR5 is known to activate predominant IL-1α secretion. In order to determine if there is a mechanistic link between IL-1α mediated S100a7 secretion, we have treated in differentiated keratinocytes with supernatant of *E.coli* at different time points (0h,12h, 24h, 36h, 48h). The concentrations of secreted IL-1α were determined by the ELISA. *E.coli* supernatant treatments show a time dependent IL-1α secretion in differentiated human keratinocytes which follows the similar trend as S100a7 secretion.

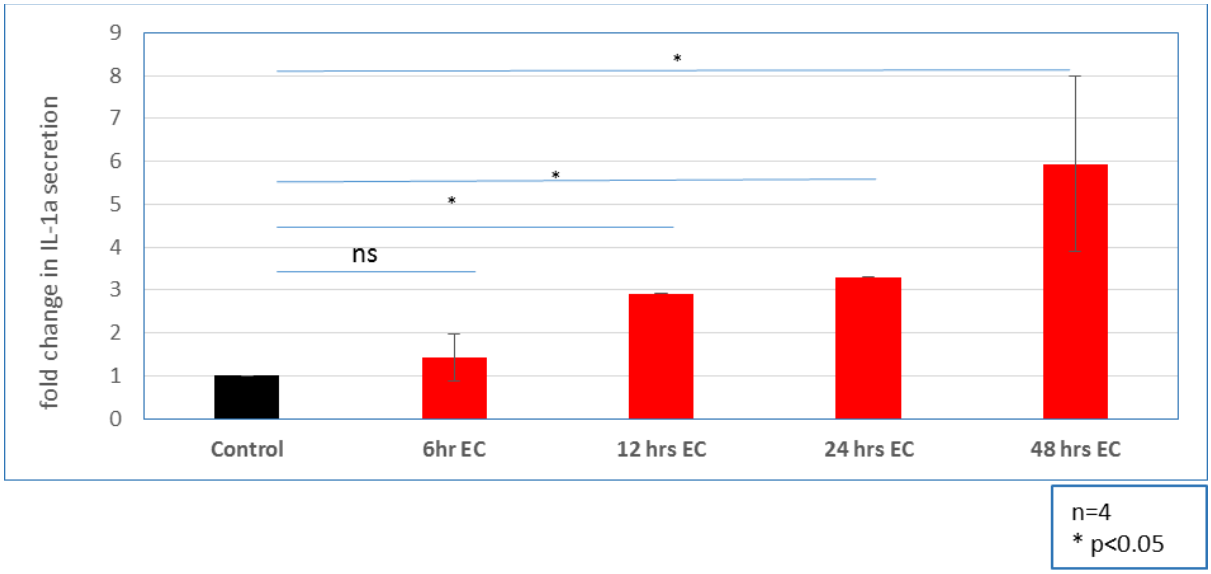


Fig.6.a) *E.coli* supernatant treatment causes IL-1α secretion with similar kinetics as S100a7 secretion. Data represents fold change, compared to respective controls, of two independent experiments and p- value was calculated by using students paired t-test. *=p<0.05

Next, to investigate whether the treatment of IL-1α is sufficient to induce S100a7 secretion, differentiated keratinocytes were treated with recombinant IL-1α protein. Cultured medium was collected at 24 and 48hrs of treatment and the secreted S100a7 was measured by ELISA.

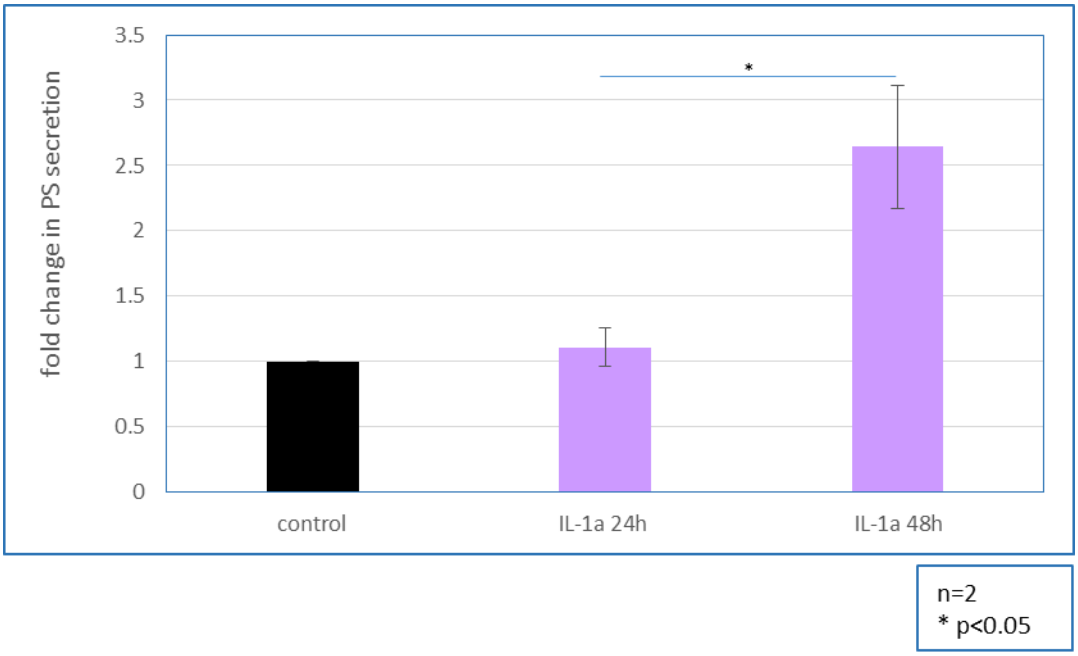


Fig.6.b) IL-1α is sufficient to induce S100a7 secretion. Data represents fold change, compared to respective controls, of two independent experiments and p- value was calculated by using students paired t-test. *=p<0.05

4. *E.coli* supernatant response utilizes wound-response pathway via CASP8 downregulation

Downregulation of CASP8 at mRNA levels has been seen in the wounded conditions where it leads to increased IL1α secretion (Lee et al., 2009). To understand if CASP8 follows a similar trend at mRNA level after *E. coli* treatment, differentiated keratinocytes were treated with *E. coli* supernatant for 24h and 48h. Cell lysates collected were processed for RNA isolation followed by qPCR analysis.

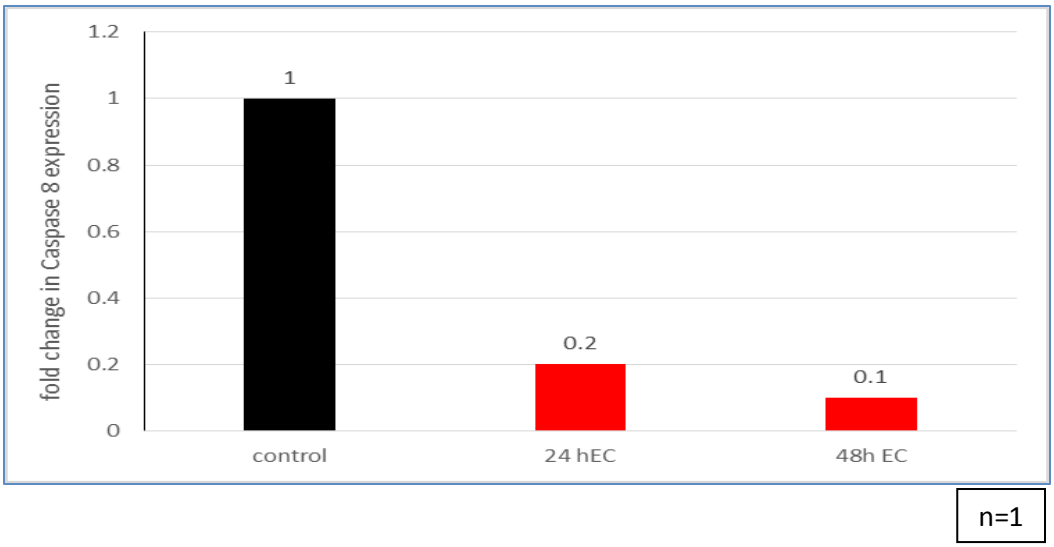


Fig.7. a) Downregulation of CASP8 mRNA upon *E. coli* supernatant treatment[#]

To understand the dynamics of CASP8 regulation with *E.coli* supernatant treatment at the protein level, keratinocytes were treated with *E.coli* supernatant for different time points (12h, 24h and 48h). Cell lysates were collected after 48hrs of treatment and processed for western blot analysis. Though a slight increase was observed in the early time points, CASP8 was seen to be downregulated at 48h. Fig.6a and 6b showing *E.coli* trigger leads to downregulation of CASP8.

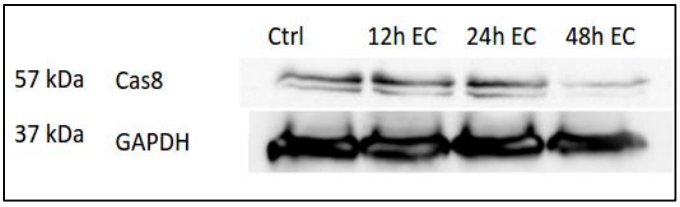


Fig. 7.b.) Western blot of CASP8

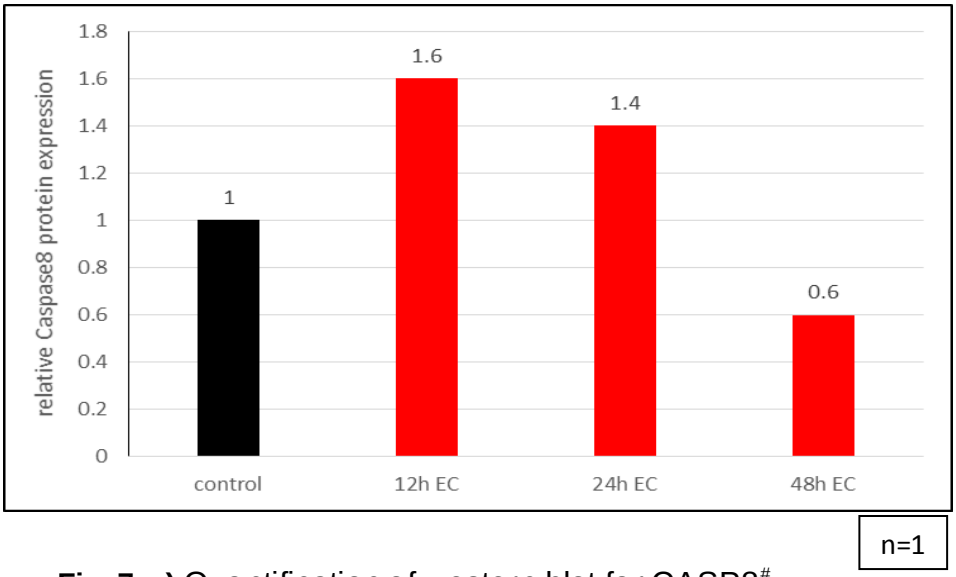


Fig. 7.c.) Quantification of western blot for CASP8[#]

Fig. 7.b).CASP 8 protein is downregulated upon *E.coli* supernatant treatment (b) western blot for CASP8 , (c) Quantification of western blot for CASP8

5. CASP1 activation can induces S100a7 secretion.

To understand if the down-regulation of CASP8 with *E.coli* supernatant treatment activates the downstream regulator of the inflammasome complex, i.e, CASP1, differentiated keratinocytes were triggered with *E.coli* supernatant and cell lysate collected at different time points (0h, 12h, 24h, 36h, 48h). The cell lysates were collected in a hypotonic buffer and the CASP1 activity was checked for with the enzyme substrate activity kit and the read outs were measured by Spectramax m5. As a positive control, we used keratinocytes exposed to ultraviolet light CASP1 showed maximum activity at 24h of *E.coli* treatment (Fig 8).

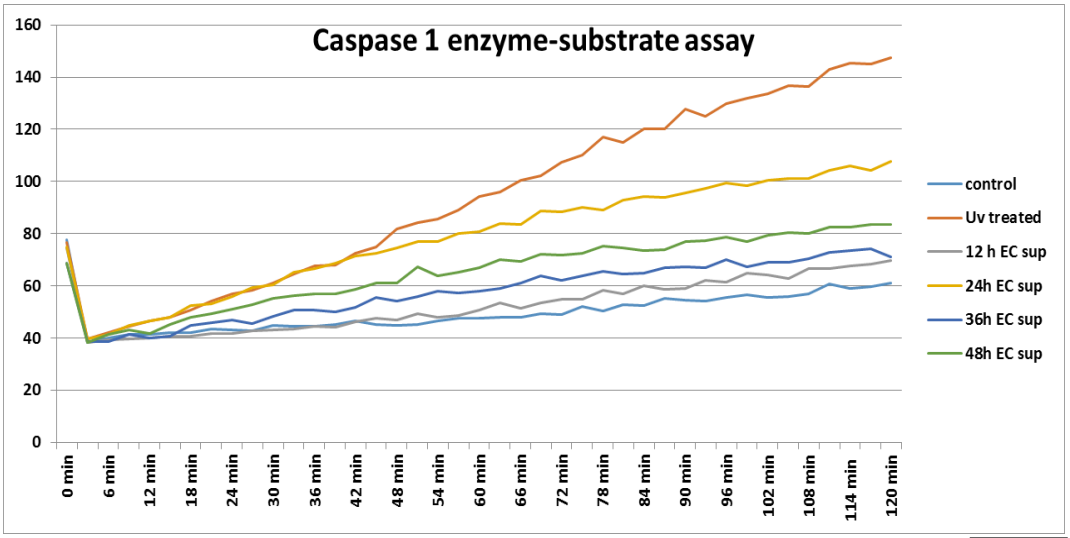


Fig.8.a) CASP1 activity increases with *E.coli* supernatant treatment.[#]

Further, to check whether the functional activity of CASP1 is necessary to induce S100a7 secretion we treated differentiated keratinocytes with chemical inhibitor of CASP1 (ICE-Inhibitor I,YVAD-CHO, membrane permeable) prior to *E.coli* supernatant treatment. Cultured medium was collected at 48h of treatment and measured for secreted S100a7 by ELISA. We saw that the inhibition of CASP1 does in fact decrease the S100a7 secretion.

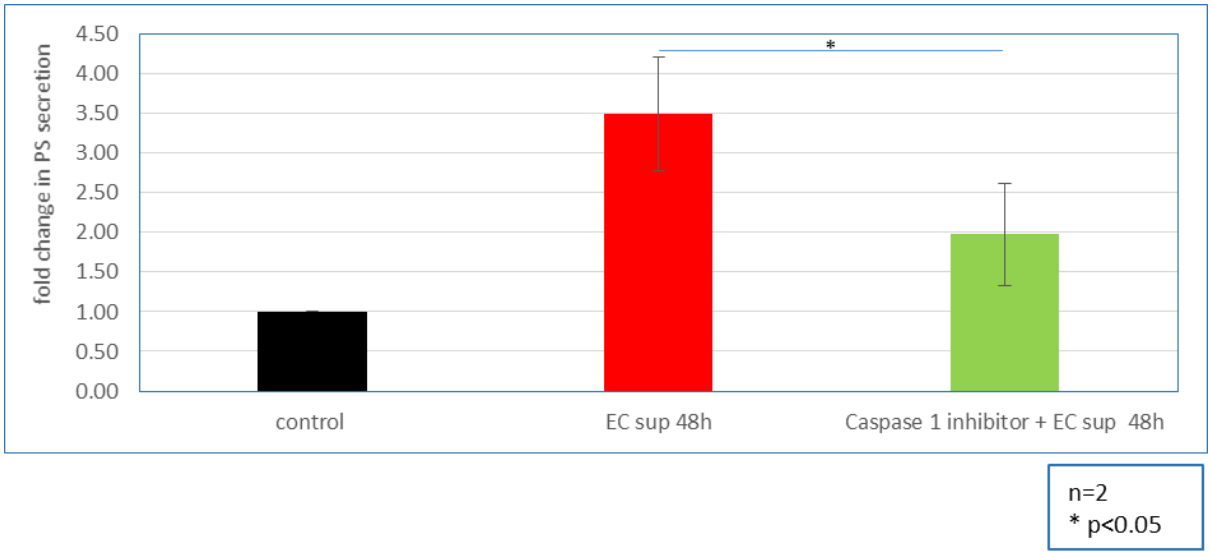


Fig. 8.b) Catalytic inhibition of CASP1 reduces the S100a7 secretion. Data represents fold change, compared to respective controls, of two independent experiments and p- value was calculated by using students paired t-test. *=p<0.05

6. p38-MAPk and NFκB activation is required for NLRP3 inflammasome mediated S100a7 secretion.

We then tried to understand the mechanistic link between the upstream regulators of inflammasome activators like p38-MAPk and NFκβ and S100a7 secretion via CASP1 activation. Cell lysates were collected according to different time points (0h, 12h, 24h, 48h). Samples were processed for western blot analysis to check the phosphorylation (and thus the activation) status of p38-MAPk and NFκB. In the case of p38-MAPK, we see an increase in the total phosphorylated p38-MAPk protein levels. The ratio of phosphorylated p38-MAPK to total p38-MAPK also shows a slight increase with time.(Fig.9(a),9(b)) However, in the case of NFκβ, the ratio of phosphorylated NFκβ to total NFκB shows a greater increase with time indicating more activation of NFκB upon E.coli supernatant treatment.(Fig.9 (c),9 (d))

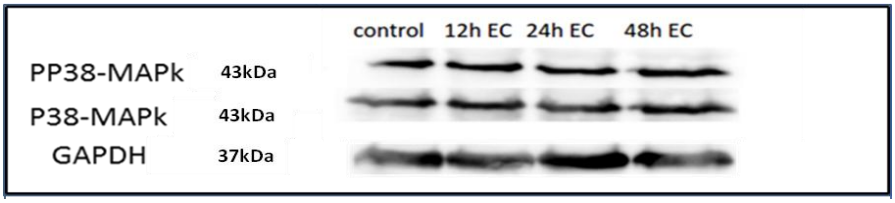


Fig. 9.a (a) Western blot for p38-MAPk

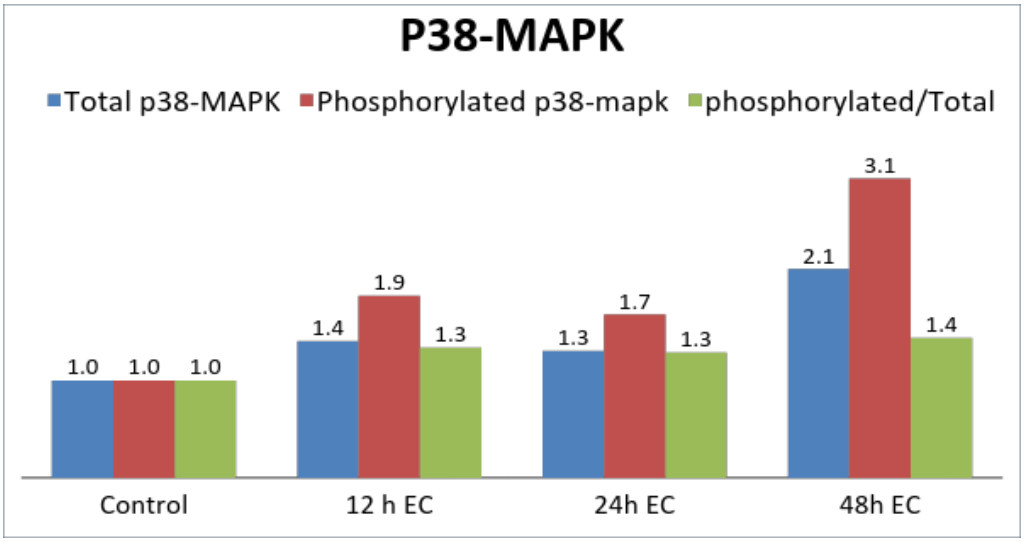


Fig. 9.a (b) Quantification of western blot for p38-MAPk#

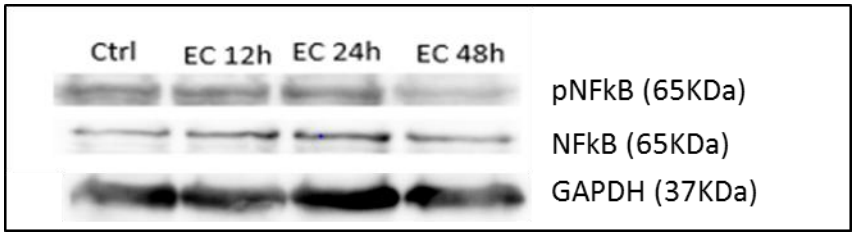


Fig. 9.a (c) Western blot for NFκB

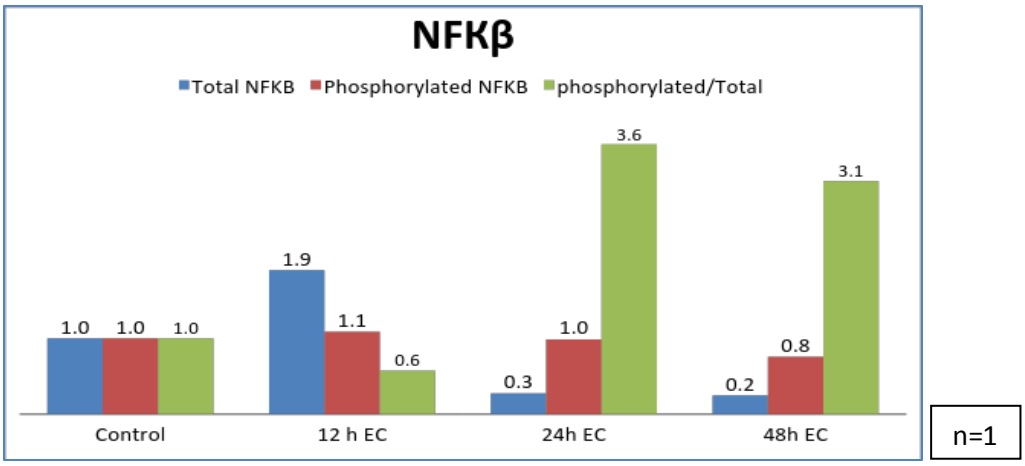


Fig. 9.a (d) Quantification of western blot#

RANTES is a common known target gene for promoter regions of NFκB and p38-MAPK. To find out the expression of RANTES, differentiated keratinocytes were

treated with *E.coli* supernatant and qPCR was performed. We see an increased expression of RANTES.

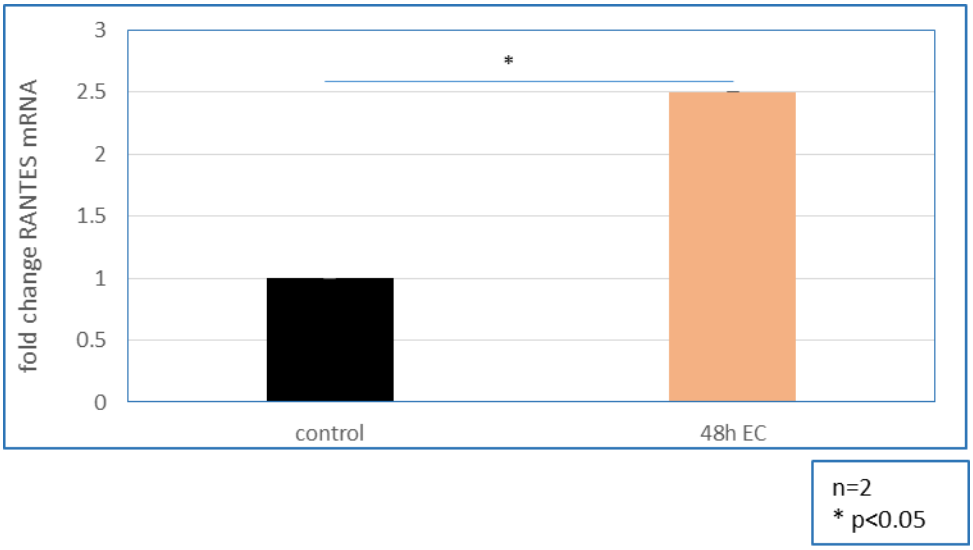
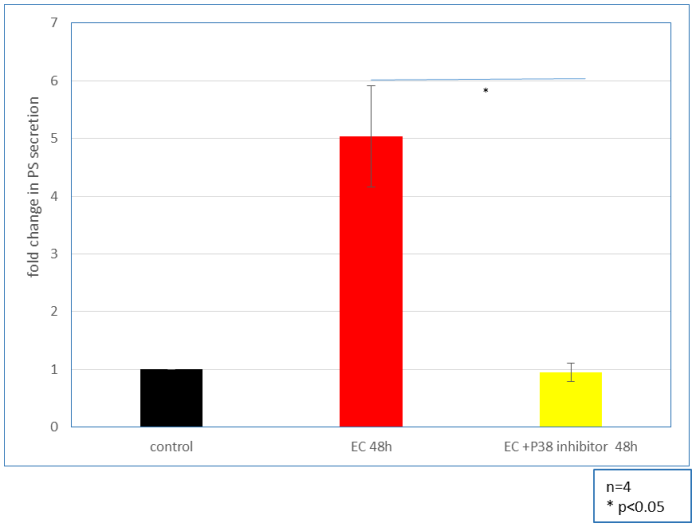


Fig.9.b) NFκB and p38-MAPk target genes are upregulated in EC sup treatment cells. Data represents Data represents fold change, compared to respective controls, of two independent experiments and p- value was calculated by using students paired t-test. *=p<0.05

Next, to check whether the functional activity of p38-MAPK and NFκB are necessary to induce S100a7 secretion we treated differentiated keratinocytes with chemical inhibitor of p38-MAPk and NFκB prior to *E.coli* supernatant treatment. Cultured medium was collected at 48h of treatment and measured for secreted S100a7 by ELISA. We saw that the inhibition of p38-MAPk and NFκB decreases S100a7 secretion.



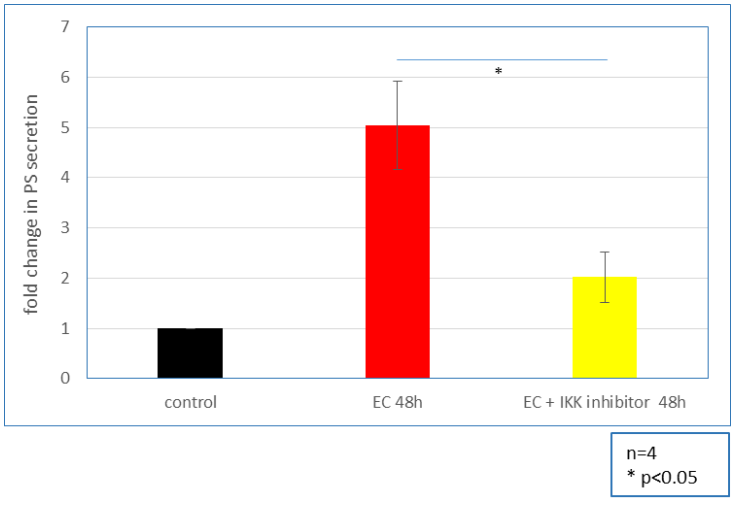


Fig.9.c) p38-MAPk and NFκB inhibitors reduces S100a7 secretion Data represents Data

represents fold change, compared to respective controls, of four independent experiments and p- value was calculated by using students paired t-test. *=p<0.05

Note: [#]*These data show results from only one biological sample. In future more experiments will be performed to fill the lacuna in the data set.*

Discussion

AMPs are a major component of the defense mechanism of the body against external pathogens. Amongst these, the role of S100a7 has been well recognized as an antimicrobial agent. There are several reports of its activity against the growth of *Escherichia coli* on human skin surface. It has been shown that S100a7 is usually known for targeting *E.coli* by Zn^{2+} sequestration (Gläser.et.al., 2005). Although a lot is known about its mechanism of action, the regulation of this molecule has not been studied in detail. Therefore, in our study, we aim to uncover the key molecular players of the host cells that come forward to tackle pathogen invasion and help restore homeostasis. In order to probe the mechanistic details of the regulation of S100a7 secretion, we first started to determine the time kinetics of its induction upon *E.coli* supernatant treatment.

Our results suggest that the secretion of S100a7 from differentiated human skin keratinocytes is time dependent. We have observed that an initial trigger of *E.coli* supernatant is sufficient to upregulate S100a7 secretion after 24 hours. This hints at an acute response to the *E.coli* sup which might involve activation of the innate immune response in the skin. However, the surge in S100a7 levels after 24 hrs makes us question if there exists another pathway that is bringing in the “chronic” effect into the picture. Given that the *E.coli* supernatant is a cocktail of various components such as lipopolysaccharides, flagellin and tryptic soy broth (Masjedian.et.al., 2011) we first wanted to narrow down on the actual trigger. Previous reports suggest that flagellin, a component of the *E.coli* supernatant is necessary and sufficient in triggering the secretion of S100a7 in human epidermal keratinocytes (Schroder.et.al., 2008). We corroborate this finding that flagellin recapitulates the time kinetics of s100a7 secretion observed upon *E.coli* supernatant treatment.

Flagellin can bind to Toll-like Receptor 5 (TLR5) on the host’s cell surface. This pathway initiates the production of specific proinflammatory cytokines by NFκB activation (Miao.et.al., 2007) that can generate an early (acute) innate response.

TLR5 inhibitor studies provide us enough evidence to confirm its activity in sensing and signaling in response to our *E.coli* supernatant treatment.

TLR5 signaling has been shown to activate Interleukin-1 family of cytokines such as IL-1α. So our next question was whether IL-1α could be the link between TLR5 and AMP secretion. It is well established that AMPs secretion is regulated by cytokines. In this context, S100a7 secretion is also known to be mediated by IL-1α (Bando.et.al., 2007). Even in our experimental system, we see exogenous IL-1α induces S100a7 secretion. Interestingly, we see IL-1α secretion follows similar time kinetics as S100a7 secretion which brings us to the question if S100a7 secretion could be dependent on IL-1α signaling, either by an autocrine or a paracrine fashion. Interestingly, the TLR5 pathway is known to be activated in the early time points (Schroder.et.al., 2008) but what drives this process for the chronic response is still unknown. This suggested that another pathway could be involved in the upregulation of IL1α as well as S100a7 at the later time point.

Previous research on IL-1α has reported that it is secreted in the wounded skin conditions via the downregulation of CASP-8 by activating the inflammasome complex (Lee.et.al.,2009). Our *E.coli* supernatant treatment studies with healthy human skin keratinocytes also showed a similar downregulation of the CASP8 protein. This finding introduced the possibility of CASP8 being an intermediate player in the regulation of S100a7 secretion. The downregulation of CASP8 could be necessary event to initiates the chronic response of cells to *E.coli* supernatant that stretches the secretion of S100a7 beyond 24 hours. CASP8 has been shown to regulate NLRP3 inflammasome activity via p38-MAPk and NFκB (Lee.et.al., 2015).The NLRP3 inflammasome complex comprises of ASC and procaspase-1.

E.coli supernatant treatment in differentiated human keratinocytes also shows that the upstream regulators of the inflammasome complex namely, p38-MAPk and NFκβ are activated via phosphorylation. Furthermore, inhibition of these molecules causes a loss of S100a7 secretion, validating our hypothesis that activity of these proteins is important for this process. Interestingly, we see maximum catalytic activity of CASP1 at 24hr, followed by 48hr of *E.coli* supernatant treatment. This hints that early (24hr) activation of CASP1 may be responsible for acute secretion of S100a7 and this activity is further maintained via downregulation of CASP8 as a chronic

response. In fact, the catalytic inhibition of CASP1 reduces S100a7 secretion at 48hr of *E.coli* supernatant treatment. In future, we will be inhibiting CASP1 for 24hr to check whether that reduces S100a7 secretion even in earlier time-points.

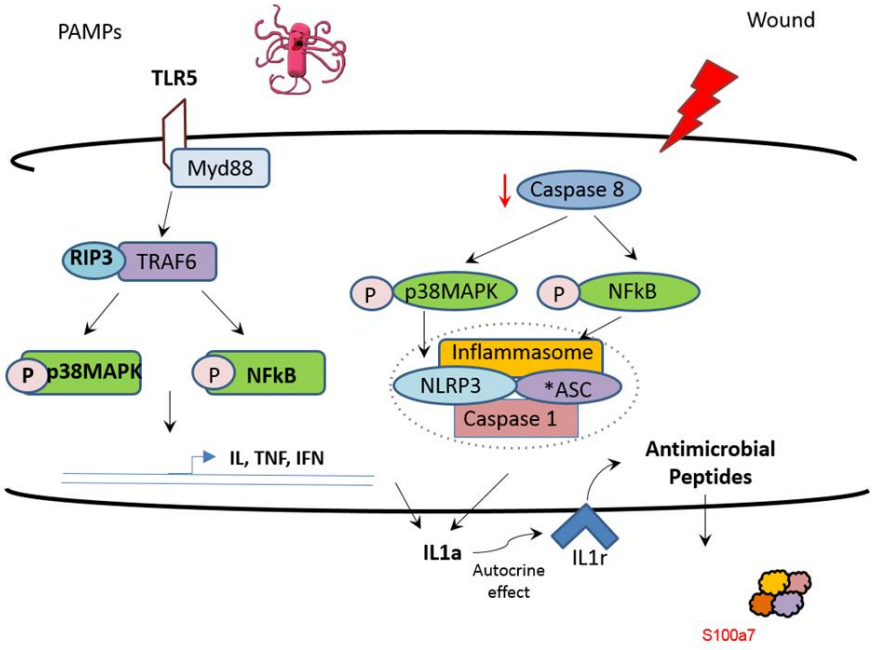


Fig. 3: Hypothetical model of S100a7 secretion.

With our experimental data, we find a novel role for CASP8 in mediating the antimicrobial response generated by the host cells in addition to its indispensable role in wound healing pathway. Thus we have established a mechanistic link between the pathways triggered by pathogen invasion and wound healing, and their convergence at CASP8 downregulation. The conservation of these molecular players of wound healing in the AMP response provides us a better mechanistic understanding of the regulation of S100a7 in diseases like psoriasis.

References

1. Mann, E.R. (2014). Review: Skin and the Immune System. J. Clin. Exp. Dermatol. Res. *4*.

2. Chu, C.Y. (2012). Skin as an immune organ. Dermatologica Sin. *30*, 119–120.

3. Blaydon,D. , and Kelsell,.D.,(2014). Defective Channels leads to an impaired skin barrier. J Cell Science.ens ,*127,* 4343-4350.

4. Fuchs, E., and Raghavan, S. (2002). Getting Under the Skin of Epidermal Morphogenesis. Nat. Rev. Genet. *3*, 199–209.

5. Bangert, C., Brunner, P.M., and Stingl, G. (2011). Immune functions of the skin. Clin. Dermatol. *29*, 360–37

6. Mogensen,Trine.H. (2009).Pathogen recognition and inflammatory signal in innate immune defenses. Clin.Microbiol.Rev. 22, 240-273

7. Anderson, K.S. (2009). Clinical Laboratory Investigation. Detection of psoriasin / S100a7 in the sera of patients with psoriasis. 325–332.

8. Elinav, E., Strowig, T., Henao-Mejia, J., and Flavell, R.A. (2011). Regulation of the Antimicrobial Response by NLR Proteins. Immunity *34*, 665–679.

9. Diamond, G., Beckloff, N., Weinberg, A., and Kisich, K.O. (2009). The roles of antimicrobial peptides in innate host defense. Curr. Pharm. Des. *15*, 2377–2392.

10. F., Pushpanathan, M., Gunasekaran, P., and Rajendhran, J. (2016). Antimicrobial Peptides : Versatile Biological Properties. *2013*, 1–23.

11. Bahar, A.A., and Ren, D. (2013). Antimicrobial peptides. Pharmaceuticals *6*, 1543–1575.

12. Ganz, T. (2003). The role of antimicrobial peptides in innate immunity. Integr. Comp. Biol. *43*, 300–304.

13. Jinquan, T., Vorum, H., Larsen, C.G., Madsen, P., Rasmussen, H.H., Gesser, B.,

Etzerodt, M., Honoré, B., Celis, J.E., and Thestrup-Pedersen, K. (1996). Psoriasin: A Novel Chemotactic Protein. J. Invest. Dermatol. *107*, 5–10.

14. Gläser, R., Harder, J., Lange, H., Bartels, J., Christophers, E., and Schröder, J.-**15.**M. (2005). Antimicrobial psoriasin (S100A7) protects human skin from Escherichia coli infection. Nat. Immunol. *6*, 57–64.

16. Kawasaki, T., and Kawai, T. (2014). Toll-like receptor signaling pathways. Front. Immunol. *5*, 1–8.

17. Murphy, J.E., Robert, C., and Kupper, T.S. (2000). Interleukin-1 and cutaneous inflammation: A crucial link between innate and acquired immunity. J. Invest. Dermatol. *114*, 602–608.

18. Bando, M., Hiroshima, Y., Kataoka, M., Shinohara, Y., Herzberg, M.C., Ross, K.F., Nagata, T., and Kido, J. (2007). Interleukin-1alpha regulates antimicrobial peptide expression in human keratinocytes. Immunol. Cell Biol. *85*, 532–537.

19. Li, Q., Valerio, M.S., and Kirkwood, K.L. (2012). MAPK Usage in Periodontal Disease Progression. J. Signal Transduct. *2012*, 1–17.

20. Lee, D.-J., Du, F., Chen, S.-W., Nakasaki, M., Rana, I., Shih, V.F.S., Hoffmann, A., and Jamora, C. (2015). Regulation and Function of the Caspase-1 in an Inflammatory Microenvironment. J. Invest. Dermatol. *135*, 1–31.

21. Guo, H., Callaway, J.B., and Ting, J.P.-Y. (2015). Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat. Med. *21*, 677–687.

22. Zhao, Y., Yang, J., Shi, J., Gong, Y.-N., Lu, Q., Xu, H., Liu, L., and Shao, F. (2011). The NLRC4 inflammasome receptors for flagellin and type III secretion apparatus. Nature *477*, 596–600.

23. Lee, P., Lee, D.-J., Chan, C., Chen, S.-W., Ch'en, I., and Jamora, C. (2009). Dynamic expression of epidermal caspase 8 simulates a wound healing response. Nature *458*, 519–523.